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Model Organisms to Study Biological Activities and Toxicity of Nanoparticles

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Preface

Past 70 years had witnessed how the way we live and work, transformed by some tiny inventions. The entry of nanotechnology into the world of science has revolutionized all its fields. Nanotechnology is known as an ever-evolving domain since ancient times and is being embraced into a wide variety of disciplines. Nanotechnology delivers an innovative picture of the destiny of biology and medicine. It was fascinating that nanomaterial can be tailored at extremely small scales to achieve their specific properties. Hence, it was possible to make the materials extremely stronger, more durable, more reactive, or lighter through extending the nanoscience toolkit. Nanoscience has the potential to modify every part of our lives. Nanoworld has a positive impact on all materials field such as polymers, metals, biomaterials, and ceramics. These new materials are believed to the foundation of major technological advances. So, the coming era will be enriched with enormous impact of nanotechnology. Future developments in the field of electronics, manufacturing, communications technology, information technology, biology, and medicine could change our earlier approaches. Our world also witnessed the nanotechnological revolution through the powerful combination of this new frontier area, nanoscience, and biotechnology. This nanotechnological revolution emerged in the nanobiotechnology field and positioned India among the world leaders owing to their creation of entirely new processes and industries. Research on nanoscience anticipated that it can deliver priceless set of tools and helpful devices in research in the forthcoming days. Scientists have initiated and received valuable outputs regarding the commercial applications in pharmaceutical industry along with new therapies, in vivo imaging and advanced drug delivery system.

However, nanotoxicology is a new research domain to study and evaluate the detrimental effects of nanomaterials on human health and to protect environment. Utmost importance should be given to depict the exposure risk of nanomaterials. Understanding the toxicity of nanoparticles is challenging and critical. Studies regarding nanotoxicology will reveal the new biological mechanisms which will give new life to nanoscience and nanotechnology. With the advent of nanoparticle applications in broad areas, mode of action of nanoparticles on health and ecosystem is unpredictable but can be scrutinized and evaluated. If possible, several regulatory guidelines can be instigated prior to their utilization. It has been suggested to nullify the toxic effects of nanoparticles prior to their use in different fields. Developments in the nanoworld have resulted in innumerable possibilities for

implications in medicine. Still, research will continue long into the future concerning the potential effects to the human health and environment.

Here, we introduce a book that imparts knowledge on recent advances in the utilization of model organisms to understand the biological activities and toxicity of nanosized particles. As nanotechnology is evolved as a multi-trillion-dollar business sector which covers a wide range of industries with a rapid commercial transition of nanoscience from research to industrial level, model systems at cellular level and organism level are prerequisite to potentially evaluate their biological activities and toxic effects. Model systems, including prokaryotes, cell-based systems, and laboratory animals are important to evaluate the biological activities and toxicity of nanoparticles. This book familiarizes the reader to different possible and novel model organisms and their potential use to understand various biological activities such as antimicrobial, antibiofilm, anticancer, and antidiabetic of nanoparticles. This book also provides a diverse sequence of potential model organisms to evaluate and understand the toxicity of nanoparticles. Model systems and their utilization in nanotechnology can provide insights in the validation and utilization of nanoparticles in medicine with less toxic effects. The chapters also describe state-of-the-art applications of model organisms in research for better utilization of nanoparticles. Authors believe that the book will be of interest to biologists, materials scientists, toxicologists, pharmacologists, molecular biologists, and people working in the field of nanotechnology, nanomedicine, and nanobiotechnology.

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Pondicherry, India — Madhu Dyavaiah
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About the Editors

Busi Siddhardha is Assistant Professor in the Department of Microbiology, Pondicherry University, Puducherry, India. He has more than 12 years of research experience in the field of microbiology, antimicrobial drug discovery, and nanobiotechnology. He has worked for the last 10 years on antimicrobial compounds and antimicrobial drug discovery. He worked at Biology Division, CSIR-IICT, Hyderabad, India, for his PhD. He completed two research projects on antiquorum sensing and antibiofilm activities of natural products funded by the Government of India. He is extensively working in the field of nanotechnology, especially applications of nanotechnology in drug discovery, drug delivery, sustained release, and photodynamic therapy. Currently, his group is working on bacterial quorum sensing, biofilms, antimicrobial photodynamic therapy, and nanobiotechnology. He is editorial board member of several reputed journals. He has also published more than 60 research articles in the peer-reviewed international journal and authored or coauthored numerous book chapters. He is a member of many national and international scientific societies. He has more than 7 years of teaching and research experience at the University level.

Madhu Dyavaiah is an Assistant Professor in the Department of Biochemistry and Molecular Biology, Pondicherry University, Pondicherry. He has earlier served as Research Scientist in the College of Nanoscale Science and Engineering, Albany, USA (2010–2012), Postdoctoral associate at Gen∗NY∗sis Center for Excellence in Cancer Genomic Rensselaer, USA (2006–2010), Postdoctoral fellow at Wadsworth Center, USA (2003–2006), and IISc Bangalore, India (2002–2003). His research interest includes DNA damage response, tRNA modification and translation regulation, and aging biology. He has worked in the area of nanotechnology in the USA and has research experience in different model systems including plant, *S. cerevisiae*, cell lines, and mice. Currently, he is working on *S. cerevisiae* and mice model to study the effect of natural compounds on the age-related diseases. He has been conferred with various prestigious awards. He has served as referee for a number of International journals. He has more than 15 years of research and 6 years of teaching experience in genomics, proteomics, molecular biology, clinical biochemistry biology, and drug discovery. He has also published more than 20 research articles in the peer-reviewed international journal and authored numerous book chapters. He is a member of many scientific societies and organizations.

Kaviyarasu Kasinathan obtained his MSc and MPhil degree in Physics from Loyola College (Autonomous), Chennai, affiliated to the University of Madras, India. Dr. Kaviyarasu Kasinathan was awarded a Senior Research Fellow (SRF) by Tamil Nadu State Council for Science and Technology (TNSCST) and completed PhD degree in Physics at Manonmaniam Sundaranar University (MSU), India. He has carried out research on multifunctional metal oxide nanoparticles for energy and biomedical applications. During the course of his research work, he has published a total number of 125 peer-reviewed publications in International and national journals and delivered 35 oral/invited talks on nanomaterials, thin films, photocatalysis, biomaterials, solar energy conversion, preparation techniques, and characterization studies in conferences, universities, and academic institutions. Currently, he is working as a Research Scientist at iThemba LABS, Cape Town; his

research is directed primarily toward developing and applying modern material design for the understanding and prediction of materials science research with ion beams that has been basically developed by nuclear/material physicists. It started with the development of accelerator-based techniques for materials characterization, leading into surface studies and depth profiling. Now the swift heavy ions are being utilized for engineering the properties of materials and are exploited in almost all the emerging new areas in materials science. His goal is to understand how to design and control the nanoscale organization of macromolecular nanomaterials and their nanocomposites in order to achieve improved structure, properties, and functionality. His research interests include bulk and nanoscaled materials for solid-state physics, and multifunctional metal oxide nanomaterial.

Nanotechnology: Application in Biology and Medicine

1

Ammani Kandru

Abstract

Nanotechnology is a novel and rapidly growing multidisciplinary field with major and multifold advances in the fields of engineering, electronics, energy, environment, biology, and medicine. The foundation of this novel science is laid down with the visionary ideas of Feynman in the 1950s. In this chapter the history of nanoscience is presented and an overview of applications of nanoparticles in biology and medicine are discussed. Several applications ranging from bioseparations, biosensing, molecular imaging, drug delivery, to hyperthermic treatment have been summarized.

Keywords

Nanotechnology · Nanoparticles · Applications · Biology · Field

1.1 Introduction

In higher animals and human beings, living process depends on interaction between cells and other smaller biomolecules that take place in nanoscale region. Organization of nanomaterials is central to biology and such intrinsic nanobiology has been noticed and investigated from the good old days. With the emerging tools and technologies in this field there is a lot of scope on the understanding of how biological systems work on the nanoscale and how these systems are integrated within the cells. Nanotechnology is a multidisciplinary field with a novel scientific approach and with a tremendous potentiality in traditional as well as advanced fields of biology, chemistry, physics, engineering, electronics, and medicine. The ultimate goal is to derive the engineering principles that govern the cellular functions, from

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growth to apoptosis. Hence, nanotechnology enables novel ways and means to detect and measure biology both in vitro and in vivo.

1.2 History of Nanotechnology

Nanotechnology has its background throughout the human history. Humans were engaged in this technology, without having appropriate knowledge on it, and even without understanding the nature of these objects and processes. The use of kajal has been prevalent in South Asia, India, North Africa, and Middle East. Indian women prepared this cosmetic from the soot of lamp burning edible oil, by holding an earthen pot above the flame. This was collected and mixed with cow ghee and is ready to use. The carbon black thus obtained is of very fine size, i.e., is nano size and the application to the eye lids gives a cooling effect. One more example is the well-known invention of Indian ink that relies on producing carbon nanoparticles in water (known around 2700 BC). Faraday prepared colloidal gold in 1856 itself. Colloidal gold has been utilized in the preparation of glasses and vases to give them color. Also, Ayurveda, the Indian system of medicine, uses gold in several preparations. Colloidal gold is believed to be a remedy for chronic inflammations and several other diseases. Paracelsus treated human diseases by using gold and other inorganic compounds. Silver in the colloidal form is also considered to be a potent natural antibiotic, used in treating several diseases for thousands of years. However, the actual concept behind nanoscience began with a lecture by a Noble Laureate physicist named Richard Feynman on December 29th, 1959. His lecture titled "There is plenty of room at the bottom" gave scope to decrease the size of things, and tiny structures could be formed by arranging in the way we need. He was the first to propose that the materials at the nano range would present future opportunities. He believed in the existence of nanostructures in the biological systems. He even imagined the use of tiny machines in medicine. He further speculated the manufacturing of nanoscale machines. However, he never used the word nanotechnology. Though the practical ideas of Feynman were not implemented, his vision awakened the interest of many scientists and paved the way for this new field of research. The term nanotechnology was actually coined by a Japanese scientist named Noris Taniguchi in 1974. He proposed that nanotechnology consisted of processing, separation, consolidation, and deformation of materials by one atom or one molecule. Later with the invention of sophisticated instruments such as electron microscope and scanning tunneling microscope (STM) that could image and manipulate atoms, and Atomic force microscope (AFM) that structures on the atomic scale could be observed (Miyazaki and Islam 2007). Several series of events came into light and the main developments were summarized in the Table 1.1.

Nanoscience is basically the study of fundamental principles of molecules and structures with one dimension between 1 and 100 nanometer. These structures are known as nanostructures, and nanotechnology is the application of these structures into useful nanoscale devices. Today, nanotechnology is a vivid and vital area of

Table 1.1 Timeline events in Nanotechnology

Year	Developments in nanotechnology
2000 years back	Sulfide nanocrystals were used to dye hair by Greeks and romans
1000 years back	Gold nanoparticles of various sizes are used to create different colors on glass windows.
1959	First concept and vision of nanotechnology - R.Feynman
1974	Taniguchi coined the term nanotechnology
1981	Invention of scanning tunneling microscope.
1986	First book on nanotechnology "Engines of Creation"—Theory of molecular engineering became popular
1986	Invention of atomic force microscope.
1987	Development of magnetic force microscope.
1991	Discovery of carbon nanotubes S. Iijima.
2000	Launching of National Nanotechnology initiative
2002	Magnetic nanoparticles were used to report hyperthermic regression of tumors in mice.
2007	First human clinical trials for the treatment of cancer by hyperthermia by Dr. Johanssen and co-scientists.
2011	Molecular nanotechnology era began

research with tremendous prospects, changing the direction of science with a variety of applications in diverse fields, in all spheres of life.

1.3 Definition

Nanotechnology is defined by the National Nanotechnology Initiative as "Research and technology development at the atomic, molecular or macromolecular scale, leading to the controlled creation and use of structures, devices and systems with a length scale of 1–100 nanometers".

The definition given by European commission is "Nanotechnology is the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale".

1.4 Importance of Size

A billionth of a meter is a nanometer or nm. Nanometer was first used by Zsigmondy for specifying particle size. Nanotechnology deals with 0.1–100 nm. The lower edge of the nano world is defined by the size of single atom; diameters vary from 0.1 nm, a hydrogen atom, to about 0.4 nm, a uranium atom. This represents the smallest structure, as we cannot create building blocks smaller than atoms. The upper edge of the nano world is 100 nm. Because of the minute size and high

surface area-to-volume ratio, they display new physics and chemistry leading to a new behavior. A few examples are:

- Inert materials become catalysts—Platinum
- At room temperature solids turn into liquids—Gold
- Opaque systems are changed to transparent ones—copper
- Insulators turn into conductors—Silicon
- Stable materials turn combustible—Aluminum

These exceptional magnetic, electrical, thermal, and optical properties are due to their spatially confined electrons (Alivisatos 2004).

- The electrical properties depend on the diameter of the material. They have very high electrical conductivity, due to the fewer defects in the crystal
- The thermal conductivity is enhanced due to the heavy vibration of covalent bonds.

Thus the nanomaterials display unique optical, biological, electrical, mechanical, and magnetic properties that are summarized in Table 1.2.

Also this size range is intimately connected with the phenomena in the biological systems. The basic building blocks of life, including cells and biomolecules fall in this range. For instance, DNA molecule is only 5–10 nm. Nanoscale devices such as nanopores (~2 nm openings), inorganic nanowires (~10 nm diameter), and spherical nanoparticles (10–100 nm diameter) are of similar size as biological entities. Nanoparticles less than 20 nm can move through blood vessels. Also nanoparticles can enter into stomach epithelium and can cross the blood brain barrier (Vinogradov et al. 2004; Lockman et al. 2003; Russell-Jones 1999). Surface charge also plays a prominent role in the ability of nanoparticles to penetrate the blood brain barrier (Lockman et al. 2003). The size of nanoscale devices also makes them readily interact with biomolecules within the cell, without changing the behavior and biochemical properties of those molecules (Bogunia-Kubik and Sugisaka 2002).

These properties revolutionized researchers from different fields and paved the way for several promising and potential applications in the following fields.

- Engineering and transportation

Table 1.2 Size-dependent properties of nanoparticles

Property	Examples
Biological	Permeability through biological barriers is increased
Electrical	Electric resistance in metals is increased
Optical	Spectral shift of optical absorption and fluorescence properties
Magnetic	Magnetic property is increased—superparamagnetism
Catalytic	Greater catalytic efficiency because of high surface-to-volume ratio
Mechanical	Increased toughness and hardness of metals and alloys Superplasticity and ductility of ceramics

- Electronics and information technology
- Energy and environment
- Physical and biological sciences
- Agriculture and industry
- Medicine and health care

However, we will confine to the applications in biology and medicine in this chapter.

1.5 Applications in Biology and Medicine

As all the biological processes are balanced by the action of biological molecular nanomachines, nanotechnology is of prime significance in biology and medicine. Nanotechnology has opened up lightning advances in biology and medicine with novel and critical tools and applications, as biological systems are highly responsive and restorative. The mechanical and chemical properties could be characterized with the available novel nano tools. One outstanding development in these fields is optical nano-biosensors, to study the single living cell in a minimally invasive manner. By this method, protein function at the single cell level can be analyzed without disturbing the chemical makeup of the cells. Cellular processes such as, functioning of proteins that occur in subseconds time, in their natural environment can be quantitated. Also apoptosis, known as programmed cell death, a cellular process usually observed in normal and diseased, is significant to both biology and medicine. To study the pathway of apoptosis, the proapoptic members, cytochrome c, caspase-7, and caspase-9, have to be detected. The in vivo detection and identification of these can be done by using optical bio-nanosensors.

The applications in biology and medicine are summarized separately.

1.6 Applications in Biology

As biological species exhibit molecular structures at the nanoscale levels, nanotechnology plays prominent role biology. Understanding the biological processes at the nanoscale level is the driving force behind the development of nanotechnology (Whitesides 2003). Nanoparticles with distinct size, shape, and surface chemistry can be engineered in a wide variety of biological applications. Thus nanoscale structures such as nanopores, nanofibers, nanowires, nanotubes, nanochannels, and nanocapacitors are investigated in many biological applications such as molecular imaging, biological separation, biosensing, bacterial detection, and sequestration as detailed below:

1.6.1 Bioseparation: Separation and Purification of Biological Molecules

Bioseparation is the separation and purification of certain biomolecules selectively from a complex mixture. In biological research, selective and efficient isolation and purification of specific cells from complex mixture is the need of the hour. The traditional methods of separation such as precipitation, filtration, centrifugation, and chromatography are time-consuming. Also they suffer from several drawbacks. To overcome the drawbacks in the traditional techniques, nanomaterials can be utilized. Thus nanotechnology offers promising applications by designing novel nanobiological objects in the bioprocessing that can be utilized in bioseparation, imaging, and sensing of several different biological compounds (Wang and Wang 2014). It plays an important role in different biological processes and in the industrial production of biological compounds.

1.6.1.1 Separation of DNA

DNA molecules are negatively charged in physiological media, whereas in acidic media they acquire positive charge due to the phosphate group's protonation. Salmon sperm was separated by means of electrostatic interactions, using magnetic mesoporous silica-magnetite nanocomposites prepared by the template-assisted method (Melzak et al. 1996). At the physiological pH, the nanocomposites acquired a positive charge that facilitated electrostatic interactions with the negatively charged phosphate backbones of DNA, paving the way for efficient separation.

1.6.1.2 Separation of Proteins

Proteins play a crucial role in cell machinery and structure. Previously, conventional protocols such as ultra-filtration, precipitation, and chromatography were of paramount importance in the separation and purification of proteins and peptides. The alternate method is the magnetic separation of specific proteins by utilizing magnetic nanoparticles (MNPs). Magnetic nanoparticles bind to different copolymers of protein by various mechanisms such as ligand binding, vanderwalls, hydrophobic, and electrostatic interactions (Churchill et al. 2004; Tenzer et al. 2013) .This can be done in samples such as blood, plasma, urine, cell lysate, or any biological fluid. The sample is mixed with MNPs with hydrophobic ligands or ion exchange groups and incubated for an appropriate period, so as to allow the affinity species to bind to the ligands anchored to the MNPs. The proteins are now separated by magnetic decantation. By using proper procedures of elution, the purified target proteins are recovered by displacement from the MNPs. When compared with conventional methods, protein separation using MNPs is advantageous for the following reasons.

(a) Sample preparation is easy and less time-consuming
(b) Purification process is simple, easy, and rapid

Table 1.3 MNPs in the separation of different biomolecules

Biomolecule	Core	Functionalization	Interaction type	Reference
Trypsin	Fe_3O_4	Carboxylic acid group	Affinity	Khng et al. (1998)
Lysozyme	Fe_3O_4/silica	Polyacrylic acid	Electrostatic	Shao et al. (2009)
BSA	Silica-coated MNPs	Alkyl chains	Hydrophobic	Chang et al. (2010)
SH-SY5Y cell	Fe_3O_4	PAA PEI	Electrostatic	Calatayud et al. (2014)
Streptavidin Protein	Silica NPs(2 nm)	Multiple layers of Fe_3O_4 Extra layers of silica Biotin	Affinity	Kyeong et al. (2015)
$CD3^+$cells from spleen	Fe_3O_4	Anti-CD3 monoclonal antibody	Affinity	Cui et al. (2011)
Salmon sperm DNA	Fe_3O_4	Mesoporus silica	Electrostatic	Melzak et al. (1996)

(c) Magnetic separation does not need equipment such as chromatographic systems or centrifuges.
(d) Small amounts are sufficient for the separation process
(e) Method is cheap and scalable

1.6.1.3 Separation of Biomolecules

MNPs are utilized in separating several biomolecules, owing to their versatility of functional groups that can be used to modify their surface (Earhart et al. 2014; Zhang et al. 2013; Intorasoot et al. 2009). T-cells from the spleen were successfully separated utilizing Anti-CD 3 monoclonal antibody bioconjugated to core/shell Fe3O4/Au MNPs (Cui et al. 2011). Some more research findings are tabulated in Table 1.3.

1.6.2 Probing of DNA Structure

In biotechnology assays structural polymorphism in DNA serves as a biological signal. Quantum dots, the semi-conductor nanoparticles, with all the three dimensions in the nano range are receiving recognition for their biological applications. These photoluminescent nanomaterials are being developed both as sensors and dyes to detect different intrinsic DNA structures (Mahtab and Murphy 2005).

1.6.3 Fluorescent Biological Labels

In the biological world, fluorescence is a commonly and widely used tool. In biological staining and diagnostics, semiconductor nanocrystals were used as fluorescent probes. In ultrasensitive biological detection, zinc sulphide-capped cadmium selenide quantum dots are coupled covalently to biomolecules. When compared with the conventional organic dyes such as rhodamine, these are brighter and have tunable, narrow symmetric emission spectrum. These features allow them to be used as a direct probe or sensitizers.

1.6.4 Biological Processes

Nanoparticles are vital tools to study and characterize biological processes. Several novel and exciting applications include the following.

- Improvement of current techniques in cellular and molecular research
- Activation of cell signaling pathways
- Regulation of protein production
- In the molecular dynamics, individual molecules in live cells can be visualized.
- Insight into molecular processes and cell functions involving complex signaling pathways

Cho et al. 2012 demonstrated that the cell signaling pathway can be controlled by using functionalized magnetic nanoparticles. When the magnetic field is applied, an apoptosis signaling pathway is promoted, which is demonstrated in vivo in zebra fish. Likewise, several such methods of noninvasive nature provide a promising tool for basic biological research.

1.6.5 Biosensing with Magnetic Nanoswitches

Weissleder along with his co-scientists was the first to propose magnetic relaxation of nanoswitches. Pathogens, proteins, DNA, and biological processes such as enzymatic function can be accurately detected using the new biosensors (Perez et al. 2002; Koh et al. 2008; Taktak et al. 2007). For the quick and quantitative analysis of unprocessed biological samples, a chip-based diagnostic magnetic resonance (DMR) system was developed (Lee et al. 2008). When compared with the conventional methods miniaturized DMR has the following advantages.

(a) DMR micro system can be prepared as disposable units.
(b) Minimum amount of sample is sufficient (micro liters)
(c) Quick screening of analytes, can be performed
(d) High detection sensitivity
(e) Screening can be performed even in opaque media

1.6.6 Single Cell Phenotypes

Single-cell phenotypes can be directly measured by using nanostructures. The mass of adherent cells were measured using resonating sensors (Park et al. 2010). In the cell cycle, at certain check points growth rates varied.

1.6.7 Delivery Vehicles

Nanoparticles serve in delivering various agents. A few of them are mentioned below.

1.6.7.1 Delivering Hydrophobic Compounds Without Solvents

Most biologically active compounds are poorly soluble in water. One traditional approach is using dimethyl sulfoxide (DMSO) as a solvent. But DMSO cannot be used for in vivo applications. Also all compounds cannot be solubilized in this solvent. To overcome these problems in delivering the hydrophobic compounds polymeric nanoparticles that possess hydrophobic cores are used. The advantages of using these particles include.

(a) The solubility of active agent is increased
(b) Safeguards the agent from the environment until it is released from the nanoparticles

1.6.7.2 Delivering siRNA for Biological Studies

In cell culture the gene functions can be studied by using siRNA. However, there are many biological obstacles in the delivery of siRNA such as difficulty in entering the cell due to its high molecular weight and negative charges, degradation by nucleases within the cell, rapid clearance, and instability in vivo (Nie and Emory 1997; Peng et al. 2009; Liu et al. 2008). Nanoparticles are a good alternative to overcome these obstacles.

1.6.7.3 Delivering Agents to Subcellular Organelles

Delivering agents to subcellular organelles throws light on certain molecular processes that are not known in the organelles. Nanoparticles are used as carriers to deliver agents to subcellular organelles as they can be easily modified. Tools for subcellular targeted delivery to the nucleus (Pouton et al. 2007) cytosol (Vasir and Labhasetwar 2007), mitochondria (Yamada and Harashima 2008), lysosomes (Lloyd 2000), and endosomes (Bareford and Swaan 2007) have been developed.

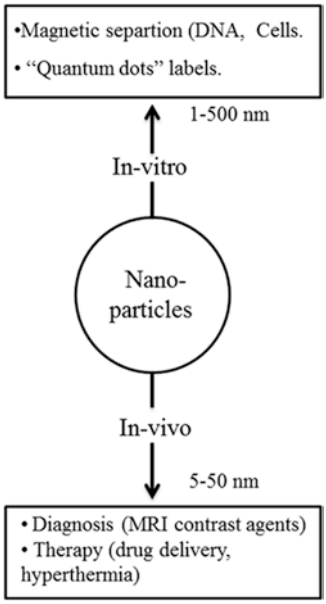

Fig. 1.1 Nanoparticles utilized in medicine

1.7 Applications in Medicine

One of the key roles of nanotechnology is for the advancement of health and medicine. This technology offers promising and potential developments in pharmaceuticals, disease diagnosis, target specific drug delivery, cancer treatment, medical imaging, tissue regeneration, implantable materials, and tissue regeneration. Nanoparticles are used to diagnose proteins and DNA, as probes for in vivo investigations of cell functions, as carriers of drugs in drug delivery system (Alivisatos 2004) for magnetic cell separations, and as contrasting agents in magnetic resonance imaging (MRI). For many applications the size of nanomaterial is very crucial (Fig. 1.1). The various applications in medicine are detailed in Fig. 1.1

1.7.1 Drug Delivery

Nanoparticles are used for new formulation of drugs and also for site-specific delivery. In this technology, the active agent of the drug is deposited in the pathological site only. Hence it reduces the drug consumption, lowers the side effects and is also cost-effective. Drugs are encapsulated in nanoshells, polymer capsules, organic dendrimers, and micelles. Also many drugs that cannot be given orally because of their lower bioavailability can be benefited by this technology (El-Shabouri 2002; Hu et al. 2004).

1.7.2 Diagnostic Applications

Detecting diseases at an early stage with greater efficiency and economy is the need of the hour. Traditional diagnostic methods depend upon the manifestation of visible symptoms. Several nanoparticles such as quantum dots, gold nanoparticles, and magnetic nanoparticles have been utilized in diagnostics.

1.7.2.1 Quantum Dots

All the three dimensions are in nano range in a quantum dot. These exhibit broad excitation spectra, high sensitivity, and stable fluorescence. Also they do not need lasers. Their infra-red colors enable whole blood assays. Visualization of cancer cells is possible with luminescent quantum dots. Intracellular imaging can be performed by labeling of target molecules with quantum dots. Thus these have several applications in genotyping, molecular diagnostics, and biological assays.

1.7.2.2 Gold Nanoparticles

Among the metal nanoparticles, gold nanoparticles are the most stable (Stroscio and Dutta 2003). Colloid gold has been used as biosensors, in disease diagnosis, and in gene expression. Gold nanoparticles are extensively used as sensors because of their surface chemistry. The gold nanoparticle-based biosensors are employed in the detection of DNA or RNA targets with single nucleotide polymorphism at a detection limit of about 50 fM (Nam 2003).

Mirkin group has developed the bio-barcode method for protein and DNA (Hill and Mirkin 2006) target detection. Also this method has been reported for a biomarker for Alzheimer's disease (Georganopoulou et al. 2005). Prostate cancer can be detected by this method, by identifying prostate-specific antigen, a common cancer biomarker. PSA gold nanoprobes are generated by conjugating DNA

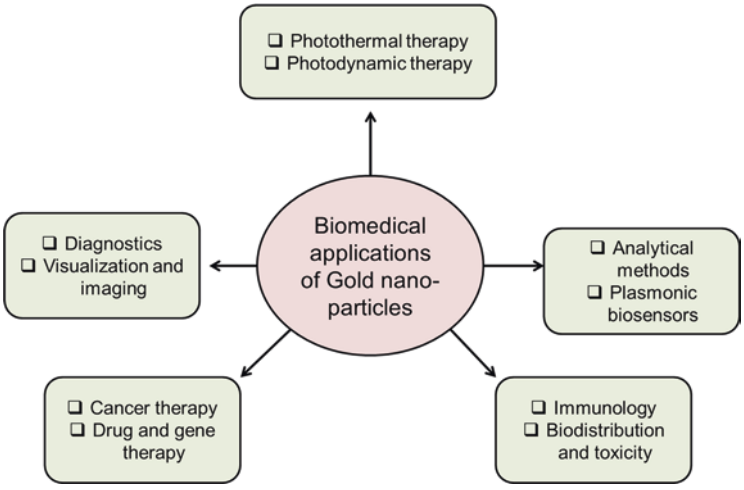

Fig. 1.2 Biomedical applications of gold nanoparticles

functionalized gold nanoparticles (30 nm) to PSA-specific antibodies (Swierczewska et al. 2012). The bio-barcodes are the DNA strands. Several antitumor substances such as paclitaxel, cisplatin, doxorubicin, and oxaliplatin were conjugated with gold nanoparticles. The biomedical applications of gold nanoparticles are depicted in Fig. 1.2.

1.7.3 Cardiac Therapy

Nano particles are widely used in cardiovascular therapy at the cellular level and play a promising role in treating cardiovascular diseases. These methods can be utilized in diagnosis, imaging and tissue engineering (Lanza et al. 2006). Miniaturized nanoscale sensors—quantum dots, nanobarcodes, and nanocrystals—can sense and monitor complex immune signals. Also critical cardiovascular diseases can be treated by the newly designed nanomachines.

1.7.4 Orthopedic Applications

Nanomaterials, nanofibers, nanotubes, nanopolymers, and ceramic nanocomposites can be utilized for the depositing of minerals containing calcium on implants. Thus nanostructures play a prominent role in improving the attachment of implant to the surrounding bone by enhancing bone cell interactions and thus improve the implant efficacy.

1.7.5 Dentistry

The role of nanotechnology in the field of dental care (West and Halas 2000; Shi et al. 1999) will ensure better oral health. Covalently bound artificial materials such as sapphire may replace the upper enamel layer to increase the durability and appearance of teeth. Thus in the maintenance of natural tooth, nanodentistry is of considerable significance (Shellhart and Oesterle 1999).

1.7.6 Magnetic Resonance Imaging (MRI)

Healthy and pathological tissues can be distinguished by using MRI, as this shows a clear contrast of the image between these two tissues. These images can be improved by adding “contrasting agents, such as gadolinium (Gd) chelates which are nonspecific and allow only a short time imaging window (Kubaska et al. 2001; Low 2001). Colloidal iron oxides, the first liver specific contrast agents, play a crucial role as MRI contrast agents (Halavaara et al. 2002).

A new contrast agent for MRI in cancer imaging was developed by (Yu et al. 2008). Interestingly this agent can deliver anticancer drugs specifically to tumors, beneficial in both cancer imaging and therapy (Yu et al. 2008).

1.7.7 Cancer Therapy

Baker and his coscientists were the first to demonstrate the delivery of therapeutics to cancer cells in vitro and in vivo. The size range used for in vivo applications ranges from 2 to 150 nm. Large particles with diameter of 300 nm are used as MRI contrast agents of gastrointestinal tract.. Large magnetite nanoparticles (40–150 nm) are suitable for imaging spleen and liver. Small nanoparticles (20–40 nm) are used to visualize tumors, whereas ultra-small, less than 20 nm, superparamagnetic iron particles are utilized for myocardial ischemic diseases and for imaging vessels in angiography.

In cancer treatment carbon nanotubes serve as a diagnostic and therapeutic tool. Cisplatin is widely used as an anticancer drug. But it is highly toxic and requires specific delivery. Scientists synthesized ultra-short carbon tubes for the delivery of cisplatin that could avoid the reticuloendothelial system.

The first clinical trial using nanoparticles for anticancer drug delivery was performed in the 1980s. From then onwards several new nanoparticles have been approved and many are under development.

The various nanosystems utilized in treating cancer are summarized as:

Nano shells—utilized in deep tissue thermal ablation and in tumor specific imaging

Nano wires—utilized in detecting DNA mutation and disease protein biomarkers

Nanocrystals: 2–9.5 nm in size

- To improve the formulation for poorly soluble drugs.
- For labeling of breast cancer marker HeR2

Nanoparticles: 10–1000 nm in size

- Utilized in MRI and as ultrasound image contrast agents.
- For targeted drug delivery
- As reporters of apoptosis and angiogenesis

Carbon nanotubes: 0.5–3 nm in diameter and 20–1000 nm length
Utilized in the detecting DNA mutation
Utilized in the identification of disease protein biomarker.

Quantum dots: 2–9.5 nm in size, helps in optical detection.

Gene Therapy

The creation of novel adenoviral vectors has revolutionized cancer gene therapy. Barker and Berk in 1987 created an oncolytic adenovirus dl1520, which has been utilized in specific targeting of tumor cells.

1.7.8 Hyperthermia

The primary goal in cancer therapy is the selective killing of cancer cells without disturbing normal cells. The use of nanomaterials in heat therapy, known as Nanoparticle Hyperthermia involves applying heat to tumor cells (Abenojar et al. 2016). The heating power of the particles is quantified as the specific absorption rate (SAR), which describes the amount of energy converted into heat per time and mass (Moroz et al. 2002). Recent studies proved that large tumors can be heated without any problem with a proper regulation of the magnetic mass used and the intra tumoral particle distribution.

Pathogen Detection and Isolation
For detecting and isolating pathogens various nanoparticles has been explored as sensors. The magnetic and optical property of the nanoparticles has been utilized. Magnetic biosensors have widely been utilized for detecting pathogenic bacteria. Magnetic nanoparticles are coated with antibodies against surface antigens (Varshney and Li 2007; Xia et al. 2006). Researchers devised a method without antibodies to detect single gene mutations. This could detect drug-resistant strains of *Mycobacterium tuberculosum* in less than 3 h from sputum samples. In the conventional system, identifying this bacteria takes long time as this bacteria grows slowly in the culture medium.

By utilizing both metallic nanoparticles and quantum dots, optical biosensing of bacteria has been possible. Many targets can be detected simultaneously by the bio-barcode assay. *Bacillus subtilis* was detected at 2.5 fM concentration (Hill et al. 2007) by this method. Also, *Salmonella enteritidis* was detected at 0.2 fM (Zhang et al. 2009). Quantum dots are also used as pathogen sensors.

1.7.9 Ophthalmology

A number of applications are available in the field of ophthalmology also. A novel nanoscale-dispersed eye ointment (NDEO) for treating evaporative dry eye has been successfully developed by Zhang et al. 2014. Some more applications are as follows:

- Scars can be prevented after glaucoma surgery
- Oxidative stress treatment
- Retinal degenerative disease can be cured using gene therapy
- Measurement of intraocular pressure

1.7.10 Tissue Engineering

Nanoscale biomaterials are utilized as carriers for artificial matrices for tissue engineering. However, the scaffold should mimic the structure and biological function

of the native extra cellular matrix not only in the physical structure but also in chemical composition. Nanotechnology can be used to create nanofiber and nanopatterns for mimicking native tissues (Chung et al. 2007). Tissue engineering is now feasible through nanotechnology and is used in stem cell tissue engineering, neural cell tissue engineering, cartilage cell tissue engineering, bone and hepatic cell engineering.

1.8 Conclusion

Nanotechnology, a multidisciplinary science with multidirectional development will provide opportunities for developing new methods, materials, and devices for more innovative applications. Nanomaterials with distinct biological properties, due to enhanced surface area and nanoscale effects, significantly affect their interaction with biomolecules and cells, creating an excellent approach for characterizing basic biological processes. Such studies can provide novel and critical insights into cellular functions and molecular processes. Also integration of proteomics and genomics with nanotechnology will throw more light in understanding biological processes.

Also, in the field of medical sciences, nanotechnology has brought a revolutionary change in diagnostics, therapy, and drug discovery. There is an immense scope and possibility to design and develop multifunctional targeted nanoparticles to diagnose and treat dreadful diseases such as cancer. Also early detection of disease, simple and inexpensive tests, sophisticated imaging methods, minimal invasive treatment, and several endless lists of potential benefits will change the medical field in future.

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Biological Activities of Nanoparticles and Mechanism of Action

2

Karan Chaudhary and Dhanraj T. Masram

Abstract

This chapter covers nanoparticles (NPs) of gold, silver, zinc oxide, copper oxide, zirconium oxide, iron oxide, and yttrium oxide, which have been recently used for antimicrobial and anticancer activity with plausible mechanism for their activity. Eco-friendly syntheses of smart metal NPs (size, shape, and morphology controlled NPs with desired modifications) through a bottom-up approach have greater selectivity and biological activity toward targeted cells without any harm to the normal cells. Selectivity is the key feature of NPs that makes possible the future use of NPs as replacements for drugs in biomedical applications. Generation of reactive oxygen species (ROS) which causes damage to cell components and membrane, interaction of released metal ions with proteins causes inhibition of enzymes activity and physiological processes, and nonoxidative mechanism are the major proposed mechanisms behind antimicrobial and anticancer activity of metal NPs which results in cell apoptosis.

Keywords

Nanoparticles · Antimicrobial activity · Anticancer activity · Mechanism of action · Eco-friendly syntheses

2.1 Introduction

Nanoparticles (NPs) are particles ranging 1–100 nm in size (Buzea et al. 2007) with unique properties due to their nano-dimensions (Hübler and Osuagwu 2010). There are two best known approaches for the synthesis of NPs; breakdown approach (or top-down approach) and bottom-up approach. Some of methods under these

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approaches are lithography, sputtering, ball milling, etching, spray pyrolysis, chemical vapor deposition, sol-gel method, and atomic/molecular condensation. The important features such as shape and size control, purity, control over aggregation, stability of NPs and mass production of particles are to be considered while synthesizing NPs (Horikoshi and Serpone 2013). And in the present era, green synthetic methods using biological precursors has gained much importance for synthesizing NPs (Fig. 2.1) (Doble et al. 2010). Cells are the building units of living organism that are having across dimension of 10 μm. Whereas, cell organelles are even much smaller in size about of sub-micron level and NPs having dimension of ~5 nm are comparable in size with these cell organelles. This size complementarity has been the idea behind using NPs as probes for studying cell machinery without interference in biological functioning (Taton 2002). Now metal NPs and their oxides, due to small size can easily approach the bio-object through which it can interact with it and contact it, which leads to biological activity by NPs. Various metal NPs and metal oxides such as copper, nickel, silver, gold, titanium, and many others have been used for biological activities due to the physicochemical properties they possess (Mamonova et al. 2015). Herein, we are discussing the anti-biological activities such as antimicrobial and anti-cancer activities of NPs.

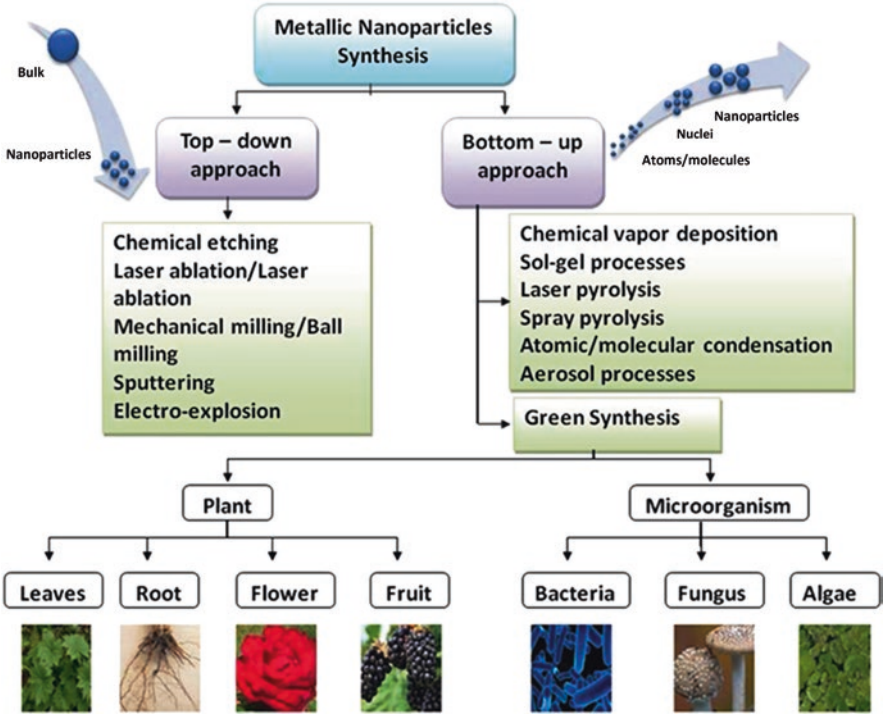

Fig. 2.1 Preparation of metal NPs by different approaches available for the synthesis. (Singh et al. 2018) (© 2018, Jagpreet Singh, Tanushree Dutta, Ki-Hyun Kim, Mohit Rawat, Pallabi Samddar and Pawan Kumar)

2.2 Green Synthesis of Nanoparticles

The green synthesis of nanoparticles aims to build an environment benign synthetic route by avoiding production of harmful byproducts and minimizing the pollution and waste production. For this, it is important to use green solvents and naturally occurring resources during the synthesis process. Moreover, to achieve this, different naturally occurring resources such as bacteria, fungi, yeast, plants, and plant extracts have been employed. Simple and easy synthesis of metallic nanoparticles has been achieved by using plant extracts among all green methods. All these plant extracts comprised phytochemicals such as terpenoids, flavones, aldehydes, ketones, carboxylic acids, ascorbic acids, amides, and phenols, which have the potential for synthesizing metal NPs by the reduction of metal salts (Doble et al. 2010). Various biological resources that are presently being used for the synthesis of metal NPs are discussed below:

Microbial synthesis: Synthesis of NPs using various microorganisms has been reported.

Bacteria: For first time in 1980, gold NPs synthesis was reported by treatment of $HAuCl_4$ with *Bacillus subtilis*. Over the years, various bacterial strains have been used for the synthesis of NPs; a few of them are *Thermomonospora* sp., *Rhodococcus* sp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Shewanella* sp., *B. sphaericus JG-A12*, *Bacillus* sp., *Enterobacter cloacae*, *Lactobacillus* spp., *Enterococcus faecium*, *Lactococcus garvieae*, *B. cereus*, *B. subtilis*, *B. amyloliquifaciens*, *B. megateriu*, *B. flexus*, *B. mycoides*, and *Enterococcus* sp. (Pantidos and Horsfall 2014; Vaseghi et al. 2018).

Fungi: Few of the fungal strains exploited for the synthesis of NPs are *Aspergillus tubingensis*, *Fusarium solani*, *A. fumigatus*, *A. niger*, *F. acuminatum*, *Colletotrichum* spp., *A. flavus*, *F. semitectum*, *Penicillium* sp., *P. purpurogenum*, *Volvariella volvacea*, *Phoma glomerata*, *Pediococcus pentosaceus*, *F. oxysporum*, *Neurospora crassa*, and *Verticillium* sp. (Pantidos and Horsfall 2014; Vaseghi et al. 2018).

Yeasts: Yeasts are also used for the green synthesis of NPs. Few examples of them are *MKY3*, *Saccharomyces cerevisiae*, *S. boulardii*, *Yarrowia lipolytica*, *Candida* sp., *Rhodotorula* sp., *Pichia pastoris* and *Schwanniomyces occidentalis* (Vaseghi et al. 2018).

Plants and plant extracts: Direct addition of metal salts solution to plant extracts at room temperature leads to the synthesis of NPs. For this various plants extracts have been used; a few of them are *Acalypha indica*, *Allium sativum* (garlic clove), *Aloe vera*, *Azadirachta indica*, *Cymbopogon* sp., *Garcinia mangostana*, *Mentha piperita*, *Nelumbo nucifera*, *Pyrus* sp., *Tanacetum vulgare*, *Coriolus versicolor*, *Jatrophacurcaslatex*, *Acalypha indica*, *Cymbopogon flexuosus*, and *Magnolia kobus* (Mittal et al. 2013; Pantidos and Horsfall 2014).

2.3 Interaction of NPs with Biological Components

In order to understand the role of NPs in biological application it is important to understand how NPs interact with the cell. Dynamic physicochemical interaction known as bio–nano interface takes place between the surface of biological component and surface of NPs. This bio–nano interface which includes NPs and biological component interactions deals with the thermodynamic and kinetic exchange that takes place between interface (Nel et al. 2009). A significant interaction takes place between NPs and biological components such as solvation, electrostatic forces, van der Waals forces, solvophobic, and depletion forces (Min et al. 2010). The importance behind this ideology is to understand how to prevent agglomeration of NPs for proper dispersal in biological component. Electrostatic forces come under the category of repulsive forces, whereas, van der Waals forces and depletion forces come under the category of attractive forces (H-Y Kim et al. 2007). These forces are responsible the interaction of NPs with the cell surface and passive introduction in the cell (Geiser et al. 2005). After passive uptake, NPs can reside anywhere in cell, which includes cytoplasm, outer membrane, nucleus, mitochondria, lipid vesicles, nuclear membrane, and DNA. NPs in the cell cause damage to the cell organelles, which lead to the death of the cell (Garcia-Garcia et al. 2005).

2.4 Antibacterial Activity of Nanoparticles

It is clearly evident that, bacteria have emerged unaffected by the use of antibiotic drugs. Even emergence of super bacteria has been observed, which are resistant to almost all antibiotics (Hsueh 2010); along with modification of cell wall and other cell components (Jayaraman 2009), modification or degradation of antibiotic by action of enzymes (Poole 2002), and expression of efflux pumps (Knetsch and Koole 2011) are the mechanisms which provide resistance against antibiotics. To overcome this issue, NPs have been chosen to target bacteria as a replacement of antibiotics, because NPs directly contact with bacterial cell wall without cell penetration. NPs demonstrate a wide spectrum of antibacterial properties. For example, antimicrobial activity of AgNPs against *E. coli* and *P. aeruginosa* and inhibition of *P. aeruginosa* by ZnONPs (Ramalingam et al. 2016). To show the antibacterial activity, it is important for the NPs to be in contact with target body. Van der Waals forces (Armentano et al. 2014), electrostatic attraction (Li et al. 2015), and hydrophobic interactions (Luan et al. 2016) are the ways for NPs to come in contact. According to research, major processes are oxidative stress (Gurunathan et al. 2012), nonoxidative mechanisms (Leung et al. 2014), and metal ion release (Zakharova et al. 2015) for antibacterial activity by NPs (Fig. 2.2).

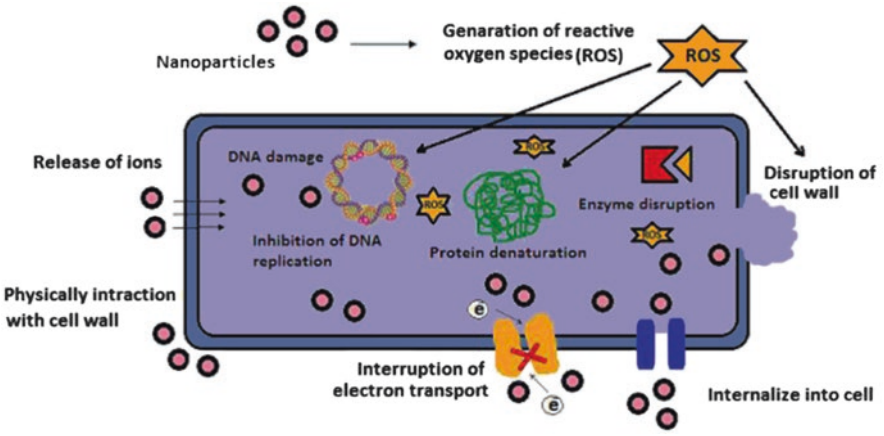

Fig. 2.2 Diagrammatic representation of proposed antimicrobial mechanism for metal NPs (Dizaj et al. 2014) (© 2014 Elsevier B.V.)

2.5 Anticancer Activity of Nanoparticles

NPs' advantage over other methods for the treatment of cancer cells is their selective cytotoxicity (Bhattacharyya et al. 2011). Various metal NPs have already been explored for anticancer therapy, such as cerium oxide, iron oxide, copper oxide, titanium dioxide, zinc oxide, gold, and silica. (Vinardell and Mitjans 2015). For example, magneto selective NPs made from iron oxide has been used to treat cancer cells selectively in the presence of magnetic field (Orel et al. 2015). Increasing oxidative stress by generating ROS by radiation using TiO_2 (Zhang and Sun 2004), CeO_2 (Wason et al. 2013), and ZnONPs (Shen et al. 2013) has been reported for the treatment of cancer cells. Even gold NPs have been used for the treatment of cancer cell by showing cytotoxic mechanism (Fig. 2.3) like production of ROS (Parida et al. 2014), DNA damage (Patil et al. 2017), mitochondrial damage and cascade caspase approach of apoptosis (Jeyaraj et al. 2014). Whereas, it was found that the soluble metal ions released inside cell are also responsible for the cytotoxic effects of NPs (Shen et al. 2013).

2.6 General Mechanism for Activity by Nanoparticles

NPs cross the cell membrane and contact with the basic components of the cell and lead to activity by the following mechanism:

2.6.1 Oxidative Stress

Oxidative stress by generating ROS species is an important mechanism for antibacterial activity. The small molecules and intermediates having strong positive redox

potential are generally known as ROS. A variety of different NPs generate ROS molecules by reducing oxygen. Superoxide radical (O_2^-), singlet oxygen, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) are the four different types of ROS generated (Malka et al. 2013). In the cell, the production and clearance of ROS is balanced under normal circumstances. But, when present in an unbalanced condition, the cell prefers oxidation by excessive production of ROS (oxidative stress) causing damage to the cell components. Due to this, the cell membrane permeability also changes, which causes the damage of cell membrane (Cheloni et al. 2016). For oxidative proteins, ROS increases the gene expression level, which leads to cell apoptosis (Wu et al. 2011). The reduction in the activity of certain periplasmic enzymes was also observed by the attack of ROS on proteins which maintain the normal morphology and are essential for normal physiological processes in cells (Padmavathy and Vijayaraghavan 2011).

2.6.2 Nonoxidative Mechanism

In nonoxidative stress, the addition of NPs does not damage the cell wall and even don't increase the amount of ROS species in the cell. Instead, the NPs reduce the critical cellular metabolic processes, which include metabolism of nucleotide, carbohydrate, amino acids and energy, which ultimately leads to damage cell (Leung et al. 2014).

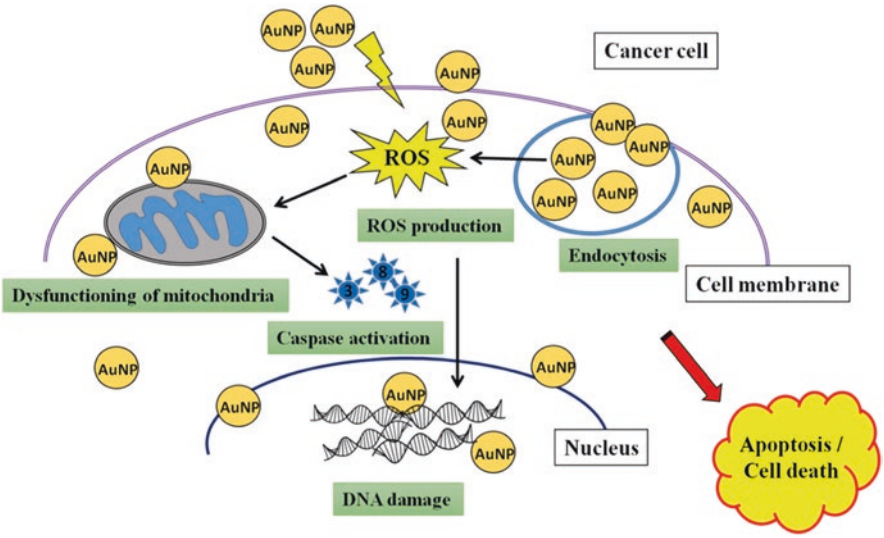

Fig. 2.3 Diagrammatic representation of proposed anticancer mechanism for AuNPs. (Patil and Kim 2017) (© 2016, Springer-Verlag Berlin Heidelberg)

2.6.3 Dissolved Metal Ions

Functional groups like –SH, –NH, and –COOH are present in proteins which directly interact with metal ions released from metal oxide NPs. The result of this interaction is inhibition of enzyme activity and physiological processes causing cell death (Yu et al. 2014).

2.7 Application of Metal NPs

2.7.1 Antimicrobial Application of NPs

Moodley et al. (2018) used extract of fresh and freeze-dried *Moringa oleifera* leaf for AgNPs synthesis in the presence of sunlight. The average size of AgNPs obtained was of 9 and 11 nm, respectively. The synthesized AgNPs showed both antibacterial and antifungal activity. Antibacterial studies shown that gram-negative and gram-positive bacteria were inhibited by as-prepared NPs. AgNPs of concentration 25 μg ml^{-1} was found to be effective in inhibiting growth in strains of *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*. Whereas, 12 μg ml^{-1} of AgNPs effectively inhibited the growth of *E. coli* and *E. faecalis.* In antifungal studies, it was found that 6.25 μg ml^{-1} of AgNPs was enough to inhibit the growth of *C. albicans*, *C. krusei*, and *C. parapsilosis*. Results showed that AgNPs synthesized using *Moringa oleifera* leaf extract have great potential as antimicrobial agent. Otari et al. (2017) used extract from the leaves of *Canna edulis* for synthesizing AgNPs having size <40 nm. When the synthesized AgNPs were used against microorganisms, they exhibited strong antimicrobial activity against both gram-negative and gram-positive bacteria along with some fungal species. As compared to gram positive bacteria, stronger resistance was shown by gram negative bacteria to the AgNPs. This showed the antimicrobial potential of AgNPs synthesized from leaves extract of *Canna edulis*.

Hariharan et al. (2016) reported the synthesis of AuNPs by the use of extract from the leaves of *Azima tetracantha* with an average size of 80 nm. The range of various bacterial pathogens used were *Aeromonas liquefaciens* (B1), *E. fecalis* (B2), *Micrococcus luteus* (B3), *Salmonella typhimurium* (B4), and the fungal pathogens used were *C. albicans* (F1), *Cryptococcus* sp. (F2), *Microsporum canis* (F3), and *Trichophyton rubrum* (F4). They were used in two different concentrations, 15 μL/disc and 30 μL/disc, for the antimicrobial activities. In the case of bacterial pathogens, AuNPs were found to be more effective and least effective against *S. typhimurium* (B4) and *M. luteus* (B3), respectively. Whereas, in the case of fungal pathogens AuNPs were most effective and least effective against *T. rubrum* (F4) and *Cryptococcus* sp. (F2), respectively. Compared to 15 μL/disc, a large zone effect was observed for 30 μL/disc against the microorganisms. In comparison, the 15 μL/disc displayed lesser activity than the 30 μL/disc where; a larger zone effect against microorganisms was observed.

The biological studies showed AuNPs as alternate source for antimicrobial activities. Sarker et al. (2019) reported the synthesis of elongated tetrahexahedral

(ETHH) AuNPs and lipoic acid functionalized ETHH AuNPs. They studied antimicrobial activity by using *B. subtilis* (Gram-positive) and *E. coli* (Gram-negative) bacteria for these AuNPs and found ETHH-LA AuNPs to be most effective in inhibiting *B. subtilis* and *E. coli*, but even more effective against *B. subtilis* as compared to *E. coli*. The antibacterial activity of ETHH-LA AuNPs could be attributed to its better interactions with the cell membrane owing to their electrostatic and hydrophobic interactions along with molecular crowding. In addition, increased oxidative stress due to formation of ROS species also leads to gold aggregation in the cell, which is the reason behind cell death. Onitsuka et al. (2019) reported the synthesis of Au (~10 nm) and Ag (~30 nm) NPs using black and green tea extracts obtained from the leaves of *Camellia sinensis*. Then, the cotton cloths were taken and NPs were immobilized over it and then dyed cloth was used for antimicrobical activity. They investigated the activity against *S. aureus* and *K. pneumonia* bacteria. The high antimicrobial activity of all NPs against *S. aureus*, but against *K. pneumonia* except AuNPs synthesized using black tea extract all other samples showed high antimicrobial activity. Along with the small size of NPs of dyed cotton cloth, the functional organic molecules from tea extracts at the surface of NPs synergistically gave high biological activity against microorganisms.

Kim and Song (2018) reported Buckwheat starch (BS) films containing ZnONPs with different N content (0, 1.5, 3 and 4.5%) which were used against *L. monocytogenes* to study the antimicrobial activity of BS/ZnO-N films. Results showed that after 8 h, the population (initially 7.20 log CFU mL^{-1}) of *L. monocytogenes* was reduced by 2.96–3.74 log CFU mL^{-1} by BS/ZnO-N(3%) showing the antimicrobial activity of the BS/ZnO-N film. The film was also applied during the packaging of freshly cut mushrooms for antimicrobial activity against *L. monocytogenes*, and after storage for 6 days showed a reduction of 0.86 log CFU g^{-1}, which demonstrated the potential of the material to be used as a biodegradable material that can be used for packing. Kaushik et al. (2019) reported antimicrobial activity of ZnONPs synthesized by wet chemical technique for wound healing which is delayed due to microbial infections. They studied the antimicrobial activity of ZnONPs against *E. coli*, *S. enterica*, *S. typhimurium*, *S. aureus*, *A. fumigatus*, *A. flavus*, and *C. albicans*. The biological studies revealed that the particle size increased on increasing annealing temperature during the synthesis of ZnONPs, and the effect was seen on the inhibition zone, which reduced with increased particle size. So, the antimicrobial activity decreased with the increase in size of ZnONPs, resulting in 60–65% cell death in the case of *S. aureus* and complete apoptosis with *S. enterica typhimurium*. The explanation behind this antimicrobial activity of ZnO was explained as follows: first the release of Zn ions in the cell results in cell death, then the generation of ROS species due to the interaction of ZnONPs with the cell which causes oxidative stress also causes cell death.

Marković et al. (2018) reported cotton fabric fabricated with CuNPs as antibacterial nanocomposite which was modified using various polycarboxylic acids. The biological studies revealed that the presence of excessive free carboxylic group on modified fabric surface resulted in large uptake of Cu ions due to which large amounts of CuNPs were obtained. Physicochemical studies show the presence of

both Cu_2O and CuO NPs at modified fabric nanocomposite. When this modified fabric composite was applied for antibacterial activity against *E. coli* and *S. aureus* bacteria, it demonstrated inhibition of bacterial cell up to 99.9%. The antimicrobial activity was due to the cell damage caused by the released Cu ions as well as cell death by oxidative stress caused due to the ROS species generated by CuNPs. Nabila and Kannabiran (2018) reported the biosynthesis of CuO NPs mediated by Actinomycetes having average size of 61.7 nm. When these biosynthesized CuO NPs were used for antibacterial activity against *P. mirabilis*, *S. aureus*, *Edwardsiella tarda*, *B. cereus*, *Aeromonas caviae*, *Vibrio anguillarum*, and *A. hydrophila* studies revealed higher activity, as larger zones of inhibition for bacterial pathogens were obtained. Antibacterial activity exhibited by biosynthesized CuONPs was much higher as compared to actinomycetes supernatant. Inhibition zone obtained for *B. cereus* was maximum (25.3 mm) among all the bacterial cells that were tested by biosynthesized CuO NPs. The mechanism behind antibacterial activity was proposed to be the interaction of CuO NPs surface with the cell membrane of the bacteria. This affects to the cellular mechanism of bacteria which inhibits the growth of bacterial cell and ultimately results in cell death.

Kumaresan et al. (2018) reported ZrO_2 NPs synthesized using marine brown alga (*Sargassum wightii*) with an average size of 4.8 nm. When ZrO_2 NPs were tested for antibacterial activity against *B. subtilis*, *E. coli*, and *S. typhi*, they showed significant inhibition of bacterial growth against all bacterial pathogens. The spherical shape and smaller size of ZrO_2 NPs possess large surface area which is the reason behind increased antibacterial activity. As, larger the surface area more is the activity. Saravanan et al. (2018) used spherical AgNPs with an average size range of 41–68 nm which were biologically synthesized using extract of *B. brevis*. The antimicrobial activity of AgNPs was investigated against *S. aureus* and *S. typhi*, and the results show that antimicrobial activity was maximum against *S. aureus* and moderate against *S. typhi*. The difference in the antimicrobial activity was related to the different cell wall structure and composition. The mechanism behind this antimicrobial activity of AgNPs against bacterial cell was the disturbance in cell functioning caused due to the penetration of AgNPs through bacterial cell membrane (Table 2.1).

2.7.2 Anticancer Application of NPs

Nakkala et al. (2018) used aqueous rhizome extract of *Acorus calamus* for the synthesis of AgNPs, with an average size of 31.83 nm, which were used for the evaluation of anticancer activity by using Hep2 (human epidermoid carcinoma), SH-SY5Y (neuroblastoma), and COLO 205 (human colon adenocarcinoma) cancer cells. The biological studies revealed the apoptotic effect caused due to AgNPs. Hep2 was found to be most susceptible to AgNPs whose effects were observed through AO/EB, PI/DAPI staining, Rhodamine 123, DCFH-DA, Western blotting, and oxidative stress markers. The feature of cell death observed in cancer cell was shrinkage of cell and rounding up of nuclei. It was due to increased level of ROS species and followed by loss of MMP. Al-Sheddi et al. (2018) synthesized AgNPs

Table 2.1 Various ways to synthesize eco-friendly metal NPs and application in antimicrobial activity

Route of synthesis of NPs	Metal	Average size (nm)	Application	References
Green synthesis (leaf extract of *M. oleifera*)	AgNPs	9 and 11	Antimicrobial activity against *E. coli*, *E. faecalis*, *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *C. albicans*, *C. krusei* and *C. parapsilosis*	Moodley et al. (2018)
Green synthesis (leaf extract of *Canna edulis*)	AgNPs	<40 nm	Antimicrobial activity against *B. cereus*, *S. aureus*, *E. coli*, *S. typhimurium*, *E. faecalis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. guilliemondii* and *P. chrysogenum*	Otari et al. (2017)
Green synthesis (leaf extract of *Azima tetracantha*)	AuNPs	80 nm	Antimicrobial activity against *A. liquefaciens* (B1), *E. fecalis* (B2), *M. luteus* (B3), *S. typhimurium* (B4), *C. albicans* (F1), *Cryptococcus* sp. (F2), *M. canis* (F3), and *T. rubrum* (F4)	Hariharan et al. (2016)
Chemical method (using CTAB and $NaBH_4$)	AuNPs (elongated tetrahexahedral nanorods)	Length 117 ± 9 nm and width 58.3 ± 5.5 nm	Antimicrobial activity against *B. subtilis* and *E. coli*.	Ranjan Sarker et al. (2019)
Green synthesis (leaves extract of *C. sinensis*)	AuNPs AgNPs	10 nm 30 nm	Antimicrobial activity against *S. aureus* and *K. pneumonia*.	Onitsuka et al. (2019)
Chemical synthesis	ZnONPs	<50 nm	Antimicrobical activity against *L monocytogenes*.	Kim and Song (2018)
Chemical synthesis (wet chemical synthesis)	ZnONPs	82–420 nm	Antimicrobial activity against *E. coli*, *S. enteric*, *S. typhi*, *S. aureus*, *A. fumigatus*, *A. flavus*, and *C. albicans*.	Kaushik et al. (2019)
Chemical synthesis	CuNPs (Cu_2O and CuO)	–	Antibacterial activity against *E. coli* and *S. aureus*	Marković et al. (2018)

(continued)

Table 2.1 (continued)

Route of synthesis of NPs	Metal	Average size (nm)	Application	References
Green synthesis (biosynthesis by Actinomycetes)	CuO NPs	61.7 nm	Antibacterial activity against *P. mirabilis*, *S. aureus*, *E. tarda*, *B. cereus*, *A. caviae*, *V. anguillarum* and *A. hydrophila*	Nabila and Kannabiran (2018)
Green synthesis (using marine brown alga *S. wightii*)	ZrO_2 NPs	4.8 nm	Antibacterial activity against *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhi*.	Kumaresan et al. (2018)
Green synthesis (extract of Bacillus brevis)	AgNPs	41–68 nm	Antimicrobial activity against *S. aureus* and *S. typhi*	Saravanan et al. (2018)

having an average size of 33 nm using *Nepeta deflersiana* plant extract. On evaluating the anticancer activity of as-synthesized AgNPs on Human Cervical Cancer Cells (HeLA), the observations made were increase in ROS and lipid peroxidation (LPO) followed by decreased levels of MMP and glutathione (GSH), which resulted in the death of cancer cell. This result indicates the anticancer potential of synthesized AgNPs. Anoop et al. (2018) synthesized silver nanorods by using the leaf extract of *M. indica*. These nanorods were used for anticancer activity on MCF 7 (breast cancer) and HCT 116 (colorectal carcinoma cells). The biological studies revealed that anticancer activity depends upon the concentration of nanorods i.e. cytotoxicity increased with increased nanorods concentration and up to 50% reduction in cancer cell growth was achieved when used 10% w/v solutions of silver nanorods. Anticancer activity of Ag nanorods was explained on the basis of generated ROS species in cell mitochondria, which leads to cancer cell death by the apoptotic route.

Barai et al. (2018) used the extract of *Nerium oleander* stem bark for synthesizing AuNPs of average size 20–40 nm. When these AuNPs were utilized for anticancer activity against breast cancer MCF7 cells found significant inhibition of cancer cell at 74 μg/mL by generation of ROS species in the cell showing efficiency of AuNPs in selective apoptosis of cancer cells. Khandanlou et al. (2018) synthesized AuNPs having spherical shape with an average size of 8.4 nm using leaf extract of *Backhousia citriodora*. On evaluation of anticancer activity of synthesized AuNPs, cancer cells of MCF-7 and HepG2 were inhibited in a dose-dependent manner with 116.65 and 108.21 μg as IC_{50} values, respectively. The promising anticancer activity could be attributed to the synergetic effect of AuNPs and phenolic moieties. Nosrati et al. (2018) synthesized L-tyrosine modified Fe_3O_4 NPs loaded with tamoxifen (TMX) with an average size of 22.19 nm. The anticancer activity of modified magnetic NPs was studied on MCF-7 breast cancer cell. The results revealed that the activity directly depends upon the concentration of TMX and modified magnetic

NPs found to have significant anticancer activity against MCF-7 breast cancer cell. Nagajyothi et al. (2018) used aq. Fruit extract of *Forsythiae fructus* for synthesizing Y_2O_3 NPs (~11 nm). On evaluating the anticancer activity on renal tumor cells, it was found that rate for cancer cell death increased on increasing the concentration of NPs. The cancer cell cytotoxicity achieved was 40% at an NPs concentration of about 1 mg/mL. Along with this, NPs were found to be nontoxic for normal cells and highly toxic for cancer cells, which proved potential of the prepared NPs as anticancer agents (Table 2.2).

Table 2.2 Various ways to synthesize eco-friendly metal NPs and application in anticancer activity

Synthetic route	Metal	Average size (nm)	Application	References
Green synthesis (aqueous rhizome extract of *Acorus calamus*)	AgNPs	31.83 nm	Anticancer activity against Hep2 (human epidermoid carcinoma), SH-SY5Y (neuroblastoma) and COLO 205 (human colon adenocarcinoma) cancer cells.	Nakkala et al. (2018)
Green synthesis (*Nepeta deflersiana* plant extract)	AgNPs	33 nm	Anticancer activity against Human Cervical Cancer Cells (HeLA)	Al-Sheddi et al. (2018)
Green synthesis (leaf extract of *Mangifera indica*)	AgNPs (nanorods)	500–900 nm (cross-sectional dimension)	Anticancer activity against MCF 7 (breast cancer) and HCT 116 (colorectal carcinoma cells)	Anoop et al. (2018)
Green synthesis (extracts of stem bark of *Nerium oleander*)	AuNPs	20–40 nm	Anticancer activity against MCF-7 cell	Barai et al. (2018)
Green synthesis (leaf extract of *Backhousia citriodora*)	AuNPs	8.4 nm	Anticancer activity against MCF-7 and HepG2 cancer cell	Khandanlou et al. (2018)
Chemical synthesis	Modified Fe_3O_4 NPs	22.19 nm	Anticancer activity against breast cancer cell	Nosrati et al. (2018)
Green synthesis (aqueous fruit extract of *Forsythiae fructus*)	Y_2O_3 NPs	11 nm	Anticancer activity against renal carcinoma cells	Nagajyothi et al. (2018)

2.8 Conclusion and Perspectives

Eco-friendly synthesis of smart NPs by using methodology in which size, shape, and morphology can be controlled, NPs will have greater selectivity and biological activity toward targeted cells without any harm to normal cells. Selectivity is the key feature of NPs used in biomedical applications. It is also possible that NPs could replace the drugs. The present chapter focused on the synthesis of metal NPs by eco-friendly route and its application in antimicrobial and anticancer activity. The probable mechanism behind antimicrobial and anticancer activity was proposed to be the generation of ROS species which causes damage to cell components and membrane, and another being the release of metal ions in the cell which interacts with proteins causing inhibition of enzyme activity and physiological processes which ultimately leads to cell apoptosis.

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Application of Nanoparticles in Drug Delivery

3

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Abstract

Microorganisms develop resistance to antimicrobial compounds, which is an important global health threat. As per the World Health Organization (WHO) report, antimicrobial resistance (AMR) is one of the leading causes of mortality worldwide. To overcome antibiotic resistance, nanoscale antimicrobial agents can be used as an alternative strategy. Different types of nanoparticles (NP) such as solid lipid (SL) NPs, liposomal NPs, polymer-based NPs, inorganic NPs, magnetic NPs, mesoporous silica NPs, and carbon nanomaterials are used for drug delivery. Metal nanoparticles (NPs) such as copper (Cu), titanium (Ti), silver (Ag), gold (Au), and zinc (Zn) have antimicrobial activity. The antimicrobial properties of NPs depend on size, chemical composition, and shape of these NPs. The present chapter reviews the application of various nanoparticles as antimicrobial agents and their potential application against multidrug-resistant microbial pathogens in public health. Advancement in nanomedicine is an important aspect for diagnosis and treatment of diseases induced by drug-resistant microorganisms.

Keywords

Nanoparticles · Antimicrobial action · Antimicrobial resistance

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3.1 Introduction

Disinfectants, antiseptics, or antibiotics are used as antimicrobials which inhibit cell wall synthesis, DNA replication, and translation of bacteria. Synthesis of peptidoglycan layer of both gram-positive and gram-negative bacteria is inhibited by antibiotics, such as penicillin, cephalosporins, fosfomycin, bacitracin, cycloserine, vancomycin, and teicoplanin (Awad et al. 2013). Antibiotics such as chloramphenicol, puromycin, tetracyclines, aminoglycosides, fusidic acid, lincosamides, macrolides, streptogramins, mupirocin, and oxazolidinones inhibit protein synthesis in bacterial cell, whereas quinolones, novobiocin, rifampicin, diaminopyrimidines, sulfonamides, and 5-nitroimidazoles inhibit DNA replication of bacterial cell (Drlica et al. 2008). Microorganisms develop resistance to antimicrobial compounds, which is an important global health threat. The antimicrobial resistance develops due to inactivation of the drug, decreased permeability, and reduction of antimicrobial effect of drugs (Schmieder and Edwards 2012). As per the WHO report, AMR is one of the leading causes of mortality worldwide (https://www.who.int/news-room/fact-sheets/detail/antibioticresistance). To overcome antibiotic resistance, nanoparticles can be used as alternative antimicrobial agents (Laxminarayan et al. 2013). Different types of nanoparticles (NP) such as solid lipid (SL) NPs, polymer-based NPs, liposomal NPs, inorganic NPs, magnetic NPs, mesoporous silica NPs, and carbon nanomaterials are used for drug delivery (Wang et al. 2017). The NP carriers enhance the level of antibiotics in blood serum and protect drugs from resistance microbes. Metal nanoparticles (NPs) such as copper (Cu), titanium (Ti), silver (Ag), gold (Au), and zinc (Zn) have antimicrobial activity (Malarkodi et al. 2014). Use of NPs can be considered as a valuable health approach against the emergence of antibiotic-resistant microbes.

3.2 Nanoparticles

Due to the high surface-to-volume ratio in nanoparticles, nanoparticles have property to interact with microorganism. Nanoparticles are considered as antimicrobial agents. Nanoparticles are 0.2–100 nm in size. Low-resolution and high-resolution transmission electron microscopies (TEM) are used to characterize nanoparticles. NPs are both organic and inorganic (Hajipour et al. 2012). Different types of NPs such as silver (Ag), gold (Au), Ag oxide (Ag_2O), zinc oxide (ZnO), titanium dioxide (TiO_2), calcium oxide (CaO), copper oxide (CuO), magnesium oxide (MgO), and silicon dioxide (SiO_2) are used (Maleki Dizaj et al. 2015). The inorganic nanoparticles produce ROS such as hydroxyl radicals, superoxide anions, and hydrogen peroxide that drive lipid peroxidation of bacterial cell membranes, inhibit oxidative phosphorylation, and DNA replication (Fig. 3.1). Cu^{2+} ions interact with amine and carboxyl groups on the surfaces of *Bacillus subtilis*. Ag and ZnONPs disrupt lipids and proteins of membrane of bacterial cell. Ag^{+} of AgNPs interacts with negatively charged lipopolysaccharide in the bacterial membrane and inhibits cytochromes of the electron transport chain. Ag and Au NPs prevent protein translation by

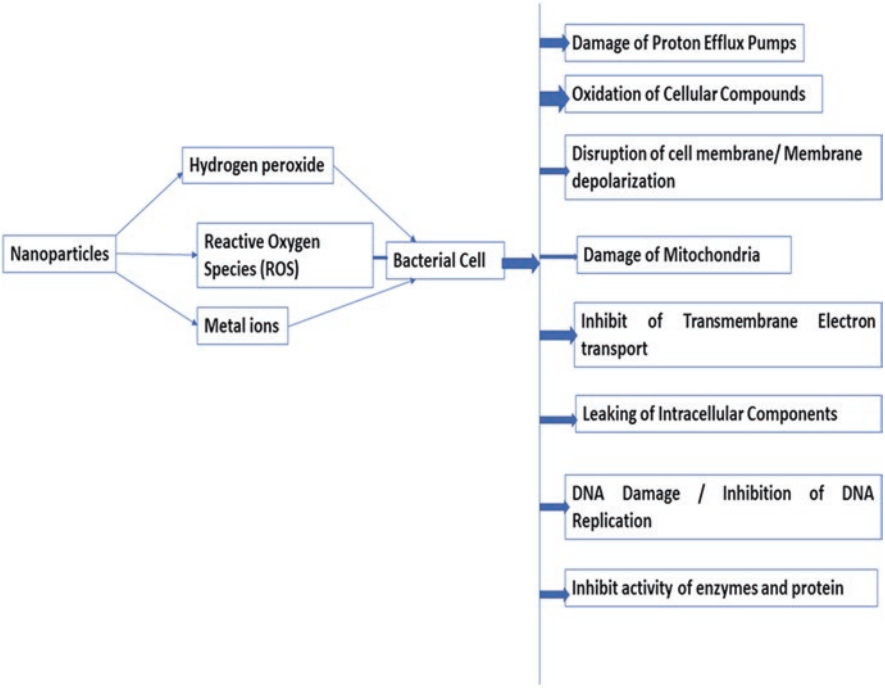

Fig. 3.1 Schematic representation of mode of action of nanoparticles on bacterial cell

denaturing 30S ribosomal subunit. Magnetic NPs such as superparamagnetic iron oxide NPs coated with Ag or Au showed inhibitory activity against bacterial biofilms (Hemeg 2017) (Table 3.1).

3.3 Antimicrobial Mechanism of NPs

Lipopolysaccharides (LPS) is an important structural component of Gram-negative bacteria. LPS has negatively charged groups which attract NPs. Peptidoglycan and teichoic acid are important characteristic features in the cell wall of Gram-positive bacteria. Gram-positive bacteria have more negative charge on the cell wall surface as compared to Gram-negative bacteria. NPs have greater affinity towards Gram-positive bacteria than against Gram-negative bacteria. NPs produce different types of ROS such as superoxide radical, hydroxyl radical, hydrogen peroxide, and singlet oxygen which penetrate the cell membrane by diffusion to kill bacteria (Li et al. 2012a, b). The cell wall thickness of Gram-negative bacteria alters the antimicrobial effects of NPs. The metal ion NPs bind with negatively charged functional groups such as carboxyl and phosphate group of bacterial cell membrane. Zinc ions have high affinity with –SH groups of proteins (Padmavathy and Vijayaraghavan 2011). Superparamagnetic iron oxide induces bacterial cell death through denaturation of

Table 3.1 Role of inorganic NPs as antimicrobial agents

NPs	Mode of action	Target microorganisms
AgNPs	• Cell wall lysis and alterations of cell permeability. • Disruption of metabolism. • Inhibition of DNA replication. • Interaction with sulfur- and phosphorus- containing compounds of bacterial cell.	*E. coli*, *B. subtilis*, *S. typhi*, *V. cholera*, *S. aureus*, Methicillin-resistant coagulase-negative *Staphylococci*, Vancomycin-resistant *Enterococcus faecium*, *K. pneumonia*, HIV-1, Influenza virus, Herpes Simplex virus
AuNPs	• Alteration of membrane potential. • Reduce the level of ATP. • Prevent binding of tRNA to the ribosome	*E. coli*, *P. aeruginosa*, Methicillin-resistant *S. aureus*, Vancomycin-resistant *E. faecium*, HIV virus, Influenza virus
TiO_2 NPs	• Production of ROS. • Peroxidation of membrane lipid particularly polyunsaturated phospholipid	*E. coli* 0157:H7, *S. aureus*, *S. enteritidis*, *L. monocytogenes*, *P. fluorescens*, HSV-1 (Herpes simplex virus), Influenza virus
ZnONPs	• Production ROS. • Morphological alteration of membrane. • Ionic interaction drive internalization of NPs into cell	*E. coli* 0157:H7, *B. subtilis*, *P. fluorescens*, *L. monocytogenes*, *S. enteritidis*, *S. aureus*, *S. typhimurium*, Herpes simplex virus type 1 & 2
CuO NPs	• Internalization of NPs prevent the functional activity of essential enzymes in bacteria	*B. subtilis*, *E. coli*, *S. aureus*, *L. monocytogenes*
SiO_2 NPs	• Alteration of cell differentiation and adhesion	*E. coli*, *S. mutans*, *B. subtilis*
MgO/CaO NPs	• Induction of death of bacterial cells. • Releasing the intracellular contents of bacterial cell by damaging the membrane	*E. coli*, *B. subtilis*, *S. aureus*, *S. mutans*, *S. epidermidis*, *B. megaterium*
Al_2O_3 NPs	• Increase permeability of bacterial cell wall through electrostatic interaction.	*E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *K. aerogenes*, *P. desmolyticum*
Iron-containing nanoparticles	• Production of ROS such as superoxide radicals, singlet oxygen, hydroxyl radicals, and hydrogen peroxide	*S. aureus*, *S. epidermidis*, and *E. coli*.

DNA, lipids, and proteins of bacterial cell by Fenton reaction (Leuba et al. 2013). NPs such as copper oxide NPs regulate the expression of proteins that are involved in nitrogen metabolism and inhibit functional activity of enzymes such as nitrate reductase and nitrite reductase (Su et al. 2015). AgNPs prevent the biofilms

formation by antibiotic resistant strains of *E. coli* and *Klebsiella pneumoniae* through inhibition of EPS production (Ansari et al. 2012).

3.4 Application of Silver Nanoparticles as an Antibacterial and Antifungal Agents

AgNPs are synthesized by physical methods such as the "top-down" method by grinding the bulk metal, chemical methods such as the "bottom-up" method, and biological methods. Chemical methods such as reduction, electrochemical processes, and decomposition by ultrasonic waves are utilized for the synthesis of AgNPs (Prabhu and Poulose 2012; Swamy et al. 2015). The physical and chemical processes for the synthesis of AgNPs are very expensive and produce toxic products. In biological methods, extracts of bacteria such as *Pseudomonas stutzeri*, *Bacillus megaterium*, *Escherichia coli*, fungi such as *Aspergillus fumigatus* and *Fusarium solani*, and plants such as *Aloe vera* and *Piper betle* leaf are used to synthesize NPs, and enzymes from microorganisms are used in oxidation or reduction reactions. AgNPs synthesized by biological methods have a size of 1 and 600 nm (El-Shanshoury et al. 2011; Bhainsa and D'Souza 2006). AgNPs showed antimicrobial activity by inhibiting replication of DNA and electron transport chain in bacteria and fungi. Ag binds with the sulfhydryl (thiol) groups in the cell wall enzymes of bacteria. The antimicrobial activity of AgNPs depends on the production of numbers of active Ag^+ ions and their interaction with the bacterial cell wall (Franci et al. 2015). AgNPs exhibit antimicrobial activity through the production of reactive oxygen species (ROS) such as hydrogen peroxide (Prabhu and Poulose 2012). Due to large surface-to-volume ratio, AgNPs easily penetrate into bacterial cells for complete destruction as compared to Ag^+ (Ramalingam et al. 2016). The Ag^+ ions which are released from NPs react with sulfur-containing proteins of bacterial cell surface and phosphate groups of nucleic acids that drive cell death through the production of ROS (Reidy et al. 2013). AgNPs with a diameter of ≤10 nm induce the death of microorganism through formation of pore in the cell wall (Keat et al. 2015). The minimum inhibitory concentration (MIC) of NPs having 25 nm in size is 6.75–54 μg/mL. The MIC of 25-nm AgNPs is 1.69–13.5 μg/mL against methicillin-resistant *S. epidermidis* and *S. aureus*, and vancomycin-resistant *K. pneumonia* and *E. faecium* (Franci et al. 2015). AgNPs with higher concentration of 100 μg/mL inhibit the growth of a Gram-positive bacterium, *S. aureus* (Yamanaka et al. 2005).

Ruparelia et al. (2008) reported that minimum bactericidal concentration (MBC) of AgNPs varies between 40 to 180 μg/mL for different strains of *E. coli* (MTCC 1687, MTCC 1302, MTCC 739, and MTCC 443). Lara et al. (2010) reported that AgNPs showed antimicrobial activity against ampicillin-resistant *E. coli* O157:H7, erythromycin-resistant *S. pyogenes*, and multidrug-resistant *P. aeruginosa* with concentration of 83.3 mM. AgNPs also inhibited growth of bacteria, such as *E. coli*, *P. aeruginosa*, *V. cholera*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Enterobacter aerogenes*, *Bacillus subtilis*, *Streptococcus viridians*, *Streptococcus pneumonia*, *Streptococcus mutans*, *Listeria monocytogenes*,

Acinetobacter baumannii, *Micrococcus luteus*, *Proteus mirabilis*, *Brucella abortus*, *Moraxella catarrhalis*, *Proteus mirabilis*, *Serratia proteamaculans*, and *Shigella Flexner* (Morones et al. 2005; Swamy et al. 2015; Pérez-Díaz et al. 2015). AgNPs showed inhibitory activity against various fungal pathogens such as *C. albicans*, *C. tropicalis*, *T. rubrum*, *Penicillium brevicompactum*, *Cladosporium cladospor cladosporioides*, *A. fumigatus*, *Chaetomium globosum*, *Mortierella alpina*, and *Stachybotrys chartarum* (Pereira et al. 2014; Mallmann et al. 2015; Ogar et al. 2015; Panáček et al. 2009).

AgNPs showed antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa* at low concentration when combined with antibiotics. AgNPs enhanced antimicrobial effects of antibiotics such as penicillin G, amoxicillin, erythromycin, vancomycin, and clindamycin against *S. aureus*, *E. coli*, and MDR bacteria (Naqvi et al. 2013). The antibacterial activity of AgNPs is enhanced in combination with compounds such as polyethyleneimines, chitosan, and glucosamine (Azevedo et al. 2014). Nanocomposition of Chitosan/TiO2/Ag showed antibacterial activity through production of ROS and lactate dehydrogenase which inhibits bacterial adhesion (Natarajan et al. 2016).

Nanocomposition of Chitosan/Ag inhibited the growth Salmonella sp. Nanocomposition of Chitosan/calcium silicate doped with Ag^{+} inhibited the growth of *S. aureus* and *P. aeruginosa*. AgNPs capped with lipoic acid inhibited the growth of biofilm formation by *S. epidermidis* and *S. mutans* (El-Nahrawy et al. 2016). Birla et al. (2009) reported about antibacterial effect of AgNPs against foodborne bacteria such as *E. coli*, *P. aeruginosa*, and *S. aureus*. Zarei et al. (2014) reported the antibacterial effect of AgNPs against foodborne pathogenic microbes such as *S. typhimurium*, *L. monocytogenes*, *V. parahaemolyticus*, and *E. coli*.

Bera et al. (2014) reported that smaller AgNPs penetrated the cell wall of bacteria and enhanced antimicrobial activity against gram positive bacteria such as *S. epidermidis* and *B. megaterium* and gram negative bacteria such as *P. aeruginosa*. Rajeshkumar and Malarkodi (2014) reported antibacterial effect of AgNPs against *E. coli*, *B. subtilis*, *K. planticola*, *K. pneumoniae*, and *S. nematodiphila*.

3.5 Application of Magnesium Oxide (MgO) and Calcium Oxide NPs (CaO) NPs as Antimicrobial Agents

MgO NPs showed antibacterial activity through the production of ROS, lipid peroxidation of bacterial cell wall, electrostatic interactions with bacterial cell surface, and alkaline effects that drive death of bacterial cell (Jin and He 2011; Huang et al. 2005). Activity of gram-negative bacteria such as *E. coli*, *S. aureus*, and *P. aeruginosa* is inhibited by MgO NPs with minimum inhibitory concentration of 1000 μg/mL, 500 μg/mL, and 1000 μg/mL, respectively (Krishnamoorthy et al. 2012). Sawai et al. (2000) reported that superoxide on the surface of MgO NPs showed inhibitory activity against *E. coli* and *S. aureus*. Jin and He (2011) reported that MgO NPs in combination with niacin could be used as an antibacterial agent in food safety.

Yamamoto et al. (2010) reported the antibacterial activity of CaO NPs against *E. coli*, *S. typhimurium*, *S. aureus*, and *B. subtilis* O.

3.6 Application of Silica NPs (SiO_2) as Antimicrobial Agents

Cousins et al. (2007) reported that Si NPs inhibited oral biofilm formation. Nanocomposition of Cu/SiO_2 showed bactericidal activity against *S. aureus*, *E. coli*, *E. cloacae*, *C. albicans*, and *P. citrinum* (Kim et al. 2006). Nanocomposition of Ag/SiO_2 showed bactericidal and antifungal activity against *S. aureus*, *P. aeruginosa*, *E. coli*, *E. cloacae*, *C. albicans*, *A. niger*, and *P. citrinum* (Kim et al. 2007). Si NPs inhibit cell differentiation, adhesion, and spreading of bacteria. Ag-conjugated Si nanowires were reported to be biocompatible, whereas Cu-conjugated Si nanowires showed high cytotoxicity (Mohammadi et al. 2011).

3.7 Application of Titanium Dioxide (TiO_2) NPs as Antimicrobial Agents

Nontoxic and chemically stable TiO_2NPs showed antimicrobial activity against *E. coli*, *S. aureus*, and fungi through the generation of ROS, which interact with the phospholipids of bacterial cell surface (Rudramurthy et al. 2016). Bacterial endospores, fungal spores, and protozoan cysts have thick cell wall as compared to vegetative forms. Photocatalytic properties of TiO_2 produce highly reactive hydroxyl radicals and superoxide ions which induce cell death of microorganisms through oxidation of organic compounds/cells adsorbed on the TiO_2 surface. The photocatalytic efficiency and antibacterial activities of TiO_2 are enhanced by the modification of the surface of TiO_2 with metal or semiconductor, increase of surface area of TiO_2, and pore-size distributions (Khezerlou et al. 2018). Roy et al. (2010) studied the effect of TiO_2 NPs against methicillin-resistant *S. aureus* (MRSA). Gumiero et al. (2013) studied the effect of photocatalytic activity of TiO_2 in a HDPE-based food active packaging to inhibit the growth of lactic acid bacteria and coliforms. Chorianopoulos et al. (2011) reported about photocatalytic activity of TiO_2 NPs against *L. monocytogenes* in food processing. Haghighi et al. (2013) reported that TiO_2 NPs inhibit the fungal biofilms of *C. albicans*. Sani et al. (2017) reported that photocatalytic properties of TiO_2 NPs inhibited the growth of gram positive (*L. monocytogenes*, *S. aureus*) and gram negative (*E. coli* O157:H7, *S. enteritidis* and *P. fluorescens*) bacteria in packaged lamb meat samples.

3.8 Application of Zinc Oxide (ZnO) NPs as Antimicrobial Agents

ZnONP showed antimicrobial activity against *E. coli*, *L. monocytogenes*, *Salmonella*, and *S. aureus* through the production of highly reactive oxygen species such as hydrogen peroxide, Zn^{2+} ions, and OH^- which can penetrate the bacterial cells through the disruption of bacterial cell membrane integrity and inhibits the transcription of bacterial genes (Tayel et al. 2011; Liu et al. 2009). Reddy et al. (2007) reported that *E. coli* was inhibited by ≥3.4 mM concentration, whereas *S. aureus* was completely inhibited at ≥1 mM concentration. Polyethylene glycol (PEG)-capped ZnONPs inhibited the growth of *E. coli* at above 5 mM concentration (Nair et al. 2009). *Campylobacter jejuni* was inhibited by ZnONPs with a concentration of 0.05–0.025 mg/mL. ZnONPs induce morphological changes, membrane leakage, and enhance expression of oxidative stress gene in *C. jejuni* (Xie et al. 2011). Espitia et al. (2013) reported the significant antimicrobial activity of ZnONPs against *E. coli*, *S. choleraesuis*, *S. aureus*, *Saccharomyces cerevisiae*, and *A. niger*. Liu et al. (2009) and He et al. (2011) reported the antibacterial activity of ZnONPs against *E. coli* O157:H7, *Botrytis cinereal* and *P. expansum*. Jin et al. (2009) reported the inhibitory activity of ZnONPs against foodborne pathogenic bacteria including *L. monocytogenes*, *S. enteritidis*, and *E. coli* O157:H7. Emami-Karvani (2012) reported that the antibacterial activity of ZnONPs depend on concentration and size. Sun et al. (2014) reported the antibacterial properties of titanium-doped ZnO powders against *E. coli* and *S. aureus*. ZnONPs were used against *C. albicans* infections in food safety (Espitia et al. 2012). ZnONPs were used against pathogenic fungi such as *Botrytis cinerea* and *P. expansum* in agricultural and food processing industry (He et al. 2011).

3.9 Application of Iron Oxide (Fe_3O_4) NPs as Antimicrobial Agents

The Fe_3O_4 NPs showed antimicrobial activity against various bacteria including *S. aureus*, *S. epidermidis*, *E. coli*, *Xanthomonas* sp., and *P. vulgaris* through the production of ROS such as superoxide radicals such as singlet oxygen, hydroxyl radicals, and hydrogen peroxide (Behera et al. 2012). Chen et al. (2008) reported that antibiotic-resistant pathogenic bacteria such as *S. saprophyticus* and *S. pyogenes* were inhibited by immunoglobulin G-bound Fe_3O_4/titania core/magnetic shell NPs. Chitosan-coated iron oxide NPs showed enhanced inhibitory activity against *P. aeruginosa* (Mukherjee and De 2016).

3.10 Application of Gold (Au) NPs as Antimicrobial Agents

The size of biologically inert AuNPs is 0.8–250 nm. AuNPs conjugated with specific antibodies are used to kill *S. aureus* (Zharov et al. 2006). AuNPs inhibit the growth of bacteria through the cell death and transcription (Rai et al. 2010). AuNPs prevented the growth of multidrug-resistant uropathogens such as *E. coli*, *E. cloacae* complex, *P. aeruginosa*, *S. aureus*, and *S. aureus*-MRSA with 8–32 nM concentrations (Li et al. 2014). *S. aureus* is inhibited by Au nanospheres conjugated with gentamicin in very minimum inhibitory low concentration (0.0937 mg/mL) as compared to free gentamicin (0.18 mg/mL MIC) (Ahangari et al. 2013). *S. epidermidis* and *S. haemolyticus* were inhibited by AuNPs conjugated with gentamicin, ciprofloxacin, rifampicin, and vancomycin as compared to antibiotics alone (Roshmi et al. 2015). Shareena Dasari et al. (2015) reported that *E. coli*, *S. typhimurium* DT104, and *S. aureus* were inhibited by AuNPs. AuNPs immobilized with arginine, tryptophan, and cysteine showed inhibitory activity against antibiotic-resistant strains of *Staphylococci* and *Enterococci* (Kuo et al. 2016). AuNPs showed antibacterial activity through reduction of ATP level, alteration of bacterial membrane potential and blocking the binding of tRNA to ribosome (Cui et al. 2012). Tiwari et al. (2011) showed that 5-fluorouracil-conjugated Au NPs' inhibitory activity against *M. luteus*, *S. aureus*, *P. aeruginosa*, *E. coli*, *A. fumigatus*, and *A. niger*. Fluconazole conjugated with Au NPs inhibited the growth of fungi such as *A. niger*, *C. albicans*, and *A. flavus*. Ciprofloxacin conjugated with Au NPs inhibited the growth of *S. typhimurium*, *E. coli* O157:H7 and *P. aeruginosa* (gram negative bacteria) and *L. monocytogenes*, *B. cereus* and *S. aureus* (gram positive bacteria) (Zawrah et al. 2011). Poly-allylamine hydrochloride-conjugated Au NPs showed antibacterial activities against *E. coli* and *Bacillus* Calmette-Guerin (BCG) (Zhou et al. 2012).

3.11 Application of Copper Oxide (CuO) NPs as Antimicrobial Agents

Copper NPs showed antibacterial activity against gram-positive bacteria such as *B. subtilis* and *S. aureus*, and gram-negative bacteria such as *E. coli* (Chatterjee et al. 2012; Ruparelia et al. 2008). The antibacterial activity of CuO NPs depends on the adhesion of NPs to bacterial cell walls; ROS induced DNA and membrane damage to bacteria (Raffi et al. 2010). CuO NPs interact with the amines and carboxyl groups of cell surface of *B. subtilis*. CuO NPs effectively inhibited the growth of gram-negative bacteria such as *K. aerogenes*, *P. desmolyticum*, *E. coli* and gram-positive bacteria *S. aureus* (Naika et al. 2015). Khashan et al. reported that CuO NPs showed inhibitory activity against *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *S. aureus* as well as antifungal activity against *C. albicans* in combination with fluconazole. Usman et al. (2013) reported that Cu-chitosan NPs showed inhibitory activity against *B. subtilis*, *P. aeruginosa*, methicillin-resistant *S. aureus*, *S. choleraesuis*, and *C. albicans*. Ren et al. (2009) reported the antibacterial activities of CuO NPs against *S. aureus* EMRSA-16 (epidemic methicillin- resistant *S. aureus*),

EMRSA-15, methicillin-resistant *S. aureus* (MRSA) 252, *S. aureus* "Golden" and *S. aureus* Oxford (NCTC 6571); *S. epidermidis* SE-51 and SE-4; *E. coli* NCTC 9001; *P. aeruginosa* PAOI, and *Proteus* spp. Azam (2012) reported that antibacterial activity of CuO NPs depends on size, stability, and concentration. CuO NPs inhibited the growth of *S. aureus* and *B. subtilis* (Gram-positive bacteria) and *P. aeruginosa* and *E. coli* (Gram-negative bacteria) by passing through the nano pores present on their membrane. Ahamed et al. (2014) reported the antimicrobial activity of CuO NPs against *K. pneumonia*, *E. faecalis*, *Shigella flexneri*, and *S. typhimurium*.

3.12 Application of Aluminum (Al) NPs as Antimicrobial Agents

Jing et al. reported that Al NPs showed highest toxicity against *B. subtilis*, *E. coli*, and *P. fluorescens* through ROS-induced disruption of bacterial cell walls (Mukherjee et al. 2011; Ansari et al. 2014). Positive-charged Al NPs showed adhesion with the negatively charged microorganisms (Li and Logan 2004). Ansari et al. (2013) reported about bactericidal activity of Al NPs against methicillin-resistant coagulase negative *S. aureus*.

3.13 Application of Bismuth (Bi) NPs as Antimicrobial Agents

Hernandez-Delgadillo et al. (2012) reported that BiNPs showed antibacterial activity with a concentration of <1 mM and antifungal activity with a concentration of 2 mM. BiNPs prevented the growth of *Helicobacter pylori* through modification of the Krebs cycle and amino acid and nucleotide metabolism (Nazari et al. 2014).

3.14 Application of Carbon-Based NPs as Antimicrobial Agents

Carbon-based NPs induce disruption to bacterial membrane through oxidative stress. Single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) showed bactericidal activity against both Gram-positive and Gram-negative bacteria. These showed inhibitory activity against *S. enterica*, *E. coli*, *E. faecium*, *Salmonella* sp., *Streptococcus* spp., and *Shewanella oneidensis*. Complex of Carbon NPs and Ag showed inhibitory activity against multidrug-resistant *Acinetobacter baumannii*, *Burkholderia cepacia*, *K. pneumoniae*, and *Yersinia pestis* (Leid et al. 2012). Drugs are immobilized to carbon nanocarriers through encapsulation and chemical adsorption by electrostatic, hydrophobic, p-p interactions and hydrogen bonding. Encapsulation is more advantageous because it protects drug from degradation and transports drug to the target cells (Wilczewska et al. 2012).

3.15 Application of Organic NPs as Antimicrobial Agents

Quaternary ammonium compounds (QAC) such as cetrimonium chloride, benzalkonium chloride, and stearalkonium chloride showed antimicrobial activity through ionic interaction between anionic bacterial membrane and cationic QAC. The hydrophobic tail of the QAC denatures structural proteins and enzymes of the bacterial membrane through its interaction with hydrophobic membrane. Nanoscale materials conjugated with N-alkylated polyethyleneimine (PEI) showed inhibitory activity against Gram-positive and Gram-negative bacteria. The growth of *S. aureus* and *E. coli* was inhibited by QAC-PEI-based NPs at 80 g/mL and at 320 g/mL concentration respectively (Xue et al. 2015; Yudovin-Farber et al. 2010). Siloxane copolymers have antimicrobial activity against *E. coli* and *S. aureus* (Sauvet et al. 2003). Chitosan NPs showed antifungal activity against *C. albicans* and *Fusarium solani* (Yien et al. 2012).

3.16 Application of Eco-friendly Green NPs as Antimicrobial Agents

AgNPs synthesized from leaf extract of *Rosmarinus officinalis* showed inhibitory activity against *E. coli*, *B. subtilis*, *P. aeruginosa*, and *S. aureus* (Ghaedi et al. 2015). AgNPs synthesized from *Ficus benghalensis* and *Acalypha indica* inhibit the growth of *B. subtilis*, *E. coli*, *P. aeruginosa*, and *Vibrio cholerae* (Nayak et al. 2016). AgNPs synthesized from leaf extract of *Skimmia laureola*, root extract of *Delphinium denudatum*, and flower extract of *Rosa chinensis* showed antibacterial activity. ZnONPs from *Solanum nigrum*, CeO_2 NPs from *Olea europaea* leaf extract, and Fe_3O_4-Ag core shell magnetic NPs from stem extract of *Vitis vinifera* showed inhibitory activity against both gram-positive and gram-negative bacteria (Hemeg 2017). Au NPs synthesized from aqueous extract of seed of *Abelmoschus esculentus* showed antifungal activity against *Puccinia graminis tritci*, *A. flavus*, *A. niger*, and *C. albicans* (Jayaseelan et al. 2013) (Table 3.2).

Table 3.2 Role of green AgNPs as antibacterial agents

Plant source of green synthesized NPs	Target bacteria
Phyllanthus amarus extract	MDR *P. aeruginosa*
Helicteres isora fruit extract	Drug-resistant *P. aeruginosa* isolates
Artemisia cappilaris extract	Methicillin-resistant *S. aureus*
Acalypha indica leaf extracts	*E. coli*, *V. cholerae*
Rhizopus oryzae	*E. coli*, *P. aeruginosa*
Cocus nucifera	*V. alginolyticus*, *K. pneumoniae*, *P. aeruginosa*, *B. subtilis*, *Plesiomonas shigelloides*

3.17 Application of NPs as Antiviral Agents

Metal NPs showed antiviral activity against human immunodeficiency virus 1 (HIV-1), hepatitis B virus, influenza virus, herpes simplex virus type 1 (HSV-1), and H1N1 influenza A virus (Rudramurthy et al. 2016). AgNPs inhibited replication of HIV-1 at early stage and CD4-dependent virion binding (Lara et al. 2010). AgNPs capped with mercaptoethane sulfonate inhibited infection of Herpes Simplex Type 1Virus through binding to the viral heparan sulfate in their sulfonate end groups (Baram-Pinto et al. 2009). Gianvincenzo et al. (2010) reported that amphiphilic sulfate-ended ligand-conjugated Au NPs inhibited HIV infection. Sametband et al. (2011) reported the inhibitory activity of anionic AuNPs against several influenza strains. The density of anionic charge and functional groups determined the antiviral activity of the AuNPs. Au NPs penetrate virus through the endosome vesicle. The NPs which contain Au core and mercaptoethanesulfonate molecules inhibited the growth of influenza virus strains.

3.18 Compatibility of Nanoparticles (NPs) in Biological System

Cytotoxicity, hemocompatibility, histocompatibility, and neurotoxicity of NPs are studied before used in gene delivery, drug delivery, or as biosensors. The biological compatibility of NPs depends on size, structure, and the surface properties of the NPs. Silica-based NPs have no effect on cell survivability (Li et al. 2012a, b). Munger et al. (2014) reported that commercial AgNP solution does not alter human metabolism. The cytotoxicity level of AgNPs in in vitro cell line model was in the range of 5–50 g/mL (Espinosa-Cristobal et al. 2013). AgNP-biopolymer complex did not show any toxic effect in mouse fibroblasts (NIH-3T3), human osteosarcoma cells (MG63), and human hepatocarcinoma cells (HepG2) (Travan et al. 2009). AuNPs are less toxic to RAW264.7 cells as compared to AgNPs (Hashimoto et al. 2014). Fe_3O_4-AuNPs complex showed minimum toxic effect in cell lines (Barnett et al. 2013). The toxic effect of NP at cellular level depends on the route of administration of NPs and its site of accumulation. Polymeric NPs are biocompatible in nature (Sosnik et al. 2010). The biocompatibility of NPs depends on the half-life of NPs, types of cells and tissues, and risk–benefit ratio (Naahidi et al. 2013). To avoid toxic effect, less toxic biodegradable NPs (BNPs) are used in biomedical sciences. BNPs are applicable for controlled release of drugs, genes, and other bioactive agents. Proteins, polysaccharides, and synthetic biodegradable polymers are used for the synthesis of BNPs. Biodegradable polymer matrices such as polylactic acids (PLA), L-lactide-co-glycolides (PLGA), polyalkyl-cyanoacrylates (PAC), gelatins, and chitosans,. Lactic and glycolic acids are produced due to biodegradation of PLGA NPs. Lactic acid is produced due to the biodegradation of PLA in the human body. PEG, polysorbate 80, polysorbate 20, dextran, and tocopheryl polyethylene glycol 1000 succinate are used for the surface modification of NPs. This increases the half-life of the NPs in blood

circulation (Kumari et al. 2010). Leroux et al. (1996) reported that PEGylated PLGA NPs are less interacts with mononuclear phagocytes.

3.19 Stimuli-responsive Therapeutic Nanoparticles and Antibiotic Delivery Systems for Microbial Infection

Metallic inorganic nanoparticles (NPs) such as AuNPs, AgNPs, Fe_2O_3 NPs, and Fe_3O_4 NPs with liposomes, polymersomes and hydrogels are used for stimuli-responsive release of antibiotics. Liu et al. reported that polydopamine (PDA)-coated gold nanorods (GNRs) with high silver-ion loading efficiency and glycol chitosan (GCS) was used against drug-resistant Gram-positive methicillin-resistant *S. aureus* (MRSA) or Gram-negative *E. coli*. The bacterial inactivation percentages for the Ag^+-GCS-PDA@GNRs + near-infrared (NIR) treatment group were 99.6% (Liu et al. 2018). Superparamagnetic iron oxide nanoparticles (SPIONs) are used to release antibiotics from drug-delivery vehicles upon thermal stimulation (Mahmoudi et al. 2011). Geilich et al. (2017) showed that complete biofilm formation by *S. epidermidis* was eradicated by using iron oxide polymersome (IOPs) which contained SPIONs and methicillin at a lower concentration. Radovic-Moreno et al. (2012) studied effect of vancomycin-encapsulated, pH-responsive, surface charge-switching poly(d,l-lactic-co-glycolicacid)-b-poly(l-histidine)-b-poly(ethylene glycol) nanoparticles on infection of *S. aureus*. In acidic pH, NP–bacteria interactions reduce the activity of vancomycin, which increases the potentiality of drug-delivery system. pH responsiveness is used for the treatment of resistant bacteria localized in acidic environment of cell. Sémiramoth et al. (2012) developed bioconjugate complex of penicillin G (PNG) with squalene (SqPNG) to release penicillin G in an acidic environment of cell which was used against infection of *Staphylococcus aureus*. SqPNG-pH NPs were more effective to kill *Staphylococcus aureus* as compared to free PNG or SqPNG NPs. Chang et al. (2010) used pH-sensitive hydrogel that contained chitosan/PGA NPs with amoxicillin for the treatment of infection of *Helicobacter pylori* in a human gastric adenocarcinoma cell line. This system prevented the destruction of amoxicillin from acidic pH of gastric environment. Lu et al. (2017) developed pH-sensitive hydrogel which was composed of poly(acrylic acid) (PAA) and p-(2-(dimethylamino) ethylmethacrylate) (PDMAEMA). The vancomycin and levofloxacin were released from this hydrogel at pH 5.5 to kill *S. aureus* in 6 h. The application of nanoantibiotics (nAtbs) is more advantageous in antimicrobial activity. The antimicrobial activity of nAbts depends on the production of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radicals, superoxide, and hydroperoxyl radicals that can affect both gram-negative and gram-positive bacteria (Muzammil et al. 2018).

3.20 Factors Controlling Antibacterial Activity of Metal NPs

The size, charge, zeta potential, surface morphology, and crystal structure of NPs control the antibacterial action of NPs on bacterial cells. Other than physicochemical properties of NPs, bacterial strain and exposure time are key factors to determine the antibacterial effects of NPs (Wang et al. 2017). Smaller NPs with large surface areas have higher probability to penetrate bacterial cell membrane as compared to larger NPs (Gurunathan et al. 2014). Different shapes of NPs showed different degrees of damage on bacterial cell (Cha et al. 2015). Cube-shaped AgNPs showed stronger antibacterial activity as compared to sphere-shaped and wire-shaped AgNPs (Actis et al. 2015). Rough-surfaced NPs enhance the adsorption of bacterial proteins that reduce bacterial adhesion (Sukhorukova et al. 2015). The zeta potential of NPs have an effect on bacterial adhesion. Positively charged NPs showed more electrostatic attraction towards negatively charged bacterial cell membrane as compared to negatively charged NPs. Positively charged NPs release more ROS as compared to negatively charged and neutral NPs (Arakha et al. 2015).

Doping modification regulates the interaction of NPs and bacteria. Doping of ZnONPs with fluorine produce more damage to bacterial cells through the production ROS as compared to ZnONPs (Guo et al. 2015). Stimulation of ZnONPs by temperature enhances antimicrobial effectiveness of ZnONPs. Acidic pH increases the dissolution rate of ZnONPs, which results in greater antimicrobial properties of ZnONPs (Saliani et al. 2015). The NPs along with carbapenems, fluoroquinolones, ceftazidime, and tobramycin showed synergistic effects against *P. aeruginosa* and *A. baumannii* (Bayroodi and Jalal 2016).

3.21 Nanoparticles Used in Drug Delivery

The recent development of organic nanocarriers such as polymer-based micelles, liposomes, and dendrimers and inorganic nanoparticles such as carbon nanotubes, gold nanoparticles, and quantum dots are the main focus for drug delivery applications. Natural polymers such as chitosan, dextran, heparin, and hyaluronan are used for drug delivery (Elsabahy and Wooley 2012). Biodegradable synthetic polymeric (bio)materials such as poly(α-hydroxy esters), including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolide) (PLGA) copolymers are used for drug delivery (Tyler et al. 2016; Wang et al. 2018; Ding and Zhu 2018). Doxorubicin- (DOX-) conjugated PLGA–PEG micellar nanocarriers showed sustained release of DOX as compared to physically incorporated DOX in PEG–PLGA micelles (Yoo and Park 2001). Liposomes are first reported as drug carriers and are composed of phospholipids and steroids (e.g., cholesterol). SLN (solid lipid nanoparticles), NLC (nanostructured lipid carriers) and LDC (lipid drug conjugates) are used as a drug delivery system. SLN protect drugs from degradation and release drug in a controlled manner. Polymeric nanoparticles (PNPs) are synthesized from polyacrylamide, albumin, DNA, chitosan, and gelatine. PNPs may be classified as biodegradable, i.e., poly(L-lactide) (PLA), polyglycolide (PGA), and

non-biodegradable, e.g., polyurethane. Glycogen, amylopectin, and proteoglycan have dendritic structure. Polyamidoamines, polypropyleneimines, poly-l-lysines, and carbosilanes are some examples of dendrimers. The surface of dendrimers provides binding sites of folic acid, antibodies and arginine-glycine-aspartic acid peptides, and silver salts complexes. Poly(amido amide) (PAMAM) dendrimers are used for incorporation of anti-inflammatory drugs, e.g., ibuprofen, piroxicam, or indomethacin (Wilczewska et al. 2012).

US Food and Drug Administration (FDA) approved antifungal drugs such as lipid formulations of amphotericin B [AmB]), amphotericin B lipid complex (ABLC), amphotericin B colloidal dispersion (ABCD), liposomal AmB (L-AmB) (Carrillo-Muñoz et al. 1999). L-AmB which showed lower toxicity as compared to AmB formulations is used for the treatment of cryptococcal meningitis, blastomycosis, coccidioidal meningitis, mucormycosis, and pulmonary aspergillosis (Voltan et al. 2016). Conjugation of lipid with drug is developed to increase the loading capacity of a drug. PLNs are combination of liposome and PNPs, which have high encapsulation efficiency, serum stability, and loading capacity of poor water-soluble drugs (Zhang et al. 2008). Several antifungal agents such as itraconazole-loaded SLNs, miconazole nitrate-loaded NLC, itraconazole-loaded NLC, and econazole nitrate-loaded NLC are used in medicine (Voltan et al. 2016). PNPs are useful to protect encapsulated drugs from degradation in acidic pH of gastrointestinal environment (Pridgen et al. 2014). Nanospheres and nanocapsules are two types of PNP. In nanocapsules, drug is present inside an aqueous or oily cavity, whereas in nanospheres, drug is physically and uniformly distributed in the matrix (des Rieux et al. 2006). To reduce the cytotoxicity of dendrimers, dendrimers are coupled with PEG, carbohydrates, and acetyl groups. To enhance the solubility of clotrimazole, polyamidoamine dendrimers are used against different species of *Candida* (Winnicka et al. 2011). Janiszewska et al. reported that dendrimeric lipopeptides are used to inhibit the enzyme activity of 1,3-β-D-glucan synthase in *Candida* (Janiszewska et al. 2012).

3.22 Conclusions

Due to their biological and physiochemical characteristics, NPs are used as antimicrobial agents. NPs conjugated with functional groups showed enhancement of their antimicrobial activity. NPs doped with ion and polymer showed reduced toxicity. Development of low-cost inorganic NPs is an alternative for traditional antibiotics and may be used in pharmaceutical, food, and medical industry. The antimicrobial properties of NPs depend on size, chemical composition, and shape of these NPs. Organic or inorganic NPs made up of metal, polymer, ceramic, latex, or carbon-base particles. Higher surface area of NPs allowed them to interact with bacterial cellular structures and enhanced the production of oxidative stress. The ROS produced by NPs induce apoptosis. The effect of NPs on biological systems should be carefully investigated. Bacteria develop antibiotic resistance properties which present a challenge for medical sciences. NPs provide hope for the development of antimicrobial

agents. NPs are also applied in drug delivery, gene delivery, cancer therapy, and bioimaging. Advancement in nanomedicine is an important aspect for diagnosis and treatment of diseases induced by drug-resistant microorganisms.

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4 Antimicrobial Activity of Metallic Nanoparticles Using Prokaryotic Model Organisms

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Abstract

Infectious diseases has remained one of the leading causes of morbidity and mortality in the past decade. The problem has further exacerbated due to the lack of newer antibiotics and emergence of antimicrobial drug resistance among the pathogens. Bacteria have evolved diverse mechanisms which make them resistant to many antimicrobials simultaneously. These alarming situations have triggered worldwide initiatives in the direction of developing novel strategies, effective antimicrobial agents, and efficient targeting systems. One of the fields which holds promise to provide solutions as efficient antibacterial agents is nanotechnology. The field of nanotechnology is rapidly evolving with more applications being developed in the pharmaceutical and biomedical domains. Nanoparticles such as metallic and metal-oxide, have gained tremendous attention owing to intrinsic antibacterial properties. These properties have been further enhanced by their surface functionalization approaches. They are being explored as delivery agents to inhibit bacterial population and also to combat drug resistance mechanisms in pathogens. The present chapter summarizes recent scientific advances on metal, metal oxide nanoparticles, and nanocomposites-preparation methods along with their antibacterial potential evaluated in prokaryotic bacterial model systems.

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Keywords
Metal nanoparticles · Metal oxide nanoparticles · Nanoparticle conjugates · Bacteria · MDR

4.1 Introduction

Metals whose dimensions fall in the range of 1–100 nm with respect to their length, width, or thickness are categorized as metal nanoparticles. Various kinds of nanomaterials are produced using physical, chemical, and biological (green synthesis) methods. With the unique properties acquired due to their dimensions, nanomaterials find varied applications in the fields of electronics, cosmetics and personal care, food & beverage industries, pharmaceutical & healthcare, and other industries such as textile, wastewater treatment, aerospace, glass, & paints (Willems 2005). Based on the metal, the global metal nanoparticles market has been segmented into silver, gold, iron, copper, platinum, graphite, titanium, and others (Willems 2005).

Owing to their antimicrobial properties, gold and silver nanoparticles are used in pharmaceutical & healthcare segment (Willems 2005). Silver nanoparticles have accounted for the largest share of the metal nanoparticles market. Recently they were valued over USD 1.3 billion in 2017 and would continue to lead the market in the next 5 years. Silver metal's exceptional therapeutic antimicrobial properties against pathogens and other microorganisms attribute to its growing popularity, which is being extended to food packing industry (Willems 2005). Moreover, the application of metal nanoparticles in electrical & electronics industry is projected to impact the market. This segment is projected to grow at the second-highest rate in the next 5 years (Willems 2005).

Other metal nanoparticles such copper, zinc, and graphite are being used as catalysts in different chemical and electrochemical reactions. They are also being used in multilayered ceramic capacitors, in conductive layers, and wire printing. Carbon, graphite, and copper nanoparticles find applications as lubricants in engine oils to impart wear resistance and reduce friction (Willems 2005). The ever growing diverse applications of metal nanoparticles along with their engineered and improvised versions are further going to boost the entire metal nanoparticle segment.

Nanoparticles (NPs) owing to their extremely large surface area in proportion to their size gather strategic advantages over their normal-size counterparts. Decreasing and bringing the size of the material in the nano-range may provide higher activity at smaller doses, thereby decreasing the effective dose to control pathogens (Hazan et al. 2015). The use of nanostructure-based materials as potent antibacterials to combat bacterial infections and moreover control drug resistances among microbes has gained tremendous importance in recent years (Baranwal et al. 2018).

Soon after the antibiotics were put to clinical use as drugs to treat and combat microbial infections, a remarkable feature was being evidenced among microorganisms i.e., to persist, survive, adapt, and to evolve in the presence of antibiotics, a phenomenon now well known as antimicrobial resistance. Lack of new antimicrobials

together with the emergence and spread of multidrug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Seil and Webster 2012) or vancomycin-resistant *Enterococcus* (VRE) (Hamilton and Wenlock 2016), are currently a global threat to public health and livestock ((Rudramurthy et al. 2016); (Baptista et al. 2018). These pressing issues have prompted research worldwide to develop novel antimicrobials along with development of effective delivery or targeting strategies. Approaches such as use of nanomaterials and their engineered versions are being currently explored as a plausible solution to overcome the growing problem of antibiotic resistance in microbes.

In this chapter, we present an overview on various metal nanoparticles and their forms used as antibacterial agents. Recent advances in the form of nanoparticle conjugates have also been summarized in the subsequent section. Though it is difficult to give a comprehensive overview and discuss all forms of metal NPs, representative methods used of synthesis for NPs along with their characterisitcs have been briefly summarized. Main focus has been to summarize recent findings on the antibacterial effect as a function of size, morphology. Also focus on the mechanism of activity of different metal NPs forms against multi-drug resistant (MDR) pathogens along with challenges and future prospects.

4.2 Metal and Metal Oxide Nanoparticles

Metals and their oxides in the form of nanoparticles, renowned for their intrinsic and potent antibacterial properties, include silver (Ag), iron oxide (Fe_3O_4), copper oxide (CuO), magnesium oxide, titanium oxide (TiO_2), zinc oxide (ZnO), and others (Hazan et al. 2015).

Different synthetic strategies are employed to generate metal NPs. Conventionally, these techniques have been classified into physical (by use of vapour deposition, microwave, ultrasound, etc.), chemical (by sol-gel, precipitation, etc.), and biological, economical and eco-friendly (by use of microbes, plant extract) methods (Willems 2005; Baranwal et al. 2018; Prasad et al. 2016).

4.3 Metal Nanoparticles and Antibacterial Mechanisms

NPs act in the complex growth environment comprising high protein contents, salts, polysaccharides, and the media components. These factors in turn can affect the stability of NPs causing them to aggregate at the concentrations at which they are studied (Schwegmann and Frimmel 2010). It appears that zero-valent metals greatly affect microorganisms during their growth phase. Due to the ionic properties and surface charge of metallic NPs, the forces of attraction & repulsion dominate the interaction between the NPs and the microorganisms. Such electrostatic interactions are integral and they promote adhesion of NPs to the cell surface of the microorganism to exert its toxic effect (Schwegmann and Frimmel 2010). A study demonstrated this phenomenon by the use of AuNPs that were tagged with anionic and cationic side chains.

Phosphatidylcholine and phosphatidylserine vesicles representing a reference system for cell membranes that carried overall negative charge and another preparation containing only phosphatidylcholine that had no net charge were used. The positively charged AuNPs lysed the negatively charged vesicles more efficiently than the one with no charge (Schwegmann and Frimmel 2010). For different metal NPs or their engineered forms, three broad antibacterial mechanisms have been proposed (1) by liberation of ions; (2) by generation of reactive oxidative species and (3) interaction of NPs with the cell membrane (Schwegmann and Frimmel 2010).

Once the metal NPs are brought in contact with the microbial cell, the metallic nanoparticles attach to the cell via transmembrane protein. Attachment to the membrane proteins triggers conformational changes in the protein, thus blocking the transport channels. Smaller sized NPs are found to be more efficient in causing this than the larger sized NPs. Larger surface area of the smaller NPs permit superior binding forces and better adhesion followed by internalization. After internalization the NPs undergo ionization within the cell that further damage intracellular structures causing cell death (Schwegmann and Frimmel 2010).

The other mechanism observed with use of metal NPs is the generation of reactive oxidative species (ROS) to cause the antibacterial effect. ROS are group of oxidants that are highly reactive but short-lived and they include hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), and hydroxyl radicals (•OH). Due to their reactive nature, they cause damage to the cellular components like DNA, RNA, ribosomes, proteins and also to peptidoglycan present in cell membrane. ROS acts by altering and halting the processes of transcription, translation, enzyme activity, or the electron transport chain (Schwegmann and Frimmel 2010). Further some metal atoms can cause damage to the enzyme structure and deactivate them by attaching to the thiol group of the enzyme. It has been proposed that they are also capable of attaching to the purine and pyrimidine bases. They too disrupt the hydrogen bonding between to the helical strands of DNA causing its structural damage (Baptista et al. 2018).

Expression studies using reverse transcription-qPCR techniques are being performed for a better understanding of the impact of metal NPs at the cellular level. Recent findings of such molecular mechanisms strongly indicate upregulation of different stress response genes in the microorganism. When subjected to MgO treatment, the expression of oxidative stress response genes such as *katA* (expressing catalase), *ahpC* (expressing alkyl hydroperoxide reductase), and *dps* (expressing bacterioferritin) were upregulated 44-, 5-, and 4- fold, respectively along with a 22-fold higher expression of general stress response gene *spoT* in *C. jejuni* cells (He et al. 2016).

Table 4.1 summarizes representative examples of metal NPs, their methods of synthesis, morphological characteristics and antibacterial effects.

Table 4.1 Representative metal nanoparticles, their forms, syntheses and antibacterial mechanisms

Metal/ Metal oxide NPs	Method of synthesis	Size	Shape	Model organisms	Antibacterial mechanism	Reference
Silver nanoparticles (AgNPs)	Nitrate-reducing *Bacillus subtilis L1* (KT266579.1)	~28.30 nm	Spherical	Gram-positive and Gram-negative organisms	DNA fragmentation, cell shrinkage and increase in surface roughness	Muthukrishnan et al. (2019)
Silver nanoparticles (AgNPs)	Extracellular filtrate of the epiphytic fungus *B. ochroleuca*	8–21 nm	Coated on fabrics of polyester and Cotton	*S. aureus* and *E. coli*, *C. albicans, C. glabrata,* and *C. parapsilosis, P. aeruginosa*	Inhibition of biofilm by *P. aeruginosa* and damage to the intracellular contents of other bacteria tested	Rodrigues et al. (2019)
Ag–TiO_2 nanocomposites	Silver nanoparticles deposited on titanium dioxide nanoparticles by a chemical reduction approach	15–30 ± 5 nm diameter	Spherical	*B. subtilis* and *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* and *C. albicans*	Anti-biofilm activity, more intensive for Gram-positive bacteria	Lungu et al. (2014)
Zinc oxide (ZnO) and titanium dioxide (TiO2) NPs	Commercially available	ZnO NPs <100 nm and TiO_2 NPs <50 nm	Spherical	Biofilm and non-biofilm-forming MRSA	Electromagnetic attraction between the microbe and treated surface, microbe oxidization and death	Jesline et al. (2015)
Titanium dioxide (TiO_2) nanoparticles	Aqueous TiO_2	–	–	*E. coli*, *P. aeruginosa*, *S. aureus*, *Enterococcus hirae*, and *Bacteroides fragilis*	Photo-inactivation & cellular damage	Tsuang et al. (2008)

(continued)

Table 4.1 (continued)

Metal/ Metal oxide NPs	Method of synthesis	Size	Shape	Model organisms	Antibacterial mechanism	Reference
Nano-tubular Cu arrays	Template-based electrodeposition	Height 13±1 mm and diameter 300 nm	Tubular	*S. aureus, E.coli*, *Shigella sonnei, Salmonella enterica, and Candida albicans*	Over 4 log reduction for *S. aureus* via cellular damage	Razeeb et al. (2014)
Nickel nanoparticles	*Ocimum sanctum* leaf extract	12 and 36 nm	Spherical	*E. coli*, *K. pneumoniae*, and *S. typhi*), *B. subtilis*, *S. epidermidis*, and fungi (*C. albicans*, *C. tropicalis, A. fumigatus*, *A. clavatus*, and *A. niger)*	ROS generation, protein leakage & damage	Jeyaraj Pandian et al. (2016)
Zinc oxide nanostructures	Zinc acetate, dihydrate and NaOH	Length of petal ~800 nm	Hexagonal nanorods assembling into flower shape	*Staphylococcus aureus* and *Escherichia coli*	Overall antimicrobial effect	Mohan Kumar et al. (2013)
Magnetic binary nanocomposites: (1) Ag@Fe3O4 (magnetite) type and (2) g-Fe2O3@Ag (maghemite) type	In situ chemical reduction of silver ions of a magnetic phase by maltose, polyacrylate molecules as spacer among iron oxide and silver NPs.	(1) Ag@ Fe3O4: 5 nm (2) g-Fe2O3@ Ag: 20 and 40 nm	Spherical geometry	Ten tested bacterial strains and four *Candida* species	Overall strong antibacterial and antifungal activities	Prucek et al. (2011)
CuO nanoparticles	A gel combustion method	20 nm	Spherical geometry	Gram-positive and -negative bacteria	Overall strong antibacterial activities	Azam et al. (2012)

4.4 Metal Nanoparticles as Antimicrobials

4.4.1 Silver Nanoparticles (AgNPs)

Among different metal NPs, silver nanoparticles (AgNPs) have gained wider usage in varied fields. Silver has been known since ancient times to possess antibacterial action (Baptista et al. 2018). At present there are over 100 consumer products that use AgNPs for its superior antibacterial properties (Baranwal et al. 2018). Conventional chemical or green synthesis approaches have been used for preparing AgNPs. AgNPs can cause damage to the bacterial cell by inducing disruption of cell wall, oxidation mechanisms, or formation of ROS species (Baptista et al. 2018).

Synthesis of silver NPs (AgNPs) with an aqueous solution of $AgNO_3$ in the presence of culture supernatant of phenol-degraded broth was reported by Otari et al. (2014). The resultant AgNPs showed strong antimicrobial effects against Gram-positive, Gram-negative, and fungal microorganisms tested (Otari et al. 2014). In a similar study, cell-free extract of *Citrobacter* spp., *Escherichia* spp., and *Pseudomonas* spp. isolates were used to synthesize AgNPs which exhibited strong antibacterial effect (Bogdanović et al. 2014). In another study, synthesis of a silver–clay nanohybrid was performed by sodium borohydride reduction, followed by calcination and UV- irradiation method to uniformly precipitate silver on clay platelets. The resultant nanohybrid structure showed synergistic antimicrobial activity with a magnitude fourfold higher than the silver particles alone (Girase et al. 2011). The studies using AgNPs have been reviewed (Baptista et al. 2018; Khan et al. 2016).

4.4.2 Iron Oxide Nanoparticles (Fe_xO_yNPs)

Iron oxide nanoparticles have also been explored for their antibacterial effects. Iron oxide nanoparticle (IONPs) with magnetite (Fe_3O_4) like atomic arrangement and carrying negative surface potential (n-IONP) were prepared by co-precipitation method. These NPs were coated with positively charged chitosan molecule and caused reversal of the surface potential of n-IONP, i.e. positive surface potential IONP (p-IONP). The nanocrystals of spherical shape and with 10–20 nm diameter were obtained. At the concentrations <50 μM of n-IONP a 30% decrease in viability was observed. IOPN (p-IONP) after appropriate coating resulted in higher 70% reduction in cell viability of *B. subtilis* and *E. coli* strains. The antibacterial effects were attributed to increased ROS production in the presence of p-IONP (Arakha et al. 2015).

Another study proposed a different method to generate iron oxide NPs. The magnetic iron oxide nanoparticles (IONPs) were initially coated with polyvinyl pyrrolidone conjugated catechol (PVP-CCDP) followed by deposition of silver nanoparticles (Ag NPs) onto PVP-CCDP-coated IONPs in the presence of catechol. The resultant NPs were of 72 nm size and demonstrated a strong antibacterial effect against the two model microbes *E. coli* and a high *S. aureus* (Mosaiab et al. 2013).

4.4.3 Copper- and Copper Oxide Nanoparticles (Cu- & CuO-NPs)

The activity of copper as antibacterial has been known since ancient period (Cioffi and Rai 2012). Copper demonstrates antibacterial effect due to its ability to donate & accept electrons and thus generate hydroxyl radical causing damage to cellular components like proteins and lipids (Sánchez-Sanhueza et al. 2016). Small and bare CuNPs prepared by reduction method were active against test bacteria by interacting with cell membrane in a concentration-dependent manner (Bogdanović et al. 2014).

Duffya et al. (2018) reported the effectiveness of copper oxide (CuO) nanoparticles against *Salmonella*. Also, evaluated a combined effect of silver (Ag) and CuO nanoparticles against *Campylobacter* isolated from poultry. The MIC against *Campylobacter* was in the order of Ag ≥ CuO ≥ ZnO nanoparticles. AgNPs were the most active against *Salmonella* (Duffy et al. 2018). In another study, authors reported the use of copper NPs as antibacterial against *E. coli* strain. The NP caused formation of ROS, protein oxidation, lipid peroxidation along with DNA damage (Chatterjee et al. 2014). The bioactivity of copper-based nanomaterials have been reviewed in literature (Cioffi and Rai 2012; Baptista et al. 2018).

4.4.4 Magnesium Oxide Nanoparticles NPs (MgO-NPs)

Importance of another inorganic material, magnesium oxide (MgO) in nano-form, is not less. It has many diverse applications in the field of adsorbents, toxic waste remediation, as additives in heavy fuel oils, catalysis, as catalyst supports, superconducting & ferroelectric thin films as the substrate, reflecting & anti-reflecting coatings, as superconductors and in lithium ion batteries. Though there are fewer reports, nano-forms of magnesium oxide (MgO-NPs) alone and in combination are being explored as antibacterial agent (Jin and He 2011). In a study, the use of magnesium oxide nanoparticles (MgO-NPs) alone and paired with other antimicrobials (ZnO-NPs & nisin) against *Salmonella* Stanley and *Escherichia coli* O157:H7 were evaluated. The MgO-NPs showed over 7 log reductions in bacterial counts. Only when combined with nisin, MgO-NPs showed synergistic effect. However, combination with ZnO-NPs did not show any enhancement of antibacterial effect of MgO-NPs. Scanning electron microscopy (SEM) images depicted cell membrane damage and leakage of intracellular contents in MgO-NPs-treated cells that finally lead to death of pathogen (Jin and He 2011).

One of the study evaluated antibacterial efficacy of MgO-NPs against three important foodborne pathogens *E. coli* O157:H7, *C. jejuni,* and *Salmonella enteritidis*. Results indicated that MgO-NPs with 20 nm diameter, a MIC of 2 mg/mL MgO-NPs was required against *C. jejuni* with 2 h exposure, whereas complete inhibition of both *E. coli* O157:H7 and *S. enteritidis* required minimum 8 mg/mL nanoparticles in 4 h. Evaluation of oxidative stress genes, membrane permeability, and hydrogen peroxide levels indicated interaction of nanoparticles with pathogens triggering cell membrane damage, oxidative stress, and ultimately causing cell death (He et al. 2016). MgO nanowires were synthesized of 6 nm diameter by

reaction between magnesium acetate with urea by a microwave hydrothermal technique. The obtained nanowires showed concentration-dependent antibacterial activity, against both *Bacillus* sp. and *E. coli*. The production of superoxide anions (O_2^-) in large quantities on the surface of MgO nanowires interacted with peptide linkages in the bacterial cell walls to finally destroy them (Al-Hazmi et al. 2012).

4.4.5 Titanium Dioxide Nanoparticles (TiO_2-NPs)

Titanium dioxide (TiO_2) NPs are produced in large quantities worldwide for use in a wide range of applications such as pigment and cosmetic products for their ultraviolet blocking ability (Trouiller et al. 2009). They have also been used for their photocatalytic properties for applications such as the removal of contaminants, & recently for disinfection of surfaces such as clothes/ glass, air and water remediation (Foster et al. 2011). Few reports have published the effects of photoactivated TiO_2 on microorganisms as potential antibacterial agent. They have been tested against bacteria, fungi, algae, and viruses. The killing mechanism involving production of ROS, degradation of the cell wall and membrane have been proposed. Titanium dioxide NPs decorated with Ag and Cu pronounced their antibacterial effects. The findings from such reports are reviewed further in detail in literature (Foster et al. 2011).

In one study, the commercially available zinc- and titanium dioxide nanoparticles showed considerable activity against biofilm-producing methicillin-resistant *S. aureus* isolates (Jesline et al. 2015). The antimicrobial activity of silver doped with titanium nanoparticles was determined in one study. Silver-coated nanoparticles of 20–25 nm size were effective among different sizes of nanoparticles and did not cause significant cytotoxicity (Martinez-Gutierrez et al. 2010).

4.4.6 Zinc Oxide Nanoparticles (ZnO-NPs)

Another category of metal oxide NPs that has gained recent interest as an antibacterial agent due to its nontoxic and biocompatible nature to human cells is zinc oxide (Sirelkhatim et al. 2015). Zinc oxide quantum dots (ZnO-QDs) as nanoparticles were evaluated against *S. enteritidis*, *L. monocytogenes*, and *E. coli* O157:H7. The ZnO-QDs were used as a powder, bound in a polystyrene film (ZnO-PS), or suspended in a polyvinylprolidone gel (ZnO-PVP). The ZnO-PVP caused a 6.0 log reduction of *E. coli* O157:H7 and a 5.3 log reduction of *L. monocytogenes* at 3.2mgZnO/mL concentration after 48 h of incubation. ZnO powder and ZnO-PVP showed remarkable antimicrobial activities against all three pathogens (Jin et al. 2009). ZnO antibacterial effect was more evident with the Gram-positive than the Gram-negative bacteria (Premanathan et al. 2011). The ZnO-NPs were prepared by sol-gel method and the effect of particle size & surface modification on its antibacterial activity was studied. Different reaction times were used to control the NPs' size and this was followed by the addition of (3-glycidyloxypropyl) trimethoxysilane (GPTMS) as a surface modifier. The smaller ZnO-NPs of the dimension 5 nm had significant

bactericidal activity when tested against *Staphylococcus aureus.* The smaller NPs were more efficient in causing damage to the bacterial cell wall than the larger NPs as confirmed by SEM studies (Lallo da Silva et al. 2019).

In a study, authors studied the expression of few important superfamily class efflux pump genes found in MDR *S. aureus* in the presence of ZnO nanoparticle conjugates. The ZnO nanoparticles (NPs) conjugated to thiosemicarbazide (TSC) under amine functionalization by glutamic acid (ZnO-Glu–TSC) and ciprofloxacin (CIP) were used for the study. The results showed significant 2–8-fold decrease in MIC of ZnO-Glu–TSC NPs compared to CIP alone. Moreover, the combination of ZnO-Glu–TSC NPs and CIP (at their ½ MIC) significantly attenuated the expression of efflux genes- *norA*, *norB*, *norC*, and *tet38* compared to the CIP alone (Nejabatdoust et al. 2019). The authors screened thirty-six metal ions to inhibit pyocyanin production and biofilm formation of *P. aeruginosa* in LB medium at a concentration of 1 mM. Of the metal ions evaluated, 10 metal ions, namely Fe^{2+}, Ag^{+}, Ga^{3+}, In^{3+}, Pt^{4+}, Cd^{2+}, CrO_4^{2-}, Co^{2+}, Zn^{2+}, and TeO_3^{2-}, inhibited pyocyanin production and thus biofilm formation. Of these metal ions, three ions (Pt^{4+}, Ag + and TeO_3^{2-}) showed antibacterial activity by abolishing cell growth while, Zn^{2+} ($ZnCl_2$) was found to be the most active in hindering production of pyocyanin and formation of biofilm without displaying bactericidal effect (Lee et al. 2014).

Another study supported the above findings in which zinc and ZnO nanoparticles significantly inhibited the formation of biofilm and pyocyanin production, *Pseudomonas* quinolone signal (PQS), pyochelin, and hemolytic activity of *P. aeruginosa* without affecting the growth of planktonic cells. Further, the expression studies showed that ZnO-NPs induced the zinc cation efflux pump *czc* operon along with other transcriptional regulators (porin gene *opdT* and type III repressor *ptrA*), but repressed the pyocyanin-related *phz* operon (Lee et al. 2014).

4.4.7 Metal/Metal Oxide Nanocomposites

Instability and tendency of aggregation that often lead to loss of nano-size and special properties are considered as significant drawbacks for nanomaterials. Therefore, it is essential to prevent the tendency of aggregation and to stabilize nanomaterials (Zare and Shabani 2016). The coating of nanoparticles, fabrication of nanocomposites, or surface modification of NPs are some of the techniques adopted to engineer the nanoparticles to retain or enhance their properties such as antibacterial effects.

Metal and metal-based NPs are used as such or are coated with doping organic or inorganic substances or chemical and biological agents such as surfactants, peptides and proteins, polymers, antibiotics, or other metallic counterparts. All which in turn enhance their properties and impart better stability to the suspension. The overall surface properties and their resultant activities are thus greatly dependent on the composition and nature of these coatings (Mukherjee et al. 2011). Figure 4.1 indicates different functionalization strategies adapted to surface modify the metal- and metal oxide nanoparticles for their further antimicrobial effect.

Fig. 4.1 Metal−/Metal oxide nanoparticle functionalization strategies

The authors demonstrated the photodynamic effect of Zn phthalocyanine-e-polylysine conjugates along with silver and gold nanoparticles (NPs) against *S. aureus*. The photoinactivation was observed to increase with concentration for conjugates. Phthalocyanines exhibited the singlet oxygen pathway causing photooxidative destruction of cellular components like sterols, peptides, and phospholipids (Nombona et al. 2012).

Zinc-based nano-metal organic frameworks (nMOFs) were fabricated and further explored for their antibacterial activities—both as mixture in the presence of antibiotics ampicillin & kanamycin and antibiotics alone. The nMOF/drug mixtures exhibited synergistic and additive effects compared to nMOFs or antibiotics alone, when tested against *E. coli*, *S. aureus*, *Staphylococcus lentus*, and *L. monocytogenes* (Bhardwaj et al. 2018).

Rhamnolipid (RL)-coated silver (Ag) and iron oxide (Fe_3O_4) NPs were prepared and evaluated against biofilm-forming *P. aeruginosa* and *S. aureus* strains. Silver of the dimension 35 nm and Fe_3O_4 NPs of the dimension 48 nm were used for this process. The presence of RLs on the NPs had significant impact in reducing the cell adhesion and biofilm forming properties of the pathogens by modifying the surface hydrophobicity of the bacterial strains. Moreover, the metal NPs generated ROS which potentiated antimicrobial effects along with RLs (Khalid et al. 2019).

Silver nanoparticles (Ag-NPs) anchored to graphene oxide (GO) were fabricated with different ratios and evaluated for their antibacterial potential. The activities of Ag-GO nanocomposites against *E. coli* and *S. aureus* model microorganisms were studied. Ag-GO nanocomposite was very effective at very low dosages of 14 μg/mL against *S. aureus* and 4 μg/mL against *E. coli*. The results also revealed Ag-GO

nanocomposite to be bactericidal causing cell wall disruption for *E. coli* while bacteriostatic for *S. aureus* by inhibiting cell division (Tang et al. 2013).

Authors reported the synthesis of a composite nanomaterial made of silver nanoparticle (AgNPs) implanted in nanofibers of poly-epsilon-caprolactone that were tested against different drug-resistant Gram-positive and Gram-negative microorganisms. Polycaprolactone-silver composites (PCL-AgNPs) showed dose-dependent increase in the antibacterial activity against *K. pneumoniae*, *E. coli*, *P. aeruginosa,* and *S. aureus* but not for *B. subtilis* and *S. mutans* (Pazos-Ortiz et al. 2017).

A study demonstrated the use of biocompatible halloysite nanoclay (HNTs) to intercalate silver nanoparticles (AgNPs) in the presence of curcumin as reducing agent. The resultant AgNPs showed good antimicrobial activity against *Bacillus cereus* and *Escherichia coli* cells (Sudhakar et al. 2017).

Polyvinyl alcohol (PVA) nanofibers containing Ag-NPs were synthesized and investigated for antibacterial activity in wound healing applications. The antibacterial activities of PVA/AgNO3 nanofibers against *S. aureus* and *K. pneumoniae* showed significant reduction of >99.9% after 18 h of incubation (Marega et al. 2015).

Dendritic Fe_3O_4 magnetic nanoadsorbents (DMNA) as spherical particles with 10 nm diameter were synthesized. At low concentration they showed around 97% inhibitory effect against *S. aureus* and good inhibitory activity against *B. subtilis*, *E. coli,* and *P. aeruginosa* strains (Singh and Bahadur 2019).

Sol-gel-based method was used to generate well-distributed hybrid copper nanostructures supported directly on the surface of the silica nanoparticles. These nanostructured particles formulated into a film that eliminated 99% of bio-burden at 0.01% in 6 h. These hybrid Cu-based nanoparticles liberated copper ions giving resultant effect against model organisms *S. aureus* and *E. coli* (Palza et al. 2015).

Graphene oxide was used as a carrier for metal- and metalloid-based nanoparticles (Zn, Cu, Mg, Ag, Se, AgP) to generate nanocomposites. These materials were evaluated for their antibacterial activity against *S. aureus*, MRSA, and *E. coli* strains as model organisms. A highest inhibition of 87.4% was observed with graphene oxide composite made with selenium nanoparticles versus control followed by silver and silver phosphate NPs against *S. aureus*. A dose-dependent increase in the antibacterial response was observed with Se-NPs against test organisms, while the same NPs caused inhibition at highest concentration against Gram negative *E. coli* (Richtera et al. 2015).

One-pot biosynthesis of four nanocomposites (NCs) by using TiO_2–Ag, Ag–TiO_2, Cu–Ag, and Ag–Cu combination was reported, and the NCs were tested for their possible antiquorum sensing, antiplanktonic, antiswarming motility, and antibiofilm activities against MDR strains. Ag–TiO_2 NCs showed strong evidence of decrease in the biofilm roughness, pyocyanin synthesis, and lowering of swarming motility of *P. aeruginosa* than control samples. Antibiofilm strength for these NCs were in the order of Ag–TiO_2>TiO_2–Ag>Cu–Ag>Ag–Cu. Agar diffusion method was used to determine MIC and MBC (Alavi and Karimi 2018).

Au-nanoparticles exhibiting smaller geometry, well-developed surface chemistry, chemical stability, and structural rigidity offer as an ideal tool to study the effects of conjugation on its properties. In a study, authors selected vancomycin as an antibiotic

to load on to Au-nanoparticles (AuNPs) and evaluated their activity against vancomycin-resistant enterococci (VRE). They devised a chemical route to synthesize Au-Van nanoparticles, which showed significantly improved activity against vancomycin-resistant *E. faecium* and *E. coli* (Gu et al. 2003).

In a recent study authors demonstrated synergistic effect of silver-minocycline combination. In the study, a metabolite of minocycline, 4-epi-minocycline, was detected as an active antimicrobial against resistant *P. aeruginosa* by use of a high-throughput screen. Nanoparticles loaded with silver and minocycline were tested against *P. aeruginosa* clinical isolates. Minocycline and silver when used alone were very effective while the combination was superior to individual entities, allowing reduction in dose for the both therapeutics to obtain similar antimicrobial effect (Chen et al. 2019).

In another study, authors proposed use of biocompatible nanomedicines as an alternative to existing antibiotics in which a novel antibacterial system against MDR strains was fabricated. They made use of modified versions of naturally occurring antimicrobial peptides in conjugation with Ag-NPs to create these novel materials. For this, a cysteine residue was introduced either at one of the terminal of the parent peptide as a strategy to enhance binding and antimicrobial potential of the resultant peptide with the AgNPs. The cysteine-tagged NCs showed MIC values of 5–15 μM as compared to 50 μM for peptides devoid of the cysteine residues. NMR spectroscopy and molecular simulations revealed a hydrophobic collapse mechanism triggering pore formation in the bilayer membrane for the improved antibacterial effects of cysteine-tagged NCs. The microbial strains chosen for the study were *K. pneumoniae*, *P. aeruginosa*, and *S. typhi* (Pal et al. 2019). Similar advantages of nanoparticle tagging and as a function of size and shape were studied by Liu et al. (2013) where the importance of cysteine in the conjugation process was demonstrated (Liu et al. 2013).

4.5 Metal Nanoparticles & Multidrug-Resistant Model Strains

Many types of metal NPs, metal oxide nanoparticles and their conjugates are being developed and evaluated against multidrug-resistant pathogens (MDRs) for their antimicrobial potential. In some cases, metal nanoparticles not only showed activity against the MDR strains but also displayed synergy by carrying and delivering cargo such as antibiotics or other natural antimicrobials (Linlin et al. 2017).

In a study, ZnO-NPs of 15 nm size were obtained through biogenic route by use of leaf extract *of Aloe barbadensis* Miller *(A. vera*). Through surface binding and subsequent internalization, the synthesized ZnO-NPs inhibited *S. aureus* and *E. coli*. Significant antibacterial and anti-biofilm potential were also detected against clinical isolates of MRSA and extended spectrum beta-lactamases (ESBL) positive *E. coli, P. aeruginosa*, with the MIC and MBC values of 2400, 2200 μg/ml and 2700, 2300 μg/ml, respectively (Ali et al. 2016).

Two carbapenems (a β-lactam antibiotic)- imipenem (Ipm) and meropenem (Mem) were individually conjugated to surface of Au-nanoparticles by citrate reduction method to maximize its therapeutic antibacterial potential against

drug-resistant pathogens. The nanoparticles of 35 nm size each thus obtained showed maximum activity against carbapenem-resistant Gram-negative clinical isolates - *K. pneumoniae, Proteus mirabilis* and *Acinteobacter baumanii*. A four-fold decrease in MIC of Ipm and a three-fold decrease in MIC of Mem were observed (Shaker and Shaaban 2017).

Gold nanoparticles prepared with the help of chicken egg white protein (CEW) as reducing and stabilizing agent were further coated with 2-mercapto-1-methylimidazole (MMT) molecules. The resultant Au-CEW-MMT nanoparticles were highly active against MDR pathogens. Further in vivo study using a rabbit model proved the nanocomposite to have effective wound healing properties (Lu et al. 2018).

In an unconventional approach, a synergistic effect was observed when antibiotic-tagged gold nanoparticles (AuNPs) and a targeted pulsed laser therapy enhanced antibiotic efficacy against MDR strains. AuNPs- in combination with gentamicin or amikacin antibiotics and targeted pulsed laser therapy caused a significant 4 to 5-log reduction in the viability of methicillin-resistant *S. aureus* and *P. aeruginosa* biofilms, respectively, compared to ~1 log reduction of when treatments were used alone (Kirui et al. 2019).

Spherical silver nanoparticles of 20–50 nm dimensions were synthesized (AgNPs) through a rapid, single pot bio-reduction method making use of *Nocardiopsis* sp. GRG1 (KT235640) biomass. The synthesized AgNPs showed antibacterial and anti-biofilm activities against a clinical isolate *Staphylococci,* at 5–60 µg/mL (Rajivgandhi et al. 2019a).

Silver nanoparticles produced using marine *Streptomyces* sp. Al-Dhabi-89 showed activity against *E.coli, P. aeruginosa*, *K. pneumoniae* and clinical drug-resistant microbial isolates MRSA and *P. mirabilis*. The NPs thus produced had cubic dimensions with 11–21 nm size range (Al-Dhabi et al. 2018).

Chang et al., reported use of glucose and trimethyl chitosan nitrate as reducing and stabilizing agent for the biosynthesis of trimethyl chitosan nitrate-capped silver nanoparticles (TMCN-AgNPs). The TMCN-AgNPs possessed antibacterial activity against *P. aeruginosa*, *E. coli*, and *S. aureus* at a concentration lower than 6.13 µg/mL and showed MIC of 12.25 µg/mL against multidrug-resistant *A. baumannii* strain (Chang et al. 2017).

Bacterial exopolysaccharide stabilized AgNPs were synthesized of 2–15 nm size range and were tested to be active against multidrug-resistant pathogens *P. aeruginosa* and *K. pneumoniae* (Kanmani and Lim 2013). In another study, biosynthesis of AgNPs was carried out using leaf extract of *Mukia scabrella* to obtain nanoparticles of 18–21 nm size with spherical shape. MDR- strains of *Acinetobacter* sp., *K. pneumoniae*, and *P. aeruginosa* were found to be sensitive in presence of these AgNPs (Prabakar et al. 2013).

A study reported synergistic action of chitosan along with zinc oxide nanomicelles (CZnO-NPs) against MDR strain for their complete elimination of test pathogens such as *E. coli* BAA-2471 and *E. faecium* 1449 (Bui et al. 2017).

Mehta et al. (2019) performed mechanistic studies using LIVE/DEAD viability assay, fluorescence imaging, 3D confocal microscopy and cytotoxicity assay to determine effective dose (ED50) of the miceller CZnO-NPs for elimination of the

MDR *E. faecium* 1449 as model. The results revealed that within 24 h the CZnONPs achieved 50.22% biofilm reduction compared to 15.66% with chitosan and 13.94% of ZnO alone. The chitosan coated ZnO-NPs (CZnONPs) showed promising action on MDR bacterial biofilms. The synergistic action has been attributed to the chitosan shell of CZNPs for controlled release of ZnO in the bacterial cell, whereby ZnO showed antibacterial property against Gram-positive species and also to chitosan's inherent antimicrobial activity mainly against Gram negative strains (Mehta et al. 2019).

Authors in a recent study showed for the first time pectin-capped biogenic platinum NPs (PtNPs) to cause selective loss of plasmid from capsulated strain of *E. coli* U3790 strain. These pectin-coated PtNPs acted as plasmid curing agent and showed a significant decline in MIC of 16-64 fold for meropenem and ceftriaxone in carbapenem-esistant *Escherichia coli* (CREC). Both in vitro and in vivo the plasmid cured strain showed smaller colonies and slower growth. It also lead to drastic reduction in bacterial bioburden by 2.4 log CFU compared to meropenem treatment alone. Acquisition of plasmid from wild-type strain into cured strain restablished drug-resistant phenotype. Mechanistic studies revealed that nanoparticles did not induce ROS formation but interacted with cell surface and perturbed inner membrane integrity (Bharathan et al. 2019).

In another study, nano-antibiotics (AgNAs) were prepared by the self-assembly of ultra-small silver-nanoclusters and biofilm-responsive polymeric ligands. The resultant AgNAs were responsive to the acidic conditions prevalent in the biofilm which caused ligand protonation and later their disaggregation. This feature of the AgNAs allowed enhanced retention, better penetration, and accelerated leaching of silver ions thus effectively killing the bacteria inside the biofilm, in comparison to regular antimicrobial agents which show limited permeation and therapeutic activity. These effects were confirmed on a methicillin-resistant *Staphylococcus aureus* (MRSA) infection model both in vitro and later in vivo which significantly reduced mortality rate of mice with biofilm-induced severe pyomyositis (Wu et al. 2019).

Authors in their study combined the antimicrobial effects of silver ion with selective toxicity of branched polyethylenimine (bPEI) to effectively kill MDR pathogenic strains by fabricating branched polyethylenimine-functionalized silver nanoclusters (bPEI-AgNCs). The MIC of bPEI-AgNCs was 10–15 folds lesser than that of PEI alone and 2–3 fold lower than $AgNO_3$ alone when determined against uropathogenic MDR strains (Huma et al. 2018).

In another study, glutathione-stabilized silver nanoparticles (GSH-AgNPs) were synthesized and their activity was evaluated against multidrug-resistant *Campylobacter* strains isolated from the chicken and patients. The MIC and minimal bactericidal concentration (MBC) were found to be 4.92–39.4 μg/mL and 9.85–39.4 μg/mL, respectively and the GSH-AgNPs remarkable activity against all MDR *Campylobacter* strains tested (Silvan et al. 2018).

ZnO nanoparticles (ZnO-NPs) of 30 nm dimension were synthesized and evaluated using dilution and disk diffusion assays to confirm their antimicrobial potential against carbapenem-resistant *A. baumannii*. The resultant ZnO-NPs demonstrated good antibacterial activity against the strain selected. Production of ROS triggering

leakage of cellular contents was proposed as the mechanism of reduction in cell viability in the pathogen (Tiwari et al. 2018). In another study, ZnO-NPs of 70 nm size with spherical or rod-shapes were prepared and tested at different concentrations as antimicrobial agent against a notorious foodborne pathogen *E. coli* O157:H7. The microbial growth inhibited at the MIC of 12 mmol/L. ZnO- NPs showed significant antimicrobial activity against *E. coli* O157:H7 by causing leakage of cellular contents and lysing the bacterial cells (Liu et al. 2009).

Bankier et al. (2018) engineered nanoparticle combinations (NPCs) by using silver-, copper nanoparticles (Ag-, Cu-NPs), and tungsten carbide (WC). Several literature is available on strong antimicrobial activity of NPCs against vast number of bacterial species. Different methods like plate count assay, viability assay by flow cytometry, and qPCR were used to evaluate the antimicrobial activity of prepared NPCs on *S. aureus* and *P. aeruginosa* bacteria at 0.05–0.25% concentration. The plate assay and flow cytometry confirmed the antimicrobial effects of NPCs to the extent of >8 log reduction (Bankier et al. 2018).

Ouyang et al. (2018) evaluated the use of black phosphorus (BP) together with AgNPs against methicillin-resistant *Staphylococcus aureus* (MRSA) model. Black phosphorus was used as a substrate, a stabilizing and a reducing agent and BP nanosheets were coated with AgNPs through an in situ growth method. Combination of near-infrared (NIR) light irradiation triggered the photothermal effect of the black phosphorus in Ag-BP nanohybrids and released Ag+ ions gradually to cause rapid disruption of bacterial membrane with enhanced the antibacterial effect. Further, the in vivo studies in mice model proved Ag-BP nanohybrids to be efficient in decreasing the MRSA bio-burden and thus reduce infection-related tissue lesions (Ouyang et al. 2018). The recent results of use of nanoparticles and nanomaterials against multidrug-resistant bacteria have been reviewed in detail by (Baptista et al. 2018). The use of some NPs targeting against MDR efflux pumps have been recently reviewed by Hasani et al. (2019) (Hasani et al. 2019). The Table 4.2 summarizes few examples of metal and metal oxide NPs against MDR strains as models.

4.6 Metal Nanoparticles and Plausible Toxicity or Resistance

Metal, metal oxides and other engineered forms of nanoparticles are an emerging class of antibacterials, finding their way to provide alternatives to antibiotics and also acting against resistant pathogens. Though, these materials are produced in large quantities globally, their impact on environment and life-forms warrants detailed evaluation studies in this direction. A study was carried out on various metal and metal oxide particles (TiO_2, Cu, CuO, Zn, ZnO, Fe_2O_4, Fe_3O_4, Fe_2O_3), and the toxicity was evaluated to that of multiwalled carbon nanotubes and carbon nanoparticles with the use of human lung epithelial cell line A549. The CuO-NPs were labeled to be most toxic and could cause severe DNA damage along with oxidative lesions. The toxicity was further pronounced in the presence of ZnO and TiO_2

Table 4.2 Representative examples of metal/ metal oxide NPs against MDR strains

Metal/Metal oxide NPs	Method of synthesis	Size	Shape	MDR Model organisms	Antibacterial mechanism	Reference
AgNPs	Single-pot biological reduction method- *Nocardiopsis* sp. GRG1 (KT235640) biomass	20 and 50 nm	Spherical	Positive methicillin-resistant coagulase-negative *Staphylococci*	Cellular damage	Rajivgandhi et al. (2019a)
Ag	Green synthesis- trimethyl chitosan nitrate-capped silver nanoparticles (TMCN-AgNPs)	81.2 nm	–	Gram-positive, Gram-negative, and *A. baumannii* MDR strains	Cellular damage	Chang et al. (2017)
AgNPs	Biosynthesis- silver tolerant mediated by bacteria (AgTB)	17.51 nm	Spherical	ESKAPE pathogens	Cell damage	Khan et al. (2019)
AgNPs	Biosynthesis- extracts of *Citrus maxima* plant	~120–400 nm	–	*B. cereus* and MDR- *S. enteritidis*	Cell damage	Jha et al. (2017)
AgNPs	Green synthesis- aqueous leaf extract of *Corchorus capsularis* (CRCP)	Average size of 20.52 nm	Spherical	*P. aeruginosa*, *S. aureus*, isolates from postsurgical wound infections	Cell damage	(Prabakar et al. (2013)
ZnO	Green synthesis- *Aloe vera* extract	15 nm	Spherical	Extended spectrum beta lactamases (ESBL) positive *E. coli*, *P. aeruginosa*, and MRSA clinical isolates	Cell damage	Ali et al. (2016)
CuO	Green synthesis- *Camilla japonica* plant leaf extract.	~15–25 nm	Spherical	Extended-spectrum beta-lactamases	Damage to bacterial membrane	Rajivgandhi et al. (2019b)

particles. While no or low toxicity was observed for remaining NPs. The carbon-nanotubes showed cytotoxicity at the lowest tested dose (Karlsson et al. 2008).

Another study investigated TiO_2 nanoparticles-induced genotoxicity, inflammation and oxidative DNA damage in a mice model. The results indicated that the NPs induced genotoxicity in vivo thus raising concerns about health hazards due to TiO_2 NPs exposure (Trouiller et al. 2009). Moreover, there are some findings that reported development of bacterial resistance to metal NPs. For instance, a recent study provided insights on impact of NPs on spread of antibiotic resistance. The results indicated that both metal and its metal oxide- i.e., copper (Cu^{2+}) and copper oxide nanoparticles (CuO-NPs) were able to stimulate multiple-drug resistance genes via the conjugative transfer. At sub-inhibitory or environmental concentrations, the plasmid-mediated antibiotic resistance genes were found to be significantly upregulated (Zhang et al. 2019).

Such evidences are a matter of greater concern and need elaborate studies to quantify and postulate mechanisms of resistance and toxicity in future.

4.7 Conclusion

The recent scientific advances in the nanotechnology domain with special reference to metal/metal oxide nanoparticles and nanocomposites as antibacterial agents have opened up new opportunities to combat infections that are caused by bacterial pathogens. The findings summarized in present body of work as a function of - nature of metal, shape, size, concentration, and nature of conjugation, show promising results in support of these nanomaterials to act against microbial species and their multidrug-resistant variants. Likewise, some observations reported recently suggest plausible health hazard based on their capability to cross cellular barriers and risk of development of resistance among microbial pathogens against metal NPs. As some of these nanomaterials are already in the consumer market, for other diverse applications, the future research and actions should be directed towards systematic evaluation of its benefits and its effects on human health. It thus demands caution to be exercised before the large-scale exposure of these wonder materials in an environmental sphere and food-chain continuation of nature.

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Modelling Nanoparticles Parameters for Antimicrobial Activity

5

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Abstract

The current study reveals the antimicrobial activity of various nanoparticles (NPs) against numerous microorganisms through statistical models that define suitable parameters to improve the antimicrobial efficacy of NPs. The antimicrobial data on NPs were collected from previously published studies, focusing on parameters such as the NPs type and size (nm), microbial strains and their initial density

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($O.D._{600nm}$), inhibition zone (IZ) size (mm), contact time (h), well and disc diffusion size (mm) and minimum inhibitory concentration (MIC) (μg/mL). A correlation between these parameters was modelled by using a multiple correspondence analysis (MCA) and a principal component analysis (PCA) for qualitative and quantitative analysis, respectively. Results showed a significant positive correlation between the IZ size and the following parameters: MIC, well size and disc diffusion size with a Pearson ratio of 95.98%, 93.99% and 94.82% ($\alpha = 0.5$), respectively. Antimicrobial efficacy by Ag, SiO_2 and ZnO NPs with a significant IZ for various gram positive bacterial strains was demonstrated. In addition, gram negative bacteria and fungi were deactivated by La-ZnO and AgNPs. Antimicrobial tests with NPs could be improved by varying the NPs concentration for improved efficacy. The NPs type should also be chosen as a function of the target bacteria characteristics, i.e. gram staining, for higher efficacy.

Keywords
Antimicrobial activity · Multiple correspondence analysis · Nanoparticles · Principal component analysis · Statistical modelling

5.1 Introduction

The increase in anthropogenic activities such as industrialisation has serious impacts on the environment and its natural resources (Mao et al. 2019). Natural resources are the primary receptors of anthropogenic activity. Water and soil are the most affected resources by these activities and their quality and pristine nature deteriorates and subsequently results in adverse impact on human health (Razanamahandry et al. 2017). For instance, pathogenic microorganisms easily grow in the modified ecosystems with degraded natural resources (Mekuto et al. 2018). Thus, the remediation of these resources using various technologies is advisable. Bioremediation and phytoremediation are among the most applied technologies (Razanamahandry et al. 2016), as they are classified as environmentally benign.

Recently, nanosciences and nanotechnology have been developed to restore the pristine condition of the polluted environment with an aim to minimise the human health challenges (Wang 2012; Webster and Seil 2012). As an example, photocatalytic methods using nanomaterials in numerous studies demonstrated the removal of several pollutants affecting water and soil resources. Rhodamine B, methyl orange and methylene blue were removed by using HAp-TiO_2 nanocomposites (Kaviyarasu et al. 2017b), ZnO nanoparticles (NPs) (Siripireddy and Mandal 2017), and SnO_2 including CuO-NPs (Diallo et al. 2016; Mbu et al. 2018), respectively. In addition, Nwanya et al. (2019) have also reported the effectiveness of CuO-NPs to remediate industrial textile effluents. In terms of human health, growth of pathogenic microorganisms was inhibited by different types of nanomaterials. *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* are among some pathogenic microorganisms responsible for various human diseases such as diarrhoea, meningitis,

septicaemia and many other infections (Makvana and Krilov 2015; Tong et al. 2015). Several nanoparticles were reported as effective against these microorganisms' proliferation. Ahmad et al. (2018) have conducted research on the inhibition of the growth of *Escherichia coli* using ZnO NPs; albeit, a small inhibition zone (13 mm) was obtained. Although, the same microorganism was tested by Karthik et al. (2017) using the same nanoparticle type and the results showed that the obtained inhibition zone was large (30 mm). Besides, several authors have shown the effectiveness of the following NPs; Co(Vox), Cu, Fe_3O_4, Ag and CdO to inhibit the growth of *E. coli*, *B. subtilis* (Yoon et al. 2007), *S. epidermidis*, *Vibrio cholerae* (Morones et al. 2005) and *P. fluorescens* (Jiang et al. 2009), respectively. These research studies were conducted under different conditions. Webster and Seil (2012) has reported that bacteria species, gram straining and minimum inhibitory concentrations (MIC) are among the main parameters that should be considered for nanoparticles' antimicrobial applications. Besides, antimicrobial effectiveness of NPs could be evaluated by a well or disc diffusion assay (Valgas et al. 2007). In addition, the average size, the initial optical density of the pathogen and the contact time could influence the effectiveness of the NPs for antimicrobial activity. Nevertheless, there is a lack of studies conducted to modelling these interlinked complex parameters to assess key variable parameter variation and the role they play for inhibition effectiveness against pathogenic microorganism proliferation. These variables are complex and must be explored as combinatorial factors using mathematical and statistical modelling. Available statistical tools could therefore be used to model the identified parameters, which must be considered to obtain the highest antimicrobial effectiveness of different nanoparticles.

As such, qualitative and quantitative input variables for various research studies were resolved by using statistical tools, i.e. multiple correspondence analysis (MCA) and principal component analysis (PCA). Dungey et al. (2018) highlighted the application of MCA to modelling qualitative variables into relational moieties of significance. Moreover, PCA can be applied to reduce quantitative variables in order to optimise and to only consider variables that are highly beneficial to human health (Yacoub et al. 2013). Various applications were conducted by using MCA and PCA analyses in hydrological studies (Van Stan et al. 2016) and in human health survey (Ayele et al. 2015). The lack of statistical modelling application was perceived as a limitation for nanomaterials application in antimicrobial efficacy studies. Therefore, the aim of this research was to find nanomaterials suitable for the elimination of pathogenic microorganisms and to identify the suitable parameters which should be considered for high antimicrobial efficacy in future applications.

5.2 Materials and Methods

5.2.1 Input Parameters

Recent data (n = 123) on the antimicrobial efficacy of nanoparticles was sourced from previously published studies (Tables 5.1 and 5.2). Parameters, which are related to the antimicrobial application of different nanoparticle types, via surface

Table 5.1 Qualitative input data for MCA analysis

NPs	Gram staining of bacteria		Fungi
	Negative	Positive	
ZnO	1[a,c,m]	1[b,n]	1[d]
Cu28-Fe72	1[a,c,]	1[b,e]	0
Ta_2O_5	1[a,]	0	0
Ag	1[a,c,j,k,l]	1[b,f–i]	1[d,]
La-ZnO	1[j,o]	0	0
Co(Vox)	1[a,k]	1[b,e]	0
ZnO/TiO_2	1[a,q]	1[b,p]	0
CeO_2	1[a,m]	1[b,n]	0
β-$CoMoO_4$-Co_3O_4	1[a,c]	1[b]	0
CeO_2/CdO	1[c]	0	0
HAp-TiO_2 (8 dip)	1[a]	1[r]	0
Cu	1[a]	1[h]	0
Fe_3O_4	0	1[b,s]	0
Al_2O_3	1[a,t]	1[h]	0
TiO_2	1[a]	0	0
SiO_2	1[a,t]	1[b]	0
CuO	1[a,c]	1[b,y]	0

Microorganisms (references). a: *E. coli* (Ahmad et al. 2018; Jiang et al. 2009; Karthik et al. 2017; Kaviya et al. 2011; Kaviyarasu et al. 2017a, b, c; Kennedy et al. 2017; Kim et al. 2007; Maria Magdalane et al. 2018; Meidanchi and Jafari 2019; Mobeen Amanulla et al. 2018; Morones et al. 2005; Nair et al. 2009; Nwanya et al. 2019; Padmavathy and Vijayaraghavan 2008; Pal et al. 2007; Simo et al. 2018; Simon-Deckers et al. 2009; Sondi and Salopek-Sondi 2004; Webster and Seil 2012; Yoon et al. 2007; Zhang et al. 2019). b: *S. aureus* (Ahmad et al. 2018; Jiang et al. 2009; Jones et al. 2008; Karthik et al. 2017; Kaviya et al. 2011; Kaviyarasu et al. 2017a, b, c; Kennedy et al. 2017; Kim et al. 2007; Maria Magdalane et al. 2018; McCarthy et al. 1992; Mobeen Amanulla et al. 2018; Nair et al. 2009; Nwanya et al. 2019; Reddy et al. 2007; Salomoni et al. 2015; Simo et al. 2018; Tran et al. 2010; Webster and Seil 2012; Zhang et al. 2019). c: *P. aeruginosa* (Bakina et al. 2019; Kaviya et al. 2011; Kennedy et al. 2017; Maria Magdalane et al. 2017; McCarthy et al. 1992; Mobeen Amanulla et al. 2018; Morones et al. 2005; Nwanya et al. 2019; Webster and Seil 2012). d: *Candida albicans* (McCarthy et al. 1992; Webster and Seil 2012; Zhang et al. 2019). e: *MRSA* ATCC 43300 (Bakina et al. 2019; Simo et al. 2018). f: *B. cereus* (Kennedy et al. 2017). g: *Micrococcus luteus* (Kennedy et al. 2017). h: *B. subtilis* (Jiang et al. 2009; Kennedy et al. 2017; Webster and Seil 2012; Yoon et al. 2007). i: *Enterococcus sp* (Kennedy et al. 2017). j: *Salmonella typhi* (Kennedy et al. 2017; Manikandan et al. 2017; Morones et al. 2005; Webster and Seil 2012). k: *Klebsiella pneumoniae* (Kennedy et al. 2017; Simo et al. 2018). l: *V. cholerae* (Morones et al. 2005; Webster and Seil 2012). m: *E. hermannii* (Kaviyarasu et al. 2017a, b, c; Maria Magdalane et al. 2018). n: *S. pneumoniae* (Kaviyarasu et al. 2017a, b, c; Maria Magdalane et al. 2018). o: *Proteus mirabilis* (Manikandan et al. 2017). p: *Streptococcus mutans* (Kaviyarasu et al. 2017a). q: *Salmonella* sps (Kaviyarasu et al. 2017a). r: *Bacillus* spp (Kaviyarasu et al. 2017b). s: *S. epidermidis*[6,17]. t: *P. fluorescens* (Jiang et al. 2009; Webster and Seil 2012)y: *Bacillus licheniformis* (Nwanya et al. 2019)

1 data available, *0* data not available

Table 5.2 Quantitative input data for PCA

NPs	Average size (nm)	Inhibition zone (mm)	Inhibition ratio (%)	Well size (mm)	Disc size (mm)	Contact time (h)	Optical density initial (CFU)	MIC (μg/mL)
ZnO	20	25.13 ± 0.0	–	7	10	24	–	10^5
	20	5[a]	–	7	10	24	–	10^5
	90	30.17 ± 0.0	–	–	–	13	5×10^6	–
	90	3[b]	–	–	–	13	5×10^6	–
	9	13 [a]	–	–	–	24	–	400
	9	16 [b]	–	–	–	–	–	200
	9	16[m]	–	–	–	–	–	200
	9	15 [b]	–	–	–	–	–	200
	13	5[n]	95[b]	–	–	–	–	80
	60	4.5[a]	50[b]	–	–	–	–	400
	40	–	99[b]	–	–	–	–	400
	40	–	99[a]	–	–	–	–	400
	12	–	90[a]	–	–	–	–	400
	–	–	100[c]	–	–	–	–	1917
	–	–	100[b]	–	–	–	–	9
	–	–	100[d]	–	–	–	–	39
		–						
		–						
Cu28-Fe72	70	–	–	–	–	16	–	125[a]
	70	–	–	–	–	16	–	125[b]
	70	–	–	–	–	16	–	250[c]
	70	–	–	–	–	16	–	250[e]
Ta_2O_5	12.5	–	–	–	–	24	7.05 ± 0.05[a]	–

(continued)

Table 5.2 (continued)

NPs	Average size (nm)	Inhibition zone (mm)	Inhibition ratio (%)	Well size (mm)	Disc size (mm)	Contact time (h)	Optical density initial (CFU)	MIC (μg/mL)
Ag	–	–	90	–	6	–	10^7	15.84[a]
	–	–	80	–	6	–	10^7	31.68[c]
	–	–	70	–	6	–	10^7	31.68[d]
	–	18[f]	–	–	–	24	–	–
	–	16[b]	–	–	–	24	–	–
	–	16[g]	–	–	–	24	–	–
	–	20[h]	–	–	–	24	–	–
	–	18[i]	–	–	–	24	–	–
	–	21[c]	–	–	–	24	–	–
	–	17[j]	–	–	–	24	–	–
	–	22[a]	–	–	–	24	–	–
	–	17[k]	–	–	–	24	–	–
	–	21[a]	100	–	–	–	–	75
	–	21[l]	100	–	–	–	–	75
	–	21[j]	100	–	–	–	–	75
	–	21[c]	100	–	–	–	–	75
	–	50[a]	99	–	–	–	–	0.1
	–	12[a]	70	–	–	–	–	
	–	13.5[a]	100	–	–	–	–	0.356
	–	13.5[b]	100	–	–	–	–	3.56
	–		100[b]	–	–	12	–	5
	35	12.5[a]	–	10	–	24	–	50
	35	11.7[c]	–	10	–	24	–	50
	35	7.8[b]	-	10	–	24	–	50
	10	16[a]	–	10	–	24	–	50
	10	13.4[c]	–	10	–	24	–	50
	10	9.2[b]	–	10	–	24	–	50

La-ZnO	12.91	22[o]	–	3	–	24	–	86.53×10^6
	12.91	7[j]	–	3	–	24	–	86.53×10^6
Co(Vox)	150	–	50	–	–	24	–	1.8×10^3 [a]
	150	–	50	–	–	24	–	1.8×10^3 [e]
	150	–	50	–	–	24	–	1.2×10^3 [b]
	150	–	50	–	–	24	–	1.7×10^3 [k]
ZnO/TiO_2	24.6	16	–	–	–	24	–	15×10^3 [a]
	24.6	17	–	–	–	24	–	15×10^3 [c]
	24.6	20	–	–	–	24	–	15×10^3 [p]
	24.6	15.5	–	–	–	24	–	15×10^3 [q]
CeO_2	20	10	–	–	–	24	–	200[n]
	20	15	–	–	–	24	–	200[m]
	20	4	–	–	–	24	–	200[b]
	20	4	–	–	–	24	–	200[a]
β-$CoMoO_4$-Co_3O_4	–	20[a]	–	6	–	24	–	50×10^3
	–	19[c]	–	6	–	24	–	50×10^3
	–	18[b]	–	6	–	24	–	50×10^3
CeO_2/CdO	10	33[c]	–	–	–	–	–	200
HAp-TiO_2 (8 dip)	10	15[a]	–	–	–	24	–	–
	10	15[r]	–	–	–	24	–	–
Cu	–	100[a]	90	–	–	–	–	33.40
	–	100[h]	90	–	–	–	–	28.20
CuO	54.5	15[a,b,c,y]	–	–	–	24	–	–
	18	10 [a,b,c,y]	–	–	–	24	–	–
	60	7 [a,b,c,y]	–	–	–	24	–	–
Fe_3O_4	–	9[b]	100	–	–	24	–	3×10^3
	–	8[s]	65	–	–	24	–	2×10^3

(continued)

Table 5.2 (continued)

NPs	Average size (nm)	Inhibition zone (mm)	Inhibition ratio (%)	Well size (mm)	Disc size (mm)	Contact time (h)	Optical density initial (CFU)	MIC (μg/mL)
Al_2O_3	–	11	35[a]	–	–	24	–	10
	–	11	70[a]	–	–	24	–	100
	–	11	68[a]	–	–	24	–	500
	–	60	36[a]	–	–	24	–	20
	–	60	57[h]	–	–	24	–	20
	–	60	70[t]	–	–	24	–	20
TiO_2	–	17	0[a]	–	–	24		10
	–	17	35[a]	–	–	24		100
	–	17	80[a]	–	–	24		500
SiO_2	–	20	58[a]	–	–	24		20
	–	20	40[h]	–	–	24		20
	–	20	70[t]	–	–	24		20

Microorganisms (references). a: *E. coli* (Ahmad et al. 2018; Jiang et al. 2009; Karthik et al. 2017; Kaviya et al. 2011; Kaviyarasu et al. 2017a, b, c; Kennedy et al. 2017; Kim et al. 2007; Maria Magdalane et al. 2018; Meidanchi and Jafari 2019; Mobeen Amanulla et al. 2018; Morones et al. 2005; Nair et al. 2009; Nwanya et al. 2019; Padmavathy and Vijayaraghavan 2008; Pal et al. 2007; Simo et al. 2018; Simon-Deckers et al. 2009; Sondi and Salopek-Sondi 2004; Webster and Seil 2012; Yoon et al. 2007; Zhang et al. 2019). b: *S. aureus* (Ahmad et al. 2018; Jiang et al. 2009; Jones et al. 2008; Karthik et al. 2017; Kaviya et al. 2011; Kaviyarasu et al. 2017a, b, c; Kennedy et al. 2017; Kim et al. 2007; Maria Magdalane et al. 2018; McCarthy et al. 1992; Mobeen Amanulla et al. 2018; Nair et al. 2009; Nwanya et al. 2019; Reddy et al. 2007; Salomoni et al. 2015; Simo et al. 2018; Tran et al. 2010; Webster and Seil 2012; Zhang et al. 2019). c: *P. aeruginosa* (Bakina et al. 2019; Kaviya et al. 2011; Kennedy et al. 2017; Maria Magdalane et al. 2017; McCarthy et al. 1992; Mobeen Amanulla et al. 2018; Morones et al. 2005; Nwanya et al. 2019; Webster and Seil 2012). d: *C. albicans* (McCarthy et al. 1992; Webster and Seil 2012; Zhang et al. 2019). e: *MRSA* ATCC 43300 (Bakina et al. 2019; Simo et al. 2018). f: *B. cereus* (Kennedy et al. 2017). g: *M. luteus* (Kennedy et al. 2017). h: *B. subtilis* (Jiang et al. 2009; Kennedy et al. 2017; Webster and Seil 2012; Yoon et al. 2007). i: *Enterococcus sp* (Kennedy et al. 2017). j: *S. typhi* (Kennedy et al. 2017; Manikandan et al. 2017; Morones et al. 2005; Webster and Seil 2012). k: *K. pneumoniae* (Kennedy et al. 2017; Simo et al. 2018). l: *V. cholerae* (Morones et al. 2005; Webster and Seil 2012). m: *E. hermannii* (Kaviyarasu et al. 2017a, b, c; Maria Magdalane et al. 2018). n: *S. pneumoniae* (Kaviyarasu et al. 2017a, b, c; Maria Magdalane et al. 2018). o: *P. mirabilis* (Manikandan et al. 2017)

p: *S. mutans* (Kaviyarasu et al. 2017a). q: *Salmonella* spp (Kaviyarasu et al. 2017a). r: *Bacillus* spp (Kaviyarasu et al. 2017b). s: *S. epidermidis*[6,17]. t: *P. fluorescens* (Jiang et al. 2009; Webster and Seil 2012).y: *B. licheniformis* (Nwanya et al. 2019)

– data not available

active ($m^2\ g^{-1}$), average size (nm), maximum inhibition zone size (mm), inhibition Ratio (%), initial (O.D. initial) and final (O.D. final) microorganism optical density, well and disk diffusion size (mm), contact time (h), nanoparticle concentration (μg/mL) and the microorganism species, were considered for the multi-criteria analyses. Statistical methods such as the MCA and PCA were used for the 123 observations to follow the association and the variation of qualitative and quantitative data, respectively. These parameters were chosen based on the considered criteria for testing the effectiveness of nanomaterials to inhibit microbial growth.

5.2.2 Multiple Correspondence Analysis (MCA)

MCA is a statistical approach to study the association and affinity, between categorical data (Dungey et al. 2018). This method uses a binary code matrix (0 or 1) to evaluate the standard correspondence between the rows and columns of the categorical data (Van Stan et al. 2016). The association between the qualitative variables, which are formed by the nanoparticles and the gram stain type of the microorganism species, was studied by choosing the MCA. A map was drawn to visualise the distances between these categories of the qualitative variables in to two dimensions with $F1$ and $F2$ axis for X and Y axis, respectively. The association between the qualitative variables was appreciated by their affinity to the axis F1 and F2 and by their distance from the axis origin. MCA considers the ($d_I^2\left(i,0\right)$) squared distance of the ith row profile from the axis origin O, which is the ith row profile Euclidean distance (Eq. 5.1) (Costa et al. 2013):

$$d_I^2\left(i,0\right)=\sum_{m=1}^{M*} f_{im}^2 \tag{5.1}$$

Where, M is the dimension correspondence plot; when the distance of the ith row profile is great, the profile of the category i would deviate the column and row categories average profile. Variables that have a same direction from the origin are highly associated (Dungey et al. 2018). f_{im}^2 is the (i, m)th element of F. F is the derivation of the coordinates of row profile for singular ordered correspondence analysis using BMD[26]. Table 5.1 enlists qualitative input data for MCA.

5.2.3 Principal Component Analysis (PCA)

PCA is a statistical approach to study the correlation and the variance between continuous variables (Ayele et al. 2015). PCA was used to study the variance between the quantitative variables such as the nanoparticle size, the inhibition zone size, eliminated microorganisms' characteristics, the well size, the disc size, the contact time, the microorganism initial density and the initial concentration of the nanoparticles. The PCA method uses an orthogonalisation technique than a distance measure which is used in the MCA (Dungey et al. 2018). Two hypotheses, which are the null (H_0) and alternative hypothesis (H_a), were considered in the PCA. H_0 defines

the absence of significant correlation between the variables. H_a states that at least there are some correlations between the studied variables. Bartlett's sphericity test was run to evaluate the validity of each hypotheses. A Pearson (n) matrix was chosen to show the correlation between these quantitative variables. A Pearson ratio α_{ij} for each (i, j)th cell entry as described by Goodman (1985) was used to measure the departure from row i and column j independence, according to Eq. 5.2:

$$\alpha_{ij} = \frac{p_{ij}}{p_{i.}, p_{.j}} \tag{5.2}$$

The (i, j)th matrix Δ cell value is the Pearson ration α_{ij}. Δ is defined by Eq. 5.3:

$$\Delta = D_I^{-1} P D_J^{-1} \tag{5.3}$$

Where, D_I: is the $I \times I$ diagonal of Δ with (i, i)th cell entry named p_i; and D_J is the $J \times J$ diagonal of Δ with (j, j)th cell entry named $p_{.j}$.

If $\Delta = I \times J = 1$, J columns and I rows are independent. A Pearson ratio value with is significantly different from 1 defines the dependence between I rows and J columns. Table 5.2 enlists quantitative input data for PCA.

Software XLSTAT v.15.1 was used to treat the data for PCA and MCA with 95% as confidence interval.

5.3 Results and Discussion

5.3.1 Microorganism Affinity with Nanoparticles

MCA showed the affinity between the microorganisms and the nanoparticles, indicating a variables matrix coefficient belonging to each $F1$ and $F2$ axis as shown in Table 5.3. Bold values are significant at the level $\alpha = 0.05$. $F1$ axis has significant values, which regroups bacteria, which are gram negative and gram positive with nanoparticles Ag, La-ZnO and SiO_2. However, bacteria which are gram negative were determined to be deactivated by La-ZnO NPs. A similar trend was observed for gram positive bacteria and Ag-including SiO_2-NPs. Axis $F2$ was defined by the following significant parameters: fungi, gram positive bacteria, Ag-and ZnO-NPs. Nevertheless, a similar trend was observed for fungi and AgNPs including for gram positive bacteria and the ZnO-NPs. A correlation was deemed feasible for the parameters which have a similar trend (Costa et al. 2013).

Figure 5.1 presents the MCA plot for the qualitative variable for MCA. A majority of the variables belonging to the $F1$ axis explained 80.86% of the data and only 19.14% of the data were explained by the $F2$ axis. The data spread was homogenous around the axis centre. Only fungi were far from the axis centre, which meant that this parameter behaved differently to the mean of the observable data (Dungey et al. 2018). However, fungi and Ag-NPs had a similar trend, which formed the first group (encircled with large dash line pink colour). In fact, Fig. 5.1 confirmed the affinities between the microorganisms and the nanoparticles by regrouping the parameters which have the same trend (Ayele et al. 2015). Three groups were observed in Fig. 5.1. The first

Table 5.3 Variables matrix coefficient for multiple correspondence analysis (MCA) of each qualitative parameter

Qualitative variables	*F*1	*F*2
Fungi	1.5427	**−5.3448**
Gram bacteria-negative	**−5.9618**	−0.5980
Gram bacteria-positive	**5.5575**	**2.0782**
NPs-Ag	**2.8783**	**−4.8522**
NPs-AgNPs nanocellulose	−1.8836	−0.3883
NPs-CeO_2	0.2536	1.2868
NPs-CeO_2/CdO	−1.8836	−0.3883
NPs-Co (Vox)	−1.8148	0.2431
NPs-Cu28-Fe72	−0.9646	0.5066
NPs-HAp-TiO_2 (8 dip)	0.1757	0.8915
NPs-La-ZnO	**−2.6903**	−0.5546
NPs-SiO_2	**2.1296**	1.6367
NPs-Ta_2O_5	−1.8836	−0.3883
NPs-ZnO	1.7893	**3.1015**
NPs-ZnO/TiO_2	−0.9646	0.5066
NPs-β-$CoMoO_4$-Co_3O_4	−0.9646	0.5066

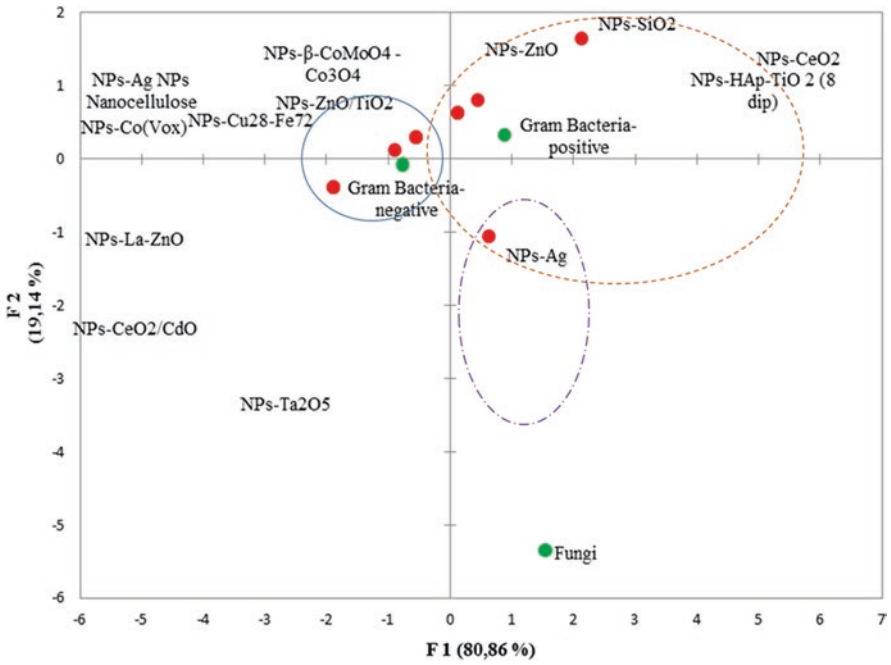

Fig. 5.1 Principal multiple correspondence analysis (MCA) plot for the variable qualitative of the nanoparticle's antimicrobial activities

group was previously described, which showed a regrouping between the fungi and the NPs Ag. The second group is encircled in orange colour (short dash line), which regroups the gram positive bacteria with the following NPs: Ag, SiO_2, ZnO, CeO_2 and TiO_2. The last group, encircled by a blue colour (continuous line), is formed by the gram negative bacteria with NPs: La-ZnO, CeO_2CdO, TaO_5, Co (Vox), Cu, CuO, TiO_2. Each type of microorganism was directly linked with several associated NPs, but only the matrix coefficient of each qualitative variable in Table 5.3, confirms the NPs highly correlated with specific microorganism. NPs that have bold values have significant antimicrobial properties towards gram positive bacteria, which are the NPs: Ag, SiO_2 and ZnO with NPs of La-ZnO having an affinity for gram negative bacteria with only the Ag-NPs having antimicrobial activity against Fungi.

5.3.2 Antimicrobial Test Conditions

The scree plot of the PCA displayed as a 2-dimensional (2D) depiction for the comparative location of the quantitative variables to each other is presented in Fig. 5.2. Data variability was computed in eight dimensions as illustrated by the axis from *F*1 to *F*8 in comparison for the cumulative variability and the Eigen values as shown in Fig. 5.2. Based on the scree plot, PCA revealed that the treated data was reliable in a 2D spacing when comparing *F*1 against *F*2 = 45.50%.

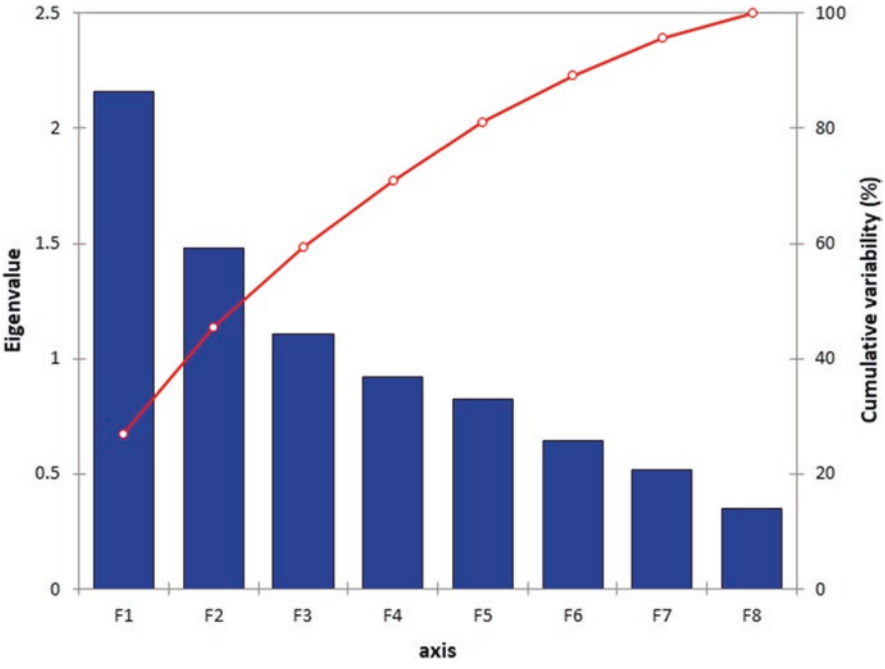

Fig. 5.2 Principal component analysis (PCA) scree plot depicting the variability of the quantitative data in the eight dimensions against the cumulative variability comparative to the eigenvalues

The Pearson correlation matrix table (Table 5.4) shows the correlation coefficient between each considered parameter to NPs test conditions. The inhibition zone size was demonstrably correlated to the MIC, the well size and the disc size with correlation coefficient (r^2) of 0.9598, 0.9399 and 0.9482, respectively. Besides, the well size was positively correlated to disc sizes (r^2 = 0.9787, α = 0.05) and to MIC (r^2 = 0.2783, α = 0.05). The disc size as well was shown to have a positive correlation with the MIC with a correlation coefficient (r^2) of 0.4814 and α of 0.05. The values of the correlation coefficient have a confidence interval of 95% which agrees with the hypothesis alternative (Ha), which states that at least one of the correlations between the variables is significantly different from 0.

Correlation of the PCA is displayed in Fig. 5.3, which depicts the projection of the NPs antimicrobial activities and quantitative variables in the 2D space. A positive correlation was observed between the IZ of the microorganism growth and the MIC of the NPs. Besides, the PCA revealed a positive correlation between the well size and disc diffusion size created on the agar plates during the NPs antimicrobial activity tests. A negative correlation was observed between the average size of the NPs and the contact time with the test microorganisms. These correlations were similar to the correlation matrix of Pearson (Table 5.4). The IZ size is defined by not only the MIC but also the well and disc size containing the NPs on the agar plates. Therefore, the necessity to vary the NPs concentration to improve the antimicrobial test was retained as reported in Webster and Seil (2012). Effectively, when the MIC is high, the IZ size would be large as reported in Simon-Deckers et al. (2009). Besides, the NPs average size plays a major role on the contact time with the microorganism.

Table 5.4 Correlation matrix (Pearson (*n*)) of quantitative variables for nanoparticles' antimicrobial activity

Variables	Average size (nm)	IZ (mm)	Ratio (%)	Well size (mm)	Disc size (mm)	Contact time (h)	OD (initial)	MIC (µg/mL)
Average Size (nm)	**1**	0.0321	−0.1438	−0.0641	0.0200	**−0.9893**	−0.1410	−0.1448
IZ (mm)	0.0321	**1**	0.0000	**0.9399**	**0.9482**	0.1550	0.0232	**0.9598**
Ratio (%)	−0.1438	0.0000	**1**	0.0000	−0.0032	−0.1267	0.0321	−0.0039
Well size (mm)	−0.0641	**0.9399**	0.0000	**1**	**0.9787**	0.0000	0.0000	**0.2783**
Disc size (mm)	0.0200	**0.9482**	−0.0032	**0.9787**	**1**	0.0116	−0.0497	**0.4814**
Contact time (h)	**−0.9893**	0.1550	−0.1267	0.0000	0.0116	**1**	**0.2706**	**0.2034**
OD (initial)	−0.1410	0.0232	0.0321	0.0000	−0.0497	**0.2706**	**1**	−0.0725
MIC (µg/mL)	−0.1448	**0.9598**	−0.0039	**0.2783**	**0.4814**	**0.2034**	−0.0725	**1**

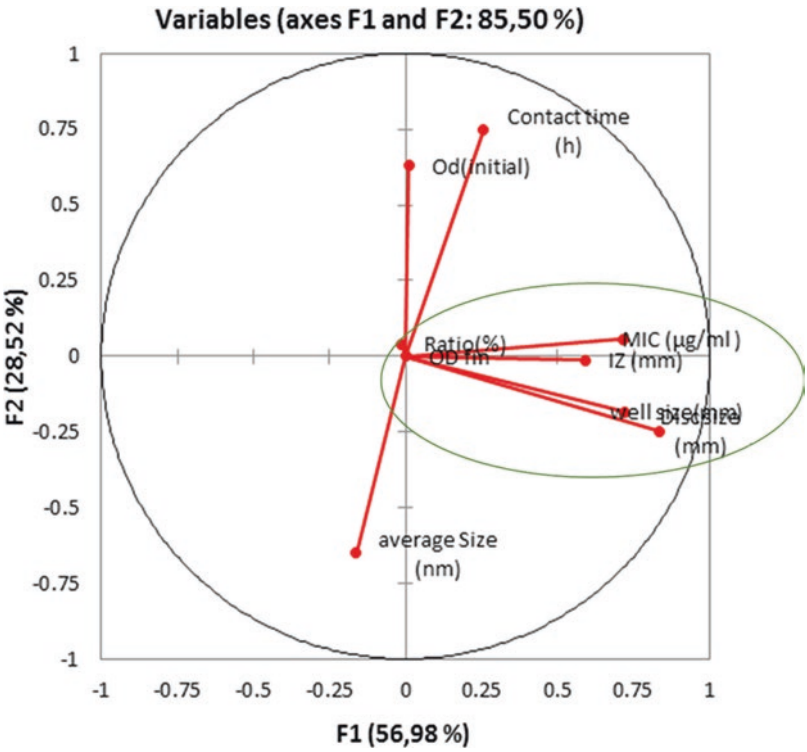

Fig. 5.3 Principal component analysis (PCA) correlation circle depicting the projection of the quantitative variables for nanoparticles antimicrobial activities

Similarly, when the NPs average size is large, the contact time is reduced. The NPs with a large average size effectively inhibited a few microorganisms growth.

5.4 Conclusion

MCA and PCA were investigated to study the correlation between the qualitative and quantitative variables, respectively, on the antimicrobial activity effectiveness of numerous NPs. Qualitative variables through the MCA showed the affinity between the gram positive bacteria and Ag-, SiO_2- and ZnO-NPs, while for the gram negative bacteria it was the NPs of ZnO. For the Fungi, this was observed in AgNPs. The PCA further highlighted a positive correlation of the inhibition zone (IZ) size with the well size, disc size and the minimum inhibitory concentration (MIC) with $r^2 > 0.90$, $\alpha = 0.05$. A negative correlation was observed between the NPs average size and the contact time. Therefore, the choice of NP types for antimicrobial activity effectiveness depends on the microorganism type and the MIC, with some influence being associated to the well size and the disc size. It is necessary to vary the concentration of the NPs to improve the effectiveness of NP against pathogens.

Also, research on the NPs antimicrobial application on fungi should be investigated in future studies to ascertain the effectiveness of other NPs for antifungal efficacy.

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6 *Saccharomyces cerevisiae* as Model Organism to Study Biological Activities of Nanoparticles

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Abstract

The species of yeast (*Saccharomyces cerevisiae*) is being used as an experimental model organism by many researchers to study various genetic and biochemical processes and to study the association among different biological processes. In the emerging field of nanotechnology, the study of stabilization of nanoparticles is gaining the attention of researchers. For this purpose, the biological active compounds from *S. cerevisiae* are presenting excellent scaffolds. The secretion of extracellular enzymes from yeast and rapid growth with usage of few and simple nutrients in the culture media provides more benefits over other microorganisms. A huge number of reviews have been conducted to examine the synthesis

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of metallic nanoparticles employing *S. cerevisiae*. In this chapter, we will review the use of *S. cerevisiae* as a model organism to study the biological activities of nanoparticles. The cumulative efforts are recorded tools for genetic tractability of *S. cerevisiae* in the field of growing science and technology.

Keywords
Saccharomyces cerevisiae · Nanoparticles · Extracellular enzymes · Ecotoxicity

6.1 Introduction

Saccharomyces cerevisiae, eukaryotic unicellular microorganism, is generally termed as baker's yeast. It is appropriately designated under fungi kingdom. The wild strains of yeast family are commonly found inside the gastrointestinal tract, outer lining of ripe fruits and on the body surface of warm-blooded animals and insects (Alberghina et al. 2012). Besides this, laboratory strains are considered to be the model organisms constituting of globular shape (Botstein and Fink 2011). Significantly, yeast consists of cytoskeleton. As a eukaryotic microbe, the locomotory activities are taken care by the actin filaments, namely, actin cables and actin patches, which are understood as actin bundles and actin patches, respectively. The practice of division is seen by the process of budding where there is asymmetric division allowed by the actin cytoskeleton (Alberts et al. 2008). Yeast being a facultative anaerobe is potent enough to grow in oxygenated and deoxygenated environment. It follows the process of oxidative phosphorylation and mitochondrial electron transport chain which leads to the conversion of water and carbon dioxide to water (Ishtar Snoek and Yde Steensma 2007). Various proteins originate differently that are expressed in yeast along with simple DNA manipulation with inducible gene expression (Bekatorou et al. 2006; Guthrie et al. 1991). These techniques involve the yeast analysis.

S. cerevisiae, eukaryotic organism, reproduces via budding. It divides in a cycle of 90 mins, is easy to manage and grow and shows maximum stability in its diploid and haploid forms (Botstein and Fink 1988). Haploid genome is of small size and less complex where it is packed into 16 chromosomes (Cherry et al. 1997). Because of its versatile nature, it became the first organism whose genome was sequenced, and it had 6466 open reading frames which are available for usage. Yeast is the most prioritized model for mammalian pathways and diseases. Approximately 31% of the proteins in the genome of yeast are encoded by human orthologues. In addition, it has acted wisely in being the model organism in the advent of recombinant DNA technologies, like drug-induced phenotypic responses, drug-induced haploinsufficiency, gene profiling of drugs (Lum et al. 2004) and synthetic lethal screens, that have been validated and implemented in yeast (Menacho-Marquez and Murguia 2007). Moreover, yeast has been friendly in its gene mapping and acquiescent for altering gene marking, gene disruption and mutation or dosage effect on genes. Such advantages make yeast a suitable choice for the researchers for inculcating it into experimental testing. For instance, yeast study has added great knowledge in eukaryotic cell regulatory division which includes cancer-related disturbances

(Hartwell 2002). Yeast acts as the functional platform for the disease-related protein determination that has no homologous similarity in the organism. These are termed to be humanized form of yeast that functions in the dissection of molecular processes of human for medicinal inventions (Mager and Winderickx 2005). The advent of *yeast* study has expanded for the study of toxicological investigation of chemicals like heavy metals (Schmitt et al. 2004), anticancer drugs, herbicides (Cabral et al. 2003), or monocarboxylic acids which act as preservatives (Kasemets et al. 2006). Similarly the bacterial and unicellular algae function in the discharge of toxic metals from metal which instigates their toxicity towards *S. cerevisiae* because the rigid structural wall of the yeast prevents its direct uptake of the particles (Kasemets et al. 2009). The nanoparticle cyto-toxicity of NPs is barely understood because there is still unstudied on *S. cerevisiae* and other yeasts. Nanomaterials such as ZnO, CuO, TiO_2 and fullerene are studied intensively using model organism (García-Saucedo et al. 2011; Hadduck et al. 2010; Schwegmann et al. 2010).

Yeasts are a kind of eukaryotes which are easy to handle as they can be cultured properly and wisely in suitable condition unlike other eukaryotes whose complex model causes difficulty in their culturing (Estève et al. 2009). *Saccharomyces cerevisiae* tester strains are designed for toxic and genotoxic models (Schmitt et al. 2005). The use of yeast is preferred generally for locations where pesticide usage is extreme in order to perform preliminary toxicity screening (Braconi et al. 2016). However, being a model organism, it holds some drawbacks which are enlisted as follows:

1. With high toxicant concentrations, it is essential to attain toxicity because of multidrug resistance machinery and cell wall, for example, the concentrations of dieldrin must be high for yeast screenings than human cell.
2. The metabolic pathway differences allow an easy comparison with mammals.
3. The target organ identification is difficult (Gaytán et al. 2013).

6.2 General Features of *Saccharomyces cerevisiae*

S. cerevisiae, being brewing yeast, is well known and a popular model organism because of its industrially beneficial features such as its fast/ease of replica plating, growth, well-structured genetic system, extremely flexible DNA transformation system isolation of mutated cells and cell dispersion. Yeast is easily grown on liquid as well as on solid media and can also be purified as single colony from the solid media. It acquires generation time of 90 min which makes them easy to grow in industrial applications. In 1996, the unicellular fungus whole genome was sequenced. Its nuclear chromosome is enriched by 16 chromosomes and 16 million base pairs. The mitochondria contain an extra pair of chromosome. The budding cells of yeast (of any species) sustain 6000 genes as its genetic information. The total number of overall genes and size of population is extensively minute, and the gene density is very high (Doke and Dhawale 2015). *S. cerevisiae* have important conserved biochemical and cellular mechanisms (Zhang et al. 2013), and on top of that, its rapid multiplication makes it more useful in nanotoxicity investigations. However, literature have few proves of the effects of different NPs on yeast cell

(Bayat et al. 2014). The *S. cerevisiae* grows easily in stress conditions for growth, and moreover they can be preserved in refrigerated stocks. Moreover, there are an ample number of genetic tool strains and vectors that support researchers to develop bioassays based on yeast (Guthrie and Fink 1991). Apart from all the newly discovered microorganisms, *S. cerevisiae* has been the most consistent in the field of science by acting as a superior microbe that would be highly utilized for the production of a useful product. *S. cerevisiae* has a feature of surviving in circumstances where it includes flocculation, biofilm development, deleterious circumstances, etc. (Fidalgo et al. 2006). The flocculating strains are not desirable for the ethanol industry as these do not provide high amount of yield, but in few industries, these strains have been proven to be successful for distilled beverages like wine and beer (Bauer et al. 2010). Yeast flocculation is asexual and homotypic and forms a multicellular mass called floc (Stewart et al. 2009). In *S. cerevisiae*, there is failure of the separation of young bud to form new cells after sexual aggregration process where they are covalently linked to each other and does not re-aggregate after mechanical dispersion (Zhao and Bhai 2009). Therefore, these rough strains are not considered as flocculent for any study aiming at sedimentation. *S. cerevisiae* strains have varied response towards external stimuli thus show contrasting colony and its morphology. Out of thousands of other strains, 2.5% are rough colonies (Casalone et al. 2005). The morphology of *Candida albicans* is similar to smooth colonies as it contains blastopores, whereas the rough colonies contain pseudohyphae or false hyphae (Novak et al. 2003). The rough colonies of the yeast hinder the fermentation process (Cabrini and Gallo 1999).

6.3 *S. cerevisiae* as a Model Organism for Pharmacological Screening

Yeast is considered to be an essential tool for drug and reagent testing which is also called as pharmacological screening or testing. The yeast strains are used for the production of human drugs like hirudin, artemisinin hydrocortisone, vaccines and insulin infused in hepatitis B and cancer-related drugs (Ardiani et al. 2010). *S. cerevisiae* provides a cumulative platform for researches based upon G-protein-coupled receptor (GPCR) (Minic et al. 2005). Effects of harmine were investigated to check recombinogenicity, mutagenicity and putative genotoxicity on yeast. Harmine can induce breakage of double- or single-stranded DNA. A test named brine shrimp lethality investigates the cytotoxicity level of harmine and along with it the determination of minimum inhibitory concentration and minimum bactericidal concentration of any compound using microdilution plate method (Patel et al. 2012). In another study, Payette et al. (1990) investigated *S. cerevisiae* for the mammalian GPCR expression with the help of cloned gene encoding HM1 (human muscarinic receptor).

This organism is essential in reviewing the basic aspect of cellular biology in diseases like Huntington's, Parkinson's and Alzheimer's for heterologous or endogenous protein study that is the main cause of the diseases (Pereira et al. 2012). *S. cerevisiae* maintains the balance between the forces of fission and fusion which acts

on inner mitochondrial membranes (Bertholet et al. 2013). The mitochondrial dynamics is important as the mitochondrial DNA is maintained in the cellular parts of *S. cerevisiae* where it suppresses the mtDNA loss and morphological defects (Fekkes et al. 2000). The mtDNA deterioration is supported by the inactivated fission which is present in yeast lacking certain genes like *Mgm1p*/*Msp1p* or *Fzo1p* in mammalian cells devoid of *OPA1* or Mitofusin 2 (*MFN2*) (Wong et al. 2000; Chen et al. 2010). Such kinds of defects are searched by pharmacological suppressors between mtDNA maintenance and mitochondrial fusion (Gao et al. 2017). Yeast-based assay has predicted the invasion of Epstein-Barr virus (EBV). The EBV immune evasion is dependent on the capacity of Gly-Ala repeat domain of EBNA1 which functions in inhibiting the mRNA translation. Furthermore, the prion-based diseases are lacking an effective treatment where the prions do not hold a fully understood solution. The formation of PrPc is eradicated by yeast (*S. Cerevisiae*) in mammalian-cell-based assay (Doh-Ura et al. 2000; Archer et al. 2004).

The different strains of yeast are also used for the expression of yeast *Gpa1*/human Gα protein chimera to determine the activity of various ligands for the coexpression of human A_1 adenosine. Adenosine is purine nucleoside which regulates the physiological processes in central nervoussystem and cardiovascular system (Haskó et al. 2008). The A1 receptor is cosmopolitan in nature and has been found in high content in the brain, spinal cord and atria (Kourounakis et al. 2001; Baraldi et al. 2000). The yeast is also compatible to express the single type of GPCR after the activation of the system that will stimulate the pheromone response pathway by coupling heterotrimeric G protein with it (Dowell and Brown 2002). It is also reported that yeast inculcates the mammalian GPCR signaling. Similarly, Brown et al. (2000) have also reported the same existing pathway that expresses the yeast Gα/human protein chimera that consists of five C-terminal amino acids of the presented human Gα protein which are infused by *Gpa1*/Gα, Gα protein and *Gpa1*(1-467) (Brown et al. 2000). This process permits the binding efficiency of the mammalian GPCR as they maintain the coupling capacity to endogenous yeast Gβγ subunits (Dowell and Brown 2002).

6.4 *S. cerevisiae* as a Model Organism for Toxicological Screening

There are a lot of reasons due to which *S. cerevisiae* is considered to be the most important model organism for studying toxicological studies. Enormous pathways are available that function along the various cellular processes which are conserved in human also. A large number of yeast genes have been reported and identified in human homologues which are complimented by many other conserved genes that are disease linked in humans (Wood et al. 2002). This model organism is functional in investigating toxicological evaluations of various contaminants including anticancer drugs, heavy metals, herbicides, etc. Unfortunately the NPs to yeast cytotoxicity is reviewed very less because effect of nanoparticles on yeast are poorly understood without any specific mechanism. The nanomaterials evaluated in various studies include fullerene/

TiO_2, CuO, ZnO and iron oxides (García-Saucedo et al. 2011). Schmitt et al. (2002) reported in vivo DNA response reporter technique using *S. cerevisiae*. They investigated test system for the diagnostic tool of aquatic biota when compared to the tests performed in unicellular organisms like ciliates, algae and bacteria population. EC_{50} values were checked using high and low concentrations of heavy metals (4.5–5.5 g/cm^3) and Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} Cr^{6+}, dimethyl sulfoxide (DMSO), ethanol, 2-aminoanthracene, 4-nitroquinoline-1-oxide and methyl-*N*-nitro-*N*-nitrosoguanidine toxicants.

Genetic manipulation in yeast is running from a very long time, and it aims at selecting a specific target where they examine the conserved genes and pathways in order to run a facilitating functional analysis. The ease is attained because of easy approach towards software resource availability as it lowers down the difficulty in handling molecular protocols which boosts the value of yeasts in toxicology (Gaytán and Vulpe 2014). In the budding process, barcoded mutant collections are generally generated (Giaever et al. 2002) which would examine the best culture among the other co-cultures (dos Santos 2012; North and Vulpe, 2010). Few techniques, such as functional genomics, chemical genomics, chemical-genetic profiling and functional profiling, can estimate the genetic requirement for the strain so that it can tolerate the stress or other inadequate conditions for the sake of survival (Gaytán and Vulpe 2014). Apart from the heavy usage of engineered nanoparticles (NPs) in numerous industries and their manufactured products, these are meant to be assessed by toxicological screening of synthetic NPs. In such cases, *S. cerevisiae* acts as a promising unicellular organism which functions in studying the oxidative stress and aging of cancer cells. The mutant collection of *S. cerevisiae* with broad genomic study is a unique tool for the toxicological profiling of various toxicants. *S. cerevisiae* BY4741 wild type has resulted in showing toxicities of IC_{50} value for nano-CuO and bulk CuO when they were grown in YPD-rich media and deionized water. The nano-CuO toxicity is more than bulk CuO toxicity as the bulk CuO lacks the inhibition potential of the growth of single-gene deletion mutant. The study has shown that CuO and its nanoparticles were extensively toxic to cells of *S. cerevisiae* when grown in distilled water than YPD media. Similarly, the same strains in YPD media have 629 times more IC_{50} value than in distilled water and the YPD media.

6.5 Monitoring the Biological Activities of Nanoparticles of *S. cerevisiae*

Production of Cu nanoparticles by electrochemical processes is used to attain stabilizing environment. These nanocomposites are used as coating material that secrete copper species in yeast and mold broth, preferably *S. cerevisiae* (Cioffi et al. 2005). The technological advancement in the nanoparticle study has shown an area of interest in AgNps for the idea of antimicrobial agent (Silver 2003). Since a long time, silver and its components are capable of making strong inhibitory and bactericidal effects on a wide range of microorganisms captivating antimicrobial activity, namely, bacteria, fungi and viruses (Lok et al. 2006). Silver has high potential to

show toxicity against microbes, but it shows less toxicity against mammalian cells (Nasrollahi et al. 2011). Silver complexes were also used as common alloy in dental practices (Yang and Pon 2003). AgNPs are considered to be essential antimicrobial agent (Shahverdi et al. 2007; Elechiguerra et al. 2005) which are employed in healing wounds, burns and other infections (Paladini et al. 2012). AgNPs are used as disinfectant against various microbial populations by embedding the sulphur-containing protein of the cell membrane to the cell membrane of the microbe (Zheng et al. 2008; Sondi and Salopek-Sondi 2004). The study itself is supporting the size- and shape-dependent interaction of AgNPs with bacterial cells, altering the bacterial cell structure and properties (Pal et al. 2007). The in situ investigation of AgNPs interaction is done by the hybridization technique called FISH which actively detects the chromosomal abnormality and RT-PCR for reviewing the gene expression changes along with the nanoparticle movements. The signals released after Ca^{2+} transient in nanoparticle-treated cells are detected to construct a model which will explain AgNP toxicity mechanism.

6.6 Sensitivity of *S. cerevisiae*-Based Toxicity Bioassays

The literature is a living evidence supporting the evaluation of pesticides (azoxystrobin, cymoxanil and diuron) to observe its harmful effect upon *S. cerevisiae* for the bioassay growth which will lead to the yeast inhibitory metabolic activity by the sake of (ATP) synthesis, as contrary to the toxicity tests of *Vibrio fischeri* activity (NF EN ISO 11348) and *Daphnia magna mobility* (NF EN ISO 6341) based on inhibition. The yeast bioassay in comparison to *D. magna* toxicity bioassay is easier and a lot faster because of its lower sensitivity (Estève et al. 2009). The standard guidelines of quality and safety allow a precise way to determine the toxicity level in wastewater samples which would incorporate the use of *S. cerevisiae* (Schmitt et al. 2005). Heavy metal could be easily accumulated in the biosphere, especially in the human body. Though trace heavy metals are essential to cell signalling, metabolism and enzymatic catalysis, they can be harmful to living cells at higher concentrations (Singh et al. 2010). For example, Cu2þ is considered as a carcinogen since its inhibition on electron transport chain and Naþ/KþATPase activity (Gumpu et al. 2015; Sheline and Choi 2004; Einicker-Lamas et al. 2002)

6.7 Major Evolutionary Perspectives in Ecotoxicology Testing Using *S. cerevisiae*

The ecotoxicity in the environment is balanced by the eradication of certain endocrine disruptors due to which colorimetric-based assay has been constructed to evaluate the chemicals like pesticides that have the capability to cause endocrine-mediated effects (Kaur et al. 2018; Singh et al. 2018; Datta et al. 2018; Singh et al. 2017) (Table 6.1). The two extensively used receptors or reporter assays are Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS) (Purvis et al. 1991; Routledge and

Table 6.1 Effect of pesticides on *Saccharomyces cerevisiae* by Omics

Compound name	Activity of compound	Experimental	New advents	Reference
2,4-dicholorophenoxyacetic acid (2,4-D)(Organochloride herbicide)	Dismantles the cellular outer coverings and hinders the metabolic activity	*S. cerevisiae* BY4741 or its single deletion mutant strain, regulated gene clusters, yeast genome S98 GeneChip arrays	14% yeast transcriptome has been changed. 2,4 -D is responsible for altering the genes which produces antioxidant enzymes, HSPs or chaperons.	Teixeira et al. (2006)
Alachlor (chloroacetanilide herbicide)	Functions in the elongase inhibition along with geranylgeranyl pyrophosphate cyclisation enzyme which is a constituent of gibberellin pathway	*S. cerevisiae* BY4741	Treatment with LOEC alachlor results in 8 repressed and 36 induced genes.	Gil et al. (2015)
Dieldrin (organochlorine insecticide)	It alters electrophysiological and its related enzyme properties of nerve cell membrane	*S. cerevisiae* BY4743 treated with dieldrin for 25 generations	427 mutants were found sensitive out of which 320 mutants acted as resistors towards minimum dieldrin dosage. It alters the leucine availability	Gaytán et al. (2013)
Mancozeb (manganese-and zinc-ethylene-bis—Dithiocarbamates [Mn:Zn, 9:1) fungicide)	Presence of sulfhydryl in amino acid is inactivated therefore, it directly affects the activity of protein which acts as pro-oxidant	*S. cerevisiae* derived deletion strain and haploid parental strain	286 genes (3 essential gene) holds tolerance against fungicide	Dias et al. (2010)
Toxaphene (organochlorine insecticide)	Disbalances the ion level of sodium and potassium in neurons	The strain BY4743 is homozygous diploid strain processed at IC20 will be 60 μM, 50% IC20 and 25% of IC20 of the drug for continuous 15 generations	A total of sensitive 130 mutants were obtained. Notably, 542 mutants showed resistivity towards minimum dosage of toxaphene	Gaytán and Vulpe (2014)

Sumpter 1996). Hydroxylated derivatives and polychlorinated biphenyls (PCBs) are observational responses obtained after the use of receptors that detect endocrine responses towards both of them (Layton et al. 2000; Schultz 2002), while other polycyclic aromatic hydrocarbons (PAHs) and biphenyls (Schultz and Sinks, 2002), pesticides (Wani et al. 2017; Kumar et al. 2017; Mishra et al. 2016; Singh et al. 2016; Sohoni et al. 2001), etc. are detected in the context of estrogen and androgen in water bodies (Thomas et al. 2002), aquifers (Conroy et al. 2005), treatment of wastewater bodies (Layton et al. 2002; Kumar et al. 2016, 2015; Kumar et al. 2014) and dairy manure (Raman et al. 2004). In recombinant receptor transcription assays, the yeast cells that show eminent response against the element-regulated reporter genes have been proven to check out effective response of first-stage screening of chemicals resulting in endocrine activity. The only one merit of bioluminescence assay is that it is very rapid in comparison to colorimetric assay. Quantified BLYES and BLYAS are observed for a time period of 3–4 h (Eldridge et al. 2007; Sanseverino et al. 2005). In BLYES assay, compounds showed dose-response activity against $EC_{20.}$ Yeast eukaryotic model is used for preliminary toxicity screening of pesticides which hold critical biomonitoring with long-term relevant assays (Kapoor et al. 2019; Bhati et al. 2019; Singh et al. 2019a; Kumar et al. 2019a). Apart from holding merits, it also deals with some demerits as being a model organism, for example, it is essential to reach a high level of toxicity concentration because of the cell wall and multidrug resistance activity (Singh et al. 2019b; Kumar et al. 2019a, b; Sidhu et al. 2019). Secondly, it is difficult to reach to the particular organ of a system which shows severe systemic effects (Gaytán et al. 2013; Kumar et al. 2018a; Kumar et al. 2018b). *Saccharomyces cerevisiae* has huge impact on the fermentable food products and also plays a major role in the agro industry.

6.8 Conclusion

S. cerevisiae has been a promising microorganism which is versatile in nature and is ubiquitous. The budding feature of yeast is a boom for itself that it divides rapidly and could increase its population. It is also beneficial as it can survive with or without oxygen. It is groomed with rich actin and myosin network. Its genome has been the first to be sequenced. The application of yeast has been expanded to anticancer drugs. It has been a successful model organism for the study of nanomaterials. It is a promising microorganism in brewery industry as one can rely on its industrially beneficial features. The toxicity investigation is strong when *Saccharomyces* species is used as a model organism for drug or reagent testing. The IC_{50} values of a drug were investigated via brine shrimp lethality method. The pharmacological testing using *S. cerevisiae* is brilliantly handled because it provides ease in regulating the coupled proteins, and moreover the drug testing is convenient in order to bring mutagenic alterations. The overall result obtained is beneficial. Further the toxicity bioassays are quite complicated as they required high amount of drug concentration that does not help the microbe to show its high level of work potential as it requires high note of optimization based upon the standard regulatory protocols because

otherwise it would hinder the biosignalling pathways of the organism itself. The presence of heavy metals in wastewater is investigated very efficiently. Therefore, the optimum use of *S. cerevisiae* is a great deal to investigate the heavy metal load and other pesticide/insecticide drugs. It is useful in aquatic as well as terrestrial habitat samples. Therefore, the usage of *S. cerevisiae* is possible but could show more valuable results if it is genetically manipulated.

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Investigation of Biological Activity of Nanoparticles Using Cell Lines

7

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Abstract

This review is provided with a detailed overview of the types, structures, properties, nano-bio interactions, positive and negative biological effects, assays and models for identifying nanoparticles, and applications of nanoparticles in biological systems especially using the cell lines. Nanoparticles are tiny particles with a size range of 1–100 nm. Particles having similar structure will have similarity in their chemical and biological properties. There are different varieties of nanoparticles with varying physical and chemical properties and geometry. The properties varying are size, shape, morphology, surface area, aspect ratio, chemical composition, chemical reactivity, and zeta potential. Due to these properties, these particles are being used in commercial and domestic applications including medical, energy research, catalysis, imaging, and environmental applications. Interactions of nanoparticles with biological systems will lead to certain desirable and undesirable effects. The mechanisms of these biological effects are investigated thoroughly to understand the structure activity relationships. The present study will provide more information on the sustainable development of nanotechnology.

Keywords

Types of nanoparticles · Structure · Properties · Biological interactions · Cell lines · Biological effects · Toxicity · Applications

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7.1 Introduction

The field of nanotechnology and nanoscience gained much attention since the last decade. Significant advancements in such fields can be attributed to their utilization in biological sciences (Liu 2006). The idea of using nanoparticles in biomedical research has drawn much attention and interest due to the fact that nanoparticles (NPs) can be easily engineered to attain unparalleled functions and unique compositions, thus rendering novel tools and techniques useful for biological research (Wang and Wang 2014). The manipulation and subsequent engineering of matter at atomic, molecular, and supramolecular levels to produce different nanomaterials useful for the advancement of various fields are comprehensively termed as nanotechnology. The merging of biology and nanotechnology has conceived a new field, nanobiotechnology, which involves the utilization of different organisms including bacteria, fungi, yeast, and plants to produce NPs at an economical and environmentally friendly manner when compared with chemical processes (Shah et al. 2015). These nanoparticles play a significant role in diverse applications ranging from biological sciences to mechanics. A rise in the usage of nanoparticles has led to the evolution of novel fields such as nanobiotechnology and bionanotechnology apart from their usage in biomedicine and biosensors where NPs are engineered and manipulated to achieve desired results (Anirudh et al. 2018).

7.2 Types of Nanoparticles

On a broad scale, nanoparticles are classified into metallic, semiconductor, and polymeric nanoparticles. Based on their application on whether they are used for diagnosis or for therapeutic purposes or for their usage in imaging, nanoparticles are categorized into inorganic and organic nanoparticles. Organic nanoparticles include micelles, liposomes, dendrimers, and polymeric and albumin-bound nanoparticles. Inorganic nanoparticles include silver, gold, iron oxide, alloy, magnetic, and quantum dots.

Unilamellar liposomes are one of the first platforms used due to their small size ranging from 100 to 800 nm. Usage of liposomes as a model for cell membranes in biophysical research was first reported in the year 1965, after which they were used for gene delivery as platforms for nanoparticles. Liposomes have the unique ability of carrying and delivering many types of biomolecules through biological membranes into cells, thus establishing themselves as a widely used type of transfection agents in biological research. Liposomes also offer several advantages such as being leak-proof, economical, non-toxic, and non-immunogenic apart from being biocompatible and biodegradable (Orive et al. 2009). Micelles are composed of either polymers or lipids that work on the principle of hydrophilic-hydrophobic interactions. Biomolecules that are water-insoluble can be embedded into the hydrophobic part of micelles, whereas water-soluble molecules can be loaded into the hydrophilic part. When transferred into the bloodstream, they have a higher life due to the presence of a hydrophilic shell surrounding them. The biomolecules bound to

albumin (albumin-bound nanoparticles) serve as a useful nanoparticle drug delivery vehicle (Hawkins et al. 2008). Non-covalent binding and endogenous albumin pathways are utilized for carrying the hydrophobic molecules such as Abraxane into the bloodstream, for treating metastatic breast cancer (Harries et al. 2005). Dendrimers are branched structures with an internal core surrounded by branched structures made up of amino acids, sugars, and nucleotides followed by an external surface (Medina and El-Sayed 2009). Based on the number of generations formed over these cores, the size, shape, and extent of branching can be described. These dendrimer cores can be embedded with small-sized biomolecules with the help of hydrogen bonding or hydrophobic interactions. The only disadvantage of these nanoparticles is their synthesis, which is time-consuming and the loading of biomolecules into the core (Adair et al. 2010). Polymeric nanoparticles are synthesized by utilizing the copolymers of different hydrophobic levels. A unique feature of these nanoparticles is that they are synthesized from biodegradable and biocompatible polymeric substances. These NPs can be loaded with small biomolecules, which may be either hydrophilic or hydrophobic in nature and has an added advantage of being more leak-proof and stable than liposomes. In general, metal nanoparticles can be made thermodynamically stable by adding capping agents such as oligosaccharides and polysaccharides, which helps to increase their solubility and also prevents aggregation (Sanjay et al. 2009).

Silver nanoparticles are categorized into two different types, uncapped and capped varieties. The size of uncapped NPs ranges from 20 to 50 nm, while capped NPs have a size of ~25 nm (Lima et al. 2012). These are the most widely used inorganic nanoparticles in textile industries and cosmetic applications due to their antimicrobial properties against microbes such as bacteria and viruses (Sharma et al. 2015). Gold nanoparticles are widely preferred as they are biocompatible and have a wide array of optical and chemical properties, thus offering themselves a part in bio-imaging, biochemical sensing and detection, diagnostics, and therapeutic applications. These nanoparticles are used as a tracer in the detection of DNA during DNA fingerprinting and also used for identifying protein interactions in immunological studies (Hasan 2015). Magnetic nanoparticles are produced from magnetized materials or from materials that can be attracted by magnets such as Fe, Co, Ni, etc. Some of the widely used magnetic nanoparticles are maghemite (Fe_2O_3) and magnetite (Fe_3O_4), which are biocompatible. Alloy nanoparticles are mainly structural and have a high electrical conductivity compared to other metals and thus are widely used. Some alloy nanoparticles are bimetallic in nature and are advantageous over other metallic nanoparticles such as silver, gold, etc. (Mohl et al. 2011).

When quantum effects take place in nanocrystals with extremely small diameters in addition to entrapment of electron carriers and holes at a size range lesser than Bohr's radius, they are referred to as quantum dot nanoparticles. Their size ranges from 3 to 12 nm in diameter. These are neither solid structures nor individual molecules as the number of atoms in the quantum dot nanoparticle ranges from 1000 to 100,000 making it a unique nanoparticle. There are different types of quantum dots based on III–V semiconductors, II–VI semiconductors, and silicon atoms included at the core part (Ghaderi et al. 2011). The overall structure of a quantum dot

nanoparticle can be described as having a secondary shell on the exterior followed by a primary shell with a core part at the centre. Most of the quantum dot nanoparticles are capped by ZnS, and their core is made up of CdSe. As the quantum dots are insoluble in water, they are not suitable for the transfer of biomolecules in an aqueous medium. Iron oxide nanoparticles belong to the paramagnetic type of nanoparticles. Due to the presence of paramagnetic nature, the iron oxide nanoparticles are widely used as a contrasting agent in magnetic resonance imaging due to increased magnetic susceptibility. The core of SPION is generally made up of iron oxide and is layered with a hydrophilic substance such as dextran so as to enhance its stability. Currently, there are two super paramagnetic iron oxide nanoparticles (SPION) with size 60–180 nm, namely, ferumoxides and ferucarbotran, respectively, that are used for MRI and molecular imaging (Weissleder 2006).

7.3 Structure of Nanoparticles

Nanoparticles generally exist in nature either in non-crystalline or in a low symmetry form. On the basis of nanoparticle size and composition of the aggregates, determination of the most stable form is important which in turn will determine the physical and chemical properties of the nanoparticles. Some of the cardinal factors that affect how the nanoparticles accumulate and interact with the body are size, shape, solubility, structural properties, and chemical composition (Vinita et al. 2010). Nanoparticles exhibit distinct and unique properties due to the presence of unusually high ratio of surface area to volume. Huge surface area of nanoparticles are attributed to a distinct surface chemistry in relation to their core properties. The surface properties that are highly specific and unique to each nanoparticle are generally lost in the process of aggregation and accumulation.

Structurally a nanoparticle can be divided into three components, namely, functionalized surface, shell, and core which is the cardinal part in determining the properties of the nanoparticle.

7.3.1 Surface

In order for nanoparticles to dissolve in an aqueous medium, their surface needs to have a charge. This charged surface can be obtained by covalently binding it with biomolecules having certain charged functional groups. This covalent binding of nanoparticle surface with biomolecules, metal ions and polymeric substances is referred to as the functionalization of nanoparticle surface (Christian et al. 2008). The three-dimensional structure of the nanoparticles can be measured by using the coherent X-ray diffraction technique (Moyu et al. 2011). A stable nanoparticle aggregate can be formed by using surfactants such as SDS, where the surfactant goes and binds to the nanoparticle formed in the micelle core.

7.3.2 Shell

The outer layer of an inorganic nanoparticle may have different chemical and surface properties when compared to the core part of a nanoparticle. This distinctive layer is considered to be the shell of a nanoparticle. Some nanoparticles may have either a single shell surrounding the core or two shells one layering over the other as seen in a core-shell quantum dot. The quantum dot nanoparticles having CdSe alloy in the core and ZnS in the shell are known to exhibit a quantum yield of greater than 50% (Ding et al. 2017). An event where the formation of a shell layer occurs indirectly without deliberately preparing it can be seen in the formation of an iron oxide layer during iron nanoparticle formation (Santos et al. 2017).

7.3.3 Core

All the properties exhibited by a nanoparticle can be attributed to the composition of the core. Some of the physicochemical and toxicological properties manifested by a nanoparticle are due to its core composition. During the preparation of a nanoparticle, a definite phase form may not sound plausible because an inorganic nanoparticle may have its existence in different phases and two distinct phases showing up may be common, which might reflect the physicochemical properties of a nanoparticle to deviate entirely from what is expected.

7.4 Properties of Nanoparticles

Fundamentally the amount of stability of nanoparticles during production and its efficiency in any operation can be attributed to its diverse properties, each having its own significance.

7.4.1 Physicochemical Properties

On a broader point of view, the physicochemical properties include optical, magnetic, mechanical and thermal properties.

7.4.1.1 Optical Properties

For measuring optical absorbance and other such properties, researchers have used spectrophotometers. Apart from such experimental methods, some theoretical methods such as Mie scattering method, effective medium theory (EMT) and Monte Carlo methods are also most widely used for the theoretical determination of the optical properties of a nanoparticle (Rastar et al. 2012). For the characterization of nanostructures and particles, several techniques such as Raman, differential and surface-enhanced Raman spectroscopy are used.

7.4.1.2 Magnetic Properties

The presence of a magnetic property in nanoparticles is due to the uneven electronic distribution. When the size of a nanoparticle is less than 10–20 nm, the magnetic property of the particle dominates. Thus, these nanoparticles are useful in catalysis, MRI applications, etc. Some of the properties which can be tuned and modified to suit different applications are coercivity, blocking temperature, saturation magnetization and Neel and Brownian relaxation time (Arati et al. 2013).

7.4.1.3 Mechanical Properties

There are many kinds of forces that play a crucial role in establishing the mechanical properties of NPs. They are van der Waals forces, capillary forces, electrostatic force and electrical double layer force, solvation and structural and hydration forces (Dan et al. 2014). In order to test the mechanical properties of nanoparticles such as hardness, elasticity, adhesion, friction, etc., various methods are used such as atomic force microscopy (AFM), transmission electron microscopy and particle tracking velocimetry (PTV).

7.4.1.4 Thermal Properties

The thermal conductivity of metal nanoparticles is much higher when compared to liquids. This advantage can be used by suspending the metal nanoparticles in liquids such as ethylene glycol, oils, water, etc. and preparing the nanofluids. These nanofluids exhibit higher conductivity due to the presence of high surface area to volume ratio as the amount of heat transfer is proportional to the surface area present. This also enhances the overall stability of the suspension (Khan et al. 2017).

7.4.1.5 Antibacterial Properties

The need for using nanoparticles in the treatment of bacterial infections arose because of the fact that, as resistance against a drug increases, the dosage of antibiotics also increases drastically enhancing the toxicity. Another advantage is the species sensitivity of a particular nanoparticle to a bacterial strain (Mohammad et al. 2012).

7.4.1.6 Thermodynamic Properties

Some of the cardinal properties which determine the thermodynamic status of a nanoparticle are melting temperature, elastic moduli, enthalpy and entropy of melting, specific heat capacity, etc. By obtaining the Gibbs free energy using surface free energy model, the melting and superheating behavior of nanoparticle structures can be extrapolated. Due to the suppression of the thermal vibration of atoms at the interface between the nanoparticle and Nano cavity, superheating is provoked (Xiong et al. 2011).

7.4.1.7 Immunological Properties

Once binding of nanoparticles to the proteins in the blood takes place, these proteins determine the quantity of uptake of nanoparticles into the cells, which either stimulates or suppress the immune response. One beneficial property of nanoparticles is

its ability to reduce the toxicity of a drug by increasing their solubility. All immunogenic properties mainly depend on their thickness, zeta potential, size, etc.

7.4.1.8 Melting Properties

As melting is a surface property, it is dependent on the surface area to volume ratio. As the size of the particle decreases, the ratio increases dramatically; thus the surface energy also increases resulting in an increase in the melting point. X-ray diffraction or electron diffraction is widely used to determine the melting properties.

7.5 Size and Shape of Nanoparticles

Particle size and its distribution are mainly important characteristics of a nanoparticle system. These characteristics will determine the in vivo distribution, biological destiny, toxicity and the targeting capacity of a nanoparticle system. In addition, they can also control the drug loading, drug release and strength of nanoparticles. Dispersion and distribution of nanoparticles, biological effects and amount of immune response are dependent on the size of a nanoparticle. The capacity of intracellular uptake and in vivo mobility of nanoparticles are more compared to micro particles due to their reduced size. Another beneficial feature of the nanoparticles in drug targeting and delivery is their small size, with maximum surface area, providing a large space for carrying the drug on its surface. In the case of micro particles, due to the huge surface area, the drug might diffuse through the outer shell towards the core. Some of the shapes that nanoparticles possess are cubes, nanostars, triangles and prisms (Calum 2017).

7.5.1 Surface Chemistry

Some of the most important properties such as charge, reactivity and hydrophobicity hold a crucial role in the interaction between nanoparticles and the biological systems, which can be modified upon attachment of nanoparticles. The main reason why the surface chemistry of small nanoparticles is significantly different from the bulk nanomaterial or larger nanoparticles is because of the disparities in the number of binding sites and the effect of quantum confinement. Another important surface property is zeta potential, which allows for the aggregations of nanoparticles as they carry specific charge, specific to each medium (Wang et al. 2013).

7.5.2 Surface Tension

Earlier, Du-Noüy ring method using an automatic surface tensiometer was employed for testing the surface tension valve in nanofluids. Surface tension is a major phenomenon that plays a major role in determining the heat transfer of a thermal system (Bhuiyan et al. 2015). It is basically defined as the amount of free surface energy

present per unit area of the liquid droplet. One aspect in which surface tension plays a negative role by causing core-shell interface defect is by building up of nanosized impurity particles (Shabarovaa et al. 2018). By utilizing a thermodynamic model that includes surface tension property dependent on the size of the nanoparticle, the behavior of the NPs can be elucidated. This surface tension is generally considered as the interface between the nanoparticle and the surface of the fluid, where it is associated, providing us with the required information regarding the stability of the biomolecule at that interface. It is generally reported that there are innumerable changes in the adsorption properties of Nano-sized materials because of surface tension that is resulting from applied surface strain. Another point to note is that the relative binding of adsorbents gets decreased when compared to their bonding on extended surfaces due to the effect of surface tension of a nanoparticle cluster and this is more evident on close-packed structures. As the size and concentration of nanoparticles in a nanofluid increases the overall surface tension increases (Lin et al. 2015). Surface tension plays a cardinal role in heat transfer applications where optimal utilization of nanofluids take place (Prasad et al. 2018).

7.6 Nanoparticle-Biomolecule Interactions

Application of nanoparticles can invariably improve the way in which many diseases caused by tumor cells are diagnosed and treated. They also show a promising result in the treatment of lung and heart diseases. It all depends on the interaction or interface of nanoparticle and biomolecule, where the nanoparticles come in contact with the biological components. Examples of some biological components are proteins, DNA, phospholipids, organelles and other biological membranes (Ghosh and Paria 2012). The nano-bio interface includes various physicochemical interactions, kinetics and also thermodynamic changes between the surface of biological components and the surface of nanoparticles. It makes highly essential to study these interactions that occur at the interface for attaining a keen insight regarding the utilization of nanoparticles for human health benefits. There are three main components that contribute to the interactions at the interface:

(a) The surface of the nanoparticle.
(b) The interaction of the solid particle with the surrounding liquid medium resulting in the formation of solid liquid interface.
(c) The interaction of the solid liquid interface with the biological surface.

All these interactions are dictated by surface properties of the nanoparticles. A detail of the interaction of the nanoparticles was provided in Table 7.1. Surface properties of nanoparticles listed in the table help in the interaction of nanoparticle with the medium by adsorbing proteins and various ion molecules, by dissolution in the medium and finally by the formation of double layer (Gilbert et al. 2004). The medium in which the nanoparticles get suspended may be composed of acids and bases, water molecules, detergents, ions, salts and some other large molecules.

Table 7.1 Characterization of different surface properties of all the three interacting components

Nanoparticle	Interaction between nanoparticle and medium	Interaction between interface and biological surface
• Chemical composition of material	• Hydration and dehydration of the surface	• Binding of the receptor and ligand
• Shape of the particle	• Release of free energy from the surface	• Transfer of free energy to molecules
• Porosity	• Net charge	• Change in conformation of molecules
• Aggregation	• Isoelectric point	• Reduction of ATP
• Roughness of the particle	• Zeta potential	• Damage of mitochondrion and lysosomes
• Angle of curvature	• Average aggregate size	
• Hydrophobicity and hydrophilicity	• Formation of electrical double layer	
• Surface layer valency		

Once the nanoparticle is placed in any medium, the properties of the material get modified to a great extent, and then they form an interface, which is in metastable state. This interface further will lead to the formation of a highly unstable nano-bio interface, which in turn undergoes many changes with certain changes in the composition of the surrounding medium. For example, with the release of any biological product into the medium, the nanoparticle interacts with the product and undergoes some changes that will influence the interface.

The interaction at the nano-bio interface includes many types of forces. The forces like van der Waals forces, electrostatic or ionic forces occur mainly between the colloidal particles are also applicable here but with slight variation (Min et al. 2008). During the interaction between two individual nanoparticles such as silicon dioxide when placed in water, attractive van der Waals forces occur due to the mutual induction of dipole moment in both the particles. Because of the surface negative charge, electrostatic forces are formed, which are repulsive in nature. Solvation forces occur when the water molecules form layers on the surface of the particles which retards their adherence thus stabilizing the particle. In contrast, when the same nanoparticle begins to interact with a living cell, all fundamental forces will be redefined by minor differences like (a) there is a chance for the cell membrane to get deformed, as it is non-rigid, (b) the non-uniform charge on the surface may change the energy of the surface and (c) the non-passive nature of the cell membrane may hinder the movement of silicon dioxide particles (Dobrovolskaia and McNeil 2007). Due to the changes that occur in the biological systems, it is presumed that these interactions are complicated and also difficult to predict.

To clearly investigate and acquire knowledge on how nanomaterials interact with cells, various cell lines such as THP-1 have been used as models for conducting experiments. THP-1 tumor cells are collected from patients with acute monocytes leukemia are used to test polystyrene nanoparticles. These nanoparticles do not degrade easily and their size is nearly 110 nm. It was identified that these particles

get internalized very well by the cell lines through pinocytosis, when compared to nanoparticles of size 50 nm which got phagocytosed by the phagocytic cells like macrophages and monocytes (Cornelia Loos et al. 2014). Just like polystyrene nanoparticles, gold nanoparticles are also used to study the interactions. Size and shape of gold nanoparticles can be easily altered according to our interest. Coupled with this, the inertness of gold made researchers use it for various works (Alaaldin et al. 2013). Many different cell lines such as HELA cells and mammalian cells are also used to understand interactions occurring at the nano-bio interface. These interactions will further affect the uptake, transportation and cytotoxicity of the nanoparticles by different kinds of cell lines.

7.7 Biological Effects of Nanoparticles

Nanoparticles and their interactions with the living cells and biomolecules serve as precursors for various biological effects. When a nanoparticle passes through the cell membrane overcoming all the barriers at the nano-bio interface and reaches the cytosol, it elicits numerous responses. These biological effects are both beneficial and detrimental to human health. Cancer is the leading life-threatening disease currently, and a large number of deaths have been caused by breast carcinoma. Biodegradable nanoparticles like PLGA-PEG loaded with chrysin are used for testing against breast cancer cells. PLGA is a copolymer of polyglycolic acid and polylactic acid. Chrysin is an anti-cancer drug that induces cell apoptosis. This drug is loaded on to PLGA-PEG nanoparticles and tested against the breast cell lines. This resulted in an enhanced inhibitory effect when compared to the effect caused by chrysin alone (Makadia and Siegel 2011). Radiation therapy is gaining importance day by day due to the incidence of cancer, and larger doses of radiation are needed to destroy tumor cells, but the healthy tissues cannot tolerate it. So an alternative was devised in which gold nanoparticles (AuNPs) are being used. When the level of dosage was relatively reduced, it was found out that a large number of cells got killed. Similarly, metallic nanoparticles made up of copper, gold and silver also showed some important biological responses within organisms. Copper nanoparticles are stable and can be mixed with polymers very easily. LCuNPs are one of the nanoparticles synthesized from plant latex that are efficient in apoptosis of lung cancer cells (Mayur et al. 2011). Human bones and teeth are made up of hydroxyapatite, which is used as a coat over the prostheses and also has many biomedical applications. Initially micro-sized hydroxyapatite particles (m-HAP) are prepared and tested with osteoblast cells like MG-63 cells. Later on nano-sized HAP was synthesized, and the same test was repeated. It was found that nano-HAP is many times more effective in inducing growth and inhibiting apoptosis in MG-63 cell lines (Shi et al. 2009). Further, due to the presence of glutamic acid in the surrounding medium, reactive oxygen species (ROS) are produced inside the nerve cells, and the presence of these ROS leads to cell death. To avoid this, antioxidants are used to reduce the free radicals. Nanoparticles made of cerium oxide and yttrium oxide were made to react with nerve cells like HT22 cell line to test their antioxidant

nature. Nano sizes of yttrium oxide particles showed better results in reducing the oxidative stress when compared to the ones made with cerium oxide, thus depicting their neuroprotective nature (Schubert et al. 2006).

Silver nanoparticles (AgNPs) are well known for their antimicrobial activity against many viruses. In order to assess their anti-tumor activity, analyses were performed on Dalton's lymphoma ascites (DLA) cell lines. AgNPs induced an enzyme called caspase3, which caused apoptosis of tumor cells, confirmed by the nuclear fragmentation. These nanoparticles also assisted in reducing the ascetic fluid in mice affected with cancer, which helped in getting back their body weight to normal (Muthu et al. 2010). Additionally silver nanoparticles were proved to be effective against colon cancer cells. They invigorated G0/G1 cell arrest causing DNA destruction. This leads to reduced viability due to apoptosis of colon cancer cells (Wan et al. 2009). Utilization of magnetic nanoparticles has been given much importance as they can be modified easily. Dendrimers are mixed with oligonucleotides and coated on the nanoparticles and incubated with breast cancer cells. This

Table 7.2 Biological function of nanoparticles on different cell lines

S. no.	Nanoparticle	Type of cell line	Function
1	Chrysin-loaded PLGA-PEG	Breast cancer cells: T47D and MCF7 cell lines	Effective carrier for anticancer drugs and also aids in targeted therapy
2	Copper NPs (LCuNPs)	Lung cancer cells: A549 cell lines	Apoptotic destruction of human lung carcinoma cells
3	Titanium dioxide NPs	Lung epithelial cells: A549 and H1299 cell lines	They aid in reducing the viability of H1299 cell line, but there is no effect on the viability of A549 cell line
4	Nano HAP	Osteoblast cells: MG-63 cell line	Induces growth
5	Cerium oxide and yttrium oxide NPs	Nerve cells: HT22 cell line	Reduce oxidative stress and are neuroprotective
6	Silver NPs (AgNPs)	DLA cell line	Antimicrobial and anti-tumor activity Induce caspase 3 enzyme
7	Silver NPs (AgNPs)	colon cancer cell lines	Cell cycle arrest
8	Silicon dioxide, titanium dioxide and zinc oxide NPs	Intestinal cell lines: SW480	Induce oxidative stress
9	Gold NPs (AuNPs)	Aortic endothelial cells	Best results of cell damage
10	Citrate NPs	Dermal fibroblast cell lines	Disappearance of actin stress fibers leading to decreased cell viability
11	Cobalt NPs	Endothelial cells: HepG2 cell line	Readily enter the cells to perform undesirable functions
12	Hafnium oxide NPs	Glioblastoma cancer cell lines	Deposits energy in high doses when they are exposed to ionising radiation
13	Copper NPs (LCuNPs)	T lymphocytes	Proliferation, expression of adhesion molecules and release of cytokines.
14	Selenium NPs	MCF-7 cell lines	Potential antioxidant

brought into light the ability of magnetic nanoparticles to deliver any gene into cells effectively, thus proving their potential to be used for cancer treatment (Pan et al. 2007). The biological function of nanoparticles on different cell lines has been listed in Table 7.2.

In spite of having numerous advantages, nanoparticles have equally considerable disadvantages. Understanding these disadvantages is very crucial as most of them are related to human health. Cobalt nanoparticles (CoNPs) are known to enter inside the cells very rapidly when compared to many other potential nanomaterials. Once they enter, they increase the reactive oxygen species instead of depleting them resulting in many undesirable reactions in the body (Elena et al. 2009). Titanium dioxide nanoparticles reduce cell activity and viability by depleting ATP when given in low doses, and there is an observed decrease in mitochondria when given in higher dosages (Roslyn et al. 2011). SiO_2, TiO_2 and ZnO nanoparticles are predominantly used in the food-processing industries. The major cause for gastrointestinal toxicity and immunological side effects caused by these food borne nanoparticles is due to a large production of reactive oxygen species inside the cells causing significant DNA damage and cell death by cell cycle arrest.

7.8 Toxic Effects of Nanoparticles

The size of nanoparticles is the main characteristic feature that allows them to easily pass through the cell membrane and perform important functions by eliciting biological responses. The integration of these biological effects may be both advantageous and deleterious to the human body. There are numerous mechanisms that cause nanoparticle toxicity. Usually very small quantity of reactive oxygen species (ROS) is produced in different organs, and their level is retrieved by the action of antioxidant enzymes and glutathione. But the production of these ROS is gradually enhanced in organs such as lungs, when nanoparticles are inhaled. During this process, glutathione gets oxidized and the ratio of glutathione to oxidized glutathione, a regulator of equilibrium in the cell decreases resulting in no effect on the depletion of ROS. When oxidative stress is in moderate levels, MAPK and NF-kB cascades get activated resulting in inflammatory responses. In case of high stress, electron transfer is disrupted, and apoptosis of cells takes place. This is the mechanism through which nanoparticles cause toxicity to the human body. This toxicity may be cytotoxicity, in which the cells are forced to undergo programmed cell death or genotoxicity where the genetic material like DNA and RNA is affected.

Even though this is the basic mechanism used by the nanoparticles, different types of nanomaterials modify it according to their ease. Carbon nanotubes are a type of nanomaterials known for many unique properties. These are of two types: single- and multiple-walled carbon nanotubes. Both of them follow different mechanisms of toxicity. Single-walled carbon nanotubes activate CN-KB in human keratinocytes to produce oxidative stress, whereas multiple-walled carbon nanotubes result in apoptosis of T-cells (Oberdorster 2004). Metal nanoparticle results in the intracellular toxicity, and the mechanism responsible for it is found in many

experiments. The lysosomal compartment of the cell is acidic in nature due to which the particles get internalized, and release of ions takes place leading to the intracellular toxicity. This mechanism is commonly referred to as 'lysosome-enhanced Trojan horse effect' (Stefania et al. 2014). Endoplasmic reticulum releases calcium ions due to oxidative stress and results in the mitochondrial deformation and cell death. Upon exposure to ZnO, there was a significant increase in calcium ion release (Xia et al. 2008). The mechanism underlying silver nanoparticles involves the oxidation of the nanoparticle surface by oxygen molecules, releasing silver ions that are toxic to the cell (Mc Shan et al. 2014). When silica nanoparticles are exposed to lung epithelial cells, lipid peroxidation occurs due to production of ROS, leading to enhanced activation of transcription factor and regulation of interleukin 6, 8 and MMP-9, finally leading to PCD using caspase-3 (McCarthy et al. 2012).

7.8.1 Cytotoxicity of the Nanoparticles

The interactions between nanoparticles with cells and tissues can sometimes be detrimental, causing cell cycle arrest, DNA damage, production of ROS, etc. This phenomenon where the nanoparticles elicit an intense immune response by causing toxic effects on cells is called as cytotoxicity. Intracellular organelles may also get affected due to cytotoxicity when oxidative stress, mitochondrial dysfunction and lipid peroxidation take place (Schanen et al. 2009). In a study, positively charged nanoceria caused toxicity in the MCF-7 breast cell line and negatively charged nanoceria caused toxic effects in A549 lung carcinoma cell line. Based on light scattering experiments, it was found that the size of nanoceria particles ranged from 5 to 14 nm in diameter (Asati et al. 2010). In a similar work done by Connor et al. (2005) on the leukemia cell line, K562, by application of gold nanoparticles, it was found that, if the concentration of the nanoparticles was greater than 25 μM, it was toxic. A549 cell lines of human lung carcinoma had shown cytotoxicity upon inducing silver nanoparticles of concentration 20 μg/mL. It caused necrosis and apoptosis in the affected cells and inhibited the normal functioning of mitochondria (Rasmus et al. 2011). Gold nanoparticles also were found to induce cytotoxicity in HeLa cell lines when the size was 1–2 nm. Synthesis of spherical silver nanoparticles of 17.6–41 nm using *Piper longum* leaf extract and introduction into Hep-2 cell lines showed intense cytotoxic effects (Justin et al. 2012). Zinc oxide nanoparticles of 60 nm size were synthesized by subjecting to ultrasound irradiation and were tested on different human glioma cell lines U87, A172, U251, LNZ308, LN18 and LN229. It was reported that a concentration of 10 mmol/L of zinc oxide concentration was sufficeint enough to induce apoptosis and cell death. It was also found that the reactive oxygen species are responsible for decreasing the viability of these cells (Stella et al. 2009). Silica nanoparticles of different sizes such as 104 nm and 335 nm showed less cytotoxicity effects on the human endothelial cell line EAHY926 when compared to smaller nano-sized silica particles, thus establishing the fact that smaller particles showed higher cytotoxicity (Dorota et al. 2009).

In a study, Chitosan nanoparticles (size of 65 nm and surface charge of 52 mV) were used against human gastric carcinoma cell line MGC803, which led to DNA degradation and necrosis (Li-Feng et al. 2005). In another study, silica nanoparticles of sizes 20 and 50 nm have shown significant nuclear condensation, cell shrinkage and high production of ROS when employed on human embryonic kidney stem cells, HEK293 cells (Fen et al. 2009). Silver nanoparticles of 15 nm size was used and tested against human lung epithelial carcinoma cell line, namely, A549, where there was a significant decrease in mitochondrial transmembrane potential, increased ROS and leakage of LDH (Jae et al. 2014). DNA damage, arrest of cell cycle and apoptosis of Hep-G2 cells occurred when silica particles of size 498 nm were used (Yang et al. 2010). Human hepatocyte (L02) and human embryonic kidney (HEK293) cells were tested for cytotoxicity by using zinc oxide nanoparticles (particle size of 50 nm), and it was found that morphological modifications, mitochondrial dysfunction, reduction of super oxide dismutase and glutathione and DNA damage have occurred (Rongfa et al. 2012). A549 lung and MCF-7 breast cancer cell lines were tested by using silver nanoparticles of 27 nm, and severe cytotoxic effects were observed (Ivan et al. 2014). Cerium oxide nanoparticles were toxic towards prostate cancer PC-3 cell lines. Testing of 15.6–1000 μg/mL nickel-zinc (NiZn) ferrite nanoparticles against MCF7 breast cancer, human HT29colon cancer and HepG2 liver cancer cell lines resulted in induction of apoptosis and cell death (Renu et al. 2012).

7.8.2 Genotoxicity of the Nanoparticles

Genotoxicity of nanoparticles leads to chromosomal changes, breakage of DNA strands and mutations. Genotoxicity may be direct or indirect depending on the mechanism applied. In the direct genotoxicity, the nanoparticles directly enter into the cells by passing through the nuclear pores and interact with the DNA present in the chromosomes during interphase. This nanoparticle interrupts transcription process by binding to DNA (Jin et al. 2011). In the indirect method, NPs do not interact directly with the DNA, but they cause toxicity in the following ways: (a) interaction with mitotic spindle, (b) interaction with nuclear proteins, (c) disturbing cell cycle checkpoints, (d) ROS production and (e) inhibition of antioxidant activity (Magdolenova et al. 2014). When human epidermal cells were exposed to titanium dioxide nanoparticles, they were readily taken up by the cells, and after 6 h of exposure, there is a significant damage done to the DNA. There is also a direct relationship between the concentration of nanoparticles and damage of DNA (Ritesh et al. 2011). Similarly, the entry of ZnONPs into the human body causes DNA damage by inducing oxidative stress in the human liver cells HepG2 (Vyom et al. 2011). Hepatic cancer cells (HepG2) and peripheral blood mononuclear cells have rendered varying levels of DNA damage by gold nanoparticles. Cobalt nanoparticles produce cobalt ions which compete with magnesium ions and impair enzyme proteins in binding with DNA when they are incubated with human leukocyte cells (Colognato et al. 2008). Copper oxide nanoparticles have the potential to effect

DNA damage in the A549 human lung cells, when exposed for a brief period of time (Maqusood et al. 2010). Genotoxicity of silver nanoparticles was studied using comet assay and cytokinesis-block micronucleus cytome assay. During mitosis, micronuclei found in chromosomes cannot combine with the mitotic spindle. In the comet assay, the extent of DNA damage was shown. In cancer cells, there was an increase in the DNA damage with an increase in nanoparticle concentration. But in fibroblast cells, the damage was not significant beyond certain concentration of nanoparticle (Asha Rani et al. 2009). Nickel oxide nanoparticles resulted in the nuclear translocation of proteins in the human epithelial lung cell lines (Laura et al. 2014). In a study, metal cobalt nanoparticles were readily taken up by the human peripheral blood cancer cell lines causing severe DNA fragmentation revealing its genotoxic nature.

7.9 Assays and Models for Testing the Nanoparticles

Nanotechnology is one of the rapidly developing fields, which has applications in numerous fields related to human health and welfare. Presently, nanoparticles are assayed for the cyto-, immuno- and genotoxicities. Determination of cell viability is done by using tetrazolium-based assays such as MTT, MTS and WST-1. Cell inflammation caused by the nanoparticles is analysed by testing for IL-8, IL-6 inflammatory biomarkers and tumor necrosis factor using ELISA. The solidarity of the cell membrane is obtained by using lactate dehydrogenase (LDH) assay. Nanoparticles are used in fields like electronics, biotechnology, medicine, etc. because of their tiny size. Nanoparticles can enter into the human body very easily and cause severe damage. Hence, various methods have been proposed for testing the safety of nanoparticles. US FDA has considered the issue of nanoparticle toxicity and issued that they are not completely harmless or safe for humans and each product must be subjected to regulation (Bahadar et al. 2016). Kathleen et al. (2011) presented different assays which can be used for studying the suitability of nanomaterials in cardiovascular applications, including in vitro blood compatibility studies. Nanoparticle-induced hemolysis can be evaluated by spectrophotometry, platelet activation and complement activation can be evaluated by thromboelastography (Kathleen et al. 2011). Toxicity assessment of aerosol nanoparticles in human lung cells (A549) was carried out by cell viability using MTT assay, extent of membrane damage by LDH assay and temporal dose response by measuring the size distribution of silicone nanoparticles using dynamic light scattering technique (Irfan et al. 2014).

Alzheimer's disease (AD) is the most common type of dementia which results in progressive loss of memory and thinking skills. AD rats are treated by transferring the neural stem cells and observing their improved learning and memory function. Nerve growth factor, poly (ethylene glycol)-poly(lactic-co-glycolic acid) nanoparticles (NGF-PEG-PLGA-NPs), can be used for the differentiation of neural stem cells in vitro. A study was conducted on AD rat models to evaluate the efficacy of NGF-PEG-PLGA-NPs combined with transplantation. This type of transplantation can specifically improve learning and memory functions of AD rats by

replenishing basal forebrain cholinergic neurons and can help in the formation of hippocampal synapses and AchE-positive fibers (Chen et al. 2015). Wistar rats are used as models to study the dose-dependent in vivo toxicity of silver nanoparticles and examined hematological parameters such as WBC count, platelet count and RBC count. These parameters were found to be changing with the amount of dosage (Dhermendra et al. 2011). Genotoxicity testing of Tio_2 anatase nanoparticles was performed using comet assay and wing-spot test in *Drosophila*. Nanoparticles are reported to cause cytotoxicity on midgut and imaginal disc of larvae, but there is no genotoxicity reported in wing-spot test. But, amount of DNA damage in was identified by comet assay (Erico et al. 2015). Human embryonic stem cell derived three-dimensional in vitro model allowed us to test the neurotoxicity of nanoparticles. For testing the specific toxicity of nanoparticles, chemically inert polyethylene was chosen. They found to penetrate deep into the three-dimensional structures and impacted gene expression at non-cytotoxic concentrations (Hoelting et al. 2013). Blood-brain barrier model is useful to test the permeability of nanoparticles for easy and reproducible assay. Reconstruction of this model is done by culturing both primary rat brain endothelial cells and pericytes to support the tight junction of endothelial cells (Hanada et al. 2014).

7.10 Applications of Nanoparticles in Biological Systems

Nanobiotechnology emerged as a mature biomedical field which possesses several applications in molecular biology, genetic engineering, diagnosis (biomarkers) and cancer treatment (De Jong and Borm 2008; Rathi Sre et al. 2015). Nanomedicines built on nanocarriers are used to treat microbial infections and diseases like cancer (Wesselinova 2011). Due to their physicochemical properties, they are widely used in food industry, cosmetology, agriculture and medicine for protection of human health. Metal-based nanoparticles are used in electron microscopy, bio-imaging, magnetic resonance, computed tomography for visualization due to their optical density and paramagnetic properties (Rai et al. 2016; Veronesi et al. 2016; Liu et al. 2013).

7.10.1 Pharma and Healthcare

The use of nanoparticles in medicine and more specifically drug delivery is currently gaining momentum. In pharmaceutical sciences, the use of nanoparticles mainly focused on to minimize the side effects and toxicity associated with the drugs. The use of nanoparticles made of biopolymers and polymeric biomaterials has become popular to overcome the toxic effects during the drug delivery (Duncan 2003). Biodegradable polymeric nanoparticles reach the diseased site in the body more specifically and exert their action effectively compared to other drug delivery systems (Ferrari 2005). Since the nanoparticles possess several therapeutic

properties, research is going on to find the novel biomaterial-based nanoparticles for targeted drug delivery.

7.10.2 Environment

Nanoparticles have potential applications in various areas with environmental inferences. Selective adsorption of certain compounds is needed for removing contaminants in landfills and industrial waste stream. We can use adsorbents, fabricated by nanoparticles. Nanoparticles, used as catalyst for effective oxidation, exhibit high specific surface areas, obtained by sol-gel method (Ward and Ko 1995). Photocatalysis is used for environmental remediation where there is total oxidation of compounds in gas and aqueous phase systems (indoor air problems, purifying air in industrial settings). Separations mediated by inorganic nanoparticulate filtration media, which overcome the limitations caused by ceramic membranes and polymeric membranes for activity of reverse osmosis. Another application of nanoparticles that benefits the environment is the usage of nanoparticle oxides for fabricating thin-film batteries, ultra capacitors and fuel cells, which are used as energy storage and power devices (Zeltner and Anderson 1996).

7.10.3 Industry

Mass production of nanoparticulate carbon improves strength. Abrasion resistance tyres are used as fillers and TiO_2 aerosols as key ingredient in white paints. Pigments with high refractive index such as titania allow high color depth and strong optical. Inorganic pigments based on titania and zinc oxide absorb UV radiations and thus are used in cosmetics. Nanometer-thick active ingredients applied as a coating on window glass will have self-cleaning property (Manning et al. 2011). Catalysis by nanoparticles like Ni, Pd and Pt is with industrial relevance. Nanoparticulate forms are used in improving the healing, implant adhesions and tissue response, delivering materials to the site of application due to their rapid mobility (Stark et al. 2015).

7.11 Conclusions

Nanoparticles are being used in designing different types of products, and their unique properties will guide us to attain new technological breakthroughs. Products produced by using nanoparticles are utilized in electronics, healthcare, chemicals, cosmetics and energy. Besides its advantages, there are limitations due to its toxicity towards health, which means the capacity of a substance to cause illness, injury or death of any organism. If a product contains toxic nanoparticles, it may create health and safety risks through all its stages of life cycle. As nanotechnology is an emerging field now, all the producers are focusing on safer and effective production by integration of nanoparticles into products. The five principles of SAFER

Guides are the following: (1) size, surface and structure, (2) alternative material, (3) functionalization, (4) encapsulation and (5) reduction of the quantity used as a framework for safe nanotechnology. To benefit society and acquire acceptance, there is a need of systemic investigations by considering environment and human health. To build the investigation, we need to understand the interactions with biological system. Our approach should start with the synthesis of nanoparticles by using nanoparticle libraries with combination of green chemistry. Evaluation is performed using in vivo models to assess the biological activity and toxic potentials of nanoparticles at various levels of organization of organisms. Nanomedicines are developed and evaluated by European Medicines Agency giving priority to patient safety. It is also needed to ensure next-generation nanomedicine to enter the market with time in a safe way. It is also important to assess the biological effects of manufactured nanoparticles by using a tool like Quantitative Nanostructure-Activity Relationship (QNAR).

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8 *Caenorhabditis elegans*: A Model Organism to Decipher Biological Activities of Nanoparticles

Ramatchandirane Mahesh and Kitlangki Suchiang

Abstract

In recent decades, the range of nanoparticles that have been authorized by many biomedical agencies globally has increased exponentially. In their therapeutic and treatment potentials, nanoparticles have gained greater importance in drug delivery because of their carrying capacity, stability, and specificity. Thus, understanding the broad spectrum of nano-bio interactions and the challenges of the interface may provide new opportunities for novel designing of nano-based materials for different biological applications. *Caenorhabditis elegans* (*C. elegans*) as a model organism has gained popularity, and its feasibility as an in vivo model has also been extended to nano-biotechnology. In the process, understanding the basic biology of *C. elegans* as a model system before the elucidation process of various biological activities of nanoparticles is critical for productivity and translational capability. The applicability of different high-end chemical-based and material science techniques in studying modes of nanoparticle exposure and their routes of absorption, bio-distribution, and excretion has made the process of nano-bio interaction studies in *C. elegans* model more accurate and informative. Furthermore, major factors affecting nano-bio interactions which might hinder the interactions of the model system such as the alterations in media, growth conditions, chemical composition, etc. are highlighted. The final discussion pertains to commonly reported biological activities of different nanoparticles using this model organism such as antioxidants, antimicrobial, toxicity studies, etc. The advantages and disadvantages of different reported activities of nanoparticles as elucidated from this model organism are also elaborated.

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Nanoparticle · *C. elegans* · Nano-bio interactions · Factors affecting the interactions · Techniques · Biological activities

8.1 Introduction

Modern advances in nanoscience and nanotechnology fields have seen faster development of various novel applications of nanoparticles ranging from electronic appliances to pharmaceutical industry and biomedical sciences. Nanotechnology represents new ways to perturb cells and treat patients, through novel designing of specific nano-based therapeutics that can target diseased cells directly, bypassing unwanted biological barriers. In this process, elucidation of these effects requires functional characterization of the different nano-bio interactions in a complete in vivo model organism. This will allow for an in-depth understanding of the broad spectrum of interactions in an active living organism. Thus, knowledge obtained after its safety validations in vivo would pave a way for the productivity of new nano-based materials in the market for various useful applications.

In the field of biomedical sciences, researchers are currently employing the unique design of these entities in a size range of 1–100 nm for their potential in improving novel targeted therapeutics and for accurate diagnostics. Thus, priorities should be made to identify routes of exposure and absorption and the consequences from their biological effects either after short- or long-term exposure. The basic framework for understanding nano-bio interfaces represents many challenges. An approach in measurement and detection of the interactions in nanoscale range with single-cell requires exquisite sensitivity both in vitro and in vivo. Similarly, it is equally important to have an in-depth understanding of the pharmacokinetics and pharmacodynamics of the whole process right from its uptake, bio-distribution, and translocation to excretion. Thus, translating these proofs of concept in nanotechnology from a controlled laboratory environment to widespread usage will require extensive testing and validations.

In terms of practicality, model organisms have always played a vital role in the field of discovery and research. The popularity and fitness of *Caenorhabditis elegans* (*C. elegans*) as an in vivo model in screening and evaluation of nanoparticles have increased exponentially in recent years (Zhao et al. 2013). The ability of worms to self-fertilize and generate large numbers of progeny aided with the presence of complex tissue systems is ideal for different biological studies in terms of both mechanistic and high-throughput screening approaches.

8.2 *Caenorhabditis elegans*: A Model Organism

Caenorhabditis elegans (*C. elegans*) was introduced in 1965 by Sydney Brenner as the whole model organism for studying developmental genetics. Since then, the utility of this model animal has been expanded to explore diverse areas of modern

biology and allied sciences. It is a free-living, soil-loving nematode (roundworm) measuring 1 mm in length. It is non-parasitic and likes to live and thrive in temperate/warm soil environments. In the laboratory, it is maintained and cultured on nematode growth media (NGM) plates and fed with *E. coli* OP50 as diet source (Fig. 8.2). The body of these animals is transparent that enables tracking of an individual cell and cell lineages. Due to its shorter life span, it's well suited for aging studies and is also widely used in neurological and host-pathogen interaction studies as a model organism (Horvitz and Sulston 1980). As a host model, it has been employed in studying microbial and parasitic infections. Similarly, *C. elegans* is at the forefront of high-throughput screening (HTS) for evaluations of biological activities of various chemicals, drugs, and nanoparticles (Brenner 1974; Brinke et al. 2011).

8.2.1 Origin

Rhabditis elegans was the initial name of *Caenorhabditis elegans*, and this name was coined by Maupas in 1900. In 1952, Osche later placed it in the subgenus Caenorhabditis which was later promoted to the generic status in 1955 by Dougherty. N2 wild type was an isolate reference that was first obtained from mushroom compost of England by L. N. Staniland in 1965. A laboratory strain was obtained from Bristol culture by Sydney Brenner in 1964 from Dougherty. It is assumed to share a common ancestor with humans, 500–600 million years ago during the pre-Cambrian era (Fig. 8.1).

8.2.2 *C. elegans* Tissues and Body Plan

Despite its simple body, *C. elegans* has some well-characterized and well-defined tissues. The worm body is a tubular structure, with a cuticle forming the external surface and skeleton necessary for its movement. Its basic body system which includes the nervous, alimentary, reproductive, and excretory systems is protected by a collagenous cuticle. The alimentary system of *C. elegans* consists of organs like the mouth, pharynx, intestine, rectum, and anus. The digestive system is organized in the form of a tube covering the whole length of the animal and is made up of a pairwise arrangement of 20 long polyploidy epithelial cells. The excretory system is considered to regulate osmolality and waste elimination from the body. It has muscles, a hypodermis, a cuticle (body)-protective covering, connective tissues, and basement membranes. Gaseous exchange and nutrient supply occur through the body cavity by passive diffusion especially through the cuticle layer of the gut surface, since they lack circulatory system (Altun et al. 2009; Kerr 2006).

In a mixed population of *C. elegans*, both male (XO) and the self-fertilizing hermaphrodite (XX) sexes are seen. After the L4 stage, the worm becomes an adult egg-laying organism of 1 mm long with ~959 cell nuclei, of which 302 are neurons. On the other hand, males have 1031 cell nuclei and can be produced rarely at about 0.1% spontaneously from nondisjunction of the X chromosome and/or up to 50% of the outcross progeny from a mating between a hermaphrodite and a male worm.

KINGDOM	Animalia
PHYLUM	Nemata
CLASS	Secernentea
ORDER	Rhabditida
FAMILY	Rhabditidae
GENUS	*Caenorhabditis*
SPECIES	*elegans*

Fig. 8.1 Taxonomy of *C. elegans*

Slight modifications in reproductive systems of hermaphrodite and male are observed with hermaphrodite having a uterus, gonad, spermatheca, and vulva (Sternberg 2005), whereas the male has gonad, cloaca, vas deferens, and seminal vesicle (Emmons 2005). Although both adult male and hermaphrodite have the same body length, phenotypically they can be differentiated with the presence of male worm with unique features such as blunt tail with rays, the spicules, the hook, a proctodeum, and a thinner body (Fig. 8.2).

Inside the alimentary tract lies a pseudocoelomic fluid called coelomocytes which houses the main organ systems besides its roles in endocytosis and fluid balance (Corsi et al. 2015). Furthermore, due to the lack of circulatory system, this pseudocoelomic fluid performs additional homeostatic roles ranging from lubrication of different tissues, trace metal enrichment of fluid, and maintenance of hydrostatic pressure balance in the worm body. It also serves as a medium for cell-to-cell communication, signal networking, and the passive diffusion and transport of O_2, CO_2, and nutrients (Wood 1988). Well-defined tissues with similar architectures of organs like intestines, muscles, pharynx, and hypodermis and gonads of higher organisms are also present in *C. elegans*. Thus, their presence allows for understanding the specific molecular interactions of different nanoparticles that can perturb/affect cellular physiology. Subcellular changes can also be used as parameters for an in-depth understanding of the process involved (Garigan et al. 2002; Herndon et al. 2002; Golden et al. 2007; Haithcock et al. 2005).

8.2.3 Reproductive System

The existence of two sexes, which include the most common hermaphrodite (XX) and the rare occurrence male (XO), has given rise to the phenomenon of sexual dimorphism. A highly sexually dimorphic tissue in the worm is the reproductive system which differs between hermaphrodites and males. The space for fertilization and laying eggs is provided by the hermaphrodite, and this is divided into three distinct major parts consisting of the somatic gonad, germline, and egg-laying

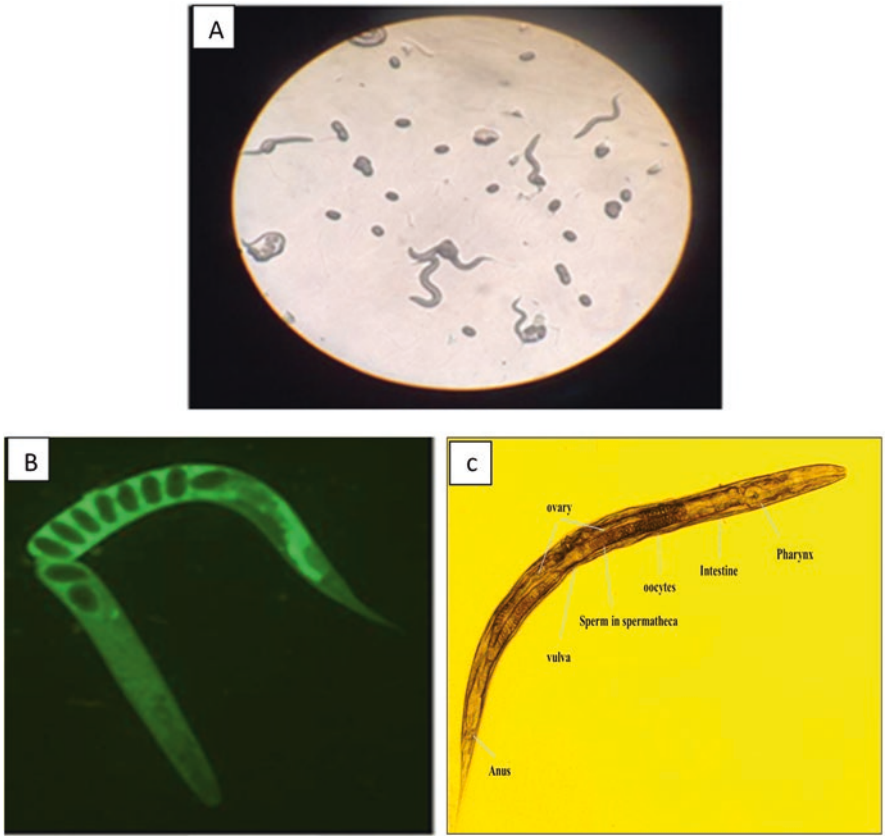


Fig. 8.2 Images of *C. elegans* depicting (**a**) mixed worm population, (**b**) transgenic green fluorescent-tagged adult hermaphrodite worm, and (**c**) bright-field image of adult worm showing organs and tissue systems

apparatus. The germline of an adult is well organized into a distal space corresponding to the distal end and the proximal space which lies near the embryo exit point from the worm. As they move to the proximal part, they can enter into different stages of meiosis. The somatic gonad comprises the spermatheca, gonadal sheath, distal tip cell, and uterus. The egg-laying apparatus is made of the vulva and the muscles of the uterine. Hermaphrodites are considered to be the self-fertilized female because the soma is female. However, the germline produces male gametes in a fixed number before the production of female gametes (L'Hernault 1997; Schedl 1997). They produce ~300 embryos through self-fertilization process. During copulation, male-derived fertilization takes place, and maturation occurs in the spermatheca (Singson 2001). A fertilized egg then passes onto the uterus, and the egg-laying apparatus makes the egg to move out through an opening ventrally called the vulva.

8.2.4 Alimentary System

The complex system in the worm's anatomy is the alimentary system with various tissues and cells (White 1988). The connections between the body and the alimentary system network are less but direct. The worm has a cylindrical body wall with an epithelial tube divided by a pseudocoelomic space which is parallel to the gonads. The system has anterior and posterior regions. The pharyngeal epithelium connecting the arcade cells of the lips is the anterior region, and the posterior region ends up in the rectal epithelium and anus. This system which comprises 127 cells is also divided into 3 major parts such as the foregut, midgut, and hindgut. During molts, the stomodeum and proctodeum (parts of the foregut and midgut) line along with the cuticle (Bird and Bird 1991). The stomodeum has the openings for the pharyngeal glands, while the proctodeum has the openings for the rectal glands. *C. elegans* intestine shares similar cellular architecture with higher animals in respect to cell polarity of the intestinal cells (enterocytes), apical and basolateral domains, cell junctions, and microvilli forming the brush border. Ingested food materials reach the intestine through anterior pumping and peristalsis of the pharynx, and the excretory materials are eliminated out through the anal opening. The muscles of the body also have a role in controlling the internal pressure and concentration of contents in the guts before excretion of waste from the body (Bird and Bird 1991). The excretory system is a unicellular tubular system comprising three different individual unicellular tubes such as the canal, duct, and pore connected to form a continuous lumen in the body (Nelson et al. 1983).

8.2.5 Nervous System

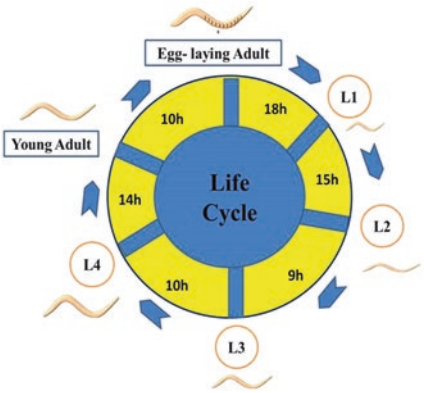
The hermaphroditic adult nervous system is comprised of 302 neurons that can be subdivided into 3 unique and independent systems, with a larger somatic system of 282 neurons and a smaller pharyngeal nervous system (Ward et al. 1975; Sulston and Horvitz 1977; Sulston et al. 1983; White et al. 1986). Majority of neurons in the somatic nervous system lie between the hypodermis and body muscles separated by the basal lamina. On the other hand, pharyngeal neurons are directly located in the pharyngeal muscles and are not divided by any basal lamina. Worm neuronal system has 1500 neuromuscular junctions, 900 gap junctions, and 6400 chemical synapses (Durbin 1987). The male population has 473 additional cells, 79 neurons, and 36 supporting cells than the hermaphrodites. These additional cells have roles in enhancing the male mating behavior (Sulston et al. 1980; Emmons and Lipton 2003; Emmons 2005). This nervous system can manage complex behaviors along with basic behaviors such as feeding, defecation, locomotion, etc. (Bono and Villu Maricq 2005). It also allows the animal to find the presence of nearby diffusible sex pheromone signals or simply to sense changes in the O_2 levels (Jeong et al. 2005). Both sexes have some sex-specific behaviors such as egg-laying and mating behaviors (Schafer 2005).

8.2.6 *C. elegans* Life Cycle

Perhaps, the best utility of this organism is due to its ease of growing and maintenance in the laboratory besides its simple and short life cycle. The reproductive life cycle of *C. elegans* is about approximately 72–120 h, and its life span is about 2–3 weeks under proper living conditions at 20 °C. During its life cycle, it has to undergo through four larval stages of development designated as L1–L4, and its reproductive phase is temperature-dependent (García-Sancho 2012). Embryonic development proceeds with the generation of an L1 larva which is about 0.25 mm long and is made up of 550 cells; later 131 cells die during the developmental process through apoptosis (Elmore 2007). On maturity, adult worms become fertile for 4 days, and each adult hermaphrodite can lay ~200–300 eggs. Alternatively, when the prevailing environmental condition is not favorable (i.e., insufficient food, temperature changes, and overpopulation), the late L1 stage nematodes can enter into dauer stage. In this stage, worms require lesser nutrients and are resistant to environmental stresses. When favorable condition returns, it reenters the normal life cycle directly to the L4 stage from the dauer stage (Klass 1977; Kenyon et al. 1993; Williams et al. 2017) (Fig. 8.3).

8.2.7 Homologous Genes and Genetic Manipulation

The first organism to be sequenced at the multicellular level (1998) was *C. elegans*. With approximately more than 20,000 genes and with the genome size of more than 100 million base pairs, there are substantial numbers of overlaps and conservative regions among *C. elegans* and human genes (Hodgkin 2005). It also exhibits greater levels of conservatives with other vertebrates in terms of gene functions and metabolic pathways. Algorithm-based studies have reported that greater than 60% of these genes are homologous to humans (Kaletta and Hengartner 2006). These characteristics make it a suitable model organism to study functional genomics and to



Stage	Hours	Size (µm)
L1	0	250
L2	15	360-380
L3	24	490-510
L4	34	620-650
Young adult	48	900-940
Adult (egg-laying)	58	1110-1150

Fig. 8.3 Life cycle of *C. elegans* at 20 °C with different body growth and development parameters as a function of time

get knowledge of the genotypic and phenotypic relationships at a genomic level globally. Furthermore, these insights on multiple biological processes and roles can be correlated to human disease genes which are homologous of *C. elegans* (Bird et al. 1999; Corsi 2006; Walhout 2006).

In a multicellular organism, an important penetration into functions of genes comes from the prevailing environmental conditions during which a gene gets expressed. At the forefront, gene expression studies and analysis in *C. elegans* have several advantages over other species. The availability of its entire genome sequence has provided a simplified functional genomic approach, where manipulations like forward and reverse genetics can be carried out with ease. Strategies that can generate valuable information on expression patterns of various genes in correspondence to different manipulations and amelioration studies have been facilitated. Additionally, by tagging a gene of interest with a reporter gene of green fluorescent protein (GFP), real-time monitoring of expression patterns can be carried out both qualitatively and quantitatively. These approaches of in vivo GFP labeling methods can also give accurate information on the localization of the translated product of a particular gene which can be visualized in live worms. As a result of the Promoterome project, GFP fusions with promoters are being created on a genome-wide scale that led to the development of promoter fusions up to 2 kb from the nematode genes (Dupuy et al. 2004). Another commonly used approach to find the unknown functions of a gene comes from the usage of available *C. elegans* mutant strains. The RNA interference (RNAi) and sequence-specific degradation of homologous messenger RNAs produced by double-stranded RNA were also regularly used to inhibit a particular gene function in *C. elegans*. Understanding of worm's biology through gene manipulation techniques like RNAi knockdowns, reporter gene assays, and protein-protein interaction networks has contributed to the proper understanding of basic and translational biology immensely (Timmons and Fire 1998).

8.2.8 High-Throughput Screening

Employing model organisms in high-throughput screening (HTS) is a useful strategy to facilitate the screening of genes or molecules related to basic biology or disease pathogenesis in humans. *C. elegans* is ideal for HTS screens because a large number of worms can go in a single well, of its transparent body, and it is easy to grow and manipulate. This allows for the design and in-depth dissections of fundamental and conserved biological processes like apoptosis (Ellis et al. 1986) and gene regulation by small RNAs (Fire et al. 1998; Wightman et al. 1993). A recent study using HTS suggests that the intensity of the fluorescent dye Nile red (lipid stain) corresponds with the rate of aging in the animals (O'Rourke et al. 2009). Worms with lesser Nile red signal age more slowly, and animals with increased Nile red signal live shorter than the wild type. Analyses of these HTS experiments were done using two types of imaging techniques including bright-field and fluorescence (FL) imaging. Bright-field images notify about developmental and gross anatomical

defects, and FL images display the distribution of Nile red dye in the worm's body (O'Rourke et al. 2009).

An all-liquid workflow to promote HTS in *C. elegans* in a 96-well form was also developed (Lehner et al. 2006). This method was originally intended to promote genome-wide RNAi screens in a high-throughput mode but now has wider applications. For example, the first HTS approach using *C. elegans* was done in 2006. Kwok and co-workers used worm transfer units of a Complex Object Parametric Analyzer and Sorter (COPAS™ BIOSORT, Union Biometrica) and semi-automated image recovery to screen approximately 14,100 smaller molecules. Molecules were evaluated for different bioactivities by assessing morphological deformities, including slowness of growth, lethality, improper movement, and other phenotypes in wild-type animals. Using this technique, ~308 bioactive molecules were identified. Since this was the first example of HTS screen using *C. elegans*, the screening was done on agar plates (24-well), and phenotypes were marked visually. Furthermore, to enhance the pace and development in *C. elegans* genetic research, libraries covering almost 94% of the 20,000 genes have been established and are also available for researchers (Kamath and Ahringer 2003; Rual 2004; Lamitina et al. 2006).

8.3 Practical Considerations for *C. elegans* as In Vivo Model

Significant delays in our understanding of the different biological activities of different nanoparticles exist. When tested in higher mammalian in vivo models, serious considerations that hinder the process include a shortage in the number of experimental animals, time factor, cost-effectiveness, and ethical procedures. Importantly, with no ethical constraints and ease of cultivation, *C. elegans* counteracts the limitations of many other in vivo models favorably. In the laboratory setting, an invertebrate model such as *C. elegans* has the advantage of getting numbers of animals assigned for the experiment because each adult hermaphrodite can reproduce about ~300 progeny with a life span of only 2–3 weeks. Thus, end numbers of different animals with different stages can be rapidly generated at the minimum cost and time involved. The ease of culturing the worms in either liquid or solid NGM and the minimum requirement of the non-pathogenic bacterium *E. coli* OP50 strain as a standardized food source have further reduced the cost involved. Additionally, for long-term usage, cultures can be easily stored and preserved at −80 °C in 96- or 384-well plates.

The blend of small size (1 mm in adult hermaphrodite) and transparent body of *C. elegans* is also ideal for the normal optical microscopic study even to the single-cell level. Their transparency permits visualization of their various anatomical structures even without dissection and permits colored materials to pass all over their body even without staining (Contag 2002; Pomper and Lee 2005). Due to their smaller size, worms in large numbers can be put in a single petri dish or 96- or 384-well plates which fits high-throughput experiment needs (Stiernagle 2006). Recently, based on their micrometric size from embryo to adulthood, microfluidic platforms have been developed where microfluidic device enables for real-time monitoring of the different parameters of the worms' population. This is advantageous from different aspects

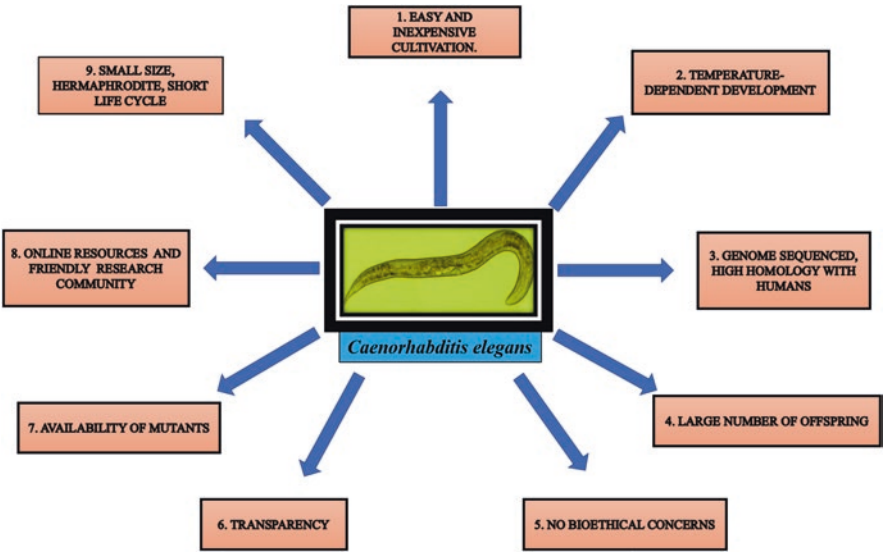


Fig. 8.4 Diagram depicting common features of *Caenorhabditis elegans* that are ideal for investigating different biological activities of nanoparticles

of time and precision. For example, toxicological profiling of a number of test materials can be done in a high-throughput and computerized manner (Altun et al. 2009; Hulme and Whitesides 2011; San-Miguel and Lu 2013) (Fig. 8.4).

Most importantly, because of its multicellular nature and the availability of complex multiple organ systems, the chances of identifying a particular nanoparticle target and its interactions with biological molecules can be correlated. Further confirmation studies can be carried out in the complex multicellular organisms like humans. For example, with its functions and composition similar to that of human skin, the external part of worms epithelial and cuticle layer can be used as a simple epidermal model to assess nanoparticle routes of entry and absorption and for an in-depth analysis of its positive or negative effects (Chisholm and Xu 2012). Similarly, the *C. elegans* intestinal cellular architecture, patterning, and growth allow for the detailed examination of delivery or entry of nanoparticles orally (Bossinger and Hoffm 2012). Therefore, *C. elegans* is a sensitive and feasible whole animal model system to study the interactions of nanoparticles with various biological barriers like cuticle and intestine and through various levels of organizations ranging from a single cell to the whole organism (Fig. 8.4).

8.4 Nano-Bio Interactions: Features of *C. elegans*

Understanding the different interactions of various nanoparticles with multicellular organisms is still in progress. Thus, assessment of interactions between different nanoparticles and *C. elegans* often serves as information-rich resources for an

in vivo behavior and biocompatibility of different nanoparticles. Processes such as uptake, distribution, aggregation, surface adherence, and agglomeration on the exposure of *C. elegans* to nanoparticles have been regularly monitored. Similarly, the roles of different physicochemical properties of nanoparticles on its interactions with *C. elegans* have been reported.

8.4.1 Exposure of Worms

Exposure of worms to nanoparticle is an essential step in studying nano-bio interactions of various nanoparticles starting from the time of its exposure till excretion. Different exposure routes for *C. elegans* can be either in the form of a direct mode of oral feeding, through topical applications, or in some organ-specific cases through microinjection. This exposure can also be in the form of acute and chronic exposure and high-dose and low-dose regimen. Thus, choosing the correct exposure route, concentration, and time is a critical step in determining successful nanoparticle bio-interactions when it is carried out for the first time. Practically, the number of worms required for the study can be obtained by rinsing the worms from NGM plates with Milli-Q water or any buffer of choice. Worm pellets can be concentrated with low-speed centrifugations to the desired number of the worm population before nanoparticle exposure. For larger-population exposure, 24- or 96-well plates with equal volumes in each well can be used to check for the general uptake and bio-distribution of particles (Moragas 2016) (Fig. 8.5).

8.4.2 Uptake, Absorption, Translocation, and Bio-distribution

The uptake of the nanoparticles mainly depends on their physicochemical properties like surface chemistry, size, and shape. In *C. elegans*, nanoparticles are commonly taken through the alimentary system during feeding. Additionally, some studies have shown that nanoparticles can diffuse in worms directly through the cuticle, vulva, anus, and excretory pores (Scharf et al. 2013). In *C. elegans*, the outermost layer of the cuticle contains a negative charge due to the presence of glycoproteins. These negative charges can electrostatically interact with a nanoparticle, which influences its effect on entry into *C. elegans* (Fig. 8.5). For example, superparamagnetic iron oxide nanoparticles (SPIONs) can be absorbed through the worm's epithelial surface (Gonzalez-Moragas et al. 2015).

In the alimentary system, nanoparticle interacts with the pharynx. The pharynx is an encapsulated organ with two muscles, and their movements are necessary for food ingestion and pharyngeal pumping (Song et al. 2013). It was reported that nanoparticle (30 nm–3 μm) can pass through the grinder to interact with pharyngeal epithelium where it will get either attached to it or taken up directly by epithelial cells (e.g., quantum dots) (Fang-Yen et al. 2009; Wu et al. 2018). Thus, the pharyngeal pumping rate can be used as an indicator for evaluating nanoparticle interactions with *C. elegans*. For example, silica (Si), silver (Ag), and titanium (Ti)

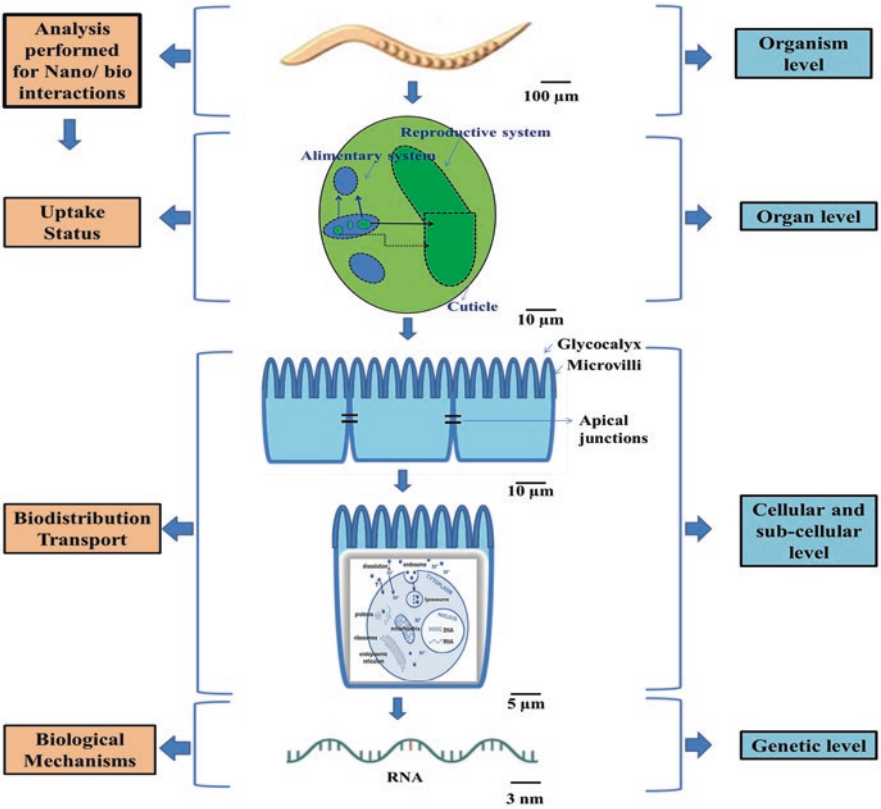


Fig. 8.5 Summary of nano-bio interactions of nanoparticles in *C. elegans*

nanoparticles have been reported to interact with the pharynx that resulted in dysfunction or pre-mature reduction of pharyngeal pumping (Scharf et al. 2013; Iannarelli et al. 2016). In the reproductive system, nanoparticles generally get localized after their direct translocation from the alimentary system and rarely get ejected from the vulva. Nanoparticles were also reported to have the capability of translocation, and this, in turn, affects the progeny and reproductive system of the worms (Laromaine 2015). In the nervous system, the interactions of Si nanoparticles with the neuronal cells have led to the segregation of proteins predominantly involved in homeostasis as reported. This observed widespread protein aggregation in axons of serotonergic HSN neurons resulted in a decline of egg-laying capacity and induces internal hatch of worms (Scharf et al. 2015).

A clear comprehension of the route of absorption and modes of bio-distribution of nanoparticles in a living organism is also crucial. *C. elegans* offers an advantage through its transparency and the oral route of entry for nanoparticles. On entry, these nanoparticles pass through the alimentary path in either the presence or absence (±) of *E. coli* OP50. Irrespective of solid or liquid media, there were speculations

suggesting that *E. coli* OP50 can absorb and decrease the nanoparticle concentration in the suspension. On the contrary, other experiments based on the discharge of metal ions from nanoparticles in both ± of *E. coli* OP50 highlighted that the live bacterial metabolism has a lesser influence on nanoparticles (Gonzalez-Moragas et al. 2017).

Notably, Ag nanoparticles and quantum dots have been reported to be diffused in *C. elegans* intestine, subcutaneous tissue, gut lumen, and gonad along with the huge amount of nanoparticles observed to be deposited in the tail (Wu et al. 2014; Luo et al. 2016; Zhi et al. 2016). For those nanoparticles that can enter the membrane layers of cells, delocalization and accumulation in the lysosomes of *C. elegans* were also reported (Wang et al. 2016; Chatterjee et al. 2017; Yu et al. 2016). Some ingested nanoparticles could be seen in the digestive system, starting from the pharynx, intestinal lumen, and rectum besides other organs, like the neurons, muscles, spermatheca, and gonad by crossing their barriers (Qu et al. 2018). In some other reports applying two-photon luminescence microscopy and absorbance microspectroscopy, it was observed that Au nanoparticles ingested by *C. elegans* get accumulated in the intestine but were not internalized by the intestinal cells (Moragas 2016).

8.4.3 Recovery and Excretion of Nanoparticles from *C. elegans*

The role of feeding the worms with *E. coli* OP50 plays a vital role in the excretion of nanoparticles. Most of the nanoparticles remain in the body even after defecation, inferring that the worms do not have a preference for the excretion of nanoparticles. In the absence of *E. coli* OP50, the defecation process ceases which leads to the accumulation of the nanoparticles in the gut. Once the animal starts to feed on *E. coli* OP50, the nanoparticles get excreted out (Mohan et al. 2010**;** Le Trequesser et al. 2014). Previous studies have shown that the excretion of nanoparticles such as nanodiamonds, Fe_2O_3 nanoparticles, and Au nanoparticles also depends on food availability and even some of the nanoparticles can increase the defecation cycle duration by 80 s. All these experimental results corroborate that caution must be taken in the evaluation of nanoparticles with or without the presence of food in the experimental setup (Fig. 8.5).

8.5 Major Factors Affecting Nano-Bio Interactions in *C. elegans*

8.5.1 Effect of Life Span and Period of the Exposure

With worms having four different larval stages, 4 days of active egg-laying period, and a life span of around ~2–3 weeks, exposure of nanoparticles to different worm's population would yield different results. Studies have shown that L3 worms were more resistant than L1 upon exposure with cerium oxide (CeO_2) nanoparticle (4 nm) (Collin et al. 2016). Similarly, the L4 or adult worms were more resistant than L1 upon exposure to titanium oxide (TiO_2) nanoparticles (10 nm), and the outcome also relies on time (Zhao et al. 2015). The effects of iron oxide (Fe_2O_3) nanoparticles

coated with dimercaptosuccinic acid (DMSA) in K-medium were shown in three assay systems such as in 24 h treatment with L4 worms, 3-day treatment (L1 to adult), and treatment from L1 to 8-day-old adult. Higher toxicity was observed with increasing time and at a concentration greater than 50 mg/ml (Wu et al. 2012). TiO_2 nanoparticles show variations in recovery response after acute and chronic treatment in *C. elegans* wherein chronically exposed worms ingested more particles and presented themselves with a reduction in movement, excretion, length (body), and pharyngeal pumping when compared to acutely exposed worms (Yang et al. 2014).

8.5.2 Effect of Exposure in Solid Media

During NGM preparation if the nanoparticles are added directly to the medium, it is difficult to confirm the uniformity of particle distribution in the medium fed with the *E. coli* OP50. The solid nature and the ionic strength of the medium can also enhance the colloidal nature of the nanoparticles. Further aggregations can prevent nanoparticles' homogenous distribution from the worms. Thus, care should be taken when the exposure medium is in the form of a solid NGM plate for non-homogenous exposure and that the desired concentration is calculated with precision. Furthermore, the role of live bacteria added as food can perturb the medium biological surface. Bacteria with active metabolism can produce variable outcomes that can change the variability of biological surfaces before and after treatment in *C. elegans*. To overcome these challenges, applications of alternative media with lesser ionic strength were suggested, and these were found to reduce nanoparticle aggregations or precipitation. Some researchers suggested for the application of K-agar over NGM agar. Its high phosphate content allows for interactions with positive charges, and this reduces its availability for unwanted interactions (Maurer et al. 2015). Similarly, acute exposures ($\leq$24 h) in liquid media without food or chronic exposures ($\geq$48 h) in liquid media (K-medium or S basal) along with food supplementation (Kim et al. 2008; Meyer et al. 2010) have also been reported (Tsyusko et al. 2012; Lim et al. 2012) (Fig. 8.6).

8.5.3 Effect of Exposure in Liquid Media

Liquid media have the advantages of uniform nanoparticle exposure and are ideal for different high-throughput studies. However, it can have its disadvantages because of varied ionic strengths that can be used in the maintenance of *C. elegans* under laboratory conditions (Stiernagle 2006). Generally, media with lesser ionic strength are preferred as these can help in maintaining the stability of nanoparticles (M9 buffer, S basal, or K-medium). Interestingly, some studies have shown that the Ag nanoparticle aggregation can take place in K-medium which immediately settles from suspension (Meyer et al. 2010; Ellegaard et al. 2012). The effect of ionic strength on the exposure media in Ag nanoparticle toxicity was also reported, in which lethal doses of 1.5–12-fold higher in low salt containing moderately hard reconstituted water (MHRW) were obtained when compared to K-medium. This

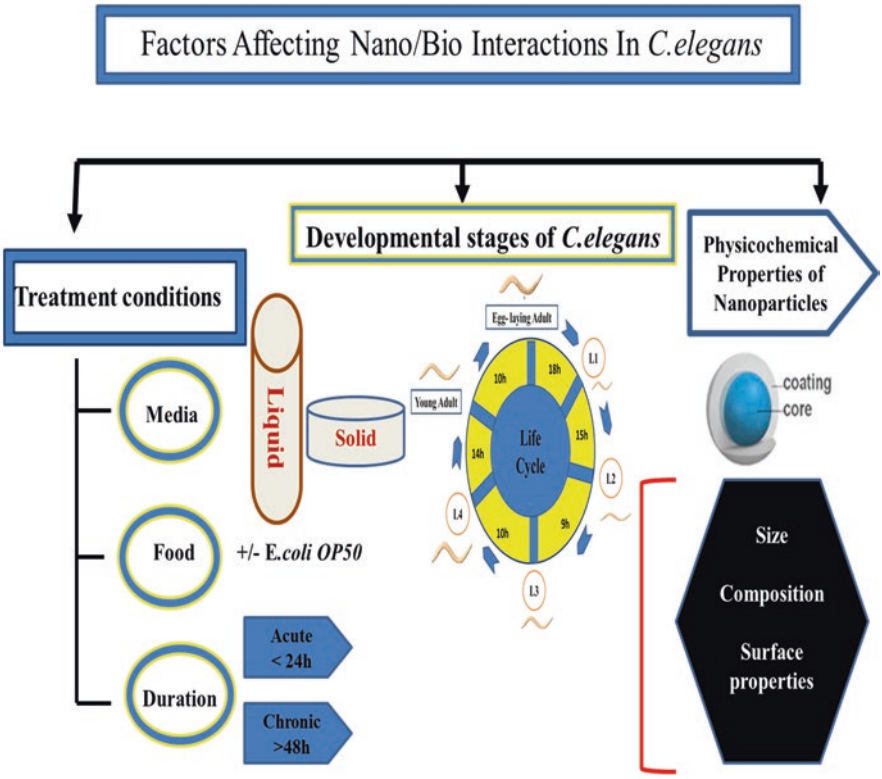


Fig. 8.6 Major factors that can affect nano-bio interactions in *C. elegans* with different parameters that are commonly employed for elucidation of nanoparticle activity in *C. elegans*

decrease in toxicity due to aggregation was followed by the decrease in surface area for their distribution (Yang et al. 2012) (Fig. 8.6).

Another study has shown lesser toxicity even in the presence of salts (ultrapure water and K-medium) when the worms were treated with ZnO nanoparticles (Wang et al. 2009) conflicting to the previous studies reported. Thus, reducing the time of exposure or the ionic strength has also a major role in preventing aggregation of nanoparticles. Precipitation and aggregation of CeO_2 and TiO_2 nanoparticles in K-medium were also reported at higher concentrations used. Exposure to a lower dose without *E. coli* OP50 for 24 h is ideal for the uniformity and stability of the particle suspensions during this experiment (Roh et al. 2010).

8.5.4 Effect of Organic Components/Food

There were conflicting reports on the effect of *E. coli* OP50 on the stability and activities of different nanoparticles during experimentation. The addition of *E. coli* in the medium, especially in the K-medium, was reported to have enhanced the

toxicity of Ag nanoparticles in the worms due to the increase in their bioavailability (Ellegaard-Jensen et al. 2012). Toxic effects of polyvinylpyrrolidone (PVP)-coated Ag nanoparticles in MHRW with organic matter (natural) along with the *E. coli* were also reported (Yang et al. 2014). On the contrary, some studies have shown that the addition of food can lead to a decreased toxicity of nanoparticles (Fig. 8.6).

8.5.5 Effect of Exposure in Standard Conditions

Exposure of nanoparticles even in standardized conditions can have different effects on different nanoparticles, and this indicates the uniqueness of a particular nanoparticle. Pluskota et al. (2009) showed that there was a monodispersion of SiO_2 nanoparticles and polystyrene (PS) nanoparticles in a suspended solution using fluorescence correlation spectroscopy (FCS). The effect was observed after the addition of nanoparticles as a solution along with the bacterial lawn (Pluskota et al. 2009). Polak et al. (2014) showed the different physicochemical properties of ZnO nanoparticle suspension in bacterial culture before the addition into NGM plates for nematode exposure. In LB broth, ZnO nanoparticles formed clusters (agglomerate) with time-dependent variations. Further, TEM investigations revealed that cluster formation resulted in decreased surface charge and weaker electrostatic repulsive force of nanoparticles. Additionally, these clusters did not affect the entry or morphology of bacteria but instead enhance the secretion of polymeric substances which coats the particles within 24 h to influence on their bioavailability (Polak et al. 2014) (Fig. 8.6).

8.5.6 Physicochemical Properties of the Nanoparticles

Smaller Ag nanoparticles (10–21 nm) were reported to have been ingested more when compared to larger-sized particle (>75 nm) in K-medium (Meyer et al. 2010). The uptake of polyethylene glycol (PEG)-coated Ag nanoparticles was shown by Contreras et al. (2014), wherein a lesser amount of Ag was internalized in worms when the worms were exposed to smaller-sized nanoparticles than the larger-sized particles. This may be due to the excretion of the smaller-sized nanoparticles. Further studies have shown that there were variations in life span and fertility of worms after frequent exposures to nanoparticles and the same treatment does not have an effect on their body length and movement (Contreras et al. 2014). Similarly, PVP Ag nanoparticles (28 nm) cause higher worm mortality than smaller-sized (1 nm) Ag nanoparticles, and this was associated with a combining effect of higher intake rates, coating, and solubility (Ellegaard-Jensen et al. 2012). On the contrary, in an assessment of Ag nanoparticles of 1–75 nm, no inhibition on growth and size was observed suggesting that there are correlations that exist between dissolved silver and its toxicity. Altogether, these investigations gave us a rough idea of

size-dependent intake, reproductive toxicity, and life span effects of nanoparticles besides its effects on motility and body size.

8.5.7 Chemical Composition

The translocation rate of nanoparticles also depends on their composition. Some comparative studies conducted on fluorescently labeled SiO_2 (50 nm), Si, and polystyrene (PS) nanoparticles have reported for the major distribution of Si-based nanoparticles in the lumen of the intestine (primary organ), while other nanoparticles like PS nanoparticles were found in secondary organs and in the cytoplasm of the immature embryos (Pluskota et al. 2009). The toxic levels of TiO_2, ZnO, and SiO_2 nanoparticles were also reported to be fully dependent on their composition (Nouara et al. 2013) (Fig. 8.6).

8.5.8 Effect of Surface Coating and Manipulations

The coating of the surface can significantly alter the toxic levels of nanoparticles by hampering the nanoparticle uptake, bioavailability, and reactivity. To access control over the nano-bio interactions, modifications have to be done properly after synthesis in the animal itself or the environment of nanoparticle engineering. Studies by Collin et al. (2014) have shown the effects of surface charge on nanoparticle toxicity and its accumulation in organs and tissues using 4 nm dextran-coated CeO_2 nanoparticles. The neutral and negative charge carrying nanoparticles exhibit lesser toxicity when compared to positively charged CeO_2 nanoparticles which can be found throughout the animal's body. This finding demonstrated that the higher toxicity of positively charged nanoparticles was due to their direct interaction and disruption of the cell membrane besides an increase in their cellular uptake (Collin et al. 2014).

8.6 *C. elegans* Assays for the Biological Activity of Nanoparticles

The contributions of different researchers with years and years of research in *C. elegans* have led to the development of various assays and standardized protocols that were time tested. They are ideal for the screening of different nanoparticles. Most of these assays are designed to evaluate on *C. elegans* organ systems, including the neural system, digestive system, immune system, and reproductive system (Handy et al. 2012; Marsh and May 2012; Hunt 2017). The information collected from these findings can be extrapolated and further confirmed in higher vertebrate systems. Thus, *C. elegans* is a reliable and valuable experimental biological

platform to evaluate the efficiency of a novel nanoparticle. With the availability of different organs and tissue systems, the targeted biological activity of a particular nanoparticle can also be evaluated specifically. For example, *C. elegans* intestinal enterocytes, cell junctions, and microvilli can be evaluated for the biological effect of nanoparticles on intestinal uptake and integrity. Similarly, to evaluate the effects of nanoparticles on the worm's physiology and metabolism, a simple experiment like monitoring of its pharyngeal pumping rate can be performed.

Generally, upon nanoparticle exposure, survival and mortality rate measurements are performed most frequently for determining the overall fitness and life span of *C. elegans*. This can generate a concentration-response and survival analysis curve upon comparison with the untreated control group of worms (Tejeda-Benitez and Olivero-Verbel 2016). Subsequently, emphasizing on the relevance of oxidative stress in regulating important signaling pathways and worm's biochemistry, different sensitive and effective methods are available for measuring different parameters like pro-oxidants, oxidized biomolecules, and its resistance. The effects of nanoparticles on the nervous system of the worms can also be monitored by different assays with the availability of specific neuron types tagged with GFP. Green fluorescent protein analysis protocols can directly or indirectly allow for the quantification, localization, and specific activity measurements of a particular neuron and/or set of neurons upon nanoparticle treatment. *C. elegans* also provide an ideal platform for whole-organism pathway and network analysis, genetic screening, and analysis through single and double mutant strains available. Recently, transcriptomics and metabolomics approaches have also been reported in *C. elegans* (Kim et al. 2017a). The details of these different assays are described in Table. 8.1 and Fig. 8.7.

To examine nanoparticle uptake and bio-distribution, the most commonly used techniques are fluorescent microscopy and hyperspectral dark-field microscopy. Additionally, transmission electron microscopy (TEM), synchrotron-based techniques, and other analytical techniques were also employed (Table 8.2). Recently, the levels of metal exposed on *C. elegans* and their uptake and bio-distribution can also be quantified using inductively coupled plasma mass spectrometry (ICP-MS). The presence of nanoparticles can also be visualized directly in various organ systems of *C. elegans*. For example, TEM and optical microscopy can detect the presence of nanoparticles in the alimentary and reproductive systems. Scanning electron microscopy with energy-dispersive X-ray analysis (SEM-EDX) is often used for the visualization of nanoparticles in the cuticular system (Moragas 2016) (Table 8.2) (Fig. 8.7).

Modern imaging techniques like magnetic resonance imaging (MRI) and synchrotron radiation X-ray fluorescence (μ-SRXRF) can be used to identify, locate, and characterize individual nanoparticle in the specific area of worm's body. X-ray spectroscopy and synchrotron X-ray absorption near-edge spectroscopy (μ-XANES) are used in confirmation of the bio-distribution and status of the nanoparticles along the body of the worm (Moragas 2016). Quantification of the nano-bio interactions between monodispersed small and large coated or non-coated nanoparticles in *C. elegans* using two-photon luminescence microscopy (TPLM) has also been reported. Other commonly

Table 8.1 Different *C. elegans* parameters and techniques involved in elucidation of nanoparticles' biological activity

Parameters	Assays	Biological effects	Techniques involved	Reference(s)
Survival	Lethality assay; classical life span assay; abiotic stress resistance assay; pathogen resistance assay; etc.	Life span decreased; sensitive to stress; increased death rate; sensitivity to pathogens; etc.	Microscopy; counting; staining techniques; statistical analysis; etc.	Kim et al. (2008); Van Voorhies and Ward (1999)
Development growth	Larval development assays; phenotypic and morphological assays; length, width, and density measurements; etc.	Changes in body length and width; dauer formation; abnormal morphology; altered survivability; etc.	Larval counting; body measurement; microscopy; image analyzer; statistical analysis; etc.	Wang and Xing (2009); Boyd et al. (2010); Cha et al. (2012); Rudel et al. (2013)
Behavioral assay	Locomotive assay; feeding and behavioral assay; growth endpoint density assay; etc.	Changes in measurements of head thrash; body bending frequency; and basic movements Food intake frequency, were test nematodes try to avoid contaminated food	Microscopy; counting; statistical analysis; image analyzer tracking; etc.	Matsuura et al. (2013); Jones and Candido (1999)
Reproduction	Fertility assay; egg-laying capacity; gonad size; progeny production; viability; etc.	Decreased or increased brood size; egg-laying capacity changes; changes in gonad morphology; progeny production; and germline alterations	Microscopy; counting; statistical analysis; image analyzer; etc.	Roh and Choi (2011); Cha et al. (2012)
Aging	Life span assays; lipofuscin content; physiological parameters; etc.	Changes in the intestinal lysosomal lipofuscin deposits with age; decreased or increased life span; changes in other physiological parameters; etc.	Microscopy; counting; statistical analysis; image analyzer; etc.	Pluskota et al. (2009); Cha et al. (2012; Wu et al. (2013)

(continued)

Table 8.1 (continued)

Parameters	Assays	Biological effects	Techniques involved	Reference(s)
Immunity	Assays to check the levels of cytokines; pro-inflammatory markers; innate immune response components; resistance to pathogens Survival assay, etc.	Changes in expression levels of different innate immune response components; susceptible to pathogen infection; etc.	ELISA; Western blots; qPCR; transcriptome and spectrophotometric analysis; etc.	Singh and Aballay (2006); Wang and Ezemaduka (2014)
Nervous	Assessment using fluorescently tagged neurons; measurement of neurons length; locomotive and behavioral assays; etc.	Locomotive and behavior changes; neuron size changes; enhanced or repressed GFP-tagged neurons' expressions; etc.	Counting; fluorescence microscopy; staining; tracking; spectrophotometric analysis; etc.	Yu et al. (2015)
Biochemical	To assess various levels of enzymes; proteins; organelles; metabolic rate assessment; etc.	Broad-spectrum changes in activity and expression levels of different biomolecules (both qualitatively and quantitatively)	Enzyme assays; Western blot; staining techniques; microscopy; spectrophotometric analysis; etc.	Jadhav and Rajini (2009); Kim et al. (2012); Lim et al. (2012)
Genetics	Assays using *C. elegans* deletion mutants Gene encoding GFP RNAi Gene expression studies, etc.	Expression studies reveal about upregulation and downregulation of genes; changes in the sensitivity and resistance patterns; etc.	Fluorescence microscopy; image analyzer microarray technology; qPCR; etc.	Hofmann et al. (2002); de Pomerai et al. (2009); Roh et al. (2009)

applied techniques to study nano-bio interactions include hyperspectral dark-field microscopy (Meyer et al. 2010; Ahn et al. 2014), fluorescent microscopy (Pluskota et al. 2009), synchrotron-based techniques, transmission electron microscopy (TEM) (Kim et al. 2012), and other chemical techniques (Table 8.2) (Fig. 8.7).

8.7 Work Flow to Study Nano-Bio Interaction of Nanoparticles in *C. elegans*

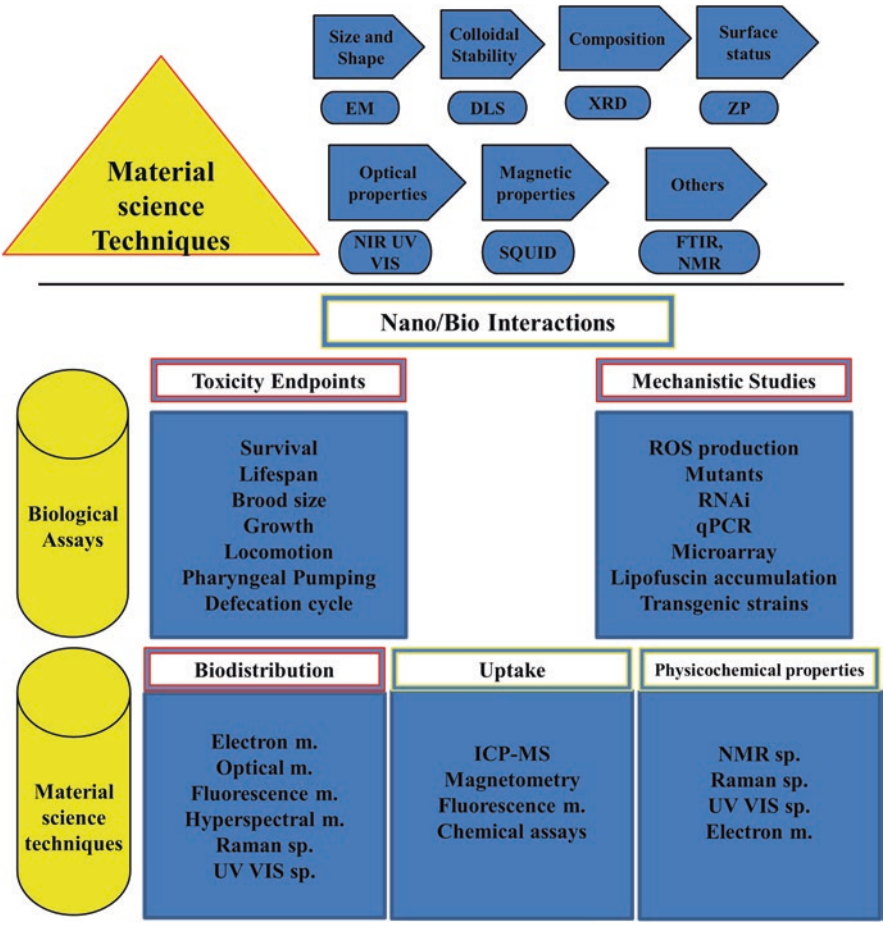


Fig. 8.7 Common methods and assays to evaluate nano-bio interactions of nanoparticles in *C. elegans*. (Legends: *m*. refers to microscopy, *sp*. spectroscopy, *EM* electron microscope, *DLS* dynamic light scattering, *XRD* X-ray diffraction, *ZP* zeta potential, *SQUID* superconducting quantum interference device, *FTIR* Fourier-transform infrared spectroscopy, *NMR* nuclear magnetic resonance spectroscopy)

Table 8.2 Common techniques to study the nano-bio interactions in *C. elegans*

Techniques	Use	Nanoparticles	Findings	Reference(s)
Absorbance microspectroscopy	For studying the plasmonic properties of Au NPs in correlation with their confinement inside the intestinal lumen	11 nm Au NPs	No effects of Au NPs on endocytosis and intestinal barrier integrity	Moragas (2016)
Hyperspectral microscopy	To find bio-distribution and localization	Citrate/ PVP-coated Ag NPs (10–75 nm) Ag NPs (8–38 nm)	Internalization of several nanoparticles/ citrate-coated Ag NPs internalized to germline	Meyer et al. (2010)
Transmission electron microscopy (TEM)	To find the integrity of the intestinal barrier and NP translocation routes	TiO_2 NP/ SPIONs	Detection of NPs on the anterior and posterior parts of worm's intestine	Yang et al. (2014)
Scanning electron microscopy (SEM)	To investigate on the morphology, external surface, the cuticle, etc.	Citrate-coated silver nanoparticles (cAg NPs)	Interaction of citrate-coated silver nanoparticles (cAg NPs) with the biological surfaces of *C. elegans*	Kim et al. (2012)
Synchrotron radiation X-ray fluorescence (μ-SRXRF)	To examine metal distribution in *C. elegans*	24 nm Cu NPs 30 nm TiO_2 NPs	Alterations in metal homeostasis	Gao et al. (2008); Le Trequesser et al. (2014)
Synchrotron X-ray absorption near-edge spectroscopy (μ-XANES)	To provide information regarding the oxidation state and coordination environment of metals	30 nm TiO_2 NPs	Alterations in metal homeostasis	Le Trequesser et al. (2014)
ICP-MS	To check for the Au-NP uptake by *C. elegans*	11 and 150 nm Au NPs	Ion release from 4 nm Au NPs in vitro at pH 4.5 and after 24 h of treatment in *C. elegans*	Sabella et al. (2014)

(continued)

Table 8.2 (continued)

Techniques	Use	Nanoparticles	Findings	Reference(s)
Light microscopy	To check for the NPs' bio-distribution (organ level)	Most of the nanoparticles	Confirmation of uptake of nanoparticles by *C. elegans*	Moragas (2016)
Scanning electron microscopy (SEM-EDX)	To study the interactions between the external part of *C. elegans* and Au NPs	11 and 150 nm Au NPs	Au NPs did not attach to the external surface of the animal	Moragas (2016)
Fluorescence microscopy	To study the uptake during feeding and translocation to several organs	50 nm polystyrene and SiO_2 NPs	Cytoplasmic uptake of 50 nm polystyrene NPs was observed in early embryos/two entry portals of silica and PS NPs	Scharf et al. (2013); Pluskota et al. (2009)
Hyperspectral dark-field microscopy (HDFM)	To study Au NP distribution inside treated *C. elegans* at both larval and adult stages	11 and 150 nm Au NPs	Au NPs were constrained to the alimentary system, located inside the intestinal lumen but apparently not internalized by the enterocytes. Translocation to secondary organs such as the reproductive system	Moragas (2016)
Two-photon luminescence microscopy (TPLM)	To find the localization of Au NPs/with better contrast than bright-field or dark-field microscopy	11 and 150 nm Au NPs	Confirming the absence of Au NPs in the cuticle and exclusion of topical entrance of Au NPs	Moragas (2016)

8.8 Examples of Common Biological Activities of Nanoparticles Elucidated Using *C. elegans*

8.8.1 Nanoparticles in Drug Delivery, Control Release, and Targeting

Delivery systems have been used to enhance the effectiveness of drugs and to decrease the dosage required. Polymeric nanoparticles were often used as particle carriers in various fields because of their subcellular size and sustained release properties that are compatible with tissues and cells. This has led to the applications of

nanoparticles as promising vehicles for drug delivery for various diseases with site specificity in the host system. Nanoparticles provide significant advantages over traditional drug delivery in terms of specificity, stability, drug-carrying capacity, and sustained release. Their ability to deliver both hydrophilic and hydrophobic drug molecules is also feasible for use in various routes of administrations.

Recently, a comparative study on the efficacy of cinnamaldehyde (CNMA) as broad-spectrum antimicrobial agents was tested via its conjugation to the surface of Au nanoparticles (CNMA-GNPs). Different parameters were evaluated which include delivery, compatibility, biofilm formation, and *C. elegans* survival rate. Interestingly, results showed that this antimicrobial nanodrug delivery system (CNMA-GNPs) successfully reduced pathogenic biofilms and antibiotic-resistant strains and significantly ameliorates pathogenic infections. Similarly, it reduced the *C. elegans* mortality rate, whereby a twofold increase in worm survivability was reported on *S. aureus* infection (Ramasamy et al. 2017). A similar study reported that pre-treatment of irradiated *C. elegans* with resveratrol-loaded nanoparticles (RESNPs) enhances the overall life span of the worms by reducing its injury from γ-ray radiation and toxicity from amyloid-peptide overexpression. Additionally, there were enhanced radical scavenging and enhanced expression of SOD-3 observed in the worms that confirmed the successful development of antioxidant nanoparticles (Yin et al. 2014). The significance of peptide multifunctionalized gold nanorods in reducing the toxicity of β-amyloid peptide in the *C. elegans* model of Alzheimer's disease has also been reported (Morales-Zavala et al. 2017).

In another interesting study that highlighted the significance of this model as an effective delivery system is in the form of nanoemulsion-based delivery systems. The incorporation of linoleic acid with conjugation (CLA) or hydrophobic molecules into nanoemulsion based-delivery systems was monitored using the body fat parameter of worms. Overall, their findings have shown that there were significant reductions in whole-body fat of the worms that are exposed to nanoemulsions containing CLA in comparison to the worms that were exposed to linoleic acid only. Thus, it provides a clue that this model is more appropriate in understanding methods or applications of food or drug that are lipophilic in nature (Colmenares et al. 2016). In another development that demonstrated the importance of an in vivo controlled and constant release of molecules in *C. elegans*, the application and design of laser-sensitive Ag nanoparticles from functionalized novel hydrogel shells were also reported using this model system. Thus, consistency in releasing was obtained on radiation with near-infrared light (Lengert et al. 2017).

8.8.2 Nanoparticles in Molecular Imaging/Bioprobes/ Diagnostics

Transparent body and small size of *C. elegans* are ideal for in vivo optical microscopy. Kim et al. (2013) have reported on a simple immobilization technique that can protect the worms from different toxicants' exposure, besides aiding in its recovery

for long-term imaging using nanoparticle-mediated immobilization (Kim et al. 2013). Recently, a new diagnostic method has also been developed for the accurate measurement of intracellular pH (pHi) using *C. elegans*. This is based on the pH-sensitive Si nanoparticles that can be monitored and visualized by the application of confocal microscopy. The fluorescence intensity patterns that are generated can be used for the quantitative ratio metric analysis of pHi and the overall functions of the organism's metabolic rate. The translational implication is that this economically feasible technique that was formulated in *C. elegans* can have huge applications in the higher model organism for understanding the normal metabolism and its related diseases (Mathew et al. 2014).

In another study that highlighted the significance of *C. elegans* in understanding nanoparticles, bioactivity is in the intercellular transport of lipoproteins whereby fluorescent nanodiamonds were utilized to serve as a marker for observation (Chang et al. 2008). Similarly, Arnhold et al. (2015) have reported that by enhancing Si nanoparticles through fluorescently tagged functionalization, it can be utilized as high-end probes. This has been employed to capture specific images of live worms under an active physiological state. Perturbations of the different cellular components of *C. elegans* can also be monitored in a real-time manner for different useful applications. The feasibility of *C. elegans* as an in vivo model in monitoring the progression of amyloid toxicity through enhanced fibrillation images and its accurate quantification has also been reported (Arnhold et al. 2015). A simple, sensitive, and highly specific lipid targeting Raman probe (Nile red-coated silver nanoparticles) has also been developed to image live *C. elegans* (Charan et al. 2011). This can have wider applications to a higher mammalian system and in understanding lipid droplets, dyslipidemia, or diseases related to lipid metabolism.

8.8.3 Nanoparticles as Antioxidants

The central idea of the oxidative damage (or oxidative stress) theory is that "accumulation of molecular damage caused by reactive oxygen species (ROS) which contributes significantly to aging, i.e., to the functional decline and increase in mortality that happens later in life" (Harman 1956; Beckman and Ames, 1998). Recent studies on the biological importance of metal nanoparticles have increased in the field of nano-biotechnology. Kim et al. (2008) investigated whether platinum (Pt) nanoparticles can influence the gathering of the autofluorescent lipofuscin pigment. Lipofuscin is the product of enhanced oxidative damage to cellular components. Its accumulation is most commonly observed in the intestinal cells, and this can serve as a marker for deterioration of overall physiological state with age (Brunk and Terman 2002; Garigan et al. 2002) (Table 8.3).

A study on the biological activity of Pt nanoparticle has shown that it can act as a mimetic of superoxide dismutase (SOD) and catalase in *C. elegans.* Comparative effects of Pt nanoparticle and EUK-8, a synthetic, low-molecular-weight

salen-manganese complex, were also studied based on the life span of wild-type N2 worms and the short-lived *mev-1*(kn1) mutant (Kim et al. 2008). The Pt nanoparticles were more efficient than EUK-8 in prolonging the life span of *C. elegans* (Kim et al. 2008) suggesting that Pt nanoparticles can have a positive effect by reducing enhanced levels of ROS that is mostly seen in diseased conditions. However, it is important to know and monitor the dosage of nanoparticles used because the excess amounts can have harmful effects (Kim et al. 2008).

8.8.4 Nanoparticles as Antimicrobial/Anti-Virulence Activity

The basic principle in assaying and underpinning the use of *C. elegans* in anti-infective/antimicrobial drug discovery is that some pathogens that cause infections in humans infect *C. elegans* too. Indeed, it is estimated that more than 40 human pathogenic strains reported so far can infect *C. elegans* (Sifri et al. 2005). *C. elegans* has become an excellent model for studying the antimicrobial effect of nanoparticles. For example, Au, Ag, and Pt nanoparticles have been extensively used as antimicrobial, antiviral, and anti-inflammatory agents (Hu et al. 2006; Jain et al. 2008). Ramasamy and his colleagues (2017) have shown the antimicrobial effect of Au nanoparticles in the *C. elegans*, where they have conjugated the cinnamaldehyde with Au nanoparticles, which eradicated the biofilm formation of Gram-positive bacteria and Gram-negative bacteria. Besides, these Au nanoparticles attenuate various virulence factors of *S. aureus* and protected *C. elegans* from infection (Ramasamy et al. 2017). Silver nanoparticles have also been reported to have both bactericidal and bacteriostatic properties (Hunt et al. 2013) (Table 8.3).

8.8.5 Nanoparticles' Roles in the Regulation of Metabolism

Feasibility of the worms allows them to be an ideal model for studying energy metabolism, wherein starvation and excess energy can affect the overall physiology of the worms. Recent reports suggest that exposure of worms to nanopolystyrene particles can change various metabolites related to energy metabolisms, such as tricarboxylic acid cycle intermediates, glucose, and lactic acid. Similarly, studies have shown that upon exposure, it reduces the levels of important amino acids that can serve as metabolites for energy metabolism such as glutamic acid, isoleucine, valine, and lysine (Kim et al. 2018; De Lorenzo et al. 2002).

8.8.6 Nanoparticles in Aging and Developmental Studies

During the reproductive cycle, the worm's vulva is the major organ involved in progeny production. The lacking of normal vulva and bag-of-worm (BOW) phenotypes with egg-laying deficiency was seen in reproductive-age worms when treated with plain or

Table 8.3 Various biological activities of different nanoparticles as reported from *C. elegans* model

Nanoparticle	Metal stress	Oxidative stress	Average size range	Mechanisms	Reference(s)
Pt	–	+	2.4–7 nm	Prevents cellular damage by scavenging endogenous ROS	Kim et al. (2008); Kim et al. (2010)
Ag	+	+	10–21 nm	Impairments in metabolic processes Stress-mediated mitochondrial and DNA damage Dermal effects Cellular damage Early-endosome formation	Scharf et al. (2016); Starnes et al. (2015); Ellegaard-Jensen et al. (2012); Ahn et al. (2014); Kim et al. (2012); Maurer et al. (2016); Ahn et al. (2014)
SiO_2	–	–	50 nm	Aging phenotypic changes	Scharf et al. (2013); Pluskota et al. (2009)
TiO_2	+	+	7–21 nm	Neuronal control over defecation	Zhao et al. (2014)
CeO_2	±	±	15–45 nm	Growth defects by inhibition on feeding	Arnold et al. (2013)
GO	–	+	~6 nm	Inhibition of spermatogenesis Modulation in fat metabolism	Kim et al. (2018); Chatterjee et al. (2014)
Al_2O_3	±	+	60 nm	Neurotoxic effects and behavioral changes	Li et al. (2012)
Au	±	±	~0.5–6.45 nm	Locomotion and axonal neuron growth	Hu et al. (2018)
CuO	±	–	28 nm	Heat response and unfolding protein function	Mashock et al. (2016)
ZnO	–	–	1.5 nm	Protection against metal toxicity	Ma et al. (2009)
MWCNT	±	±	10–20 nm	Involves in molting cycle Regulation of metabolic pathways	Zhao et al. (2017)
Nanodiamond	–	–	~120 nm	Role in resistance to arsenite and oxidative stress	Mohan et al. (2010)

labeled Si nanoparticles. BOW phenotype is characterized by fertilized embryos that come from their shells within the body of hermaphrodite and feeds on the body of their parent worm itself. Further, they ruled out the post-larval stage applications and organ-specific interactions of nanoparticles that affect the reproductive organs. These findings prove that Si nanoparticles can mediate the degeneration of neural and reproductive systems concerning their age (Scharf et al. 2013; Pluskota et al. 2009).

The various effects of nanoparticles on the growth and development of the worms were also linked to defective food intake. Arnold et al. (2013) have seen a decline in *C. elegans* growth on CeO_2 nanoparticle treatment due to a reduced dietary intake mediated by the interactions between CeO_2 and *E. coli* (Arnold et al. 2013). CeO_2 has a greater affinity to bind to *E. coli* (Thill et al. 2006). The decrease in growth and development of the worms may be due to the treatment with CeO_2 nanoparticles. Also, the developmental delay of *C. elegans* is a common physiological response to stress and has been observed after exposure to copper sulfate ($CuSO_4$), TiO_2, and ZnO nanoparticles (Wu et al. 2013).

The effect of SiO_2 nanoparticles on enhancing the aging of cells at the molecular and organism level was also reported (Scharf et al. 2013). Treated animals exhibit enhanced accumulation of ubiquitinated proteins when compared to controls, which resembles the accumulation of endogenous insoluble proteins in older worms. Additionally, fine structures in the intestinal cell nuclei (amyloid-like) were observed in SiO_2 nanoparticle-treated worms. Also, SiO_2 nanoparticle is reported to have a direct influence with pharyngeal use in worms by premature induction of an age-related reduction in pharyngeal motor activity through pharyngeal pumping (Scharf et al. 2013).

8.8.7 Nanotoxicity Assessment

C. elegans has become a favorable model organism for toxicity assessment. Nanoparticles can have surprisingly both beneficial and toxic effects on macromolecules of cells. Nanoparticle-mediated toxicity in *C. elegans* can be assessed using different standard methods and protocols. For example, assays that determine the worm's growth, mortality rate, reproductive capability, and locomotive changes can provide accurate measurements and predictability when applied to higher mammalian systems. In comparison to different in vitro assays, toxicity assays in *C. elegans* are reproducible. Scientific data can be generated easily from the different lethal and sublethal endpoints of an intact and metabolically active animal with different tissues and organ systems (Boyd et al. 2010; Corsi et al. 2015) (Fig. 8.8).

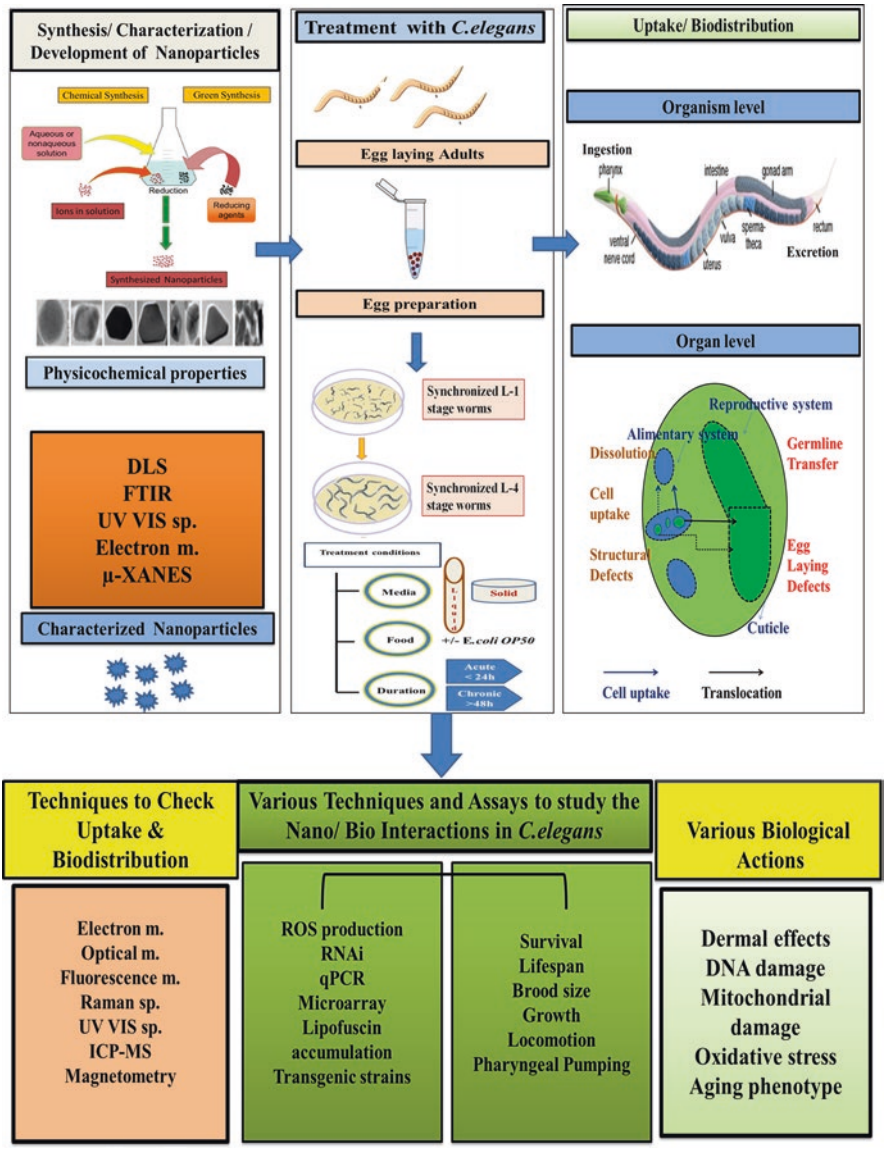


Fig. 8.8 Summarized and simplified diagram highlighting the overall process involved in the elucidation of nanoparticle biological activity

8.9 Conclusion

Underrating the importance of nanoparticles in research for the usage of mankind has steadily increased the scope of nanotechnology. To serve this purpose, the synthesis and formulations of various nanomaterials should focus on the elimination of as

much of the toxic effects on the biological systems. Thus, a feasible and proper in vivo model system such as *C. elegans* represents an ideal platform for fast and reproducible results for the elucidation of nanoparticles biological effects. At the same time, in learning the effects of nanoparticles, it is necessary to examine their interactions since accurate outcome depends on various factors such as media, exposure, physicochemical properties, growth conditions, etc. Different mechanistic studies of nanoparticles for uptake, bio-distribution, and excretion were simplified with the availability of different high-end and sensitive techniques. Biochemical assays and genetic and molecular analyses such as RNAi, qPCR, and microarrays have also enriched our understanding of the whole process of nanoparticle biological activities using *C. elegans*. Lastly, realizing the significant contributions of *C. elegans* in the elucidation of various biological activities of nanoparticles, their safety designs and development can be further improved for various translational applications and human therapeutic values.

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Zebrafish Model System to Investigate Biological Activities of Nanoparticles

9

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Abstract

Globally, the zebrafish as an experimental animal has been welcomed, and utilization increased successively. Zebrafish is a common name of *Danio rerio* fish. It belongs to the Cyprinidae family, within the order of the Cypriniformes. The fish is named for the five uniform, parallel blue-colored long narrow bands on the body side. The stripes are extended up to the tail end of the fish. The successful implementation of zebrafish as a trial animal majorly depends upon the key features like its genotypic and phenotypic similarities to human and their easy maintenance at laboratory scale. There is much similarity between the major organ systems like nervous, cardiovascular, and digestive systems of human and zebrafish. It is possible to identify and study the physiological and pharmacological responses of drugs and other bioactive compounds with therapeutic value. The zebrafish model is a powerful, well-established research platform for the testing of activities of new drug molecules. The nano-sized materials have opened up various possibilities in a variety of industrial issues and scientific endeavors. Nanomedicines are an effective way of drug delivery systems as they enhance drug absorption by improving the solubility characteristics of the drug. Bioactive nanoparticles can be easily and successfully studied on both embryos and adult zebrafish. Nanoparticles having biological activities like anti-convelsant,

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anti-melanogenic or other activities which affect the cardiovascular system, nervous system, reproduction system, etc. have been successfully studied on zebrafish as an experimental animal model. The zebrafish also plays an important part in toxicological studies of the nanomedicines. The zebrafish has proven its extensive promises as an in vivo animal model for screening of nanomaterials. The zebrafish can be employed in the process of drug development at the stage of pre-clinical testing. Presently, research is focused on the biological activity testing and toxicological testing of newly developed medicines especially chemotherapeutic agents or nanoparticles used in the treatment of cancer.

Keywords
Zebrafish · Nanoparticles · Genotypic · Phenotypic · Bioactive compounds

9.1 Introduction

Globally, the utilization of zebrafish as an experimental animal has been increased successively. Zebrafish is the common name of *Danio rerio* fish. It belongs to the Cyprinidae family, within the order of the Cypriniformes (MacRae and Peterson 2003). It is a small, freshwater fish. It is a tropical fish and can endure a temperature range of around 24–29 °C. It is native to Southeast Asia and found in the rivers of countries like India, North Pakistan, Nepal, and Bhutan. It commonly lives in streams, lakes, canals, and moving water to stagnant water bodies, including grasslands (Chakraborty et al. 2016b). Zebrafish is popular as aquarium fish and has been introduced in aquariums in the United States and Japan. This species is also popular for decorative purpose. The application of zebrafish as an experimental model was reported in 1955 for the first time. Since then to date, the use of zebrafish had an expanding growth (Fig. 9.1).

Fig. 9.1 Representation of life stages of zebrafish (Saleem and Kannan 2018)

9.2 Morphology of Zebrafish

The fish is named for the five uniform, parallel blue-colored long narrow bands on the body side. The stripes are extended up to the tail end of the fish. Male fish have torpedo shape and gold-colored stripes in between blue-colored stripes. While female fish have silver-colored stripes in between the blue-colored stripes. Females have a whitish belly and larger in size than male (Brundo and Salvaggio 2018). The zebrafish grows up to 6.4 cm (2.5 in.) approximately. Adult zebrafish are 3–5 cm in length. Due to their small size, they can be maintained easily in massive amount in the laboratory as experimental animal for research purpose. Most zebrafish live for 2–3 years in confinement. They can live up to 5 years in ideal conditions (MacRae and Peterson 2003).

Zebrafish embryonic growth has been thoroughly distinguished. The embryos themselves are clear in appearance during the first few days of life because chorion is translucent. After 30–72 h of post fertilization (hpf), pigment deposition appears in the embryos of zebrafish. The cytoplasmic movements are triggered by fertilization. About 40 min of post fertilization, the first bifurcation of the fertilized egg happens. The first cleavage of the newly fertilized egg occurs about 40 min after fertilization (Brundo and Salvaggio 2018). But the speed of zebrafish embryos growth varies according to temperature. The larval stage of zebrafish is transparent, and as it grows to adult phase, stripes start appearing. The stripes develop along the body length and in blue shade. Male fish are outlined like torpedo. They are slimmer than female and have a pink or yellow tinge usually, while female fish are fatter as they carry eggs. They are less pink than the male. They have the ability to deposit ample of eggs during the entire year. So, this is an excellent laboratory model (MacRae and Peterson 2003; Haque and Ward 2018).

The embryo phase of zebrafish is a "stereoblastula" as embryo is developed by spiral cleavage and absence of blastocoel. The blastula stage is equal to 2.25–5.25 h after fertilization (hpf), while gastrula stage of zebrafish is equivalent to 5.25–10 hpf (Brundo and Salvaggio 2018).

The term "pharyngula" (24–48 h) was referred to the embryo that has matured to the phylotypic phase. At this period of life, zebrafish is easily compared to other vertebrates for morphologies (Mushtaq et al. 2013).

9.3 Zebrafish Evelopment

The zebrafish eggs are sturdy and evolve in the exterior of the body. The optical microscopy of zebrafish showed visual analysis, in addition to fluorescent and other markers. So, it is easy to control and influence the zebrafish for research applications (MacRae and Peterson 2003).

Zebrafish have the ability to produce plenty of offspring with transparent embryo. The development of major organs of zebrafish occurs in the larval stage within few days post fertilization (dpf) as hatching eggs and production and development of the organs occur rapidly. The development of zebrafish is magnificently rapid, as they

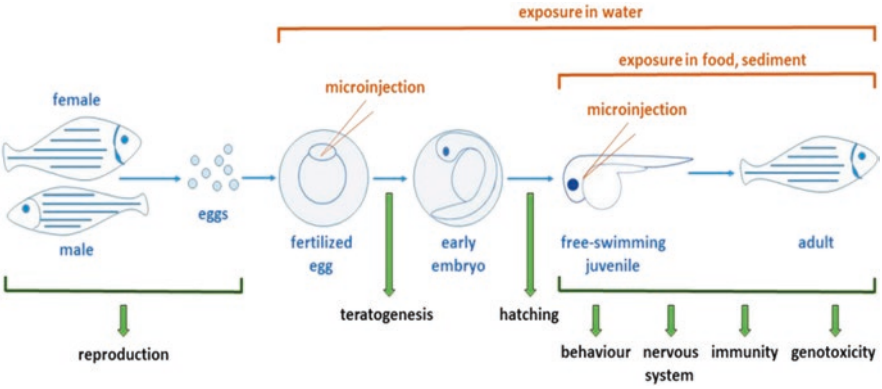

Fig. 9.2 Representation of zebrafish developmental stages and use of different stages in toxicity models (Haque and Ward 2018)

reach in adulthood in around 3 months with the well-established basic body plan by 24 hpf. The embryogenesis of well-established and completed within 72 hpf while fully developed organs by 96 hpf. This makes them susceptible to various toxicological applications through their whole lifespan (Fig. 9.2).

9.4 Zebrafish Animal: As a Perfect Experimental Model

The zebrafish (*D. rerio*) is a well-liked tropical fish pet. They are also a prime animal model for research in vertebrate development, genetics, and human biology and human disorders. The utilization of zebrafish as an investigational animal started in the 1960s. Zebrafish have features like a large family size, external development, and a relatively low cost of production and maintenance. These features make them useful in the laboratory animal model (Brundo and Salvaggio 2018). The successful implementation of zebrafish as a trial animal majorly depends upon the key features like its genotypic and phenotypic similarities to human and their easy maintenance at laboratory scale (Lin et al. 2013).

The zebrafish produces a large number of eggs. The life cycle of zebrafish is short with rapid development. The development of zebrafish occurs outside the uterus of female. These features contribute to the low cost of its production, maintenance, and care.

There are many benefits of utilizing zebrafish as a laboratory animal in research studies. Their fecundity rate is more as one female produces not less than 300 eggs. Their proficiency as an experimental animal is more (Lin et al. 2013). The adult zebrafish is tiny and has a length of 5 cm approximately. This is a critical feature for less space requirement. They can be maintained with no difficulty. It reduces housing space, housing requirements, and husbandry costs compared to the other animal models, making them cost-effective. The small size makes them easy to handle and use in laboratory experiments.

As the dimensions of the larva and adult zebrafish are tiny, it reduces the quantities of the dose of experimental or testing agent solutions. This cuts down the bulk of waste material destruction and also reduces requirement of quantities of glass wares, equipment, and chemicals used in laboratories for research purpose (Brennan 2014). As the embryos of zebrafish are small, more screening agents can be investigated simultaneously by using a multititer-well plate. As the maturation of zebrafish is quick, the trans-generational experimentation becomes easy.

The zebrafish remain transparent from the egg stage and fertilization to the embryo development. So, the development can be observed visually for the morphological development and other changes without any obstruction (Brundo and Salvaggio 2018). The unfavorable results of chemicals on the growth of the zebrafish organs can be easily assessed, with some magnification. The adverse effects can be quantified by measuring the size of the organs at every developmental stage. During mutagenesis, the identification of phenotypic traits is possible as the zebrafish have optical clarity. The advance techniques of immunochemistry allow the assessment of morphology (Sieber et al. 2017).

The genomic sequence of zebrafish and human has ~70% similarity. The progress of embryonic growth of zebrafish was reported in 1981. There was a significant improvement in genomics of zebrafish in the 2000s. In 2001, the series of mitochondrial genome of zebrafish was fully acquired. The DNA sequence of zebrafish consists of base pairs and protein coding, which are 1,505,581,940 and 26,247 in number, respectively. Their complete genomic sequence was published in 2013 (Das et al. 2013). Zebrafish is being employed as an experimental animal model for inheritable investigations. Zebrafish helps in many biological processes and muscular dystrophy. The researchers discovered the zebrafish as a wide range of resources and useful for studies like toxicity, DNA cloning, etc. (Mushtaq et al. 2013).

The zebrafish is a vertebrate animal and has been utilized to study many human diseases. They play cardinal part in understanding the mechanism and progression of many diseases like cancer. The application of zebrafish as an experimental animal has been expanded as being employed in preclinical studies, toxicology studies, and their applications (Brennan 2014). There is much similarity between the major organ systems like nervous, cardiovascular, and digestive systems of human and zebrafish. So, the developmental and physiological processes and the response to pharmacological agents are also similar (Amatruda et al. 2002). So, it is possible to identify and study the physiological and pharmacological responses of drugs and other bioactive compounds with therapeutic value. This makes zebrafish animal as a paragon model to analyze the in vivo characterization of a compound. The bioactive compounds can be diluted with distilled water and can be given to zebrafish. The compounds are easily absorbed by zebrafish through gills and skin and allow easy and rapid screening of many compounds per day. Majorly larvae and embryos are used as the experimental model (MacRae and Peterson 2003).

9.4.1 Benefits of Zebrafish

The benefits of the zebrafish (Fig. 9.3) can be summarized as follows:

- The zebrafish is a vertebrate, little in size, and robust animal model.
- The zebrafish are economical to maintain than other experimental animals.
- The cost of maintenance of animal housing and caring is comparatively low.
- The zebrafish breed tons of offspring at an interval of minimum of 7 days. This contributes to scientists with an adequate amount of study animals to carry out research investigation.
- Embryonic development of zebrafish is relatively fast (only 72 h).
- Its embryos are transparent and develop outside the body, so they can be easily observed.
- They provide genetic similarities with human.
- The progress of growth of internal organ systems of the zebrafish embryos can be easily examined as they are transparent.
- The early stages of life of zebrafish can be studied as the egg fertilization and development occur outside the mother's body.
- The zebrafish genetic structure has been thoroughly extracted to an excessive quality.
- The zebrafish have similar genomes to humans.
- The zebrafish have similar tissues and organ system as humans. As a vertebrate, they harbor several features almost identical with human systems.
- The zebrafish are able to repair heart muscle.

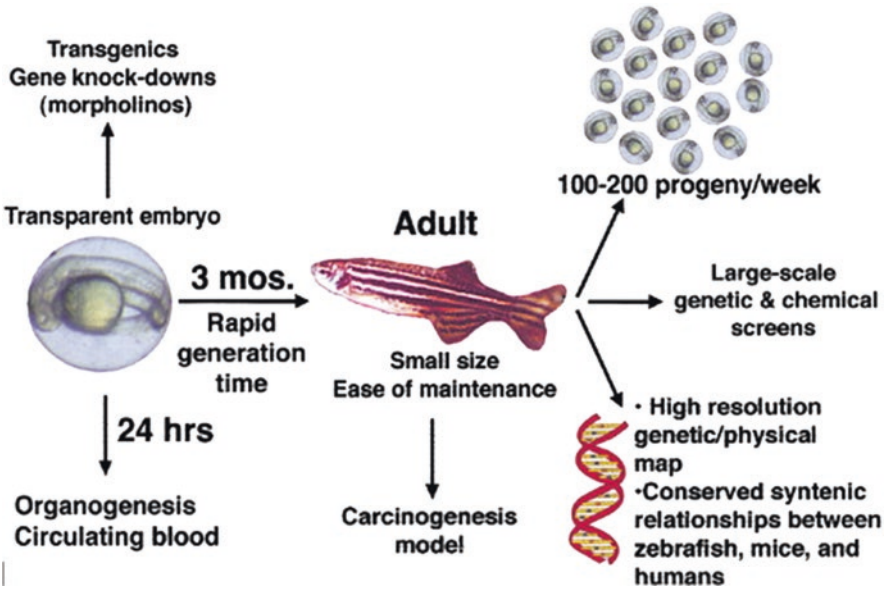

Fig. 9.3 Key benefits of zebrafish life stages (Amatruda et al. 2002)

9.5 Nanotechnology

The nano-sized materials have opened up various possibilities in a variety of industrial issues and scientific endeavors. Nowadays, nanotechnology encircles a major influence on the transformation. This is interpretive for multibillion-dollar commercial activities in industrial section (Sundararajan et al. 2016). The industrial sectors including engineering, disease diagnosis, drug delivery, biotechnology, etc. are using nanomaterials in their products. Nanotechnology is an upcoming solution for various industrial problems. It can be applied to all other science fields like chemistry, biology, physics, materials science, medicine, electronics, energy and environment, etc. (Hu et al. 2011).

Over the last few years, nanotechnology is a growing worldwide field for utilization of large variety of products. The progress in the field of medicine, engineering, and other technologies is remarkable. Nanotechnology is the science of design, development, interpretation, and implementation of materials at nanometer scale. Nanotechnology can be defined as a branch of science which deals with techniques, controlled at the level of nano-scale (Fabara et al. 2018). Nanotechnology is the study and applications of nano-scaled materials ranging from 1 to 100 nm (Fig. 9.4).

9.5.1 Nanomedicine

Nanomedicines are an effective way of drug delivery systems which include nanoparticulate carriers. The use of nanomedicines enhances the drug absorption by improving the solubility characteristics of the drug. The nanocarrier binds the drug

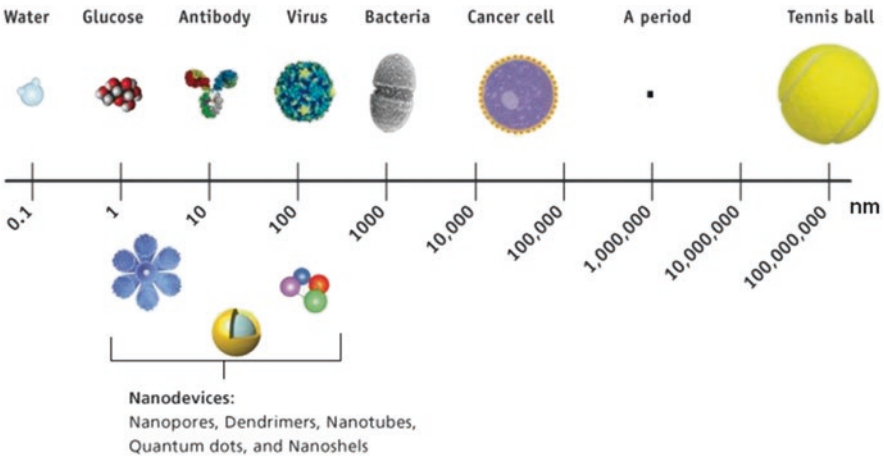

Fig. 9.4 Representation of nano-scale (Chakraborty et al. 2016a)

molecule to the specific target tissues. It minimizes the side effects (Celá et al. 2014). The physicochemical and pharmacokinetic properties of nanomedicines need to be optimized. Nanomedicines include nanoparticles, carbon nanotubes (CNT), metal nanoparticles, fullerenes, crystalline materials, nano-sized polymers, etc. (Liu et al. 2013). The nanomedicine formulations should be designed, developed, and optimized according to the properties like size, shape, chemical composition, surface charge, or modification (Harper et al. 2008). These formulations should also be tested for their half-life, absorption rate, bioavailability, cell specificity, stability, compatibility with biological environments, and toxicity study under in vivo conditions (Celá et al. 2014).

9.6 Nanoparticles

Nanotechnology deals with the design, development, and applications of nano-scaled materials. A nanoparticle is the particulate drug delivery system with nano-carriers. The size of nanoparticles ranges from 1 to 100 nm. Nanoparticles are very small-sized particles, providing large surface area for interaction of drug with the cell/tissue (Agrawal et al. 2018). The physicochemical properties of nano-scaled materials are different with the bulk compounds. On account of minute particle size and huge surface area, the nanoparticles facilitate easy absorption of drug (Bano et al. 2017). This enhances the bioavailability and reduces the requirement of dosing frequency and drug dose. They also improve the required pharmacological response and disease curing (Sundararajan et al. 2016).

The advantages of nano-particulate drug delivery are they can be used for targeting the diseased organ site. They provide enhanced therapeutic effect by enhancing drug absorption due to their nano-scaled size (Celá et al. 2014; Hu et al. 2011).

Table 9.1 Approved list of nano-pharmaceuticals (Choi and Han 2018)

Category	Marketed name	Name of drug	Indication
Liposomes	DepoCyt	Cytarabine	Brain cancer
	DaunoXome	Daunorubicin	Kaposi sarcoma by HIV
	Caelyx	Doxorubicin	Breast cancer, ovarian cancer, Kaposi sarcoma
	Abraxane	Paclitaxel	Breast cancer
Nanoparticles	Epaxal	Inactivated hepatitis A virus	Hepatitis A vaccines
	Zevalin	90Y-ibritumomab tiuxetan	Lymphoma
Nanocrystals	Emend	Aprepitant	Vomiting after surgery
	Rapamune	Sirolimus	Kidney transplantation rejection
Nanoemulsions	Norvir	Cyclosporine	HIV infection, kidney transplantation rejection
	Renagel/ Renvela	Sevelamer	Dialysis, hyperphosphatemia

Nanoparticles reduce the side effects due to drug toxicity on non-targeted organs. They provide safety and compatibility (Liu et al. 2013). Different types of nanoparticles include polymeric, inorganic, and solid lipid nanoparticles, nanocrystals, carbon nanotubes, fullerenes, liposomes, dendrimers, etc. (Mallakpour and Behranvand 2016). The physicochemical characterization and purity profile of nanoparticles can be derived by using various techniques (Epa et al. 2012). There are lots of opportunities and challenges in the development and laboratory testing of nanoparticles. Some of the nano-formulations are enumerated in Table 9.1.

The research on nanoparticle is an area of interest, because of a large variety of its implementations in biomedical, electronic, and other fields (Khan et al. 2017). These particles have applications in many fields like medical imaging, i.e., in disease diagnostic tools, nanocomposites, filters, drug delivery systems, and gene delivery. The main application of nanoparticles in cancer therapeutics is to determine physiological state of tumors and curing cancer by targeting the specific organ site (Salata 2004). Silver nanoparticles are becoming popular as the research shown its applications in numerous areas like integrated circuits, filters, sensors, biolabeling, antimicrobial toiletries fibers, cheap paper batteries, and antimicrobials (Asharani et al. 2008). Nanomaterials have an expansive range of applications in biomedical field. It includes antimicrobials, bio-detection, imaging and labeling, drug delivery, MRI agents, implants, cosmetics, and thermal spray coatings (Sundararajan et al. 2016).

9.7 Similarities of the Zebrafish to Humans

It is important to minimize the animal suffering and sacrificing during the research. The mouse is 83% similar to humans while chicken is of 64%. The zebrafish mimics the human body in 71%. All human organs are found in the body of zebrafish as they are vertebrate. While comparing the genetic characters, zebrafish have around 80% similar genes associated with human disease. Thus, zebrafish are similar to human anatomically and genetically. So, zebrafish is an ideal experimental animal and a popular choice for biomedical research (Sieber et al. 2017; Das et al. 2013).

There is ample amount of genetic information available to study the root causes of human disease. The genetic data available in public databases, and in medical records, help researchers to develop some novel treatments. But, as human genome can show only a statistical association between a particular gene and disease, this method has its limitation (Dooley 2000).

9.7.1 Human Disease Representation in Zebrafish Animal

In the latest years, the zebrafish are widely being used as recognized animal model for investigation of toxicity of nanoparticle and human diseases (Bradford et al. 2017). For toxicological studies, specific protocols should be used to use zebrafish as an animal model. For evaluation of toxicity of nanoparticles, correlation between

hatching efficiency and toxicity of embryo is a significant parameter (MacRae and Peterson 2003). Also parameters like hatching rate, developmental deformities, impairment in gills and skin, reproduction toxicity, behavioral abnormalities, mortality, etc. are used for the evaluation of toxicity of nanoparticles. Because of the unique features, zebrafish is becoming a popular animal model to study in vivo activities of nanoparticles (Chakraborty et al. 2016b).

The abnormal behavioral response of zebrafish is a sensitive indicator for toxicity study. The researchers proved that, after the chronic exposure of TiO_2 nanoparticles, the reproduction system of fish gets disturbed (Moreno-Olivas et al. 2014). To study the nanoparticle-induced toxicity, parameters like gills and skin disruption and disturbances in endocrine system are evaluated (Sizochenko et al. 2017). Some human diseases studied in zebrafish are listed in Table 9.2 as follows.

9.8 Nano-Particulate Drug Testing and Screening in Zebrafish Animal Model

The zebrafish are ideal for screening of chemical agents in their nano-particulate form as their embryos are transparent, rapidly developing, and produce externally (Sieber et al. 2017). Since the zebrafish have the permeability to small molecules, they can be used for screening and testing of drugs affecting on biological processes. Bioactive nanoparticles can be easily and successfully tested on both embryos and adult zebrafish (Bradford et al. 2017).

The zebrafish produces large quantities of eggs and embryos, and due to their small size, they can be fit in around 380-well plate. This allows testing of a number of compounds at a time, and screening can be done in a very less time frame (Sivamani et al. 2016). Automated screening techniques have been developed to minimize human error and to achieve high possible throughout. The automated techniques include embryo collection and preparation, delivery of testing compound, incubation, imaging, and analysis of results (Sieber et al. 2017). The automation allows the dissection of phenotype and evaluation of side effects. The rapid

Table 9.2 Studies of zebrafish animal models for human disease (Kari et al. 2007)

Disease	Responsible gene	Zebrafish model obtained by
Spinal muscular atrophy	Reduced levels of SMN protein	Morpholino targeting
Duchenne muscular Dystrophy	Mutation in the dystrophin Gene	Screening
Joubert syndrome	Mutations in the gene CEP290 (NPHP6)	Morpholino targeting CEP290
Severe congenital anemia	Defects in the 4.1R red blood Cell membrane protein	Screening
Erythropoietic protoporphyria	Disorder of ferrochelatase	Screening
Barth syndrome	Mutations in tafazzin	Morpholino targeting tafazzin
Nephrotic syndrome	Mutations in the PLCE1	Morpholino targeting PLCE1

development of zebrafish embryos is beneficial for drug testing and screening. So, in a short period of time, assays can be performed.

Some anticancer drug gives desired pharmacological response by interacting with a single specific target. The delivery design and development of such drugs are challenging. In zebrafish drug screening assays are reliable tool for pre-clinical safety evaluation and used routinely to evaluate toxicity (Santoro 2014).

The zebrafish provides advantages in structure-activity relationship and high-content screening. In the process of drug discovery and testing, the chemical compound selected for the screening can be modified for improvement in desired pharmacological response with minimum side effects (Mushtaq et al. 2013). The structural derivatives can be prepared and tested directly on zebrafish as animal model. By chemical modification in drug compound, improvements can be done and identified with in vivo testing. The zebrafish is becoming a prominent model organism for processes of drug invention that includes target identification, disease modeling, biological activities, and toxicology (Santoro 2014). The reference genes of zebrafish embryos used in toxicity studies are listed in Table 9.3 as follows.

9.8.1 Assessing Teratogenicity and Other Developmental Effects in Zebrafish Model

The eye development is disrupted, and pigment deposition was noticed with a simple microscope when the zebrafish embryos are exposed to gold nanoparticles, The visualization effects can be seen on pigmented cells, including RBCs, hatching, and mortality. The dose-dependent and time-dependent screening of silica nanoparticles toxicity was used in assessing the mortality rates and impacts on cardiovascular system (Chakraborty et al. 2016b).

Table 9.3 Reference genes of zebrafish embryos used in toxicity studies (Liu et al. 2018)

Gene symbol	Gene name	Accession	Function
18S rRNA	18S ribosomal	RNA generic	18S ribosomal RNA
eef1a1l1	Eukaryotic translation elongation factor 1 alpha 1, like 1	NM_131263.1	Factor for protein translation
actβ2	Actin, beta 2	NM_181601.4	Cytoskeletal structural protein
polr2d	Polymerase (RNA) II (DNA directed) polypeptide D	NM_001002317.2	Enzyme for transcription
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_200910	Enzyme in tricarboxylic acid cycle
β2m	Beta-2-microglobulin	NM_131163	Beta chain of a major histocompatibility complex I molecular

Researchers studied the fetal alcohol syndrome on zebrafish. The embryos of zebrafish were exposed to ethanol at different concentrations and different time durations. They resulted in defects in the development. These developmental defects can be directly compared with the defects in human birth (Loucks and Ahlgren 2012). Scientists also studied teratogenic effects of nitrate and nitrite by using zebrafish as animal model. High levels of nitrates result in congenital defects or miscarriages in humans. As the embryo of zebrafish got exposed to higher level of concentration of nitrite, various birth defects were observed, while that of nitrate, no defects were observed. Even so, the zebrafish act as a beneficial model to quickly determine birth and developmental defects caused by exposure to teratogens (Keshari et al. 2016).

9.8.2 Genetic Analysis in Zebrafish Model

The genetic analysis consists of gene mutations and chromosomal alteration study. Because of chemical exposure, DNA is susceptible to damage. This can be studied in the different stages of life of zebrafish like embryo, larvae, and adult. The RAPD-PCR methodology is used to determine effect of nanoparticles on genetic analysis including genotoxicity of TiO_2 nanoparticles (Moreno-Olivas et al. 2014).

More sophisticated protocols have been used to study the transgenesis to enhance the zebrafish genomic resources. This established zebrafish as a human disease model (Carpio and Estrada 2006). The recently developed targeted genome editing techniques like CRISPR/Cas9, ZFN, and TALEN have been used in zebrafish model to reproduce pathological conditions similar to human and to investigate in vivo effect. The goal of genomic study on zebrafish is to determine new targets for drug therapy (Rissone and Burgess 2018).

9.8.3 Immune System Testing in Zebrafish Model

The nanoparticles are very sensitive toward the immune system. The nanoparticles with an inflammatory response are also associated with the activation of neutrophils and macrophages. Gold nanoparticles disrupt the pathways involved in immune response. While testing on zebrafish model, silver nanoparticles caused immunotoxicity (Chakraborty et al. 2016b).

During the embryogenesis of zebrafish, the specification of both B and T cells appears. Newly developed methods like ES cell gene inactivation allow the generation of specific mutants. Thus, the zebrafish became more resourceful as an experimental animal for immunological research (Yoder et al. 2003).

9.8.4 Reproduction Analysis in Zebrafish Model

The reproduction rate of zebrafish is high. The nanoparticles with activity affecting the reproduction system can be analyzed using zebrafish. The effect of such agents on egg production, fertilization, and embryo development can be tested. Instead of the tiny size of zebrafish, there are more similarities in reproduction system and its functions to mammals. This promotes their use as a model in infertility research (Hoo et al. 2016). The exposure of zebrafish to TiO_2 nanoparticles gives reduced production of eggs and increased mortality of embryo. While being exposed to silver nanoparticles, the maturation of zebrafish embryo enhances because of the elevation in levels of oxidative stress and apoptosis of follicle cell (Gondwal and Joshi 2018).

9.8.5 Nervous System Analysis in Zebrafish Model

The effect of nanoparticle activity on nervous system of zebrafish can be determined by evaluating some sensitive parameters such as spatial recognition, color preference, locomotion, etc. By observing these parameters, the changes in complex behavior of zebrafish can be observed. The development of the brain in zebrafish is at risk of oxidative stress due to excessive content of fats and proteins and low levels of antioxidants in cells. The oxidative stress occurs as the nanoparticles activate free radicals deposited on their surface. Commonly, neurotoxicity is observed in nanoparticles which lead to the degeneration of the nervous system (Win-Shwe and Fujimaki 2011).

The neurodegenerative diseases including Alzheimer's diseases, Parkinson's disease, Huntington's diseases, etc. have been studied on zebrafish as experimental model. Researchers evaluated the effect of extract of *Alpinia oxyphylla* fruit in ethanol on PC-12 cell line and zebrafish as an experimental animal model (Saleem and Kannan 2018). The extract has neuroprotective effect and been used in Chinese therapeutics traditionally. The outcome of the screening demonstrated that this extract blocked and repaired neuro-degeneration induced by 6-hydroxydopamine. It also reduced the locomotion activity in Parkinson's disease in zebrafish model study (Vaz et al. 2018).

9.8.6 Behavioral Analysis in Zebrafish Model

The specific nanoparticles can alter the behavioral system of the zebrafish. The locomotor activity of zebrafish can be seen altered with the use of quantum dots of cadmium telluride (CdTe). The color preferences get altered because of the use of silicon dioxide (SiO_2) nanoparticles. The exposure of zebrafish to the titanium dioxide (TiO_2) nanoparticles enhances the neuron apoptosis and proliferation of glial cell. Also gene expression alteration can be seen in zebrafish model (Moreno-Olivas et al. 2014).

9.8.7 Anticonvulsant Activity in Zebrafish Model

The extract of a longa is used for the treatment of epilepsy (Krausz et al. 2015). The anticonvulsant effect of this extract can be studied on the zebrafish animal model. For the study, seizures were induced in the animal with the chemical pentylenetetrazol. The insertion of this extract exhibited antiepileptic effect (Alafiatayo et al. 2019; Nieoczym et al. 2019). Similarly, antiepileptic drug was studied by exposing the zebrafish to an increasing concentration of the drug. This resulted in the increase in seizure as increase in concentration of pentylenetetrazol-induced seizures (Gupta et al. 2014). Scientists studied the effect of an anticonvulsant drug, i.e., pterostilbene (PTE), in the larvae of zebrafish. This showed that there were no any noteworthy changes in neuromuscular power and movement (Nieoczym et al. 2019).

9.8.8 Melanogenic Activity in Zebrafish Model

Easy examination of phenotypic pigmentation process is possible in the larvae of zebrafish. So, it is considered ideal for the melanogenic-activity-related studies. The study of hanginine A activity suggested that it promotes the anti-melanogenic activity. The treatment of zebrafish with arctigenin showed that there is a decrease in pigment deposition in zebrafish after 15 days of fertilization. In another study, the extract of *Eurya emarginata* produces compound rengiolona which has anti-melanogenic activity. It was observed that there is an inhibition of pigment deposition on the body of zebrafish after the depletion of amount of melanin (Santos et al. 2016).

9.8.9 Anti-Inflammatory Activity Evaluation Using Zebrafish Model

To study the anti-inflammatory activity, the zebrafish was infected with *Staphylococcus aureus*. Then the zebrafish were treated with grape extract containing dihydrofolate reductase activity. The significant decrease in the inflammatory activity was observed.

The investigation of anti-inflammatory activity of essential oil extracted from *Rosmarinus officinalis* L. (OERO) was carried out on zebrafish as an experimental model. This study on zebrafish resulted in inhibiting the inflammatory process. In another study, abdominal edema in zebrafish animal model induced by using carrageenan was treated with methylprednisolone. This resulted in significant inhibition of carrageenan-induced inflammation.

9.8.10 Antithrombotic Activity Evaluation Using Zebrafish Model

The zebrafish can be evaluated for rare genetic blood diseases (Rissone et al. 2018). Zebrafish can be treated as animal model to examine the compounds isolated from plant extracts having anti-thrombotic activity. These compounds were examined together with other known compounds, to investigate the anti-thrombotic activity on zebrafish. Among these compounds, the eriodictyol indicated to be a potent anti-thrombotic agent which hinders the formation of thrombus. Researchers carried out studies to evaluate anti-thrombotic properties of hawthorn leaves. An extract of hawthorn leaves was prepared in ethanol and tested on zebrafish as animal model. The inhibitory activity of the drug was investigated by aggregation of platelets and anti-thrombus assessments using a zebrafish model (Gao et al. 2019).

9.9 Future Prospects

The zebrafish also plays an important part in toxicological studies of the nanomedicines. The zebrafish has proven its extensive promises as an in vivo animal model for screening of nanomaterials. The zebrafish can be employed in the process of drug development at the stage of pre-clinical testing. The future studies are necessary to determine new targets of testing agents.

They are able to survive without a fully functional cardiovascular system. If the cardiac muscles get damaged, the zebrafish has ability to develop the muscles newly. So, with cardiac muscle development property of the zebrafish, it will become common animal model for evaluating drugs used in the treatment of cardiovascular diseases (Zakaria et al. 2018). A broader research is required for novel targets of testing compounds, which are responsible for the effects on the development of the cardiovascular system.

9.10 Conclusion

The zebrafish have genotypic and phenotypic similarities as humans. The zebrafish offers advantages like rapid development, simple maintenance, egg collection, and low cost of production. The zebrafish model is a powerful, well-established research platform for the testing of activities of new drug molecules. They are vertebrate animals mimicking the human body. The zebrafish offers advantages mainly as low cost of production, maintenance and utilization as an animal model, rapid development and rapid in vivo analysis. With these features, the use of zebrafish in testing of biological activities is becoming frequent and more popular.

The zebrafish has become an experimental animal for evaluation of activities of bioactive extracts and constituents with medicinal activities derived from plants, natural resources, etc. So, the study of small molecules like nanoparticles and natural products can be carried out with zebrafish model. The main attraction of increased use of zebrafish is that a variety of tests can be carried out on this animal. The assays

of bioactive compounds derived from the plant extracts can be carried out on zebrafish. Presently, research is focused on the biological activities such as testing and toxicological testing of newly developed medicines especially chemotherapeutic agents or nanoparticles used in the treatment of cancer. The zebrafish animal model is inexpensive and more systematic. It completes the study much rapidly. By using advanced techniques, the zebrafish are becoming a remarkable possible option of other vertebrate animal models for investigating toxicity studies of nanomedicines.

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Drosophila melanogaster: A Model Organism to Understand Biological Activities of Nanoparticles

10

Bijayata Patra, Poulomi Ghosh, and Saprativ P. Das

Abstract

Nanoparticles exhibit remarkable physicochemical features not inevitably found in bulk arrangements; their size or coating modifications distinctly alter their physical, chemical, and biological attributes. Owing to these unique properties, there is a general inclination to explore nanoparticles in numerous fields, viz., in medicine and food industry. To that end, our environment and human health are also affected by its toxicity. To study the reaction of nanoparticles with human cells and complex system, a thorough understanding of the parameters is necessary for the nanoparticles to react within the cells. As human-based trials are difficult with ethical barriers, one extensively exploited laboratory model organism, viz., *Drosophila melanogaster*, is used as an in vivo model organism for the study of developmental biology, genetics, and recently host pathogenicity. *D. melanogaster* is very much genetically malleable organism with its short life-cycle, clear developmental stages and availability. *D. melanogaster*, possessing completely the experimental features like inclination of investigational manipulation proportional to vertebrate models, significant gene homology with developed and complex organisms, and simplicity of gaining mutant phenotypes, seems to be an ideal model organism for the study of nanoparticles activities in cells and nanotoxicological trials. The molecular trails with numerous developmental and behavioral factors can be assessed expending this model in different modes of throughput type tests.

Keywords

Nanoparticles · *D. melanogaster* · Host pathogenicity · Nanotoxicology · Gene homology

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10.1 Introduction

Nanoparticles exhibit remarkable physicochemical features which are not inevitably found in bulk arrangements; their size or coating modifications distinctly alter their physical, chemical, and biological attributes. Owing to these unique properties, there is a general inclination to explore nanoparticles in numerous fields, viz., in medicine and food industry. To that end, our environment and human health are also affected by its toxicity. To study the reaction of nanoparticles with human cells and complex system, a thorough understanding of the parameters is necessary for the nanoparticles to react within the cells. As human-based trials are difficult with ethical barriers, one extensively exploited laboratory model organism, viz., *Drosophila melanogaster*, is used as an in vivo model organism for the study of developmental biology, genetics, and recently host pathogenicity (Pandey and Nichols 2011). *D. melanogaster* is very much genetically malleable organism with its short life cycle, clear developmental stages, and availability (Greenspan 2004). *D. melanogaster*, possessing completely the experimental features like inclination of investigational manipulation proportional to vertebrate models, significant gene homology with developed and complex organisms, and simplicity of gaining mutant phenotypes, seems to be an ideal model organism for the study of nanoparticles activities in cells and nanotoxicological trials (Rand 2010). The molecular trails with numerous developmental and behavioral factors can be assessed expending this model in different modes of throughput type tests (Ahamed et al. 2010).

10.2 Insects and Nanoparticles

The progress of nanoparticles is now widely used in the various divisions of science to understand their toxic effects on expansion and anatomy of their organisms and environment (Ahamed et al. 2010). It has widespread applications in pest management of insects. As per research conducted, ferromagnetic substances have been recognized in the head, neck, thorax, and abdomen of *Solenopsis substituta* ant (Guan et al. 2008). The components of nanoparticles are found in the compound eyes of insects. The bright colors of wings of butterflies are nothing but the nanoparticles. In recent years, an innovative photodegradable insecticide involving nanoparticles have been developed (Garcia-Bellido et al. 1979; Guan et al. 2008).

10.3 Toxic Effects of Nanoparticles in Insects

Nanoparticles (NPs) invade by intracellular penetration through the exoskeleton and disrupt the organisms (Benelli 2016). Then, the nanoscale substances bind to sulfur or phosphorous from proteins or DNA, respectively, leading to the expeditious reconstruction of organelles and enzymes (Benelli 2016). The degradation of

Table 10.1 Toxic effects of nanoparticles on insect physiology

Insect name	Nanomaterial	Physiological effect	References
Aedes aegypti	Ag, Au, ZnO	Damages the epithelial in insects	Banumathi et al. (2017a, b, c)
		Damages the midgut, cortex, and gill	Kalimuthu et al. (2017)
		Reduces lateral hair	Sundararajan and Kumari (2017)
A. albopictus	Ag	Reduces the total number of protein in larval stage Reduces phosphatase enzyme, esterase, and acetylcholine	Ga'al et al. (2018)
Anopheles stephensi	Ag, Au, ZnO, Polystyrene, SiO_2	Damages the epithelial in insects	Banumathi et al. (2017a, b, c)
		Reduces lateral hair	Sundararajan and Kumari (2017)
		Damages the midgut and cortex	Kalimuthu et al. (2017)
		Reshapes the thorax	Abinaya et al. (2018)
		Abuses the structure of larval body	Sultana et al. (2018)
Culex pipiens	Ag	Reduces the total number of protein in larval stage Reduces acetylcholine, α and β carboxylase	Fouad et al. (2018)
Bombus terrestris	SiO_2	Midgut epithelial injury	Mommaerts et al. (2012)
Acheta domesticus	Graphene oxide	Activates catalase, glutathione, and peroxidase Activates heat shock protein (HSP70)	Dziewięcka et al. (2016)

membrane permeability and interruption in proton intent force cause reduction of the cellular function followed by cell death (Benelli 2018) (Table 10.1). A few experiments exhibit the toxic effects of nanoparticles on growth. A testing with 100 mg zinc oxide nanoparticles (ZnONPs) per litre resulted in 100% lethality in the *Aedes aegypti* mosquito production whereas, 1.57 mg/l of ZnONPs witnessed a low LC_{50} (Banumathi et al. 2017a, b, c). Several scientists exhibited that midgut epithelial injury occurred in intoxicated workers of *Bombus terrestris* due to silicon dioxide nanoparticles (SiO_2NPs) and in *A. aegypti* due to silver nanoparticles (AgNPs), respectively (Mommaerts et al. 2012; Kalimuthu et al. 2017) (Table 10.1). *A. aegypti* associated with gold nanoparticles (AuNPs), on a contaminated environment at the favorable concentration of high mortality results in harming the midgut, epithelial cells, and cortex (Sundararajan and Kumari 2017). Studies on *Acheta domesticus* revealed that the graphene oxide nanoparticles activate catalase, glutathione peroxidase, and heat-shock protein (HSP70) (Dziewięcka et al. 2016) (Table 10.1).

Table 10.2 Scientific classification of *Drosophila melanogaster*

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Drosophilidae
Genus	*Drosophila*
Subgenus	Sophophora
Species	Melanogaster

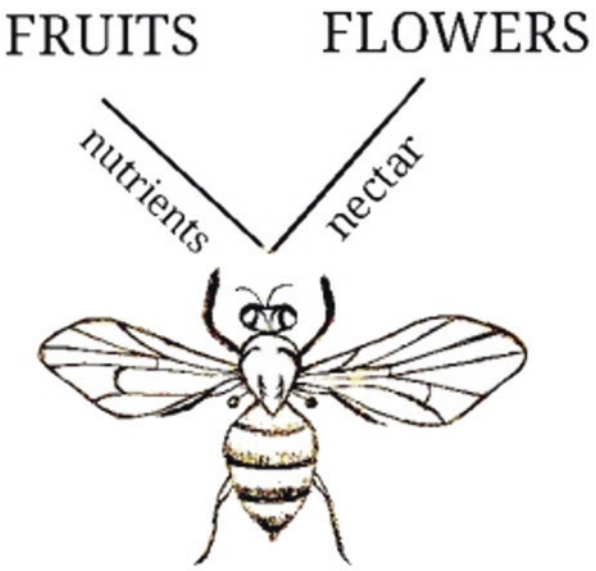

Fig. 10.1 The fruit fly, *Drosophila melanogaster*

10.4 *Drosophila* in Nanoparticle Study

Thomas Hunt Morgan first used *D. melanogaster* as a model organism in the time period of 1960–1990 (Morgan 1910). It was also used to study some human diseases and as a model organism used to study toxicology (Pandey and Nichols 2011). In the year of 2010, the term Drosophotoxicology was reported, as *Drosophila* has a short life span around 40–60 days (Rand 2010). The nanotoxicity can easily be studied for their genome stability, development, reproduction, and activity in several age periods of adult flies, utilizing any particular tissue or organ (Greenspan 2004). As an example, the *Drosophila* can be used in organogenesis to exhibit the toxins and developmental studies concerning cell determination and neurons in embryonic stage or the larval stage, during its applications in developmental and physical studies. In the stages between late larva and pupa, the toxin's mode of action to affect fictional discs is an advantage to study the unfavorable toxic effects

on end replication and physiological changes between the stages of larva to adult flies (Pandey and Nichols 2011; Stocker and Gallant 2008). Some organs of adult *Drosophila* such as the brain, heart, lungs, kidney, liver, gut, and reproductive tract are physically almost similar to humans. *Drosophila* fat body also has identical functions as the human liver (Pandey and Nichols 2011). The tracheal system of *Drosophila* (respiratory system) is nothing but a divided network of epithelial tubes, separated in every part of the body. It helps in transporting oxygen and other gases. As for these identical traits in organs between *Drosophila* and human, it can be the acceptable model organism of toxicology analysis, approved by the European Centre (Ahamed et al. 2010). Besides this, as a conclusion, we can say that the short life span, recognizable developmental stages in their life cycle, well-known genome sequence, accessible equipment, and reagents makes *D. melanogaster* an efficient in vivo model organism for toxicology. The scientific classification of *D. melanogaster* and the fly is shown in Table 10.2 and Fig. 10.1, respectively.

D. melanogaster belongs to kingdom Animalia, phylum Arthropoda, class Insecta, order Diptera, and family Drosophilidae. It is generally known as fruit fly. The first approach of using *D. melanogaster* as a model organism is registered by Charles W. Woodworth. It has a broad spectrum in genetics, physiology, and microbial pathogenesis researches. As per reports till 2017, eight Nobel Prizes are archived for researches regarding *D. melanogaster*. It has a worldwide geographic extent including islands. The flies belonging to the family Tephritidae are also known as "fruit fly," but these flies, *Ceratitis capitata*, are economic pests in Australia and South Africa.

10.5 Characteristics and Life Cycle

Female *Drosophila* is 2.5 cm in size, whereas the male *Drosophila* is shorter than them (Fig. 10.2). Male *Drosophila* appears with a darker back, black patch at the abdomen part, a row of dark hairs on the tarsus of first leg (sex-comb), and the various body colors which make a difference between male and female *Drosophila*.

Drosophila has a short life span around 50 days at the optimal room temperature of 25 °C (77 °F). But the developmental period depends on the temperature and the ectothermic species as well. The time period and the temperature increase proportionally due to heat stress. At the temperature of 28 °C (82 °F), *Drosophila* can develop from egg to adult stage just in 7 days (Ashburner and Thompson 1978; Ashburner et al. 2005); at 30 °C (86 °F), it takes 11 days; at 18 °C (64 °F), it takes 19 days; at 12 °C (54 °F), it takes over 50 days; and at 25 °C (77 °F), it takes 8.5 days, which is the ideal case. The developmental time period also depends on crowded environment. The time period gets increased (Chiang and Hodson 1950) such as female flies may lay 400 eggs at a time. They mainly lay eggs into the decaying material such as decaying fruits, mushrooms, and slap fluxes. Some specific eggs having 0.5 mm long in size hatch after 12–15 h at the ideal condition. As a result, the larvae complete their growth in 4 days. Within this period, after 24 and 48 h of hatching, molting occurs twice in the second instar larval stage and third

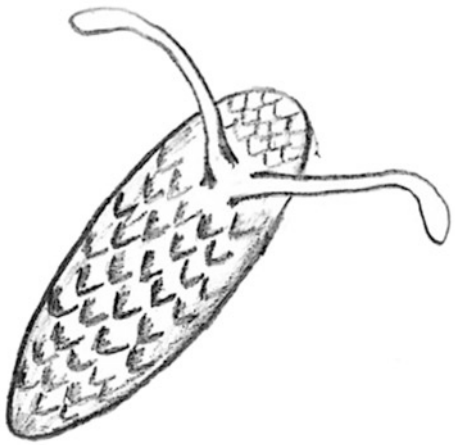

Fig. 10.2 Male *Drosophila melanogaster*

instar larval stage. They used to feed on the nutrients of decomposed materials, sugars from fruits, and nectar from flowers during their developmental period. The mother flies generally deposit their feces on the egg sacs with the intention to initiate an identical microbial configuration in the embryo's guts (Blum et al. 2013). Then the larvae get transformed into pupa, and subsequently after 4 days, the adult *Drosophila* emerges with some physiological changes (Blum et al. 2013).

10.6 *Drosophila melanogaster* Reproduction Mechanism

Male flies used to approach the female by five types of observable techniques. At first, they carol a courting song by vibrating and expanding their wings horizontally to orient them, and then the male fly places himself at the low back portion of the female fly's abdominal part with the motive to press and lick the female genitalia. And then they make their abdominal a coil-like structure to participate in mating, staying for around 15–20 min (Houot et al. 2010). Some specific neurons of abdominal nerve cord permit the female to hold their body during mating and control their mood to approach the male for mating. But the male can help them by their secret chemical substances, pheromone, to reactivate the nerves. They didn't mate in a poor environment (Dagaeff et al. 2016). Female prefer to mate with their brothers rather than other males (Loyau et al. 2012), and they used to follow the other females while choosing the male as a copulation partner. Both male and female flies can do multiple mating at the same time, to make sure about the fertilization known as polygamy (Haartman 1951). But conventionally with whom the initial mating is done, he is the 80% ancestor of the offspring. The priority of the last male can be registered over the both phenomenon dislocation and inability (Price et al. 1999). The advantages of mature males in mating are they can adopt a developed courtship dance while approaching recent females and as they are efficient in it they can complete their copulation quickly.

By this procedure, male flies can inject their 1.76 mm long sperms in the seminal fluid of the female flies (Gilbert 2006). The female flies get ready for the next mating after 8–12 h of development (Pitnick 1996). As they prefer a short-lived copulation than male, they can deny or refuse the male fly for copulation by quitting or kicking and ejecting their ovipositor (Connolly and Cook 1973). *Drosophila*'s reproduction mechanism doesn't follow the human's estrous cycle. Due to gonadotropic hormone, they used to follow a cyclic pathway which is correlated and produces stable and controlled offspring (Meiselman et al. 2017).

10.7 A Model Organism for Understanding the Biological Activities of Nanoparticles

Model organisms are a non-human species used to study easily biological phenomenon in the laboratory. *Drosophila* is one of the most efficient biological model organisms in genetics and developmental biology.

D. melanogaster as a model organism

- Adult flies are very small, 3 mm in length, so it can easily grow and be studied in the laboratory.
- It produces a large number of offspring by multiple mating (female fly lays around 400 eggs at a time).
- It has a short generation time period, around 14 days.
- Embryos grew up and get developed outside the mother's body, and the multicellular anatomy helps to study the developmental stages.
- The modified nervous system helps to study their characteristics.
- The genome sequence is well known, containing 13,600 genes (Adams et al. 2000).
- Some have physically identical organs, unlike humans.

The most acceptable technique to study the nanotoxicity is by analyzing their existence after the revelation of nanomaterials into the fly's body. The introduction of nanomaterials into the *Drosophila* can be done by the common way of ingestion. As an example, the effect of nanoparticles in *Drosophila* was studied after 6 h of fasting by influxing a specific concentration of 20 nm AgNPs through their food. After 10 days, the effects of before and after the introduction of nanoparticles into the flies were observed. As a result, it decreased the survival rate of flies. But in case of $AgNO_3$ with the same conditions, the results will be positive, concluding that specifically AgNPs (Tian et al. 2013) cause the toxic effects in *Drosophila*. It evades the capability of larva to develop in pupate. So the cycle of larva to pupation stage and then pupate to adult stage gets retarded.

Sometimes the toxicity of nanomaterials varies on their coat. Poly(maleic anhydride octadecene) and polyethylene glycol-coated nanomaterials are more venomous than the mercaptoundecanoic acid or poly(maleic anhydride octadecene)-coated nanomaterials (Galeone et al. 2012). CdSe-ZnS quantum dots exhibit toxic effects on *Drosophila* by decreasing their survival rate as CdSe-ZnS quantum dots are

coated with poly(maleic anhydride octadecene) and polyethylene glycol. By several experiments, it is considered that the integral quantities of nanoparticles are responsible for the toxicity, such as citrate-coated AuNPs, degrading the *Drosophila's* life span (Pompa et al. 2011). On the other hand, some nanoparticles play a positive role in *Drosophila*. Approximately around 20 nm silica nanoparticle did not show any toxic effects on flies, such as almost 80 nm of gallium phosphide (nanowires) (Adolfsson et al. 2013; Barandeh et al. 2012) and Gellan gum-PEI (nanocomposites) (Goyal et al. 2011) don't degrade their developments and survival rate, respectively. The insulin nanoparticles, having the function of transporting insulin, also didn't show any toxic effects to flies (Fangueiro et al. 2013). Dry nanoparticles, such as carbon black and unicellular nanotubes, can also be encountered to *Drosophila* by physiological contact. As *Drosophila* has similar traits like human, this process can be considered for the introduction of nanoparticles to the human skin. The introduction to the exterior part of the flies causes mortality to them within a couple of hours. If the nanoparticles specifically get exposure to the spherical open areas, then it causes respiratory retardation in flies, considering the main reason for death (Lehmann 2001; Liu et al. 2009).

Lastly nanoparticles can be inflamed by inhalation. Small nanoparticles can influx in *Drosophila* by a nebulizer-based technique through the spherical opened areas. These experiments conclude that some specific nanoparticles such as 24 nm-, 100 nm-, and 210 nm-sized FluoSpheres and 20 nm silver can transfer to the respiratory system of *Drosophila*, and it can be considered as the primary analysis of nanoparticle inhalation technique to the human also. As the embryo grows up outside the mother body, the various methods of introducing nanoparticles can be successfully studied in their different stages. It can therefore be concluded that *Drosophila* is an efficient model organism in nanomaterial studies (Fig. 10.3).

10.8 Gene Homology

Homology was first reported by Richard Owen. He registered that homology is nothing but the sharing of common organs or genes (Owen 1843) in various taxa, with the variety of structure and function (Gegenbaur 1878). The word "homology" is the joining of two meaningful words "homo" which means same and "logos"

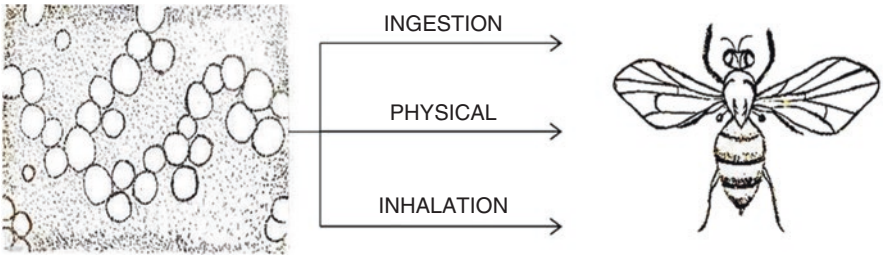

Fig. 10.3 *D. melanogaster* for nanoparticle study

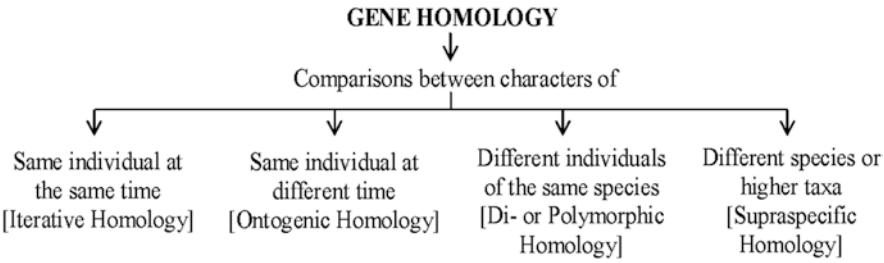

Fig. 10.4 Classification of gene homology

which means relation (Bower 1906; Williams and Forey 2004). As per developmental biology, the structure of homologs can be modified by the dynamic process of adaption to different motifs, resulting in enhanced descent from a common founder or ancestor. But later, the definition of homology restricts the homologous usage to supraspecific analogy (Ax 1989). Some examples of homologous structure are wings of bat, forelegs of dogs and horses, etc. A gene inherited in two species from a common founder is known as homologous gene. They may be having a same sequence, but it is not mandatory at all (Haszprunar 1992; Wagner and Laubichler 2000; Wagner et al. 2000). The traits of a homologous gene are known as homology. But the word "homology" applies for both homologous protein and gene in genetics. It forms the fundamental arrangements for provisional biology. Homologs vary due to mutations, as their identical founder. Homologous gene can derive their common founder. Homologous gene can derive though three specific circumstances as follows:

(a) Phylogenetic event: This helps to grow the orthologous gene.
(b) Genetic duplication event: This helps to grow paralogous gene.
(c) Horizontal gene transfer event: This helps to grow xenologous gene.

10.9 Classification of Gene Homology

The classification of gene homology is shown in Fig. 10.4.

10.9.1 Iterative Homology

Iterative homology is the specific type of homology which clarifies the typical and repetitious relationship into the fragment of the common organism (Webster 1913). Iterative homology is also known as serial homology. The occurrence of homoeotic mutations can encounter this type of homology. These individuals are as complicated as in numerous *Drosophila* mutants. But homoeotic mutation in *Drosophila* can be a matter of probabilities.

10.9.2 Ontogenic Homology

This is the type of homology in which there is specific arrangement in the common taxa, showing evolutionary similarities in growth. It can be recognized by pursuing the characteristic distinction and adaption, throughout the ontogeny (Haszprunar 1992). The phenomenon of naturally occurring neoteny as a mutation is occasional. But until now, it is considered in modifying phylogenies. It should be emphasized that this defines all characters of a definite ontogenic stage, ancestor homologue of the adult stage. As ontogeny is a part of the evolution, larval character gets reconstructed from an adult stage of a precursor in the cases of the biological and polyphasic life cycle, deduced for the pedigree of larval insect homolog along the ommatidia of the possible ancestor. But it doesn't count as a natural ancestor of the common entities. Similarly it can also be registered with protonephridia and metanephridia in larva and adults of coelomate metazoans, respectively (Ruppert and Smith 1988).

10.9.3 Di- or Polymorphic Homology

In this type of homology, species are arranged in the common taxa, which exhibit physiological similarities. This naturally occurs between subspecies, variants, and mutants, where the adaption of characters is not stable, whereas in dimorphism and polymorphism, the adaption of characters is stable. It also includes the examination of specific mutation or diagnostic experiments regarding sex or polymorphism. The instances of hypothetical polymorphism detection are generally possible in the adult bee. On the contrary, studies of polymorphism recognition in typical larvas show that they get triggered by food with the intention of becoming a queen. For example, studies of sex change along with all intermediary structures in gastropods, dog whelk (*Nucella lapillus*), and European sting winkle (*Ocenebra erinaceus*) (Gibbs et al. 1990).

10.9.4 Supraspecific Homology

This homology defines the contrast between the different species and higher taxonomy. As particularly, oncogenic and bi- or polymorphism homology is the type of phylogenetic homology, so this phase should evade for supraspecific homology. Nonetheless, it is a major part of phylogenetic reconstruction. During the meiosis cell division, the genetic conversions take place as the former varieties can obtain within the species. Although these occurrences are not within species, some exceptional examples are the procedure of transferring gene by vectors or conjunction (Stachel and Zambryski 1989).

10.10 Host Pathogenicity

10.10.1 Pathogenicity

Pathogenicity is interpreted as having the complete ability of infectious agent including bacteria, fungi, virus, protozoa, and helminth, responsible for disease in a host (Malcolm and Moore 2017). Pathogenicity means virulence, but in many cases, it is analyzed as a qualitative term. Pathogenicity is also being definite from the transmissibility of the virus, which measures the level of infection.

10.10.2 Pathogenic Treatment

Infectious diseases are inescapable disorders due to organisms (bacteria, virus, fungi, or parasites). Many of them also live in our bodies with different functions. They may be harmful or helpful for the host. There are some simple strategies, treatments, and global methods to prevent pathogenic diseases such as vaccines and medicines, antibiotics, and antivirals.

10.10.2.1 Vaccine

The vaccine is a biotic arrangement that provides an active and improved acquired immunity to a specific disease. Vaccines are basically made of by the exhausted

Table 10.3 Side-effects of vaccines

Vaccines	Side effects	Reference
1. DTaP (diphtheria, tetanus, acellular pertussis) vaccine	Redness, soreness, fever, coma, permanent brain damage, poor appetite	Regalado et al. (1990)
2. Hepatitis A vaccine	Low grade fever, headache, tiredness, soreness, redness, shoulder pain, allergic reactions	Tong et al. (1993)
3. Hepatitis B vaccine	Soreness, fever, shoulder pain, allergic reaction	Bircan and Rahmet (2017)
4. Influenza (inactivated) vaccine	Flu, soreness, redness, swelling, red or itchy eyes, fever, headache, fatigue, Guillain-Barre syndrome (GBS)	Hirve et al. (2016)
5. Influenza (live) vaccine	Runny nose, cough, fever, headache, wheezing, abdominal pain, vomiting, sore throat, chills, tiredness	Hirve et al. (2016)
6. Polio vaccine	Dizziness, ringing in the ear, shoulder pain soreness, allergic reaction	O'Reilly et al. (2012)
7. Rabies vaccine	Soreness, redness, swelling, nausea, abdominal pain, dizziness, muscle aches, pain in joints, nervous system disorder	Hsu et al. (2017)
8. Yellow fever vaccine	Soreness, redness, swelling, sever allergenic reactions and nervous system reaction	Amanna and Slifka (2016)
9. Anthrax vaccine	Tenderness, redness, itching, lump, bruise, headache, fatigue	Zai et al. (2016)

micros and their toxins and contain a factor that reorganizes the microorganisms, responsible for diseases. The factor helps the immune system to make it easier to recognize it, identify it as a foreign body, and eradicate the disease-causing microorganisms by provoking host's immune system. The vaccine itself is incapable of causing disease. But the host body reacts to vaccines as if it is a pathogen-containing agent. Many diseases are now cured by vaccine such as polio, measles, diphtheria, whooping cough, mumps, and tetanus (Table 10.3). The procedure is known as vaccination (Melief et al. 2015; Bol et al. 2016). Vaccinated host body constructs antibodies which counteract the disease-causing viruses and bacteria. As they are not much probable to being infected or transfer the infection to others, non-vaccinated people will also be saved by the immunity of the herd. The side effects of various vaccines are shown in Table 10.3.

10.10.2.2 Antibiotic

Antibiotic is an antimicrobial material containing medicine, powerful to fight against bacterial infection. It has the function to either kill or inhibit the bacterial reproduction in the host body. Antibiotics are universally used in the treatments. But it is necessary to use it properly to save lives. To prevent the development of bacterial resistance, it should be taken as directed and also after the symptoms disappear. Antibiotics are not effective for virus infections. Some antibiotics are penicillin,

Table 10.4 Side effects of antibiotics

Antibiotic	Side effects	Reference
Antibacterial		
(a) Penicillin G	Muscle spasm, nausea, vomiting, skin rash	Hitchings et al. (2015)
(b) Amoxicillin	Nausea, diarrhea, stomach pain, headache, rash	Gillies et al. (2015)
(c) Cephalexin	Dizziness, abdominal pain, joint pain, itching, diarrhea	Haberfeld (2009)
Anti-tumor		
(a) Doxorubicin	Darkening of skin and nails, puffy eyelid, eye redness, weakness, loss of appetite	Chaterjee et al. (2010)
(b) Bleomycin	Poor appetite and weight loss, phlebitis, pneumonitis, pulmonary fibrosis	Huls and Ten Bokkel Huinink (2012)
(c) Mitomycin	Pale skin, unusual bruising or bleeding, irritability, bloody diarrhea, rapid weight gain, no urinating	Charpentier et al. (2011)
Anti-fungal		
(a) Griseofulvin	Heart burn, numbness or tingling in hands or feet, stomach pain, rash	Harris (1976)
(b) Micafungin	Indigestion, constipation, trouble sleeping	Carver (2004)
(c) Nystatin	Mouth irritation, hives, skin irritation	Carver (2004)
Antiprotozoal		
Daunorubicin	Temporary hair loss, reddening within 1–2 days, mild itching, irregular heartbeat	Fornari et al. (1994)

cephalexin, etc. (Gould 2016; Foster and Raoult 1974). The side effects of various antibiotics are displayed in Table 10.4.

10.10.2.3 Antiviral Agents

As antibiotics are not effective for viral infections, antivirals are used in which it is a class of medicine, mainly used to prevent viral infections like flu, warts, cold, etc. It inhibits viral reproduction into the host and encourages body immune system to fight against viral contaminations. There are some classifications among the drugs of antivirals, specific to different types of infections such as abacavir used for HIV, amantadine used for influenza, oseltamivir, tamiflu, etc. (Rossignol 2014). The side effects of various antiviral medicines are displayed in Table 10.5.

10.11 Nanoparticles

Nanoparticles are a type of particle enclosed within the interfacial layer, ranging within 1–100 nm. Interfacial layer is a basic component of nanoscale, containing ions and organic and inorganic molecules. This inorganic nanoparticles when coated with organic fragments is known as stabilizer, surface ligand, etc. (Batista Carlos et al. 2015). The first basic researches started during the period of 1970–1980 in the USA (Granqvist et al. 1976), and in Japan, it was first studied during an ERATO project, known as ultrafine particles (UFP) (Hayashi et al. 1997).

10.11.1 Characterization

Characterization defines the physical and chemical components present in NPs. This characterization is done with the various motives like in nanotoxicology studies, exposure assessment, which shows their different nanomaterial's toxicity levels

Table 10.5 Side effects of antivirals

Virus	Anti-viral agents	Side effects	Reference
Herpes virus	Vidarabine	Burning, stinging, pain, irritation, itching, redness	Rossi (2013)
Herpes simplex	Acyclovir	Nausea, vomiting, diarrhea, headache, abdominal pain	Rossi (2013)
Retro virus (HIV)	Ritonavir	Diarrhea, stomach pain, dizziness, loss of appetite	Hayward (2017)
Influenza A	Amantadine	Dry mouth, insomnia, constipation	Singhal and Rahman (2002)
Influenza B	Relenza	Ear, nose, throat infection, nasal irritation, vomiting	Hayden (2001)
Hepatitis B and C	Interferon	Trouble sleeping, fever, nausea, weakness	Bhatti and Berenson (2007)
HCV, HSV	Ribavirin	Muscle pain, stomach pain, headache	Alvarez et al. (2006)

and constructing process control which is a fusion of control engineering and chemical engineering. These things can be estimated by the techniques of microscopy, spectroscopy, and particle counters (Hassellöv et al. 2008; Tiede et al. 2008). But many of the processes are unable to calculate the unfavorable effects on less concentrated nanomaterials. Electron microscopy and scanning probe microscopy are not that much efficient to examine nanomaterials because of their small size in visible light. Spectroscopy technique is used to calculate the concentration and morphological traits of nanoparticles by electromagnetic radiation, such as X-rays and UV rays. The chromatography, centrifugation, and filtration methods are used to separate the different sizes of nanoparticles for characterization. For some specific approaches, nanoparticles can be characterized in complicated matrices such as soil, water, food, polymers, blood, etc. (Linsinger et al. 2011).

10.11.2 Functionalization

Functionalization defines the polymers present over the nanoparticles. The stability, tragedy, physical, and chemical characterization depend on the coating over the nanoparticles, for example, coating of red blood cell can degrade the immune system. Nanoparticles coated with polymers are highly stable. If the coating will be polar, then the nanoparticles will be highly soluble in water. The coating that is highly activated produces non-specific binding. But the hydroxyl or methoxy end group that attaches to polyethylene glycol prevents non-specific binding (Prime and Whitesides 1991; Liu et al. 2010). Nanoparticles can attach to biotic components also, but it will only react on those specific organelles and the locomotion of some proteins and RNA for where it is actually tagged (Suzuki et al. 2007). Nanoparticles except monovalents contribute many selected groups. It can arrange the receptors in a gathered form, resulting in the signals that detect the cellular path getting charged and more attached. So the particular groups, monoclonal antibodies, aptamers, or peptides, must be attached sequentially and in a restricted number with the nanoparticles.

10.12 Nanotoxicology

Nanotoxicology, an important part of toxicology, defines the experiments and studies about the toxicity of respective nanoparticles (Buzea et al. 2007). But it is not mandatory to have toxic effects in all nanoparticles. Nanotoxicology experiments and analysis also explain the maximum levels of specific nanoparticles in which they will not exhibit any toxic effects or any kind of negative effects on environment and mankind (Mahmoudi et al. 2012).

10.12.1 Mechanisms of Toxicity

10.12.1.1 Oxidative Stress

Small-sized nanoparticles can have a large volume as well as a broad exterior part, helping them to actively participate in chemical and biological events. It causes the high production of reactive oxygen species (ROS) and free radicals (Jaeger et al. 2012; Ng et al. 2013). The rate of production varies on nanoparticles, such as carbon nanotubes, nanoparticle metal oxides, etc. The production of ROS and free radical can be considered as the preliminary method of nanoparticle toxicity. The greater yield of ROS causes intracellular effects on proteins, lipids, and DNA followed by cardiovascular diseases and neurological disorders (Turrens 2003). The overdose of nanoparticles can promote highly oxidative stress. But for ethical barriers, it is difficult to execute any experiment in in vivo mammalian model organisms. *Drosophila* can be the efficient model organism to study oxidative stress. After ingesting 5 nm-, 15 nm-, 40 nm-, and 80 nm-sized AuNPs, the intracellular ROS level can be measured from the 2,7-dichlorofluorescein diacetate (DCF-DA)-dyed fly's homogenate. AuNPs might be the cause of increasing production of ROS. But the various-sized AuNPs didn't affect the production of ROS. It concludes that the total exterior surface is not the major measuring factor in promoting oxidative stress (Vecchio et al. 2012). On the condition of introducing 10 and 100 mg/mL of indefinite silica nanoparticles to *D. melanogaster*, causing an increase in oxidative stress depends on time and concentration.

10.12.1.2 Cytotoxicity

Due to the negative effects of NPs, the sustainability of cells is driven by the state and naked surface of cell membrane. In case of metallic nanoparticles such as NPs, the cells in copper oxide provided 60% unfeasibility in them. An electrostatic interest attracted the positive charged metallic ions toward the cell membrane. It coats the membrane and inhibits the ability of producing basic needs such as fuels and wastes (Seabra and Durán 2015). The toxic effects on specific cells damage their mitochondria and promote oxidative stress resulting in cell death (apoptosis).

10.12.1.3 Genotoxicity

Metallic oxide nanoparticles such as copper oxide, uraninite, and cobalt oxide exhibit genetic effects. This effect on DNA causes genetic disorders that can be detected in future generations, e.g., cancer. Nowadays, it is an important study in scientific investigation. Since *Drosophila* is an excellent model organism in genetics, a well-known genome sequence, and has identical traits with human, it is used to study the interaction between nanoparticles and the genome of specific organism. The introduction of 15 nm sodium citrate-capped AuNPs to *Drosophila* affects the DNA fragments present in gastrointestinal tissue. Chronic genotoxicity can occur in future generations due to genetic disorders in germline cells by the AuNPs. As the

small particles can influx easily into the organisms, the larger particles, 40 and 80 nm of AuNPs, are less genotoxic than small particles having 5 and 15 nm size (Vecchio et al. 2012). AgNPs and CdSe-ZnS QDs are also responsible for genotoxicity. The genotoxicity can be estimated by SMART (somatic mutation and recombination test). The characterization of nanoparticles defines the reproducibility of toxicology and the mode of action of toxicity of nanoparticles (Powers et al. 2006). The properties of nanoparticles such as size distribution and agglomeration state vary on the components of toxicological studies. Among the common toxic researches in nanotoxicology, the probable toxin characteristic is challenging, but their biological methods are not yet well known. In microscopy methods, only the electron microscopy and atomic force microscopy allow to examine the nanoparticles. But, basically a specific characterization of physical (shape description, size, total quantity, vectors that are attached) and chemical components is needed to study the nanotoxicology. And this characterization should be examined on biological moist environment before its exposure to living organism (Powers et al. 2006).

10.12.2 Factors Affecting Toxicity

The physical and chemical factors affect toxicity. The size of the nanoparticles mainly defines the level of toxicity. But besides this, chemical composition, shape, surface structure, surface charge, aggregation, and solubility are also responsible for the toxic effect (Nel et al. 2006). The functional groups explaining the chemical reactions of molecules also affect toxicity. If the level of toxicity increases, then the exposure of this nanoparticle may be harmful for mankind.

10.12.2.1 Composition

Metal-Based NPs

Metal-based NPs means basically synthesized NPs, having the functions as semiconductors, thermoelectric materials, used in drug delivery mechanism. Various studies have been done on NPs, such as their small exterior region with comparison to their volume, concluding an adverse effect on biological environment (Schrand et al. 2010). But still, trials are in progress to find out such nanoparticles having toxic effects that causes genetic disorders, evade cell sustainability, necrosis, etc. after their exposure.

Carbon-Based NPs

In 2013, nanotoxicology studies were done with the carbon nanotubes, resulting in minor lung trouble. But it was observed that the introduction of nanoparticles is needed for a long time to conclude results according to pathology. But some experiments of fullerenes proved C60 as a non-toxic carbon-based nanoparticles.

Size

Size also defers toxicity. The large-sized nanoparticles can be less toxic of their small particles. The small particles can influx independently everywhere in an organism causing more toxicity.

Dispersion State

The nanoparticles can agglomerate or aggregate in environmental or biological fluid. Agglomeration and aggregation denote loosely and tightly bounded particles, respectively. Due to the excessive environmental ionic stability, the nanoparticles get agglomerated. It protects the counter-attractions, occurring due to nanoparticle alterations. But many nanoparticles may get agglomerated in the environment or on the host body, so it is necessary to study if agglomeration affects toxicity or not.

10.13 Conclusion

In *D. melanogaster*, while other nanoparticles display scanty effective reaction, AuNPs show an induced toxicity by decreasing fertility and life span. It was manifested by the presence of DNA fragmentation in the gastrointestinal tissue. AuNP on the basis of its size influences the surface chemistry. It also effects the localization, intracellular fate, and toxicological pathways in vivo. So, nano-safety is strongly required which is being increasingly exploited in commercialization and novel application. AgNPs are also being obtained from bio-reduction of silver nitrate. They are removed by using olive, fig, and mulberry leaf extracts. Silver nanoparticles have been characterized for using UV rays, FT-IR, and SEM analysis, which shows better stabilization (Armstrong et al. 2013). In *D. melanogaster*, the larvae, pupae, and adult mortality gets reduced by olive, mulberry, and fig AgNPs. A more detailed research in the need of the hour regarding studies on translocation and uptake at cellular and molecular level.

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Understanding the Biological Activities of Nanoparticles Using Murine Models

11

Subhaswaraj Pattnaik and Busi Siddhardha

Abstract

The advent of nanotechnological interventions in the biomedical and pharmaceutical sectors has revolutionized the current therapeutic strategies by significantly complementing the conventional approaches. Deciphering the widespread biomedical potential of engineered nanomaterials, it is highly important to develop potential model systems. Among the different model systems used to study the biological evaluations of nanomaterials, in vivo model systems gained considerable attention. Among the various in vivo model systems, exploitation of murine models including experimental rats and mice is frequently being used owing to their phylogenetic relatedness to human system, their ability to decipher the biodistribution and bioavailability profile of administered drug candidates, and their ability to determine the different physiological and biochemical responses following the therapeutic administration. Though the limitations such as ethical considerations and other technical issues are frequently being questioned on the use of the animal models in scientific research, the use of murine models remains highly essential in finding the scientific purposes as promising alternative model systems are not available in the current scenario. In this context, murine models are being exploited to decipher the extensive biomedical applications such as antimicrobial, anti-infectives, anti-biofilm, anticancer, wound healing, radioprotective, and anti-diabetic potential of engineered nanomaterials. The nano-based platforms not only provided the widespread biomedical applications but also complemented the therapeutic efficacy of antibiotics and other drug candidates as evidenced from the in vivo studies. This chapter summarizes the advent of nanotechnological platforms in the field of biomedicines thereby improving the therapeutic efficacy of antibiotics and other drug

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candidates. The use of experimental murine models in understanding the biomedical potential of the engineered nanomaterials is also described in this chapter. This chapter will provide an in-depth understanding of utilizing appropriate model systems to decipher the biological properties of various nanomaterials and the drug-encapsulated nanomaterials.

Keywords
Nanotechnology · Murine model · Antimicrobials · Anti-infectives · Anticancer · Wound healing · Radioprotection

11.1 Introduction

11.1.1 Conventional Therapeutics and Limitations

No doubt, the invention of antibiotics and antimicrobial therapeutics in the twentieth century has revolutionized the biomedical sectors by critically avoiding the potential risks associated with conventional biomedical practices including complicated invasive processes (Ashkenazi 2013). However, the irrational and indiscriminate uses of these antibiotics lead to the development of antibiotic resistance, making it difficult to treat life-threatening infections in the twenty-first century (Chen et al. 2014; Prestinaci et al. 2015). The incidence of antimicrobial resistance not only provided serious global health issues but also contributed a significant impact on global economy in the developing countries as well as developed countries (Rather et al. 2017). In this context, it is important to quest for novel strategies which could not only bypass the resistance profile but also improve the therapeutic index of the administered drugs (Frieri et al. 2017).

11.1.2 Emergence of Nanotechnology

The nanoscience and nanotechnology are the study and application of extremely small things in multidisciplinary fields including material science, chemistry, biology, physics, and engineering. The widespread involvement of nanotechnology in the field of biology has given new dimension to the nanoscience platforms in the form of nanobiotechnology which showed promising applications in agriculture, bioremediation, pharmaceutical sectors, biomedicines, food industries, and biomedical engineering (Mostafavi et al. 2019). The promising applications of nanotechnology could be attributed to the advantageous features such as their physicochemical properties including high surface area to volume ratio, tunable size, ease in surface modification, higher reactivity, slow release efficacy, high drug payload, and high therapeutic index (Hussain et al. 2016; Hamdan et al. 2017). In particular, the nano-based platforms are frequently being used as delivery vehicle for the sustained delivery of therapeutic drug moieties, antibiotics, phytochemicals,

peptides, and genes at the target sites bypassing the different cellular barriers suggesting their enhanced therapeutic index as compared to their bulk counterparts (Reddy and Couvreur 2011).

The advent of nanotechnological platforms being used as promising drug delivery systems could be promising in the development of next-generation therapeutics owing to their ability to enhance the therapeutic index, drug release efficacy, biocompatibility, bioavailability, and long-term therapeutic actions with improved stability of the loaded drug moieties (Marta et al. 2016). The advanced and unique characteristics of the synthesized nanomaterials, used as tools for widespread application in biomedical and pharmaceutical sectors, could be attributed to their typical small size. The nano-sized materials have the inherent potential to bypass the cellular and sub-cellular barriers and interact closely with the important cellular and sub-cellular components including DNA, proteins, and lipid-containing cell membrane thereby modulating the physiological process in a sophisticated and controlled way at the cellular level (Aggarwal et al. 2009). The metal-based nanomaterials as well as polymeric nanoparticles alone or functionalized with different chemical moieties or antibiotics showed promising attributes as next-generation nanotherapeutics in the fight against microbial infections and related health issues (Hemeg 2017).

11.1.3 Applications of Nanotechnology in Biomedicines

The recent concept of nanomedicines has revolutionized the current understanding of conventional therapeutics and diagnostic approaches and thus could be considered as next-generation therapeutics in healthcare settings. The undue potential and novel avenues provided by the development of nanomedicine could provide new dimensions for efficient diagnosis and treatment of diseases (Donahue et al. 2019). The unique physicochemical properties, versatility in design and synthesis, and widespread functional importance have made the nanotechnological interventions as an attractive and alternative therapeutic choice for biomedical applications (Sakhtianchi et al. 2013). The multifaceted platforms of nanomedicines enable their widespread biomedical applications ranging from biosensing, bioimaging, early molecular diagnostics, and localized drug delivery at the target sites (Fig. 11.1) (Sankar et al. 2013).

The advent of nanotechnological therapeutic approaches not only provides wide spectrum biomedical applications but also essentially complements the conventional therapeutics by critically enhancing the bioefficacy of the traditional drug moieties (Subhaswaraj et al. 2019). The nanotechnological interventions not only provided widespread antimicrobial properties but also combated the microbial drug resistance profile thereby providing an aided arsenal in the fight against chronic microbial infections and related health diseases (Baptista et al. 2018; Baranwal et al. 2018). The nanotechnological interventions provide novel avenues in increasing the therapeutic efficacy of poorly soluble drugs, in maintaining the stability of administered therapeutic drugs, and in modulating the circulation and tissue distribution of the encapsulated drugs (Bertrand et al. 2014; Subhaswaraj et al. 2018). The key aspect of any therapeutic drugs is the stability profile after administered

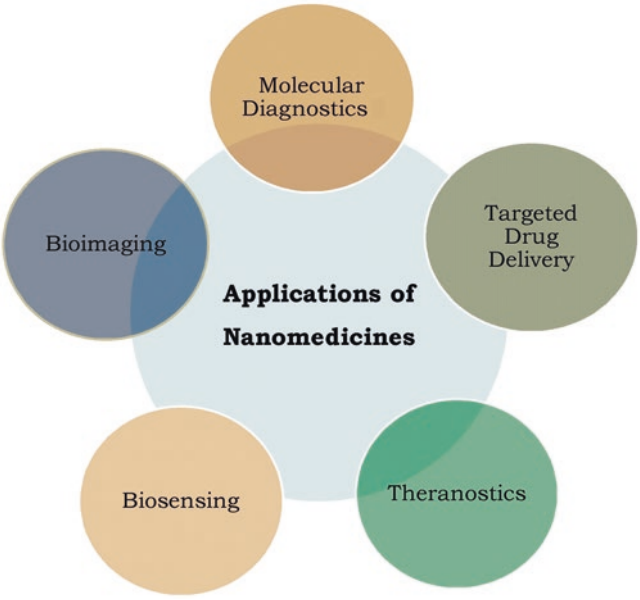

Fig. 11.1 Schematic overview of widespread biomedical applications of nanomedicines

into the biological system. The therapeutic efficacy fails when the administered drugs subjected for early degradation before being reached at the target sites. In this context, the limitations of early degradation of therapeutic drugs and their undesirable interactions could be avoided by providing novel nano-based platforms by specifically tuning the physicochemical characteristics of the encapsulated drug candidates (Zaidi et al. 2017).

11.1.4 Understanding the Mode of Action of Nanoparticles Using Model System

The exploitation of animals in scientific research and development of novel antibiotics and therapeutic drugs is an age-old process, and the involvement of animals from invertebrates to vertebrates remains an interesting buzz in the society. The use of animal models in particular the exploitation of mammalian models in developing novel therapeutics against various lethal diseases and disorders is due to the remarkable anatomical, physiological, and phylogenetic relatedness with the humans. The employment of different mammalian models owing to their greater relatedness with humans has given new dimensions to the scientific societies to discover the biological mechanisms associated with the disease progression and thus prompted to develop novel therapeutic strategies in animal models before being applied to humans (Barre-Sinoussi and Montagutelli 2015). In the development of nanomedicines, it is

important to understand the interactions of the nanomaterials with the cellular and sub-cellular components and their effect on modulating the cellular and metabolic processes. The important physiological parameters such as cellular uptake, cellular retention, biocompatibility, biodistribution profile, and exocytosis process following the uptake and toxicological aspects of the administered nanomaterials are equally important in design and development of novel nanomaterials for biomedical applications (Sakhtianchi et al. 2013).

In understanding the interactions between the nanomaterials and the biological systems, it is also important to understand the interactions of the nanomaterials with the different primary biological barriers such as skin epithelial barrier, gastrointestinal tract (GIT), and the air-blood lung barriers. Apart from these primary biological barriers, blood-brain barrier, reproductive system barrier, and circulation barrier also interact with various nanomaterials. The important function of these biological barriers is to act as primary defense system in checking the access of nanomaterials to the cellular and sub-cellular components. In this context, it is highly important to design and develop novel nanomaterials such that it could bypass these biological barriers for efficient biological actions. In this regard, the development of quantitative structure activity relationships (QSARs) as well as exploitation of promising model systems in sequestering the nano-bio interactions could provide new dimensions to the current therapeutic approaches (Meng et al. 2018).

Among the different biological barriers, the intestinal epithelial barrier proved to be instrumental in providing a check point to the oral delivery of low-permeable drug moieties. In this context, it is important to develop promising nanocarriers for the oral delivery of low-permeable drugs bypassing the intestinal epithelial barriers. The design and development of such nanocarriers should be based on their ability to follow three sequential steps including endocytosis (clathrin-mediated endocytosis/lipid raft mediated endocytosis/macropinocytosis) at the apical side, followed by transport through the cytoplasm and finally exocytosis at the basolateral side (Fan et al. 2016). In the process of understanding the abovementioned physiological processes and the effect on metabolic pathways when treated with different nanomaterials, the use of appropriate in vivo model systems is proved to be highly influential owing to their physiological and phylogenetic relatedness with human physiology.

11.2 In Vivo Model Systems to Study Biological Activities of Nanoparticles

The in vitro models are regularly used for the assessment of biological activities of different engineered nanomaterials owing to their unique features including simplicity, reliability, and cost-effectiveness. However, in vitro results could not provide reliable characteristics such as biodistribution profile, effective clearance pathways, and systemic toxicity. In this context, in vivo models could provide new dimensions in the assessment of biological actions of the nanomaterials in the biological system (Kumar et al. 2017). Despite the continuous eyebrows being raised due to ethical considerations and other limitations associated with the use of animal

models, the use of an appropriate model is essential for understanding the pathophysiology of human diseases as well as evaluating the biological significance of drugs used in in vitro conditions. The involvement of an appropriate animal model is prerequisite to establish the in vitro findings into clinical settings followed by human consumptions by critically enduring the physiological relationships during the drug administrations, their metabolism, and the mechanisms of action (Hajishengallis et al. 2015). In the assessment of biological activities of different nanomaterials, both invertebrate as well as vertebrate models are used as in vivo model systems. Among the invertebrate model systems, *Caenorhabditis elegans* (nematode model) and *D. melanogaster* (insect model) gained considerable attention in determining the biological activities of nanoparticles including the systemic toxicity profile of administered nanomaterials. Meanwhile, zebrafish (*Danio rerio*) and mammalian models including mice and rats showed immense potential in depicting the mechanism of biological actions (Fig. 11.2).

11.2.1 Invertebrate Model

Among the different invertebrate models used, the nematode model, *C. elegans*, gained considerable attention in depicting the pathophysiology of various diseases

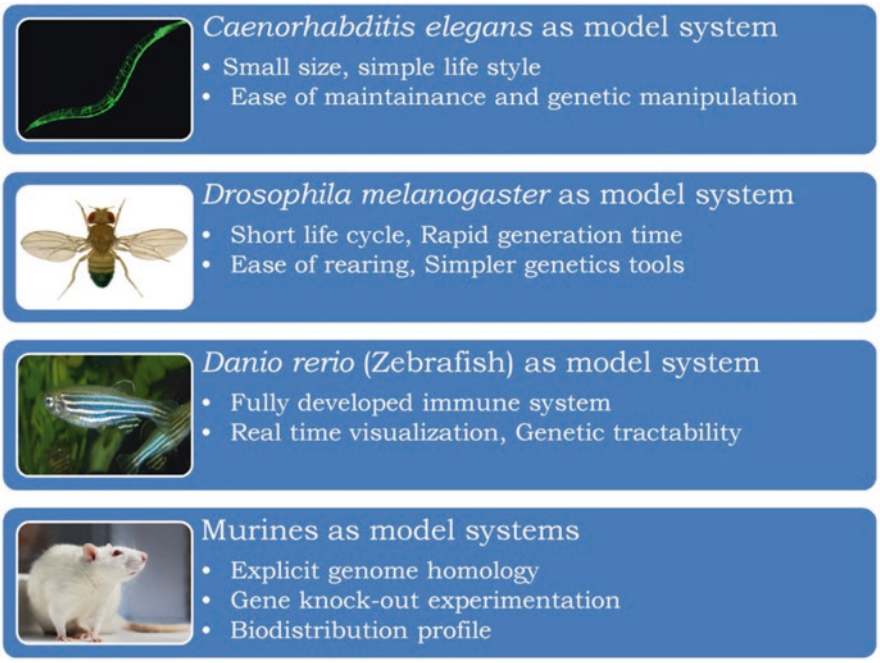

Fig. 11.2 A schematic overview of different invertebrate and vertebrate model systems exploited for understanding the biological activities of nanoparticles

and microbial infections as well as in determining the mechanism of various drug moieties and nanomaterials. The unique characteristics including small size, simple life style, cost-effectiveness, ease of maintenance, invariant developmental trajectory, conserved and well-annotated genome, and ease in genetic manipulation provide the undue importance of *C. elegans* as a convenient model (Gonzalez-Moragas et al. 2017; Hu et al. 2018). Apart from *C. elegans*, *D. melanogaster* is also considered to be influential as promising invertebrate model system in depicting the various biological functions such as tissue regeneration and developmental genetics. The characteristic attributes such as short life cycle, rapid generation time, large progeny profile, ease of rearing, cost-effectiveness, maintenance, and simpler genetics tools are considered as influential in the candidature of *D. melanogaster* as promising model organism (Markow 2015; Tolwinski 2017).

11.2.2 Vertebrate Model

The problems and limitations associated with the invertebrate model systems could be complemented with the use of vertebrate model systems which are phylogenetically closer to human beings. In this context, in the last few years, zebrafish (*D. rerio*) became the household name as promising model system in depicting the pathophysiological processes during chronic microbial infections and life-threatening diseases. The interesting attributes such as close homology with human genome, cost-effective experimentation, transparency of embryo, real-time visualization, fully developed immune system, large population size for experimentation, and genetic tractability proved to be influential in establishing zebrafish as promising model system (Fako and Furgeson 2009; Fehr et al. 2015; Chakraborty et al. 2016). Owing to the characteristic attributes of zebrafish as promising model system, zebrafish could also be used for the development of novel therapeutics and nano-based therapeutics against microbial infections as well as different life-threatening diseases (Lorenz et al. 2016). The exploitation of zebrafish could also be directed toward evaluating the toxicological aspects of engineered nanomaterials (Dai et al. 2014). Though zebrafish is considered to be an alternative model system, the use of mammalian models especially murine models remains the most extensively used model systems owing to their more relatedness with human beings with explicit genome homology. The use of murine models mice is observed to be appropriate model system in understanding the pathophysiology of disease progression and the metabolic responses of administered drugs and nanomedicines inside the biological system and in deciphering the toxicity profile of engineered nanomaterials. The unique advantage of using murine model is to understand the interactions of nanomaterials with the biological machinery, biodistribution profile of nanomaterials, and their explicit clearance from the cells as well as biological system (Yang et al. 2017).

11.3 Murine Model as Promising In Vivo Model Systems

Among the different vertebrate models used for depicting the mechanism of biological activities of therapeutic drugs or nanotechnology-based therapeutic approaches, murine models gained considerable interest. The reason behind the exploitation of murine models as promising model system is not only their evolutionary closeness to the human physiology but also due to their small size, ease of handling, inexpensiveness, and ease of gene knockout and overexpression profile (Radermacher and Haouzi 2013). One of the interesting aspects of murine model is their ability to mimic the physiological and metabolic processes in human beings thereby providing new horizons in deciphering the various pathophysiological conditions as well as development of novel therapeutic strategies (Almeida et al. 2011). Among the different murine models, because of their phylogenetic relatedness to humans, the ease of maintenance, ease of laboratory breeding, and availability of many inbred strains, house mouse, *Mus musculus*, has been exploited as promising model in understanding the human biology and related diseases. The exploitation of mouse as promising model system has genuinely provided novel avenues and powerful tools for understanding the pathophysiology of different diseases and development of new therapeutic approaches in combating the severity of disease progression (Perlman 2016).

11.3.1 Advantages and Implication of Murine Models

Though ethical considerations and other technical limitations are associated with the use of animal experimentation especially the use of mammalian models, the role of murine models remains quintessential in understanding the fundamental concept of disease progression and to discover novel therapeutic strategies in the treatment of various life-threatening diseases and infections (Vandamme 2014). The small animal models, including the murine models, have the unique advantage of phylogenetic relatedness to the human genome, ease of breeding and maintenance, availability of many inbred strains for experimentation, and affordable experimental setup. These unique characteristics provided new horizons to the use of small animal models in understanding different biological functions (Moran et al. 2016). In the development of cancer therapeutics, the exploitation of murine models has provided novel avenues in understanding the process of oncogenesis, molecular genetics in cancer cells, and metastasis and also significantly contributed in the development of novel therapeutic approaches (Kohnken et al. 2017). The extensive exploitation of different murine models remains the center of attention in the scientific community to understand the pathophysiological changes during microbial infections and diseased conditions and physiological alterations after the therapeutic administration. Mice are observed to be much more interesting as favorable models owing to the availability of various inbred, outbred, and transgenic strains as per the requirement of scientific purposes. In addition, mice are relatively easy to maintain and have a high fecundity in scientific findings as compared to rats (Stortz et al. 2017).

11.3.2 Diversity of Murine Models for Experimental Investigations

The murine models, in particular the mice model, are regularly employed in understanding the insight into the pathophysiological mechanisms during diseased conditions, to determine the efficacy of drug candidates when interacted with the biological system and to evaluate the responses on administration of therapeutic drug moieties (Justice and Dhillon 2016). The exploitation of murine models in biomedical research and preclinical drug evaluation has created a paradigm shift providing new dimensions to the current drug discovery programs (Zuberi and Lutz 2016). It is highly important to choose an appropriate model to study the highly complex interactions between the administered drugs with the biological systems thereby predicting the complexity and relatedness of the therapeutic options before being considered for human applications (Swearengen 2018).

11.4 Assessment of Biological Activities of Nanoparticles Using Murine Models

11.4.1 Antimicrobial Activity

Murine skin infection model in rats is established as a promising platform to evaluate the ability of different therapeutic drug moieties, antibiotics, or drug-conjugated nanomaterials in attenuating the bacterial burden from the infection site. In this context, biogenically synthesized silver nanoparticles (AgNPs) using *Acacia rigidula* plant as reducing and capping agent were experimentally investigated for their efficacy in minimizing the multidrug-resistant *P. aeruginosa* burden from the infection site (Escarega-Gonzalez et al. 2018). From historical perspectives, the advent of nanomaterials not only promotes intensive biomedical applications but also advocates their synergistic biomedical potential when administered in combination with therapeutic drug moieties, antibiotics, or native phytochemicals. In this context, allicin, bioactive phytochemical of garlic (*Allium sativum* L.), was used in combination with AgNPs to fight against chronic skin infection caused by methicillin-resistant *S. aureus* (MRSA). The combined therapeutic potential of allicin and AgNPs significantly decreased the MRSA burden in the skin infection site of mice model as compared to allicin and AgNPs administered individually (Sharifi-Rad et al. 2014). Apart from silver nanoparticles (AgNPs), zinc oxide nanoparticles (ZnO NPs) also showed widespread biomedical applications including antimicrobial activity, drug delivery, cancer therapy as well as diagnostic probes (Mishra et al. 2017). The biopolymer-capped ZnO NPs showed tremendous antibacterial activity against both Gram-negative and Gram-positive bacterial pathogens by altering the membrane integrity, reducing the surface hydrophobicity properties and generation of reactive oxygen species (ROS) (Fig. 11.3) (Pati et al. 2014).

11.4.2 Anti-Infective Potential

The world is currently facing a serious global issue in the form of microbial drug resistance to the conventional antibiotics (Aderibigbe 2017). In the process of development of novel therapeutic strategies to tackle the problems associated with microbial drug resistance, the advent of non-drug antimicrobials in the form of metallic nanoparticles, polymeric nanoparticles, and carbon-based nanomaterials provided promising avenues (Rai et al. 2015; Tran et al. 2019). Nanoparticles have the inherent property to be functionalized with specific chemical moieties thereby modulating the pharmacokinetic profile. These pharmacokinetic modulations have provided the target recognition and increase the efficacy of the encapsulated anti-infective drug candidates (Colino et al. 2018). The drug resistance profile of pathogenic microorganisms are basically regulated by the formation of recalcitrant biofilm as well as the activation of efflux pumps, which have the inherent property of drug extrusion thereby decreasing the drug therapeutic efficacy. In recent times, metallic nanoparticles are considered as an arsenal against these efflux pumps thereby bypassing the drug resistance profile of the encapsulated drug moieties (Gupta et al. 2017).

The advent of nanomaterials in attenuating the health consequences of infectious diseases caused by pathogenic microorganisms gained considerable attention owing to their physicochemical characteristics. Antimicrobial peptides are known for their

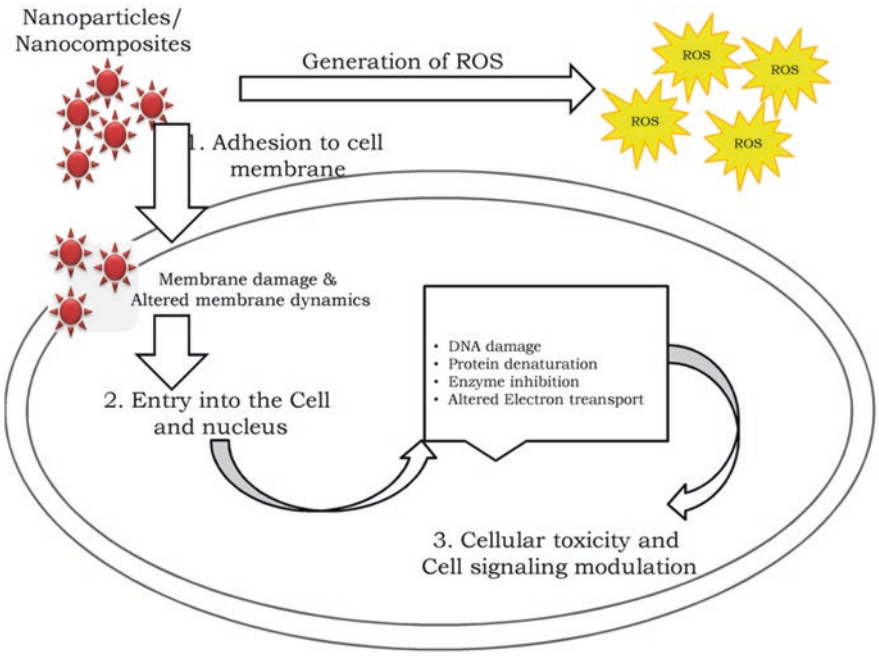

Fig. 11.3 Schematic representation of mechanism of action of antimicrobial potential of nanoparticles/nanocomposites

ability to combat multidrug-resistant microbial infections. However, when these amphiphilic antimicrobial peptides self-assembled to novel core-shell nanoparticles, they exhibited enhanced antimicrobial properties against *S. aureus* infection in mice owing to their increased therapeutic index, high drug payload, slow and sustained release profile, and their ability to cross the blood-brain barrier (Liu et al. 2009). Microbial infections are a common phenomenon found after the implantation of fixation devices for traumatic orthopedic injuries. These microbial infections in post-traumatic injuries remain a serious public concern owing to their resistance profile against wide class of antibiotics. In this context, exploitation of selenium nanoparticle (SeNPs) coatings as a potential anti-infective approach for orthopedic medical devices could be a promising alternative strategy to counteract the severity of microbial infections. SeNPs coatings significantly attenuated the methicillin-resistant *S. aureus* and *S. epidermidis* infections including orthopedic implant infections as evidenced from in vivo studies suggesting their widespread applications in counteracting the implant devices-related microbial infections (Tran et al. 2019).

11.4.3 Anti-Biofilm Activity

The emergence of multidrug resistance (MDR), extensively drug resistance (XDR), and pandrug resistance (PDR) phenomenon in pathogenic microorganisms especially ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) could be attributed to their inherent ability to form highly recalcitrant biofilm matrix (Magiorakos et al. 2012; Santajit and Indrawattana 2016). Biofilms are nothing but highly complex, self-made consortium of heterogenous microbial communities made up of extracellular substances including polysaccharides, proteins, lipid moieties, and nucleic acids. The biofilm-mediated sessile life style of pathogenic microorganisms is considered as an arsenal against hostile environments including host immune system, nutrient limiting conditions, and administration of therapeutic drugs (Wu et al. 2014; Ramasamy and Lee 2016; Mira et al. 2017; Dos Santos Ramos et al. 2018). One of the interesting aspects of biofilm matrix is its ability to enhance the complexity of the therapeutic strategies against human chronic wound infections which become a serious public health issue (Kim 2016). In the fight against microbial infections and associated drug resistance problems, targeting the biofilm dynamics could be instrumental in the current drug discovery programs.

The advent of nanotechnology has provided a unique and multifaceted platform for the scientific community in developing novel therapeutic strategies to fight against microbial biofilms associated drug resistance and related health issues. The recent development in nanotechnology-based approaches could play pivotal role in controlling the highly persistent and biofilm mediated chronic wound infections. As per recent trends, metal and metal oxide nanoparticles especially AgNPs have gained considerable attention in the treatment of wound biofilm infections. The synthesized AgNPs exhibited prolific anti-biofilm potential by significantly modulating the bacterial membrane permeability, destabilizing the biofilm matrix in the

infection site by intermolecular forces and triggering the generation of reactive oxygen species (ROS) resulting in biofilm disruption followed by eradication of microbial infections (Kim 2016). Among the metal oxide nanoparticles, ZnO NPs received substantial attention owing to their widespread antimicrobial activities. Sudheesh Kumar et al. (2013) advocated the combination of ZnO NPs along with β-chitin hydrogel bandages for the treatment of skin wound infection in Sprague Dawley rat model. The composite bandage significantly improved the wound healing rate with enhanced blood clotting ability and platelet activation in the infection site by inhibiting the growth of pathogenic bacteria. The topical administration of starch-capped ZnO NPs significantly disrupted the biofilm formation in *S. aureus* and also controlled the biofilm-mediated skin infections by minimizing the bacterial load and inflammation process in the infection site in BALB/c mice model (Pati et al. 2014).

11.4.4 Anticancer Activity

Cancer is one of the most decorated and deadliest public health issues across the globe owing to its ability to cause high mortality and morbidity in both developed as well as developing countries. Cancer, a collection of related diseases, becomes the second leading cause of death in the USA, the most developed country in the world (Siegel et al. 2016). In recent years, the development of nanotechnology-based cancer therapeutic strategies characteristically complements the conventional cancer treatment by critically providing localized and targeted therapies without any side effects. In particular, gold nanoparticle-mediated hyperthermia shows promising anticancer properties in animal studies suggesting their widespread avenues in cancer therapeutics in the near future (Kennedy et al. 2011). The introduction of nanotechnology in the cancer treatment also provided complementary therapeutic efficacy against multidrug resistant cancer (Friberg and Nystrom 2016). Owing to the unique characteristics of AgNPs, they are extensively used for widespread biomedical applications. In this context, *Melia azedarach*-mediated synthesis of AgNPs was developed to evaluate the cytotoxic potential of the engineered nanomaterials against Dalton's ascites lymphoma (DAL) mice model. The biogenic AgNPs significantly increased the life span of DAL mice model by induction of apoptotic mechanism in a dose-dependent manner suggesting their efficacy in the treatment of cancer in near future (Sukirtha et al. 2012). Gold nanoparticles (AuNPs) are exploited as promising radiosensitizers which provided the functional sites for their interaction with the radiation therapy thereby optimizing the fate of radiation therapy against cancer cells (Cui et al. 2017; Her et al. 2017; Borran et al. 2018).

Among the different nanomaterials exploited for therapeutic purposes and systemic drug delivery, polymeric nanoparticles in particular chitosan-based nanocomposites grabbed considerable attention due to their unique physicochemical properties such as high drug payload, controlled delivery profile, long-term effect, and localized delivery (Pattnaik et al. 2018). A novel nanoformulation containing chitosan nanoparticles encapsulating epigallocatechin-3-gallate (Chit-nanoEGCG) was designed for the treatment of prostate cancer. On treatment with

Chit-nanoEGCG, a significant modulation in the tumorigenic factors as well as tumor-suppressing factors such as poly(ADP-ribose) polymerase cleavage, Bax protein (Bcl-2 associated X protein), antiapoptotic Bcl-2, and caspases was observed in mice model (Khan et al. 2014). In addition to chitosan-based nanomaterials, novel FDA-approved poly(lactic-co-glycolic acid) (PLGA)-based nanocomposites are being frequently used as promising anticancer therapeutics. In this regard, PLGA-curcumin nanocomposites were designed and evaluated for their anticancer effect on prostate cancer xenograft mice model. The PLGA-curcumin nanocomposites have provided significant anticancer effect by inducing apoptotic process by activating the cellular morphological changes and membrane damage. The novel nanocomposites also exhibited promising anti-tumor effect as evidenced from lower tumor volume in xenograft mice model without any side effects (Yallapu et al. 2010). In the process of improving the bioavailability and anticancer effect of bioactive curcumin, α-tocopherol polyethylene glycol 1000 succinate (TPGS)-functionalized mesoporous nanocarriers with bamboo charcoal nanoparticles (TPGS-BCNPs) was designed and developed. The novel nanocomposites critically improved the bioavailability of curcumin in cancer cells and could improve its cancer therapeutic potential (Xie et al. 2017).

11.4.5 Wound Healing Activity

Wound infections remain one of the important healthcare issues posing a serious clinical challenge owing to their ability to cause systemic infection, sepsis, and multiple organ dysfunction. This results in enhanced mortality and morbidity among the immunocompromised individuals as well as healthy individuals, if neglected (Leaper et al. 2015). The therapeutic approaches available for the treatment of chronic wound infections become more complex and complicated when the infection sites provide suitable platforms for the establishment of co-aggregating biofilm forming microbial communities (Omar et al. 2017). The recent developmental approaches in nanotechnology-based diagnostics and treatment offer novel avenues to combat the complexity of the normal wound healing process by controlling the cell type specificity as well as the pathophysiology of chronic wound infections (Fig. 11.4) (Hamdan et al. 2017). The design and development of novel nanocomposites are achieved by encapsulating therapeutically active phytochemicals or drug-like candidates in combination with various nanoplatforms for enhancing the bioefficacy of the conjugated moieties. In this context, highly bioactive phytochemical, curcumin was encapsulated into typical nanoplatforms for the efficient delivery of curcumin at the target sites. The curcumin-encapsulated nanoparticles significantly inhibited the growth of MRSA and enhanced the wound healing process in murine wound model system (Krausz et al. 2015).

In recent years, selenium nanoparticles (SeNPs) have received considerable attention in various fields owing to their unique physicochemical properties, bioavailability, good absorption capacity, high surface area, and low toxicity. In 2015, the isolated actinobacterial strain, *Streptomyces minutiscleroticus* M10A62, was

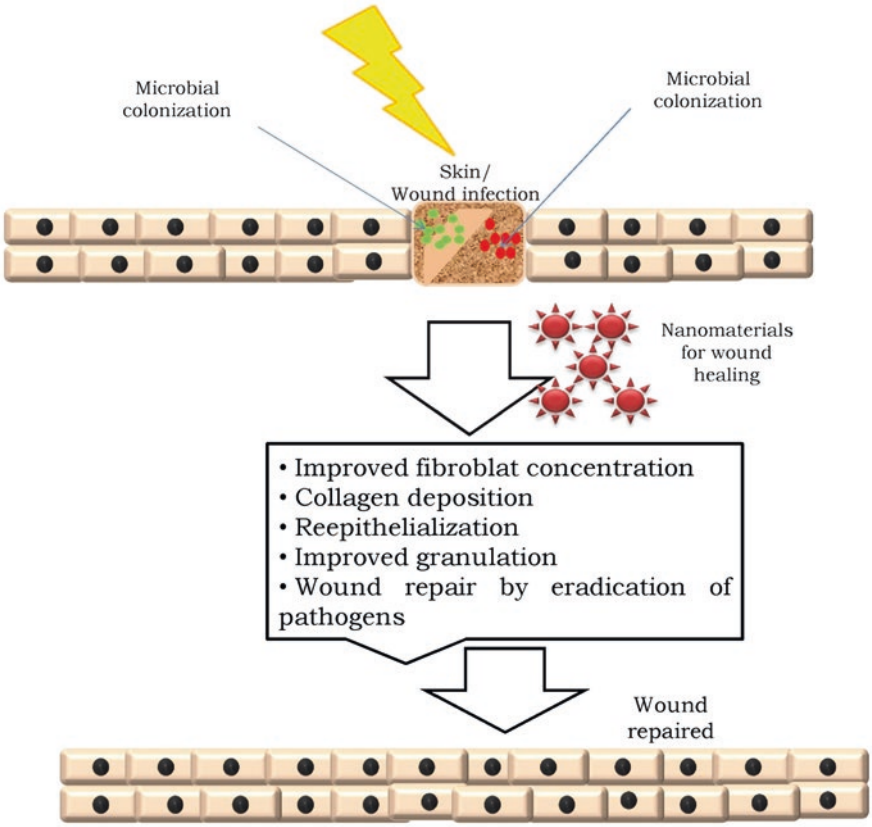

Fig. 11.4 A schematic overview of the mechanism of wound healing activity of engineered nanomaterials and specially designed nanocomposites

employed for the synthesis of SeNPs, which exhibited significant wound healing potential in the excision wound of Wistar rats as compared to the standard wound healing ointment. This result suggested the widespread avenues of using selenium based nanomaterials in the wound healing therapeutics or could be used in combination with standard drug moieties for improved and faster would healing (Ramya et al. 2015). Earlier, *Origanum vulgare*-engineered titanium dioxide nanoparticles (TiO_2 NPs) exhibited promising wound healing properties as evidenced from excision wound model in Wistar albino rats. The wound healing efficacy of topically administered TiO_2 NPs could be attributed to their bioavailability and enhanced fibroblast deposition in the wounded tissue sites (Sankar et al. 2014). In the process of nanomaterial-based wound healing applications, an antibody-targeted magnetic nanoparticle was designed to achieve an accelerated wound healing process without causing tissue injury due to highly resistant *S. aureus* infections (Kim et al. 2013).

The FDA-approved PLGA is widely considered as promising antibacterial and wound healing agent. The contribution of PLGA based nanocarriers in the targeted

delivery of poorly soluble wound healing agents also gained considerable attentions in recent years (Chereddy et al. 2016). In this context, novel biodegradable nanocomposites were designed (containing PLGA, collagen, and selected antimicrobials) and developed to enhance the wound healing activity in *S. aureus*- and *E. coli*-infected wound in rat model. The novel nanocomposites have the inherent property of sustained release profile suggesting a long-term effect in accelerating the wound healing process with improved fibroblast concentration, sparse inflammatory cells, and reepithelialized epidermis in the dermis and subcutis at the wounded site (Chen et al. 2012). In a similar experiment, fusidic acid-encapsulated PLGA ultrafine fibers also exhibited an accelerated wound healing process by sustained delivery of fusidic acid at the wounded site protecting the wounded tissues from reinfection by eradicating the *S. aureus* infections (Said et al. 2012).

In nanomaterial-based drug delivery system, micellar nanocomposites hold a special emphasis owing to their unique physicochemical properties, targeted drug delivery efficacy, and biodegradability. In this context, curcumin-loaded micelles (Cur–M) in combination with desirable wound dressing in situ gel-forming hydrogel system (Cur-M-H) were designed to evaluate their wound healing potential in linear incision as well as full thickness excision wound model. The unique nanoformulations significantly improved the wound healing process by increasing the collagen content, improved granulation, enhanced wound maturity, and increased cutaneous wound repair (Gong et al. 2013). The encapsulation of bioactive curcumin into desirable nanomaterials significantly improved the wound healing efficacy of curcumin owing to their slow and sustained release profile and enhanced bioavailability (Hussain et al. 2017). In recent years, nitric oxide (NO) has emerged as a warrior in the fight against wound infections. In this context, nitric oxide releasing nanoparticle technology was designed to improve the conventional wound healing process. The NO nanoparticles (NO NPs) significantly accelerated the wound healing process in mice model by critically inducing the fibroblast migration and collagen deposition in the wounded tissue site (Han et al. 2012).

11.4.6 Radioprotective Activity

The inadvertent exposure of living organisms to the ionizing radiations such as ultraviolet (UV) rays in general and X-rays and gamma rays in particular proved to be detrimental to public health. The continuous exposure of ionizing radiations tends to generate severe deleterious effects and more often irreversible cellular damage in the living organisms (Painuli and Kumar 2016). Protecting the living system from the radiation-induced health consequences, a variety of potent chemical and biological compounds were screened for their ability to reduce the risk to the normal tissues or those that facilitate the healing of radiation injury. Antioxidants, phytochemicals, cytoprotective agents, immunomodulators, etc. have been screened extensively for their radioprotective potential (Arora et al. 2005; Aprotosoaie et al. 2015). In recent years, the nanotechnology-based radioprotective measures are being considered as next generation therapeutics as the nanomaterials not only possess intrinsic

radioprotective properties but also stringently improved the radioprotective effect of the encapsulated therapeutic drugs (Mohamed et al. 2013; Xie et al. 2018).

Selenium nanoparticles (SeNPs) exhibited promising radioprotective effect against gamma radiation induced nephropathy in mice model by critically altering the serum creatinine, urea, β2-microglobulin, as well as in-built antioxidant enzymes (Karami et al. 2018). Recently, graphdiyne, a new emerging carbon network material, was employed for promising biological activities. Xie et al. (2019) developed bovine serum albumin (BSA)-modified graphdiyne nanoparticles (graphdiyne-BSA NPs) for the assessment of their radioprotective efficacy. From the results, it was evidenced that graphdiyne-BSA NPs significantly attenuated the radiation induced DNA damage as well as maintained the superoxide dismutase (SOD) and malondialdehyde (MDA) level in the mice model without any systemic toxicity (Xie et al. 2019). Other carbon-based nanomaterials such as water-soluble fullerenes, grapheme oxide, and carbon nanotubes also proved to be influential in exhibiting radioprotective effect (Krokosz et al. 2016). Vesna et al. (2016) advocated the efficacy of fullerenol-based nanoparticles in mitigating the radiation-induced lesions in the spleen, lungs, and intestinal tissues of rats when exposed to X-rays (Vesna et al. 2016). The unique surface regenerative property of cerium oxide nanoparticles (CeO_2 NPs) could be utilized for their ability to mitigate the acute radiation mediated lung injury in CBA/J mice model suggesting their efficacy in controlling the endogenous level of highly reactive oxygen species (Xu et al. 2016). The mitigation of radiation induced lesions by CeO_2 NPs could be attributed to their inherent ability to absorb ionizing radiations as well as their potential to neutralize the radiation-induced oxidative stress as evidenced from their ability to protect the germ cells from radiation mediated cell death in C57BL/6J mice (Das et al. 2018).

Chlorogenic acid-encapsulated chitosan nanoparticles showed significant antioxidant potential by mitigating the oxidative stress-inducing reactive oxygen species thereby suggesting their potential in combating radiation-induced health hazards (Nallamuthu et al. 2015). Tea polyphenols are known for their widespread biological activities. However, their poor bioavailability issues hinder their pharmacological potential. In this context, encapsulating the tea polyphenols into chitosan nanoparticles where BSA was used as matrix could be of promising aspect in mitigating radiation-induced hematological injuries as well as radiation-induced lesions in Swiss albino mice suggesting their ability to improve the radiotherapeutic efficacy of tea polyphenols (Kumar et al. 2016). The radioprotective efficacy of poorly bioavailable vascular endothelial growth factor (VEGF) could be improved by impregnating into chitosan nanoplatforms. The VEGF-chitosan nanocomposites significantly improved the radioprotective efficacy by critically improving the local microcirculation and mitigating the effect of radiation-induced skin damage (Yu et al. 2016).

The specially designed α-tocopherol polyethylene glycol 1000 succinate (TPGS)-functionalized mesoporous nanocarriers with bamboo charcoal nanoparticles (TPGS-BCNPs) not only improved the bioavailability of curcumin in cancer cells but also critically improved the radical scavenging potential of curcumin (Xie et al. 2017). No doubt melanin can protect the cells from oxidative stress by scavenging the highly reactive oxygen species. However, the radioprotective efficacy of melanin

could be improved by designing melanin nanoparticles (MNPs), which critically protected the cells from radiation induced DNA damage, restored the SOD level, and reduced the MDA level in the mice model (Rageh and El-Gebaly 2018). The *Withania somnifera* extract-mediated synthesis of gadolinium III oxide nanoparticles (WSGNC) was evaluated for their radiosensitizing potential. The engineered nanomaterials exhibited promising radiosensitizing properties thereby promoting the efficacy of radiation therapy against cancer cells (Abdallah et al. 2016).

11.4.7 Anti-Diabetic Activity

Through the development of high-throughput technological advancement as well as healthcare settings, the incidence of diabetes remains a global public health issue. Diabetes is a form of metabolic disorder characterized by different interlinking parameters and biochemical conditions such as hyperglycemia, altered carbohydrate, and lipid and protein metabolism (Shanker et al. 2017a). The nanotechnological intervention in the field of biomedicines has paved the way for their explicit role in combating diabetic conditions. In this context, *Psoralea corylifolia* seed extract-mediated synthesis of AgNPs was developed. The biogenically synthesized AgNPs exhibited promising anti-diabetic effect by characteristically inhibiting the protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signaling pathway (Shanker et al. 2017a, b). The anti-diabetic properties of biogenically synthesized AgNPs could also be attributed to their effect on non-enzymatic glycosylation of hemoglobin with reduced of blood glucose level in streptozotocin-induced diabetic rats (Prabhu et al. 2018). The anti-diabetic properties of *Solanum nigrum*- and *Punica granatum*-synthesized AgNPs could be attributed to their efficacy in modulating the dyslipidemic condition and also inhibiting the α-amylase and α-glucosidase activity in diabetic rats (Sengottaiyan et al. 2017; Saratale et al. 2018). The biogenically synthesized AuNPs showed promising anti-diabetic effect by improving the insulin resistance and blood glucose level in Wistar albino rats (Dhas et al. 2016). Earlier, *Gymnema sylvestre*-mediated AunPs also exhibited potential anti-hyperglycemic properties suggesting the anti-diabetic role of engineered AuNPs in alloxan-induced diabetic rats (Karthick et al. 2014). *Sambucus nigra* L. extract-functionalized AuNPs exhibited significant anti-hyperglycemia properties by mitigating the hepatic inflammation and oxidative stress conditions suggesting their potential role as promising adjuvants in diabetes therapeutics (Opris et al. 2017).

The anti-hyperglycemic potential of SeNPs also gained considerable attention in recent times owing to their ability to reduce the glucose-6-phosphatase activity, hepatic function markers, and low-density lipoprotein cholesterol (LDL-C) levels. On treatment with SeNPs, there is a marked increase in the levels of glucose-6-phosphate dehydrogenase and hexokinase activity, high-density lipoprotein cholesterol (HDL-C) levels, and liver glycogen levels suggesting their prolific anti-diabetic properties (Al-Quraishy et al. 2015). Owing to the widespread biomedical applications of ZnO NPs, biosynthesized ZnO NPs using *Hibiscus sabdariffa* leaf extract was employed to determine the anti-diabetic activity using mice model system. On

treatment with ZnO NPs, the blood glucose level was restored by modulating the expression of pro-inflammatory cytokines including tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and IL-1β. Besides, the expression of IL-4 and IL-10 was also normalized when treated with ZnO NPs suggesting their characteristic anti-diabetic properties (Bala et al. 2015). The anti-hyperglycemic potential of biogenically synthesized ZnO NPs from *Momordica charantia* extract was evaluated against streptozotocin-induced diabetes in Wistar rats (Shanker et al. 2017b). The anti-diabetic properties of ZnO NPs could be attributed to their ability to deliver zinc which is essential for the synthesis, storage, and secretion of insulin and structural integrity of insulin (Jiang et al. 2018).

Myricitrin, an antioxidant-based solid lipid nanoparticle, also exhibited promising anti-diabetic activities by critically modulating the hyperglycemia-related complications in mice model (Ahangarpour et al. 2018). A pH-sensitive polyurethane-alginate nanoparticle was developed which eventually controls the blood glucose level by critically modulating the release of insulin (Bhattacharyya et al. 2016). *Stevia rebaudiana* leaf extract was encapsulated into chitosan nanoparticles and evaluated for its anti-diabetic efficacy. The novel nanocomposites critically improved the serum levels of serum glutamic-oxaloacetic acid (SGOT), serum glutamic pyruvic transaminase (SGPT), reduced glutathione (GSH), catalase, and SOD thereby promoting the reduction in blood glucose level as compared to diabetic Wistar rats (Perumal et al. 2016).

11.5 Current Trends and Future Perspectives

The advent of nanotechnology in the biomedical sectors has revolutionized the current understanding of therapeutic strategies. The widespread biomedical applications especially antimicrobial potential of biogenic nanoparticles are considered to be influential as compared to their bulk counterparts or the conventional antimicrobial strategies. The advantages of nanomaterials in the treatment of microbial infections could be attributed to their insensitivity toward the drug resistance shown by pathogenic microorganisms. The unique properties and ease of modification approaches also significantly improved the therapeutic index of nanomaterials with a long-term effect. In addition, being small in size, the nanomaterials could bypass the biological barriers and could be impregnated deep into the cellular sites for effective therapeutics (Khan et al. 2016). The nanoparticles could also be attributed toward their efficacy in controlling the vector-borne diseases in infected mice models suggesting their widespread avenues. In this context, benznidazole nanoparticles (BNZ-nps) were developed to target the Chagas disease caused by *Trypanosoma cruzi*. The results suggested the effectiveness of engineered nanomaterials in increasing the survival rate of *T. cruzi*-infected mice and thereby controlling the Chagas disease (Scalise et al. 2016).

The nanotechnology-based platforms could also be used for enhancing the biological activities of old drug moieties suggesting their role in deciphering the undue potential of these repurposed drugs for various biological functions (Patra et al. 2018). The recent concept of antimicrobial photodynamic therapy (aPDT) also

proved to be influential in targeting recalcitrant microbial biofilms and associated drug resistance phenomenon. The combinatorial concept of aPDT along with a promising nanocarrier greatly improves the photodynamic inactivation of highly tolerant biofilms suggesting their widespread venue in the near future (Biel et al. 2011; Sharma et al. 2014). The advent of photothermal therapy and efflux pump inhibitors in combination with appropriate nanoplatforms could be influential in sequestering the widespread avenues of nanotechnology in biomedicines (Millenbaugh et al. 2015; Vyshnava et al. 2016).

11.6 Conclusion

The nanotechnology-based platforms have provided novel avenues in understanding the current therapeutic strategies. The engineered nanomaterials also characteristically improved the biological efficacy of various drug candidates as well as antibiotics. In understanding the extensive biomedical applications of different engineered nanomaterials, it is highly important to select an appropriate model system. In this context, murine models have provided the horizons to understand the physiological, biochemical, and metabolic processes during the therapeutic approaches and could also decipher the impregnable responses made after the administration of drug candidates. Though ethical consideration limits their extensive exploitation, the established murine models remain highly essential to understand the various biological functions as no promising alternative models are available. In this regard, it is important for the scientific community to quest for alternative model systems sequestering the mechanism of biological activities of engineered nanomaterials without affecting the information gained from murine models including the biodistribution profile, pathophysiological processes, and physiological responses.

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Insecticidal Activity of Nanoparticles and Mechanism of Action

12

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Abstract

The growth of population in the world and the requirement for food have urged the need to optimize the agriculture practices with minimal loss on fields. This can be achieved by the application of insecticides and pesticides. However, long-term application of these compounds has encountered serious environmental concerns of insecticide and pesticide resistance in plants and environmental deterioration. This has led to the ban of numerous deadly pesticides. However, this problem could be overcome with the development of various biological pest control agents. In recent years, nanotechnology has picked up prevalence at a fast pace in various field and disciplines with special mention in environmental and agricultural systems. In this regard, application of various nanoparticles has attracted many researchers worldwide to investigate and test their toxic potential against various insects and pests. Owing to the advantages, that is, affordability, availability, and easy synthesis, numerous inorganic and organic nanoparticles/composites, namely, titanium, gold, silver, silica, titanium dioxide, zinc oxide, iron and carbon, etc., have been successfully targeted against extensive range of noxious arthropods and agricultural pests and vectors. Therefore, the present chapter deals on different nanobased formulations employed against insects and pests, along with their mechanism of action. Based on many research reports,

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nanoparticles have been recognized as excellent candidates to combat insects and pests with their proven toxicity against mosquitoes and ticks. In addition, they are capable of exhibiting their toxicity at different stages of insects and pests. However, implementation of nanotechnology in agriculture, particularly in pest control, needs to be carefully evaluated to benefit the agricultural sector and the public health concerns of nanotoxicity.

Keywords
Nanoparticles · Insecticidal · Pesticidal · Agriculture · Environment · Mechanism

12.1 Introduction

Agriculture contributes to the crucial development toward the rise of sedentary human lifestyle. The production of crop for human consumption by agriculture began thousands of years ago. However, present-day agriculture faces huge loss in terms of crop and finance due to various biotic and abiotic factors. Biotic factors include pest invasion and resistance, whereas abiotic factors include water inadequacy or excess during growing season, extreme temperature changes, high or low irradiance and biotic stressors, and nutrient supply (Oerke 2006). Thus, the farmers had to compete with biotic factors such as plant pathogen (bacteria, virus, fungi, weeds, and chromista) and animal pathogens (mites, rodents, insects, nematodes, snails, and slugs) that are together specified as pests. Unlike abiotic factors, biotic factors are caused mostly due to anthropogenic activities of excess usage of synthetic pesticides and insecticides, due to which the crop is expected to gain resistance. Insects are the common creatures found in almost all the environment, occupying slightly more than 2/3rds of animal's space, globally. Insects feed all the types of plants, namely, medicinal plants, crop plants, weeds, and forest trees. They too exhibit the capability of infesting food products and stored grains in godowns, bins, packages, and huge storages leading to massive loss in food quality and investments (Rai and Ingle 2012). Wheat, maize, rice, barley, potatoes, coffee soybeans, and cotton are crops that face major loss due to pests and diseases (Oerke 2006).

In general, insects causing <5% damage are not categorized as pests. If the destruction is between 5% and 10%, they are termed as minor pests and with damage more than 10% are termed as dominant pests (Dhaliwal et al. 2010). According to Pimentel (2009), globally, an estimated loss of 14% was caused by insect pests and 13% loss due to plant pathogens and weeds, with a crop loss estimate of US$ 2000 billion per year. For example, the annual yield loss of potatoes ranges from 5 to 96% in France, 100% for cotton in Thailand, and 24 to 41% in Asia. Over the decades, global crop loss due to insect pest invasion varies with different crops. However, such losses are scarcer in perennial crops. Yield losses on apples and other stone fruits are much less compared with coffee yield loss in Brazil that ranged from 13 to 45% and 5% in Netherlands. However, most developing countries do not have accurate estimation of the loss caused by the invasion of insects and noninsect pests,

affecting main crops (Culliney 2014). The scarcity of crop loss quantifications and their causal analysis is mainly related to the difficulty of their evaluation.

Pest and disease attacks are quite common even during preharvest and postharvest (storage) stages, that greatly affects the crop yield along with its quality and quantity. Additionally, financial returns are also been threatened due to low production and quality that reflects in poor customer satisfaction. The most outstanding efforts taken during the last 20 years focused on the quantification of relative yield losses due to pests and diseases only by experiments and surveys. However, control or reduction of pest-associated crop losses will accelerate the agricultural production and allow us to march one step forward to accomplish the global Sustainable Development Goals (SDGs) to curb hunger, poverty, and malnutrition.

12.1.1 Emergence of Pesticides

In Ancient Roman times, sulfur was used to kill pests, and weeds were controlled with salts, ashes, and bitters. Honey/arsenic mixture was used in the 1600s to control ants. Farmers in the United States started to use certain chemicals for field-related posts such as sulfate, nicotine, sulfur, and calcium arsenate in the late 1800s. Different types of pest control agents are illustrated in Fig. 12.1. Colorado potato beetle was controlled by arsenic, and an impure form of copper, in the United States in 1867. During and after World War II, a quantum leap in the pesticide development had arisen that eventually led to the synthesis and production of numerous effective and cheap pesticides/insecticides (Mahmood et al. 2016). 2,4-D, Dieldrin, DDT, BHC, Aldrin, Endrin, and Chlordane were discovered in 1939. However, due

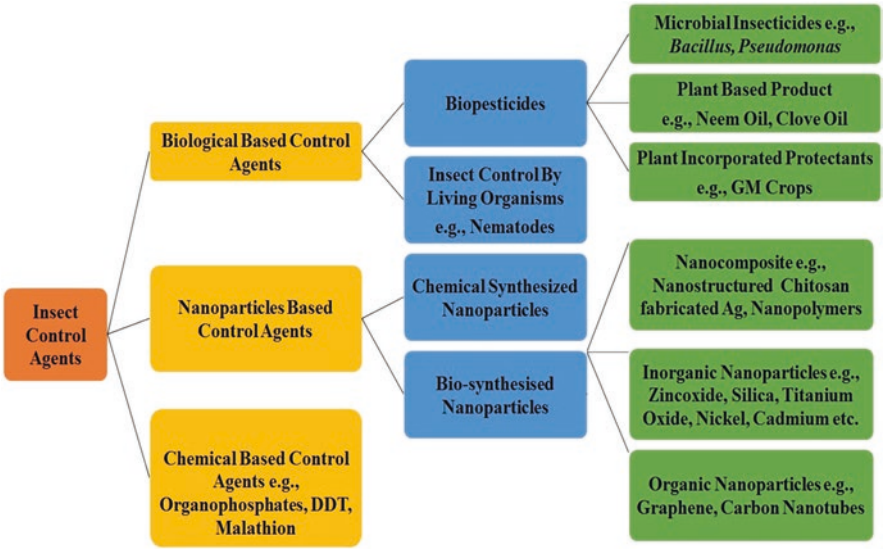

Fig. 12.1 Different types of pest control agents

to primitive application methods, most of the efforts were unsuccessful (Delaplane 2000). Despite this fact, usage of pesticides has reached its peak, worldwide in the year 1961 with 48,000 tons usage in Germany, 1.7 million tons in China, 24,000 tons in Poland, over 18,000 tons in The Great Britain, and 62,000 tons in Italy. However, in the year 1962, pesticide usage was sharply declined due to the public awareness of environmental hazards and health effects related to indiscriminate the use of chemical pesticides. This paved to a new area of "integrated pest management" (IPM) from late 1960 onwards.

12.1.2 Need for Pesticide

A pesticide is a general term which refers to herbicides, insecticides, rodenticides, fungicides, nematicides, molluscicides, and growth regulators. Pyrethroids, neonicitinoids organochlorines, carbamates, and organophosphates are the most common and widely used pesticides (Theerthagiri et al. 2017). These toxic chemical compounds are employed to kill/control/destroy rodents, weeds, fungi, insects, and other harmful pest populations, that challenge the crop production. Pesticides work primarily by attracting the pests, then seduce them, and finally destroy or mitigate them. According to Alavanja (2009), billions of kilograms of pesticides are used annually to reduce such yield losses. Over the past few decades, the practice of pesticides application has increased several folds. According to an estimate, the use of pesticides was found to be approximately 5.2 billion pounds globally per unit area/annum.

It is estimated that 90% of the pesticides will be lost during or after the application (Ghormade et al. 2011). In addition, chemical insecticides are often nonspecific and hence can affect nontarget organisms too. As a result, there is increased need to develop high-performance, sustainable, and cost-effective pesticides that are less harmful to the environment.

12.1.3 Effect of Conventional Pesticides/Insecticides

In India, pesticides are primarily used for cash crops such as cotton, paddy, and wheat to improve their production in terms of quality and quantity (Choudhary et al. 2018). Pesticides/insecticides are used not only in agricultural farmland but also in household as powders, sprays, and poisons to control cockroaches, fleas, ticks, mosquitoes, rats, and other pests and insects (Murugan et al. 2018). However unexpectedly, the risks combined with their usage have exceeded their beneficial effects. Due to continuous application, few traces of chemical pesticides are commonly detected in our food commodities, water, soil, and even in air (Murugan et al. 2017). On environmental degradation, the metabolites of the pesticides were reported to be equally harmful as the active ingredients of the pesticides. Owing to their ill effects, carcinogenicity, and roles in ozone depletion, many widely used pesticides has been banned in many countries (Rajendran and Sriranjini 2008). According to a report on 19 March 2019 by "Directorate of Plant Protection Quarantine & Storage", Govt. of India, and USEPA

(United States Environmental Protection Agency), various chemical pesticides such as aldrin, chlordane, endosulfan, lindane, DDT, DDD, etc., have been completely banned in India and throughout the world (Web sources 2019). In particular, many governments have prohibited the use of pesticides to protect stored products too. Due to continuous application, most of the pests/insects become resistant and worsen the pest management practices. Various resistance mechanisms include penetration resistance, altered target-site resistance, metabolic resistance, and behavioral resistance (Benelli 2018). Therefore, this has urged the worldwide researchers to develop new eco-friendly and sustainable options of insecticide for plant protection.

12.1.4 Nanobased Pesticide/Insecticide

A nanoparticle (NP) refers to ultrafine particle subclass with distinctive dimensions ranging from 1 to 100 nm, which seems to be uncommon with non-nanoscale particles of same chemical composition. Nanotechnology offers numerous applications in many fields such as medical, industrial, environmental, nanobiosystems, parasitology, among others. A wide number of nanomaterials, including carbon, metal oxides, metals, polymers, proteins, dendrimers, ceramics, semiconductor quantum dots (QDs), emulsions, lipids, and silicates, have been synthesized by chemical or biological means (Puoci et al. 2008). Physical characteristics such as, shape, size (irregular, rods, tubes, sphere), crystal phase (crystalline/amorphous), chemical configuration (e.g., carbon, metallic, organic, polymeric, inorganic), and surface-to-volume ratio are the vital parameters that define the outstanding characteristics of these nanomaterials and aids in determining their applications in various fields (Athanassiou et al. 2018).

Over the past decades, nanotechnology is a rapid growing and highly attractive research field among worldwide researchers. This field has shown remarkable potential for the use of nanomaterials towards food protection and crop. A variety of metal NPs, metal oxide NPs, and polymer-based nanocomposites have been developed for crop pest management. Different research attempts have been successfully reported on new approaches of constructing nanoscale materials, active insecticidal ingredients, formulation, and delivery which can be referenced as “nanopesticides” (Ragaei and Sabry 2014). The evolution of nanopesticides has been one of the most recent discoveries that address the application of nanomaterials for crop protection through nanotechnology. It includes broad research contribution that aids in the fundamental understanding of the formulation of the active ingredients into nanoemulsions, interaction between nanoscale materials and insects, and effective delivery options. Development of new formulations of nanopesticides employing nanomaterials as active insecticidal tool for delivery is collectively referred to as nanocarriers (Benelli et al. 2017). As agricultural nanotechnology develops, there will be a significant upsurge in the application prospective of the nanoparticles to provide a new generation of pesticides and other plant disease management options. Different nanobased insecticides along with their insecticidal effects are schematically shown in Fig. 12.2.

Many common economic advantages of nanobased insecticide formulations include (1) enhanced solubility of the insoluble insecticide components; (2) high

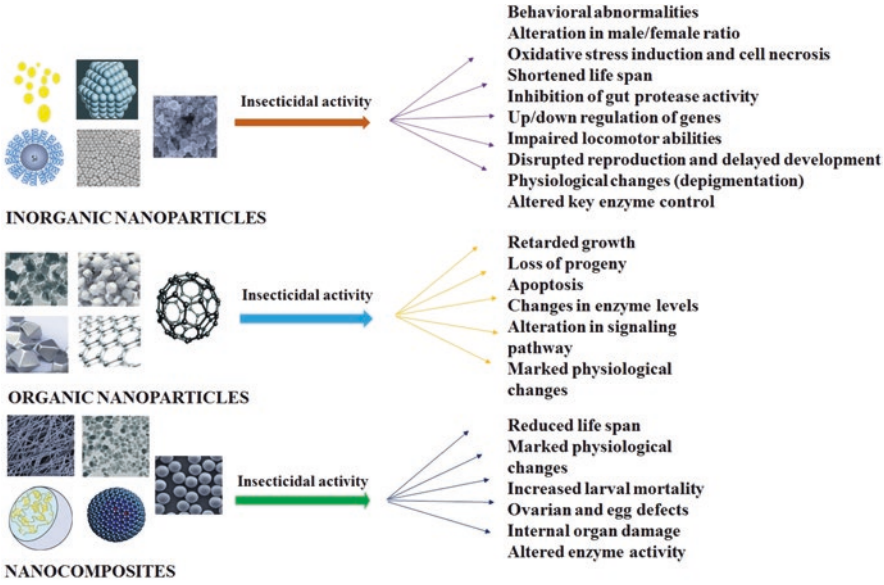

Fig. 12.2 Different nanobased insecticides along with their insecticidal effects

formulation strength; (3) active expulsion of toxic organic solvents than the conventional pesticides; (4) sustained release options; (5) improved resistance to degradation; (6) high mobility and enhanced insecticidal action, due to its small particle size; and (7) prolonged longevity, due to large surface area (Sasson et al. 2007). Additional nanocarrier benefits include increased activity efficacy and stability of the nanopesticides under extreme environmental conditions (UV and rain) and reduced toxicity and costs (Worrall et al. 2018). Using of NPs and its composites has various advantages than the other control strategies, namely, being cost-effective, exerting no adverse effects toward nontarget pests/insects, and use of low temperature, less pressure, and energy (Jayaraman et al. 2018; Arun Prasad et al. 2018; Barabadi et al. 2019). In particular, inorganic NPs, metals, metal oxides, and nanocomposites synthesized by green methods were reported to be highly efficient against various economically important pests and insect vectors.

The use of nanoparticles for plant protection was reported to be implied via two different mechanisms (Worrall et al. 2018). One way is by direct use of nanoparticles to provide crop protection, and the other one is by using nanoparticles as carriers for existing pesticides or other active ingredients. Adapting the second option ensures the application of pesticides specifically to seeds, foliar tissue, or roots by spray application or drenching/soaking. Torney et al. (2007) reported on the concept of effective delivery of NPs directly into biomolecules of plants. This concept was later expanded by other researchers too (Martin-Ortigosa et al. 2012). The available report specifies that a minimum number of NPs may be used by plant cells (Yasur and UshaRani 2013). However, the physical characteristics of the NPs were reported to play a major role in exhibiting its application. Stadler et al. (2010) reported on the

insecticidal activity of the alumina NPs toward two species of stored grains, namely, *Rhyzopertha dominica*oryzae and *Sitophilus oryzae.* Goswami et al. (2010) assessed the controlling effect on *S. oryzae* using various NPs, namely, titanium/aluminum oxide NPs (ANP), SiO_2 NPs (SNPs), TiO_2 NPs (TNP), and ZnO NPs (ZNP). By comparison, ANP and SNP showed superior activity than TNP and ZNP. In addition, based on the different spherical SNPs functionalized on their surface, they exhibited different insecticidal effects, thus confirming the significance of the physical characteristics of the NPs. Another study by Debnath et al. (2012) also pointed out that the amorphous structured nanosilica (SNP) effectually killed the larvae of *Spodoptera litura* at 0.5 mg/cm concentration. These studies evidenced the nanobased pesticide/insecticide that could surely serve the agricultural sector with huge benefits.

12.2 Inorganic Nanoparticles-Based Insecticides

Insecticides are most likely to emerge in the upcoming years with new nanoformulations of existing insecticide's active ingredients (AIs). Most of the agrochemical nanoformulations that are in use today contain structures of nanometer-sized range depending upon specific applications including nanopesticide development. Applications of inorganic NPs have been extensively reviewed over the past 20 years, for pharmaceutical formulations Benelli (2016). Nanoformulations are mostly the combination of solid inorganic NPs in the form of nanoemulsions, liposomes, and polymer NPs that provide simple production, higher loading capability, more responsive release, low cost, and better stability (Sujitha et al. 2017; Small et al. 2016). Despite these advantages, the availability of less reports toward the development of advanced pesticide delivery systems using inorganic NPs is quite surprising. Future studies of other nanoformulation combinations and AIs are in urgent need, with a particular objective of achieving longer durability on application to soil.

12.2.1 Silver Nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were considered as a primary target of a green method possessing larvicidal, pesticidal, antimicrobial, viral, inflammatory, angiogenesis, and platelet properties. These properties have found applications in various fields such as medicine, biology, plant management, pest control, and agriculture (Chhipa 2017). Many studies demonstrating the inscecticidal activity of the AgNPs were reported by researchers worldwide. A concentration of 5–25 mg/L of adult hematophagous flies, *Hippobosca maculate*, and cattle ticks, *Rhipicephalus (Boophilus) microplus*, was reported to be killed by AgNPs (Santhoshkumar et al. 2012). The synthesized AgNPs (form of nanocorns) from *E. prostrata* were reported to be effective against pests and insects, *S. oryzae* (Zahir et al. 2012). Nair and Choi (2011) reported on the insecticidal effect of AgNPs in *Chironomus riparius* (Meigen), an aquatic midge, at a concentration of 0.2, 0.5, and 1.0 mg/L. The results of the above study demonstrated the nanomaterial's impact on the genetic

expression of glutathione S-transferase enzyme, that are associated with the induction of oxidative stress in the pest/insect. Similar study on AgNPs nanomaterial induced oxidative stress against two lepidopteran species, *Spodoptera litura* (*Fabricius*), the Asian armyworm, and *Achaea janata* (L.), the castor semilooper was also reported (Yasur and Usha-Rani 2015).

AgNPs synthesized from *Manilkara zapota* were also reported to exhibit dose-dependent activity (concentration 1.25–20 mg/L) against the *R. microplus* larvae (Rajakumar et al. 2012). The insecticidal effect of nanosilver colloid and ethanol-based colloid sulfur nanosilver at a concentration of 20 ppm was reported to show almost massive mortality against case-making clothing moth, *T. pellionella* (L.) larvae, affecting wool fibers within 14 d of treatment (Ki et al. 2007). AgNPs from the aqueous extracts of *Eclipta prostrata* exhibited larvicidal activity to control the *C. quinquefasciatus* and *Anopheles subpictus Grassi* mosquitoes (Rajakumar et al. 2011). The nano-formulated AgNP biopesticide synthesized from *H. coronarium* and its rhizome extract was reported to show and pupicidal and larvicidal activity against *A. aegypti* and the adults of *Mesocyclops formosanus,* the nontarget copepod, within an exposure time of 24 h. Significant histological changes, targeting the midgut epithelial cells of mosquitoes, were also reported (Kalimuthu et al. 2017). The systemic effect of AgNPs obtained from the aqueous extracts of *Cassia fistula L* was tested against the fourth instar larvae of *A. albopictus* and *Culex. pipiens.* The study was supported by enzyme assays too (Fouad et al. 2018). Green synthesized AgNPs was reported to be active against *A. aegypti* and few human pathogens (Ezhumalai et al. 2019). The survival of the *Drosophila melanogaster* flies was found to be compromised with pathophysiological abnormalities due to the exposure of AgNPs at higher concentrations (Armstrong et al. 2013). AgNPs synthesized from the leaf extracts of *Ficus religiosa* and *F. benghalensis* were reported to exhibit insecticidal activity against *H. Amigrate.* They reported an inhibition of Ha-Gut protease by 50% and 70%, respectively, due to the activity of AgNPs (Kantrao et al. 2017).

12.2.2 Nickel Nanoparticles (NiNPs)

Nickel nanoparticles (NiNPs) synthesized from methanolic extract of *C. nucifera* exhibited pesticidal activity against the agricultural pest, *C. maculates*, with 97.31% mortality and larvicidal activity against *A. aegypti* larvae (Elango et al. 2016). Rajakumar et al. (2013) reported on 5–10 mg/L nanoparticles of this metal were active against the larvae of two species of cattle ticks, namely, *Rhipicephalus microplus* and *Hyalomma anatolicum*, and against three species of mosquitoes, namely, *A. subpictus*, *C. gelidus,* and *C. quinquefasciatus*. Phytochemically synthesized bimetallic NPs, Ni-Pd, were found to exhibit larvicidal activity against *A. aegypti*, and phyto-synthesized Ni-Pd NPs were showed antifeedant and ovicidal effect against *C. maculatus* and *C. maculatus* eggs (Ganesh et al. 2016).

12.2.3 Cadmium Nanoparticles (CdNPs)

Though CdNPs exert significant biotoxicity, not much work, they have not been much explored in pest control application. In a study by Sujitha et al. (2017), nano-CdS exhibited high toxicity against young malarial instars of *A. sundaicus* and *A. stephensi* after 16 d of exposure. In another study, marigold petal extract synthesis of CdNPs showed complete mortality (100%) toward mosquitocidal larva after an incubation period of 72 h with 10 ppm of CdNPs in comparison with that of rose petal extracts (Hajra et al. 2016).

12.2.4 Gold Nanoparticles (AuNPs)

Reports on exploration of AuNPs against insect control are less abundant as compared with research attempts of AgNPs (Benelli 2018). The biosynthesized AuNPs from *Jatropha curcas* L. *latex* showed serum trypsin inhibition in different species of insects, including *A. Aegypti*, beetles, and pests of mealybug (Patil et al. 2016). In another study, AuNPs disrupted the reproduction and development in German cockroaches, *Blattella germanica* (L.) (Small et al. 2016). Larvicidal effects of AuNPs synthesized from the zein biopolymer (Ze-AuNPs) were tested against *A. aegypti,* a Zika virus vector. Histopathological results showed remarkable physiological changes such as complete abdominal disintegration (midgut and caeca), caudal hair loss in antenna, lower, lateral, and upper head (Suganya et al. 2017). Similar study of AuNPs synthesized from the leaf extracts of *Artemisia vulgaris* L. was found to exert larvicidal effect against third and fourth instars of *A. Aegypti* (Sundararajan and Kumari 2017). They too have observed marked physiological damage in epithelial cells, cortex, and midgut along with AuNPs deposition in the midgut region.

12.2.5 Silica-Based Insecticides (SiO_2 NPs)

Silica NPs are one of the most interesting inorganic NPs employed as pesticide delivery nanocarriers, for the application of fungicides, bioinsecticides, growth, promoters, and pheromones. Silicone has already been recognized long since enhancing plant tolerance and acting against stress (biotic and abiotic) responses. It has naturally been considered as potential candidates that can offer increased protection over a wide range of agricultural insects/pests (Barik et al. 2008). Song et al. (2012) showed that the new compounds of silica NPs had previously been reported for delayed release of growth promoters and chlorfenapyr. Field testing of such nanoformulation had showed that the silica NPs-related insecticidal activity was twice as large as microparticular or particulate-free chlorfenapyr. The mechanism involved is distinct from the mixtures of insecticides without NPs, and the greater

efficacy observed is possibly associated with the slow and sustained release (up to 10–20 weeks), that provided high regionalized target activity over a long time period. The effect of hydrophobic nanosilica toward *C. quinquefasciatus, A. aegypti,* and *A. stephensi* was studied by Barik et al. (2012). The lethal and sublethal side effects of commercially available Ludox TMA silica NPs were tested against a terrestrial pollinator, *B. terrestris L.*, via a dietary exposure of drinking sugar water (Mommaerts et al. 2012). The insecticidal potential of silica NPs against various insects, namely, *Bombus terrestris, Callosobruchus maculatus, P. xylostella,* and *Lipaphis pseudobrassicae* has also been reported (Mommaerts et al. 2012).

Debnath et al. (2011) reported on higher incidence of mortality of the weevils (*Sitophilus oryzae*), affecting *Oryza sativa* grains (kept in storages) on using nano form of silica NPs, of size ranged from 15 to 30 nm than the bulk silica of size that ranged from 100 to 400 nm. On the one hand, they too have attempted the surface modification in silica NPs using hydrophobic and hydrophilic coatings and tested against the same pest and reported on fewer deaths of weevils on using bulk silica. On the other hand, no new progeny of weevils was found after the treatment of *O. sativa* grains with silica NPs. As an additional advantage, silica NPs may release silicate ions in very small quantities, which exhibit insecticidal effect that may not involve the pests, but the grain itself. It exerts this property by strengthening the cell wall by depositing solid silica on it and by promoting the biosynthesis of defense compounds (Epstein 2009). The factors such as temperature, pH, and shell thickness were found to influence the release rate of these molecules. The release profile of the encapsulated avermectin showed a multistage pattern that interrupted the AI in various parts of the particles (i.e., internal core, porous channel, and external structures) (Liu et al. 2006). According to a report, lab trials were conducted to determine the insecticidal potential of silica NPs and AgNPs on the larvae and the adults of *C. maculatus*, affecting cowpea seed which showed 100% and 83% insect mortality (Rouhani et al. 2012). A study showing evidence of silica NPs sprayed at a concentration of 3200 mg/Lwas found to show lack of phytotoxicity in several plants (Park et al. 2006). This confirmed the urge of following permissible or optimized concentration of NPs in a strict manner, in order to achieve the targeted applications.

12.2.6 Alumina Nanobased Insecticides (Al_2O_3 NPs)

Many laboratory bioassays have already evidenced the insecticidal properties of the nanobased alumina structures that were proved effective against insect pests affecting stored products. It also has the advantage of involving electrostatic and physical phenomenon in their mechanism of action. Owing to these properties, nanoalumina was reported to an effective alternative to conventional organic synthetic insecticides (Sabbour et al. 2015). The insecticidal action of nanostructured alumina on *Sitophilus oryzae* (L.) showed the binding of nanoalumina to the cuticle of the beetle due to tribo electrical forces that sorbs its waxlayer, resulting in insect dehydration (Stadler et al. 2017). Stadler et al. (2010) demonstarted the insectical property

of the nanoformulated alumina against two species, *S. oryzae* L. and *R. dominica* (F.). These two are regarded as the common insect pests that affect the world's stored food supplies. However, both the species were found to experience critical fatality after 3 d of continuous exposure to the nano-treated wheat. Buteler et al. (2015) too studied the insecticidal activity of nanoalumina dust of varying size and morphology against the above said two pests that affect the stored food supplies. Though they observed greater mortality rates, they concluded that reducing the size of the particle and increasing the surface area were not the only dominant factors that influence the effectiveness of insecticides.

12.2.7 Titanium Dioxide Nanobased Insecticides (TiO_2 NPs)

The insecticidal activity of TiO_2 NPs was reported to be the causative agent for the deaths of *R. microplus* larvae and *Haemaphysalis bispinosa* adults (Marimuthu et al. 2013). An increased insecticidal activity was noted with an increase in the concentration (4–20 mg/L) of NPs. Philbrook et al. (2011) too reported on the effect of TiO_2 and AgNPs (concentrations ranging from 0.005 to 0.05%) against the fruit fly, *D. Melanogaster*, that resulted in major progeny loss and decreased success in insect development. Sabbour (2012) demonstrated entomotoxicity testing of TiO_2 and Al_2O_3 NPs against *S. oryzae,* both under lab and sored conditions. TiO_2 NPs activity against the *Bombyx mori L.* (silkworms) was found to stimulate the biosynthesis of 20-hydroxyecdysone. This resulted in shortened time for insect growth and decreased molting period (Li et al. 2014). This study has enabled the researchers to claim and explore the potential sericulture benefits in using TiO_2 NPs. The insecticidal activity of TiO_2 NPs synthesized from extracellular *Trichoderma viride* was evaluated for their pupicidal, larvicidal, and antifeedant effect against *H. armigera.* They reported on complete 100% mortality on the first and second instar and 92.34% on third instar larvae at a concentration of 100 ppm (Chinnaperumal et al. 2018). The larvicidal activity of TiO_2 NPs against *Bombyx mori* has been much explored by various researchers (Tian et al. 2016; Xue et al. 2018).

12.2.8 Zinc Oxide Nanobased Insecticides (ZnO NPs)

Zinc oxide nanoparticles (ZnO NPs) exhibit remarkable physical, optical, and antimicrobial properties that can be used to enhance agriculture. Synthesis of ZnO NPs can be achieved by various chemical and biological methods. However, the plant extract-based biogenic synthesis of ZnO NPs is most often used in crop-pest control practices. *L. leschenaultiana*-encapsulated ZnO NPs showed 100% mortality of *A. Aegypti*. They too reported on significant morphological defects such as abdominal shrinkage, thorax shape changes, mid-parent damage, loss of lateral hair, brushes and anal gills, and deposition of ZnO NPs in thorax and abdomen (Banumathi et al. 2017). Similarly, ZnO NPs synthesized from exopolysaccharides (EPS) extracted from *Bacillus licheniformis* Dahb1 (EPS-ZnO NPs), the probiotic strain showed

complete mortality (100%) against third instar larvae of *Aedes* mosquito species even at lowest doses. Histolopathological studies too confirmed the cellular and tissue damages in the midgut of the nano-treated mosquito larvae (Abinaya et al. 2018). Another insecticidal study of *U. lactuca*-fabricated ZnO NPs showed complete mortality (100%) of the fourth instar larvae of *A. aegypti* at 50 μg/mL within 1 day with marked morphological and histological changes inthe larva (Ishwarya et al. 2018). Different concentrations of ZnO NPs synthesized via precipitation route was exposed to the *Trialeurodes vaporariorum* (greenhouse whitefly), a major pest affecting ornamental and horticultural plants and reported with a maximum mortality of 91.6% (Khooshe-bast et al. 2016). Surface coating of ZnO NPs with *B. thuringiensis* was found to act against *Apis mellifera* and *Callosobruchus maculatus* (Milivojevi et al. 2015; Malaikozhundan et al. 2017). Another study to test the efficiency of zinc oxide, aluminum oxide, and aluminum-doped zinc oxide NPs against *Cx. quinquefasciatus* larvae showed a maximum mortality of 96%. The tested NPs were found to be attached in the cuticle of different body parts of the dead larvae (Mostafa et al. 2018).

Apart from the above-discussed NPs, other inorganic metal oxide NPs and sulfide based compounds have been reported from our group too. The toxicity of bismuth oxyiodide nanoflakes was investigated and reported for the first time against *A. stephensi* and *Plasmodium berghei*. They have compared their results with malarial drug, chloroquine, via animal testing and reported on excellent antiplasmodial experiments against *P. berghei* with a prominent chemosuppression after 4 d of treatment with of BiOI (300 mg/kg/day) (Murugan et al. 2018a). Green and chemical-fabricated nano-ferric and ferrous oxide NP-based composites produced from *Ficus natalensis* were tested against larvicidal and pupicidal experiments on *Cx. Quinquefasciatus* and reported on enhanced toxicity of the iron-based composites on the mosquito vectors (Murugan et al. 2018b). Profound mosquitocidal activity of flower such as copper sulfide nanocrystals was reported against the *A. stephensi* instar larvae and plasmpodium parasites (Theerthagiri et al. 2017).

12.3 Organic Nanobased Insectides

12.3.1 Carbon Nanobased Insecticides (CNPs)

Low toxicity and readily available characteristics make carbon NPs a much preferred option in a variety of biomedicine and research developments. In addition, they exhibit good mechanical properties, extraordinary electrical conductivity, and heat conductivity. Since they are made of pure carbon, they show high stability, low toxicity, good conductivity, and environment-friendly properties (Street et al. 2007). Moreover, carbon NPs synthesized by environmentally sustainable methods were reported to be highly effectual against the arthropod pests that are economically important (Athanassiou et al. 2018). Nonetheless, most of the studies concentrated on mosquitoes with only few NPs tested on pests (Murugan et al. 2017). Martins et al. (2019) demonstrated the enduring effects of the diet containing two classes of

carbon NPs of different dimensions (i.e., oxidized form of multiwalled carbon nanotubes (MWCNT), 1D-MWCNT, graphene oxide (GO), and 2D-GO) fed to the insect *Spodoptera frugiperda (Lepidoptera: Noctuidae*). They have observed many critical influences that affected the general behaviour, fertility, nutritional physiology, and changes in enzyme activities in the moth. Similar work by Liu et al. (2009) demonstrated that a diet-fed fruit fly larvae, *D. melanogaster* coformulated with single-walled or MWCNT carbon black, fullerene C60, had no considerable effect on insect development and survival, despite the presence of carbon nanostructures in tissues of fruit fly. However, fruitfly adults exposed to dry forms of carbon-based NPs were found to strongly adhere to the flying parts of the fly, resulting in diminished motor activities and less mortality. Recently, Sultana et al. (2018) reported on the *Solanum tuberosum* L. that served as carbon-dot precursors source-based synthesis of carbon-dot silver nanohybrid was tested against the larval and pupal stages of the two mosquito vectors, *A. stephensi liston* and *Culex quinque fasciatus,* and found to be highly toxic to both the species that led to their death due to cellular-level nano-Ag toxicity. Dziewięcka et al. (2016) demonstrated the GO NP toxicity (0.1 μl/100 mg) on insertion into the hemolymph of Cricket fly, *Acheta domesticus L,* caused oxidative stress with increased activity of catalase, glutathione peroxidase, total antioxidants, and heat shock proteins (HSP 70) release.

12.3.2 Nanocomposites-Based Insecticides

Nanocomposites have wide applications in many areas such as disease diagnosis, drug delivery system, energy storage, food processing, pest detection and control, water treatment, and agricultural productivity. Among all, the potentiality of nanocomposites has been effectively employed in plant growth and plant pest management. In the present era, the role of nanocomposites in agriculture is expected to reduce the burden of chemical pesticides and fertilizers (Gupta 2018).

12.3.3 Chitosan-Fabricated AgNPs-Based Insecticides (Ch-AgNPs)

In a recent study, chitosan (using male crab shells)-synthesized silver nanoparticles (Ch-AgNPs) was tested against *A. sundaicus* vector larvae and pupae. They also evaluated the predatory activity of the mosquito's natural enemy, *C. auratus,* under lab conditions and found significant improvement after treating with sublethal dosages of Ch-AgNPs. Similar report on testing the efficiency of Ch-AgNP against the pupae and larvae of *A. stephensi,* a malarial vector with observed LC_{50} value range of 3.18–6.54 ppm (pupae) was also reported (Murugan et al. 2016). In another study, cumulative mortality was noted for larval instars of *S. litura,* thus confirming the insecticidal activity of chitosan nanocomposite. In addition, biochemical changes of midgut and hemolymph constituents evidenced the enhanced pesticidal activity of the nanocomposite against all stages of larva with high mortality rate (Namasivayam et al. 2018).

12.3.4 Nanopolymer-Based Insecticides

In recent years, polymer-based nanoformulations are gaining popularity among many researchers to employ them as plant protection molecules (mainly pesticides). Pesticide formulation prepared using polyethylene glycol (PEG) in water was found to show controlled and slow released activity that lasted for several weeks than commercial pesticides, such as betacyfluthrin, carbofuran, thiamethoxam, imidacloprid, and thiram (Kaushik et al. 2013; Pankaj et al. 2012). Few reports on bioassay research too confirm the potentiality of some of these formulations based on PEG being more effective than commercial insect and nematodes control products (Pankaj et al. 2012). Essential garlic oil loaded on PEG-coated polymer-based NPs was used to control red flour beetle adults, *Tribolium castaneum (Herbst)* (*Coleoptera: Tenebrionidae*). They too reported on the sustained efficacy of PEG-coated polymer NPs <80% after 5 months (Yang et al. 2009). Film preparation of high-density polyethylene (HDPE) fabricated with two NPs (ZnO/Ag) was reported to shield stored food supplies from the insect/pest invasion. The outcomes of the present study showed the non-penetration of *Sitophilus granarius* adults into the loaded NPs and the γ-irradiated HDPE films (Eyssa et al. 2018). Polymer-based nanoformulations were found to exhibit greater effectiveness to commercial mixtures over relatively long time period (30 d), owing to their slower release capability instead of increased absorption by the nanoformulation uptake by the target organisms of the released pesticide. However, their very slow release (in some cases) has been considered as a disadvantage of polymer-based nanoformulation as it can lead to decreased environmental flexibility, high production expenses, and high energy involving methods of preparation (Torchilin 2006). Argemone mexicana-based synthesis of TiO_2, capped with poly(styrene sulfonate)/poly (allylamine hydrochloride) was reported to exhibit both larvicidal and pupicidal activities against Zika virus vector, *A. aegypti* (Murugan et al. 2017).

12.4 Smart Delivery Systems of Nanopesticides

NPs are employed as a common delivery tool in medical therapies. Likewise, a similar "pesticide delivery system" for pest control has also been established. The nanobased deliveries are much preferred for their physical properties and specificity. The idea of nanobased formulations as effective delivery systems is adapted from its application in medical field. Many researchers believe that these nanocarrier systems can strengthen/enhance the pesticide properties and activities such as mobility, stability, solubility, dispersion, and targeted delivery. In addition, they possess the capacity to provide highly flexible loading because of the installation of single carriers, wider surface area, many distinct pesticide compounds, and an extremely rapid mass transfer toward the targeted pest (Zhao et al. 2017). Many conceptual ideas

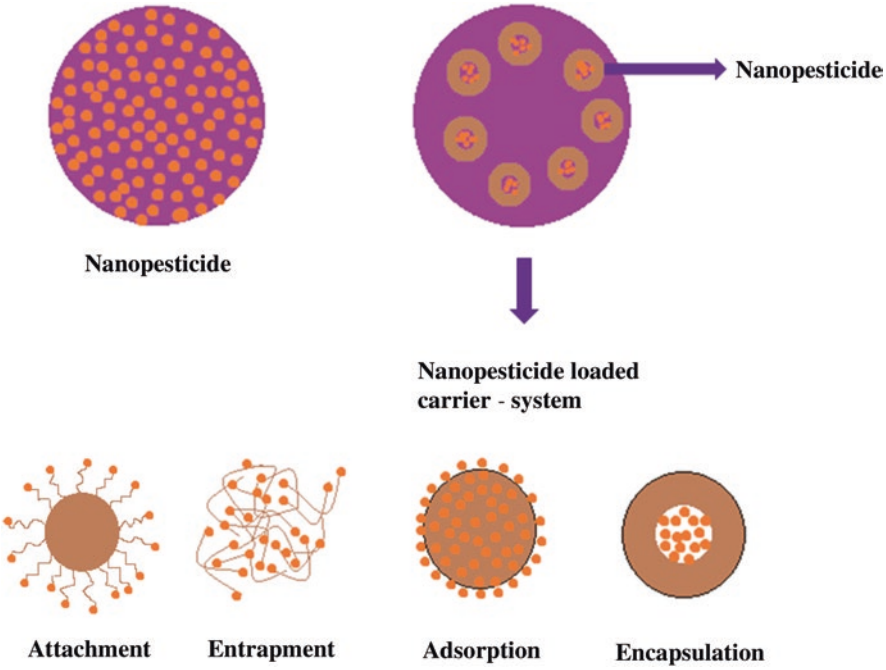

Fig. 12.3 The delivery systems of nanopesticides

were reported so far for the loading of active pesticide compounds on NPs, namely, surface adsorption, nanoparticulate polymer shell encapsulation, covalent binding by various ligands, and nanopolymer matrix entrapment (Jia et al. 2014). The delivery systems of nanopesticides are illustrated in Fig. 12.3. Of all, nanoencapsulation was reported to be an effective strategy that can be used safely to deliver pesticides with less exposure to the environment (Qian et al. 2011). Other reports on the use of mesoporous NPs such as porous hollow silica, activated carbon, and nanoclay were also found to be ideal delivery systems with controlled release options for both polar and nonpolar pesticides. These NPs were found to possess good biocompatibility, high drug-loading capacity, low toxicity, and multistage release patterns (Wang et al. 2012). According to Yu et al. (2017), the strength of the adhesion depended heavily on the size distribution and the surface functional groups on the NPs and hence can easily be controlled by varying the size and functional groups. Among all NPs, silica-based NPs had generated much interest among the researchers to employ as an effective agent for plant-based agrochemical application. This is attributed to their flexible structure that allows to form numerous shapes and sizes of NPs, as well as their ability to form pores that aid in effective loading of the biomolecules.

12.5 Mode of Action of Nanobased Insecticides

Though enormous literature is discussed on nanotoxicity to selected pests and vectors, reliable data about the possible mode of insecticidal action is lacking (Athanassiou et al. 2018). As discussed earlier, nanobased formulations possess specific morphological characteristics compared with traditional pesticide formulations, which could effectively enhance pest coverage, adherence, and permeability (Zhao et al. 2017). Owing to the influence of the size, shape, and charge of the AgNPs, various in vitro and in vivo studies have been reported on the cytotoxicity and genotoxicity using bacterial and other biological models. However, only a few research has focussed on the mechanism of action against mites and insects (Santo-Orihuela et al. 2016). In addition, their toxicity may also be due to the penetration of NPs into the exoskeleton. Few nanobased formulations were found to act similar to traditional pesticides. Basically, the mode of action would be by activating insect-target alteration pathways and triggering stomach poisons release. Pesticide ingestion in pests primarily affects its digestive system. The other way of entry is through inhalation or contact with the poisonous fluids from the host (Zhao et al. 2017). Based on the other reports, the nanobased formulations enhanced the function of stomach and contact poisoning by improving the dispersal and permeability which in turn increased the rate of pesticides entering the pest (Yang et al. 2017). Enhanced transport and conductivity of the nanobased formulations was reported to improve the insecticidal efficiency by accelerating the pesticide poisoning, pesticide efficacy, bioactivity, and dose effect. Possible mode of action of nanoinsecticides is shown in Fig. 12.4.

12.6 Future Research Challenges

By reducing the use of chemical-based crop protectants, nanotechnology is expected to make agriculture more eco-friendly and highly profitable. Despite a slow progress, nanobased technologies appear to have a great future not only in the agricultural sector but also in other sectors (Athanassiou et al. 2018). In the past few decades, NPs have gained huge prospects for their wide applications in agricultural field that will surely interest the researchers to develop safer and more efficient pest control formulations. Undoubtedly, intensive research could possibly lead to many revolutionary formulations based on nanopesticides such as nanoemulsions, nanodispersions, etc., in the near future (Murugan et al. 2015). However, research pertaining to insecticidal mechanism or mode of action remains lacking for many nanobased insecticides. Apart from very few studies of Ag, copper. and chitosan NPs, many key classes of NPs are yet to be investigated in terms of entomological and parasitological research. Likewise, there is an urgent need for a comprehensive understanding of different routes that lead to the toxic effects of chronic NPs on the vertebrate population (Benelli 2018). Similarly, experiments performed on polymer-based nanoinsecticides remain at nascent stage due to lack of knowledge on the unique characteristics of nanocomposites behavior in soil and agriculture. Hence, more studies are required to extend this concept for various commercial crop

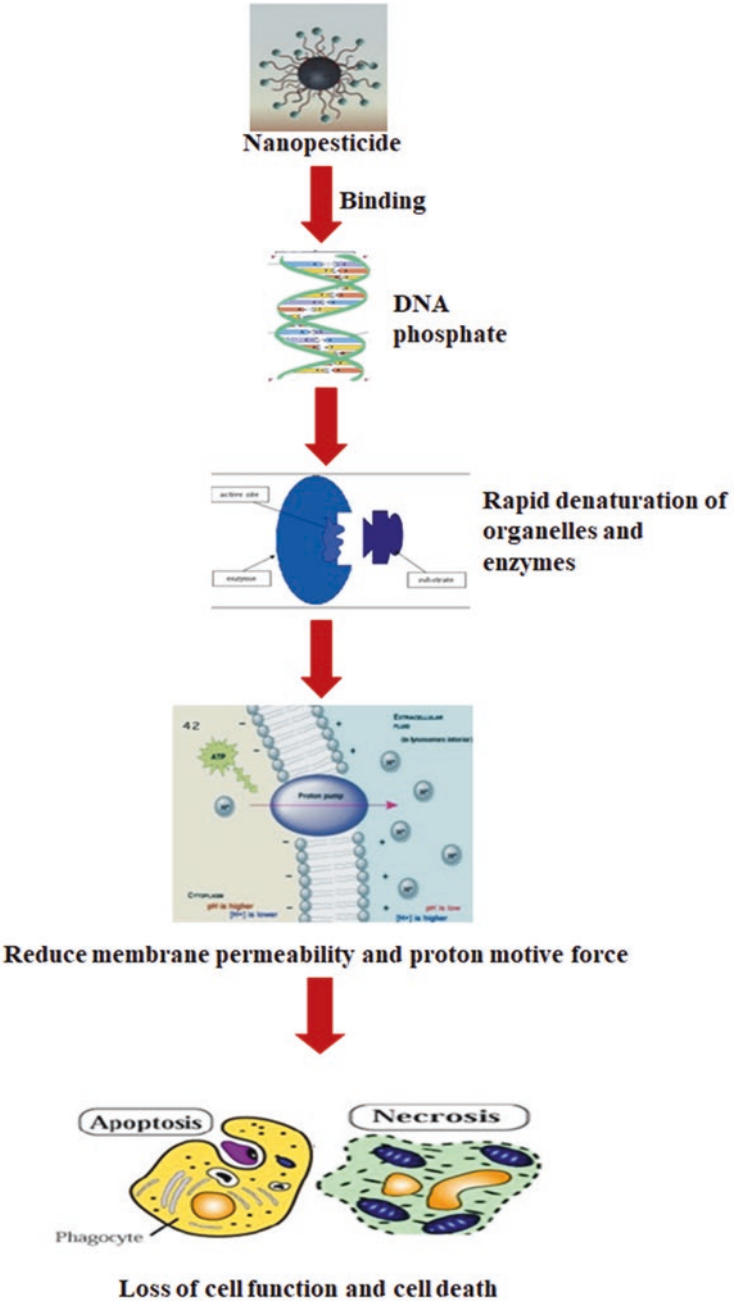

Fig. 12.4 Possible mode of action of nanoinsecticides

applications. Before the implementation of in situ pest management practices, a huge amount of work is still required on formulations of nanopesticides by integrating analytical methods which can detect, characterize (in terms of size, surface area, nature, or shape), and quantify the main ingredient and formulations of the insecticides (Athanassiou et al. 2018). Additional research efforts toward the development of smart nanodevices as effective delivery tools, that aid in the development of smart fertilizers, pesticides, and growth regulators are also of high need. Development of nanosensors for real-time monitoring of crop growth, pest invasion, soil conditions, and disease development will surely place nanotechnology in greater heights. Similarly, the development of nanocapsules for controlled and systemic release of herbicides through proper implementation means will also greatly increase their options to use against parasitic plants (e.g., contact herbicide-based mode of implementation) (Shahzad and Manzoor 2019). Therefore, intensive research on the above said issues is much needed to target the pests more precisely for the implementation of sustainable advanced agricultural nanotechnology.

12.7 Limitations of Nanopesticides

Considerable knowledge on the possible toxicity of NPs and nanoformulations toward both the target and nontarget species is one of the key issues that need to be addressed before their extensive use. More alarming is that, in many cases, the NPs claimed to be synthesized using "green" methods are not at all "green" (P'erez-de-Luqu et al. 2009). It is also equally important to notice that not all NPs pose similar toxicity risks. Serious evaluation of biocompounds is much needed to be chosen to develop less hazardous nanoformulations. In this regard, it was reported that the nanocapsules and liposomes formulated using natural organic compounds such as chitin and lipids are considered to be less toxic than those NPs formulated using heavy metals (P'erez-de-Luqu et al. 2009). Another important for nanocarrier development is the scale of production and costs. Till date, large quantities of many NPs that can be used in agriculture cannot be produced, owing to their prohibitive costs. Nonetheless, the implementation of nanotechnology for crop protection and agricultural pest combats needs to be carefully evaluated to serve public health and livestock science. Unfortunately, there are almost no data on the final destiny of nanoparticles, particularly those that have not reached their goal and leaked away from the agricultural fields. However, few researchers believe that agricultural nanotechnology research is not reaching its maximum potential due to the scarcity of commercial applications. Worrall et al. (2018) reported that out of the 84 papers investigating pesticides, herbicides, fungicides, or loaded with nanoparticles (published until October 2018), only two papers have attempted field trials. In addition, only 24 papers addressed environmental issues such as soil leaching and nontarget toxicity, and only 46 papers inspected the formulations developed against the target pest.

12.8 Conclusions

Nanobased insecticidal formulations exhibit many benefits over conventional formulations such as highly targeted delivery, high efficiency, smart controlled release options, and environment friendly. Due to technological advancements, large-scale application of nanopesticides in crop production is quite possible, nowadays. Based on many research reports, various NPs, namely, AgNPs, SiO_2, TiO_2, ZnONPs, etc., were recognized as excellent candidates for fighting against many insects and pests with their proven toxicity against mosquitoes and ticks. They were also found to display their toxicity at various stages of insects and pests. Nano-encapsulation-mediated nanocomposites loaded with herbicides, fungicides, fertilizers, nutrients, and target-specific plant site are regarded to achieve desired results. However, these nanocomposites are to be carefully evaluated, as they might pose certain health hazard and disturbances in the ecosystem. In conclusion, formulations of nanobased pesticides bring beneficial improvements to the behavior and properties of the traditional pesticides such as dispersion, solubility, controlled release of active ingredients, delivery targeting, and of course, stability. Additionally, it could not only enhance the efficiency but also improve the bioavailability properties and minimize the nontarget toxicity against wildlife, food, and environmental residues.

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Routes of Exposures and Toxicity of Nanoparticles

13

Koigoora Srikanth

Abstract

Nanotechnology is a vast growing research niche and has found its application in diverse fields. The vast applications include the environment, chemical, biological, electronics, medicine, and sports. The miniature size and high surface area of nanoparticles (NPs) do cause increased toxicological effects on various organisms. To know the effects of NPs exposure, the current chapter presents the various routes of exposures and its toxicity on different model systems. Different NPs can pass through the host system via the skin, olfactory route, respiratory tract, and oral route. The entry of these NPs in the following routes may be either during their production, use, intentional, or unintentional. The entry of NPs in the following routes may lead to negative biological effects. The key points for discussion in this chapter include the routes of exposure of different NPs and their toxicology impact at that particular point of entry and the target organ.

Keywords

Nanoparticles · Exposure · Routes · Tissues · Organs

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13.1 Introduction

Nanotechnology involves the handling of materials to near atomic scale to produce novel framework, materials, and architecture. It makes nanoparticles (NPs) whose size is below 100 nm by altering the materials at the atomic level (Srikanth et al. 2016). Nanomaterials have been extensively used in numerous applications, but their harmful effects on the environment and organisms are paid least attention (Srikanth et al. 2017).

The major point in toxicological studies is focused on the exposure–concentration–effect relevance. Usually, external exposure allows the toxicants/contaminant to enter the body. Exposure is defined as the state at which protection from any external contaminant such as NPs is poor. The crucial factor deciding the potential toxicity of NPs is the route of exposure (Sahu and Hayes 2017). The current chapter highlights the possible ways of exposure to NPs (i.e., through either the skin, olfactory route, respiratory tract, and oral route) (Fig. 13.1).

13.2 Dermal Exposure

The skin is a heterogenous structure consisting of epidermis and dermis with hair follicles and sweat glands giving access across these layers. Moreover, the peripheral blood vessels run into the dermis. The epidermis primarily consists of keratinocytes which emigrate from the basal layer approaching the skin exterior, forming the outer defensive layer stratum corneum. The stratum corneum prevents the entry of microbes and other hazardous chemicals by forming a barrier. This barrier is not absolutely permeable, and it is desirable for relatively small molecules (<100 nm) which diffuse across the stratum corneum via cellular and/or intercellular pathways. If the membrane/barrier is impaired, then movement is seen increased. Table 13.1 presents the exposure of NPs through the skin.

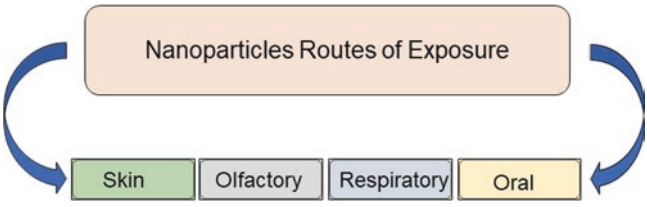

Fig. 13.1 The different exposure routes of nanomaterials

Table 13.1 Nanoparticles dermal toxicity studies

Study (year)	Nanoparticles	Exposure route	Outcome
Mohammed et al. (2019)	Zinc oxide	In vitro	Zinc oxide did not show any toxicity in the skin
Raju et al. (2018)	Gold	In vivo	Depending on the thickness of the skin, it allows the entry of NPs into the tissue and further acts as reservoir for their release into the circulation
Hadrup et al. (2018)	Silver	In vivo	Argyria was observed
Kuwagata et al. (2017)	Silver	In vivo	Silver NPs can reach the body only when it is ruptured
Pal et al. (2016)	Zinc oxide	In vivo	Induce toxicity in the skin
Holmes et al. (2016)	Zinc oxide	In vivo	Topically applied zinc oxide (ZnO) NPs do not pass through the epidermis, and the different formulations of ZnO are subjected to hydrolysis on contact with the skin
Crosera et al. (2015)	Titanium dioxide	In vivo and in vitro	Titanium dioxide NPs cannot pass through the skin and is only present in stratum corneum
Sykes et al. (2014)	Gold	In vivo	The skin is an important site for NPs accumulation
Mohanan et al. (2014)	Ferrite NPs coated with dextran	In vivo	Could not induce toxicity
Koohi et al. (2011)	Silver	In vivo	Smaller NPs highly toxic

13.3 Inhalation Exposure

Nanomaterials during their production or use are seen affecting humans mostly through dermal or inhalation toxicity. The NPs inhaled during the above said process are reaching the deep lungs and are seen deposited in the alveolar regions. Nanomaterials around 10–100 nm are usually seen deposited in the alveolar regions. The respiratory system creates a barrier obstructing most of the foreign particles from entering the airway epithelia. The respiratory wall constitutes surfactant film and mucus layer below and is followed by ciliated cells of the bronchiolar epithelium which are held together by tight junctions (Hayes and Bakand 2010). The inhaled NPs usually reach the nonciliated alveolar region (Landsiedel et al. 2012; Savolainen 2013; ICRP 1994; Bräu et al. 2012). Here these are picked up by the alveolar macrophages which then reach the mucociliary escalator and to a lesser amount to that of epithelial cells (Anttila 1986). The NPs entering the lungs cause harm to the cells, if the amount of inhaled NPs overtake the macrophage's removal rate (Morrow 1988; Donaldson and Tran 2002; Monteiller et al. 2007; Pauluhn 2009). The retention and systemic absorption of NPs is very little in the lungs, and

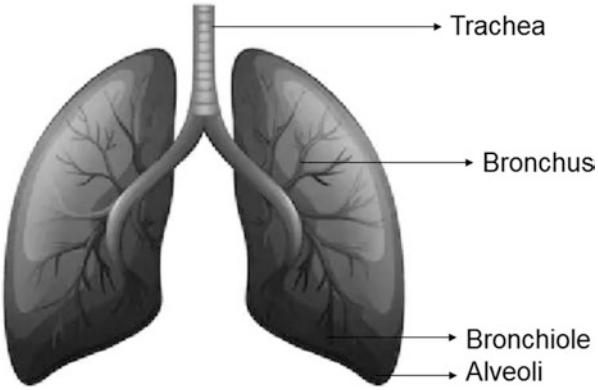

Fig. 13.2 Different sizes of nanomaterials are seen affecting the lungs and its different parts. The nanoparticles below 100 nm are seen deposited in the trachea, whereas below 10 are usually deposited in the bronchus, bronchiole, and alveoli

Table 13.2 Nanoparticles inhalation toxicity studies

Study (year)	Nanoparticles	Exposure route	Outcome
Zhang et al. (2019)	Silver	In vitro	It shows other pathways for quantitative analysis of inhalation toxicity of NPs
Nasirzadeh et al. (2019)	Graphene	In vitro	Graphene NPs were cytotoxic to A549 cells after 72 h of exposure
Hurbánková et al. (2018)	Titanium oxide	Intravenous	Titanium oxide NPs are inert after long-term incubation moved from the blood stream to the respiratory tract
Oyabu et al. (2017)	Nickel oxide and titanium oxide	Intratracheal instillation and inhalation	The bio-persistence shown in biological half time is a good hazard indicator for the two NPs used
Bengtson et al. (2016)	Graphene oxide and graphene	In vitro	No cytotoxicity was observed in FE1 cells.
Vlachogianni et al. (2013)	Engineered nanomaterials	Respiratory system	The inhaled NPs generate free radicals and cause ROS formation leading to oxidative damage and failure of the respiratory system
Srinivas et al. (2012)	Iron oxide	Head and nose inhalation	Inhalation of iron oxide NPs may cause toxicity and lead to inflammatory responses in rats
Srinivas et al. (2011)	Cerium oxide	Head and nose inhalation	Cerium oxide NPs through inhalation causes cytotoxicity through oxidative stress
Gosens et al. (2010)	Gold	Intratracheal instillation	The 50 nm Au NPs are not cytotoxic than their aggregates

their toxicity is confined to lungs only. Some of the researchers have reported that NPs cross the pulmonary barrier and reach the circulation and exhibiting toxicity. The ability of NPs to cross the lung barrier relies on the size and concentration of NPs. Figure 13.2 represents the different sizes of nanoparticles affecting the different parts of the lungs when inhaled (Table 13.2).

13.4 Oral Exposure

Nanomaterials when administered orally are passed through the entire gastrointestinal (GI) tract, and while passing, they are seen to be accumulated in the liver and caused toxicity (Chen et al. 2006; Wang et al. 2006, 2007). The NPs are entering into the GI tract of humans either during their production, use, or through the consumption of contaminated food and water (Ahamed et al. 2010; Sharma et al. 2011). Table 13.3 represents the toxicity of NPs when ingested orally.

Table 13.3 Nanoparticles ingestion toxicity studies

Study (year)	Nanoparticles	Exposure route	Outcome
Henson et al. (2019)	Copper oxide	In vitro	Oxide of copper NPs was toxic to the intestinal cells of both human and rat
Dumala et al. (2019)	Nickel oxide	Oral	Nickel oxide NPs had accumulated in various organs and caused severe effect on them
Yousef et al. (2019)	Aluminium oxide and Zinc oxide	Oral	Aluminium and zinc oxide NPs caused hepatocellular toxicity and systemic inflammation
Bugata et al. (2019)	Copper oxide	Oral	Higher doses of copper oxide NPs caused variations in antioxidant and biochemical parameters of the brain, kidney, and liver of albino Wistar rats
De Jong et al. (2018)	Copper oxide and copper carbonate	Oral	The NPs caused liver damage, and tissue-based pathological changes were observed in the liver, bone marrow, and stomach
Dumala et al. (2017)	Nickel oxide	Oral	Nickel oxide NPs when induced orally caused significant gentotoxicity
Kumari et al. (2014a)	Cerium oxide	Oral	Oral toxicity of NPs caused tissue distribution, and the histopathological studies revealed alterations in the liver
Kumari et al. (2014b)	Cerium oxide	Oral	Nanoceria induced significant changes in DNA damage in leukocytes and liver when ingested orally
Kim et al. (2008)	Silver	Oral	The silver NPs caused significant liver damage after oral ingestion

Table 13.4 Nanoparticles olfactory toxicity studies

Study (year)	Nanoparticles	Exposure route	Outcome
Sudhakaran et al. (2019)	Zinc oxide	In vitro	The NPs caused cytotoxicity in primary astrocytes. Morphological alterations, cytoskeletal arrangement, and mitochondrial membrane potential were also altered
Zheng et al. (2019)	Cobalt oxide	In vitro	Cobalt chloride and CoNPs when exposed to male Wistar rats; CoNPs were found to be more toxic than cobalt chloride. Neural loss was observed in hippocampus, cortex, and temporal lobe
Ma et al. (2019)	Iron oxide	In vitro	The cytotoxicity was evaluated on PC12 and ReNCell VM, and iron oxide NPs induced significant decrease in their viability
Greish et al. (2019)	Silver	In vivo	The effect of silver NPs on BABLB/C mice was tested for learning, memory, social behavior, and motor function. The results revealed alterations in cerebral recognition
Dąbrowska-Bouta et al. (2019)	Silver	In vitro	Male Wistar rats were exposed to silver NPs which caused oxidative stress alterations in the myelin membranes of the myelin sheath
Dąbrowska-Bouta et al. (2018)	Silver	In vivo	Wistar rats were treated with NPs for 2 weeks orally which caused significant variation in cerebral vessels leading to disruption of tight junction proteins
Węsierska et al. (2018)	Silver	In vivo	Silver NPs were applied via oral route to rats for 28 days; later it was observed that there was loss of short- and long-term memory. High concentration of silver was found in the region of xhippocampus than compared with lateral cortex
Kim et al. (2015)	Zinc oxide	In vivo	The NPs were cytotoxic to SH-SY5Y human neuroblastoma cells. Compelling decline in live cells was noted followed by morphological changes of cells
Sruthi and Mohanan (2015)	Zinc oxide	In vivo	The NPs-induced cytotoxicity in rat C6 glial cells via oxidative stress

13.5 Olfactory

Nanoparticles entering the respiratory system and reaching brain via the air pollution and occupational exposure may lead to neurodegeneration and brain damage (Calderón-Garcidueñas et al. 2002; Sunderman 2001). The path adopted by the inhaled NPs to reach the brain is uncertain, but there are reports available that the NPs depositing in the olfactory region in the nasal cavity can travel to the brain and olfactory bulb (Hopkins et al. 2014; Oberdorster et al. 2004). The nose-to-brain route of exposure studies has not been established in humans; it has been shown in

primates (Dorman et al. 2006). Workers exposed to fumes of welding are seen developing Parkinson-like neurological syndrome due to the transport of these fumes from the nose to the brain (Antonini et al. 2006). Table 13.4 represents the toxicity of NPs in the nervous system.

13.6 Conclusion

Nanomaterials have found widespread application from cosmetics to medicines, as well as in environmental bioremediation, chemical, and pharmaceutical industries. Although NPs have diverse properties, there remains to exist negative impacts of these NPs to the biota. Humans are exposed to NPs either during production or their use. These are also seen entering the body through contaminated food (oral) and cosmetics (dermal) and inhalation of polluted air (respiratory). As mentioned in the current chapter, we observed detrimental effects of nanomaterials because of their aggregation in organs. This leads to severe toxicity over a period of time due to the induction of oxidative stress. As a result, membrane, protein, and DNA are damaged. In the current chapter, a number of studies have provided interesting information on the systems causing the adverse effects of NPs.

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Toxicological Evaluation of Nanoparticles Using Prokaryotic Model Organisms

14

Pavani Sanapala and Sudhakar Pola

Abstract

The extensive application of nanoparticle (NP) synthesis and expansion in recent advances in biological and material science have been of considerable scientific interest from the last century as they possess properties to measure and influence the physical substance from an atomic and molecular point of view compared with bulk materials. NPs are usually coated with metal ions, chemical surfactants, polymers, and smaller molecules. Due to these properties nanoparticles tend to get the toxic value that is largely estimated triggering both the environmental and human health risks. The number size distribution of nanoparticles of 1–100 nm is the main cause of these substances affecting the environment and health system where the passage into the ecological food chains via microorganisms has been easy, disturbing the biological balance. Hence, it is vital to evaluate the toxicity of NPs associated with microorganisms beforehand. Though the eukaryotic model was renowned, in recent developments, the use of prokaryotic models especially bacteria is considered the most convenient, rapid, and cost effective. Evaluating the toxicity of NPs using microorganisms gives an insight into the toxic impacts of NPs. Bacterial species such as *Escherichia coli*, *Pseudomonas* sp., *Bacillus* sp., and mainly magnetotactic bacteria intracellularly can synthesize the tiny crystals referred to as nanocrystals. The mechanism associated with the toxicity of NPs is mainly the oxidative stress and generation of reactive oxygen species that results in membrane disorganization, impair reproduction, and growth inhibition. This chapter in detail will give out the different approaches to evaluate the toxicity of NPs and also the use of different prokaryotic models that produce eco-friendly nanoparticles that are of greater importance in the biological system.

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Nanoparticles · Engineered nanomaterials · Nanotoxicity · Prokaryotic models

14.1 Introduction

In recent days, the target on nanoparticles, their origin, activity, and biological toxicity by the researchers has been of much interest due to revolutionary developments in the field of nanotechnology with novel and diverge application coming into view in our daily lives. Nanotechnology is a well-known advanced technology in the field of research since the twentieth century. The word "nano" is derived from a Latin word, which means "dwarf." The productions of nanoscale level materials have become major innovatory developments in the field of nanotechnology. The nanostructures show substantial innovative and enhanced physical, chemical, and biological properties and develop owing to their size (Kumar et al. 2011). Extensive application of nanoparticle (NP) synthesis and expansion in recent advances in biological and material science have been of great scientific interest from the last century as they possess properties to measure and influence the physical substance from an atomic and molecular point of view compared with bulk materials. NPs are usually coated with metal ions, chemical surfactants, polymers, and smaller molecules. Due to these properties, nanoparticles tend to get the toxic value that is largely estimated, triggering both the environmental and human health risks. Facts of the toxicity effects of these small molecules are restricted, but according to latest research, it is swiftly budding (Ai et al. 2011).

Prokaryotes especially bacteria are the most common module of all known ecosystems. They play a significant role in biological cycles, degradation of impurities, the root of food webs or chains, and also soil health. The choice of researchers to use prokaryote models extensively is due to their simple structure and functional organization, short generation time compared with other organisms, nonpathogenic nature (harmless), an efficient genetic, and experimental model (easily manipulated) since the genetic background of these organisms is clear (i.e., the genome levels are fully sequenced and studied). Bacterial species intracellularly can synthesize the tiny crystals referred to as nanocrystals. Wiesner et al. (2009) have avowed that "microbial ecotoxicity" is predominantly an important consideration in assessing the toxicity mechanism of NPs that extrapolate to eukaryotic cells.

14.2 Nanoparticles

Nanoparticles (NPs) are a wide class of miniaturized particles with a range of 10^{-9} and 1–100 nm dimensional size (Laurent et al. 2008). Nanoparticles vary in their chemical, physical, mechanical, and electrical properties that differ significantly as of their corresponding bulk material due to their broad distribution in size (Biswas and Wu 2005; Lowry et al. 2012). For this reason, a material acknowledged to be

nontoxic in bulk can subsist toxic at the nanometer scale due to its characteristic properties (Karlsson et al. 2009). Nanosized materials are allied to various scientific and advanced application technologies in the field of biosciences, namely, environment, chemical, pharmaceutical, health, and electrical engineering and also in the area of life and applied sciences (McDonald et al. 2005; Tripathi et al. 2016; Tiwari et al. 2017). Taken as a whole, based on the shape size and structure, these materials are grouped from 0D to 3D (Tiwari et al. 2012). NPs are complex molecules with three distinct layers: (a) the topmost being surface layer (functionalized with an array of small molecules, metal ions, surfactants, and polymers), (b) the shell layer, and (c) the core layer. The shell and core layer differ chemically in every characteristic. The core, the central portion, is by itself the nanoparticle (Singh et al. 2017).

14.3 Classification of Nanoparticles

Nanomaterials (NMs) can be classified by different looms. NMs are stratified based on their dimensions, type of material, and their origin.

14.3.1 Classification Based on Dimensions

Pokropivny and Skorokhod (2007) have given out a new classification for NMs based on the dimensions of the particle structure that included 0D, 1D, 2D, and 3D.

14.3.1.1 Zero-Dimension Nanoparticles (0D NPs)

Zero nanomaterials or 0D refers to the measurement of all the dimensions within nanoscales (no dimensions larger than 100 nm). The general representation of 0D nanomaterials is the nanoparticles.

14.3.1.2 One-Dimension Nanoparticles (1D NPs)

One-dimension system has been in use for decades. The utterance of "nano" has been allocated to refer the digit 10^{-9} (Hickey et al. 2013) which means one billionth of any unit that fallouts in the development of 1D NPs resembling a thin film or monolayers (range 1–100 nm in size) which is exploited in electronics; chemical and biochemical sensors (Alivisatos 2004; Kong et al. 2000); information storage system; pharmaceuticals; bioengineering; fiber and magneto-optic systems (Kong and Dai 2001; Cui et al. 2001); construction of nanowires, rods, tubes, belts, and ribbons; and nano-hierarchical models (Tolani et al. 2009; Wang 2000; Duan et al. 2001; Cui and Lieber 2001; Huang et al. 2001; Xia et al. 2003).

14.3.1.3 Two-Dimension Nanoparticles (2D NPs)

Two-dimension structures are not confined to the nanoscale. They usually have two dimensions exterior to the range that of nanometric size with a particular shape. 2D materials habitually exhibit platelike shapes and successive exploitation as building blocks for the manufacture of nanodevices (Jibowu 2016). Two of the dimensions

have potential application in the field of nano-containers, nano-reactors, photocatalysts, and as a template for other 2D structures. The most known 2D nanostructures are the carbon nanotubes (CNTs). Carbon nanotubes (CNTs) which are cylindrical hollow fibers consist of pure graphite surrounded by a hexagonal net of carbon atoms (Novoselov et al. 2004).

14.3.1.4 Three-Dimension Nanoparticles (3D NPs)

3D nanoparticles or bulk nanomaterials are not confined to nanoscale in any dimensions. These materials are differentiated and said to have three arbitrarily dimensions beyond 100 nm. Bulk nanostructures have gained a broad interest in research. 3D nanostructures are classifed as dendrimers (highly branched, star-shaped macromolecules), fullerenes (also named as carbon 60 (C60), resemble soccer ball), and quantum dots (Bhatia 2016).

14.3.2 Classification Based on Material Type

Current NPs are grouped into four material-based categories: organic, inorganic, carbon, and composite-based materials.

14.3.2.1 Organic-Based Nanomaterials

These types of NMs are of organic substance that is eco-friendly and nonhazardous. These are mostly preferred in drug delivery systems due to their unique property as a nanocapsule which is sensitive to thermal and electromagnetic radiation (Tiwari et al. 2008). The weak bonding (noncovalent) for self-assembly and design of molecules facilitates the transformation of organic NMs into the preferred formation, for example, dendrimers, micelles, liposomes, and NP polymers.

14.3.2.2 Inorganic-Based Nanomaterials

Inorganic NMs are more often than not made of carbon. They usually include metal ions such as aluminum (Al), silver (Ag), gold (Au), zinc (Zn), copper (Cu), cobalt (Co), cadmium (Cd), iron (Fe), and lead (Pb) and metal oxides, namely, iron oxide (Fe_2O_3), aluminum oxide (Al_2O_3), titanium oxide (TiO_2), zinc oxide (ZnO), silicon dioxide (SiO_2), cerium oxide (CeO_2), and magnetite.

14.3.2.3 Carbon-Based Nanomaterials

Commonly, these nanoparticles are made of carbon, hence known as carbon-based materials (Bhaviripudi et al. 2007). Carbon NMs are classed into graphene, CNTs, C60, carbon nanofibers, carbon black, and onions (Kumar and Kumbhat 2016).

14.3.2.4 Composite-Based Nanomaterials

These nanosized materials are multiphase NPs in combination with metal-, carbon-, and organic-based NMs that can combine any NP with neighboring NP or a combination of bulk-type materials (e.g., hybrid nanofibers) or added complex structure (e.g., metalorganic frameworks).

14.3.3 Classification Based on Origin

Based on either natural or incidental or synthetic source, the NPs are classified into natural and synthetic nanomaterials.

14.3.3.1 Natural Nanomaterials

In the natural world by either biological species or through anthropogenic activities, natural nano-objects are formed. NMs occur naturally through the Earth's spheres which constitute the total atmosphere, hydrosphere, lithosphere, and biosphere that cover up micro- and higher organisms, together with humans (Hochella et al. 2015; Sharma et al. 2015).

14.3.3.2 Synthetic Nanomaterials

Synthetic nanosized materials are also called engineered nanomaterials (ENMs) since they are formed by mechanical actions (manufactured) either by physical, chemical, or biological hybrid methods by humans. These are generally toxic to the environment where researchers are highly forecasting the risk behavior.

14.3.3.3 Incidental Nanomaterials

These are by-products incidentally produced from industrial processes such as engine exhaust, smoke from forest fires, welding fumes, and combustion processes.

14.4 Nanotoxicology

Nanotoxicology is an emerging new branch of bionanoscience that deals with the study of toxicity of structures smaller than 100 nm (nanomaterials) which affects both the environment and the human health as a result from manufacturing processes (engineered NMs), natural processes such as geological processes (volcanic ashes), atmospheric actions, and combustion practices (Haynes 2010; Maynard et al. 2011). Improvement in the fields of nanotechnology has benefits, but weighing the risk against benefits is needed and evaluating the level of toxicity is to be focused on reducing the risk assessed. Early million years ago, mankind and the living beings on Earth were claimed to be exposed to naturally produced NMs that result from the natural processes taking place every day around us (Nel et al. 2006; Buzea et al. 2007). NMs turned out from industrial and manufacturing practices by man have been probably toxic. They enter the ecosystem or the food web and tend to show direct risk for exposition. Few examples of natural and human-made NP by-products are soil erosions, ocean water evaporation, volcanic ashes, biogenic magnetite, quantum dots, catalysts, cosmetics, coating, consumer products, and building demolition, respectively. Also noticeable is that merging metals cause complex toxicity, which is not shown with single metals. Earlier in 1975, a study reported oxidative stress in asbestosis and cell structure disconfirmation due to the nanoparticle asbestos.

14.5 Nanomaterials and Biological System Interaction

Engineered NPs (ENPs) produced as a result of human activities furtively makes a way into the environment through water, soil, and air as a source. Application of NPs for green management intentionally instils or dumps ENP into the soil or aqua bodies. Nearly all nanoparticles are nondegradable and live longer than years in our surrounding environment (Navarro et al. 2008). This has consequentially engrossed an increasing alarm for all the stakeholders.

Commercially synthesized NPs have pioneered a way into our daily lives. One such example is the most widely used nanomaterial, ZnO, that has a leading application with industries and commercial productions such as products of personal care, ceramic goods, and paints (Brar et al. 2010; Blinova et al. 2010; Dechsakulthorn et al. 2008; Fan and Lu 2005). Another paradigm is the most common nanoparticle TiO_2; it is extensively used in food additives and drug delivery systems in personal care products (Ray et al. 2009; Kangwansupamonkon et al. 2009).

Living beings, especially humans, are exposed to these nano-objects through inhalation, dermis, blood circulation, ingestion, and translocation to various organs and tissues (Oberdörster et al. 2005a). The passageway of nanomaterials through cell membranes and other natal barriers causing cellular dysfunction is due to their tiny size of the so-called nanostructures (Nel et al. 2006; Xia et al. 2008). The typical example in the human body is the respiratory system which is an inimitable target for the NMs toxicity as it has a dual function of inhaling and gets complete cardiac output (Ferreira et al. 2013). The inhaled nanoparticles with the help of Brownian movement are put down in the alveolar region. As the surface area of the alveoli is high and has rigorous blood contact, the target system on the subject is more likely to be exposed to environmental influences (Maynard and Kuempel 2005; Aillon et al. 2009; Chidambaram and Krishnasamy 2012). The digestive stimulation due to ingestion of NPs in the digestive tract is because of the increase in macromolecular absorption due to the massive upload of nanoparticles (Hagens et al. 2007). The skin, the body's largest organ, is the first line of defense against external aggressors. The mechanism underlying the nanoparticle's entry into the dermis is that when in contact to the outside environment, the tiny particles are expected to mount around the hair follicles and cross the threshold keen into the body (Stern and McNeil 2008).

Studies have shown that most NPs do release reactive oxygen which in turn causes oxidative stress and inflammation by the reticuloendothelial system. The outcome on inflammatory and immunological systems may perhaps result in pro-inflammatory cytotoxic activity and oxidative stress in the lungs, liver, and brain, pre-thrombosis, and paradox effects on the circulating system (Ai et al. 2011). Nanoparticles are capable of reorganizing the protein concentration which depends on the size, twist, shape and surface charge, free energy, and functionalized groups. Due to this complex binding, adverse biological outcomes arise in the course of protein unfolding, fibrillation, thiol cross-linking, and reduced enzyme activity. Another instance is the discharge of toxic ions, while the thermodynamic traits of materials favor particles suspension in a biological surrounding (Xia et al. 2008).

Though few studies have addressed the toxicity effect of nanomaterials on animals and plant cells, the mechanism relating to the toxicological studies has not yet concluded. Silver (Ag) nanoparticles produced from consumer products in the dissolved form highly sediment in aquatic bodies exerting a toxic effect on marine organisms together with bacteria, algae, fish and daphnia (Navarro et al. 2008).

NPs have a propensity to amass in the sea and hard water and are very much powered either by specific type of organic matter or other biological particles herein freshwater. The state of distribution alters the ecotoxicity; however, several abiotic factors influence the dispersion. These factors are, namely, pH and salinity, and the existence of organic substances remains to be analytically examined as a part of ecotoxicological studies (Handy et al. 2008).

14.6 Evaluation of Toxicity

Evaluation of nanomaterial effect on biological organisms and ecosystem showed no general concord on techniques and protocols despite the many efforts done (Reineke 2012). Many tools have been established in evaluating the toxicity of NPs. As discussed already in the above sections, engineered nanomaterials (ENMs) have more odds for toxicity as testing these materials necessitate special attention and contemplation (Dusinska et al. 2015). EMEA and FDA in Europe and the USA regulated chemicals under the process of REACH (registration, evaluation, authorization, and restriction) for nanomedicine and pharmaceutical products (Dusinska et al. 2009, 2015; Seaton et al. 2010). Several tools have been in existence for testing toxicity, preferably using in vitro, in vivo, and in silico approaches. Characterization, bioavailability, and uptake of NPs and mechanism of toxicity should be evaluated stepwise. However, huge sets of data are required for budding and confirming different strategies in case of ENMS risk assessment; this normally is based on grouping and read-across approaches (Oomen et al. 2015). Strong and consistent data can be issued by using high-throughput methods. The use of high-throughput methods in testing ENM toxicity allows the testing of several ENMs at different concentrations, cells, and conditions exposed, reduces inter-experimental variations effects, and makes considerable savings in rate and time (Collins et al. 2017).

The measurement of environmental hazards due to NPs is taken for ecotoxicity test, an alternative tool framed for assessing intrinsic dangers of chemical substances which may be freed into nature (Crane et al. 2008). Methods for testing of NMs and their impact on the environment and living systems are assembled into four categories, namely, chemical and physical characterization, a microbiological assay using prokaryotes, in vitro and in vivo assays.

In vitro and in vivo studies generally are used to test the toxicity of chemicals and to know their primary mechanism, for example, oxidative stress, immunotoxicity, and genotoxicity. Some of the said methods are already established and approved by OECD guidelines. Nevertheless, methodologies concerning dosimetry, dispersion, short of washout, uptake, and ENMs interaction with cells and tissues are to be concerned.

14.6.1 Physicochemical Factors of Nanotoxicology

Physical and chemical characterization of nano-objects plays a crucial role in toxicity. The size and surface area of the nanostructures function as a key cause in the occurrence of some diseases, for example, respiratory diseases. Besides the size of the particle, features such as crystallinity, surface chemistry, oxidative stress, surface coating, porosity, purity, and the longevity of particles play a significant role in nanotoxicity (Ai et al. 2011).

14.6.1.1 Size

Toxicity of a particle lies basing on the size and chemical compounds. A drop in the size of nanosized objects results in enhancing the particle surface area. Consequently, a large number of chemical substances attach to the surface which in turn increases the reactivity resulting in increased toxicity (Linkov et al. 2008). An example of this type of mechanism was seen in mucus where the absorbed nanoparticles travel through tissues before reaching the bloodstream. A different study by Hyuk et al. showed 33% of 50 nm, 26% of 100 nm, and 10% of 500 nm in mucosal and lymphatic tissues of the intestine. Nanomaterials larger than 1 and 3 μm were seen as weaker and rare, respectively, in lymphatic tissues. The conclusion drawn by the researchers on particle size is that: (a) Nanoparticles <100 nm and not ≥300 nm are absorbed by intestinal cells. (b) The absorption of smaller NPs in the lymphatic tissue is greater than intestinal cell but cannot absorb particle size of 400 nm and above. (c) Nanomaterials below 500 nm are said to enter the circulatory system. Crossing the cell membranes reaching the bloodstream via many organs is because of their small size and larger surface-to-volume ratio than bigger nano-substances. Hence, this is the sole basis for the presence of more chemical molecules on the surface; this by reason gives the more toxic effect for small NPs than larger components of the same composition (Hyuk Suh et al. 2009).

14.6.1.2 Particle Surface Chemistry

Nanoparticles cover a slightly high proportion of surface atoms basing to their geometry, and this ratio as well depends on the particle size, porosity, surface coarseness, and smoothness. For example, the biocompatibility of nanoparticle is higher for porous than nonporous silica. Furthermore, the hemolytic activity of the porous silica is considerably lower than nonporous (Slowing et al. 2009). Another study showed higher toxicity levels in case of Ag nanosheets judged against nanospheres and nanowire; this is because reactions on the surface were known to have large defects (George et al. 2012).

The presence of high or no impurities shows an effect on toxicity levels for a nanomaterial. Changing electrical property may vary the toxic effect. A study demonstrated the cytotoxic reactions by NPs as a source depend merely on purity. For instance, zinc and copper oxide were the two NPs upshot (Xu et al. 2010). Ease of surface plays a part in nonspecific bindings that enhances cellular uptake of NMs and is futile in the reaction rate of NMS with cells.

14.6.1.3 Chemical Composition

Chemical constituents have a greater impact on NMs as they respond to other metals. Any modifications in the nanoparticle surface will reduce the toxicity. A case in point is the reduction of toxicity of nanoparticle super-paramagnetic iron oxide on the coating with pullulan (Singh et al. 2007; Clift et al. 2008; Oberdo 2010).

14.6.1.4 Dose-Dependent Toxicity

The amount or quantity at which a particle or a substance enters the biological system is defined as "dose." The dose is directly proportional to exposure or concentration of a particle in the appropriate medium (e.g., air, water, food, or soil) multiplied by the duration of contact. However, the dose whether low or high is harmful to health.

14.6.1.5 Aspect Ratio

Aspect ratio is defined as the ratio of length to the diameter of a particle or a substance. The higher the aspect ratio, the higher is the toxicity (Lippmann 1990). The best exemplar is the carbon-based nanoparticles (e.g., CNTs have high aspect ratio).

14.6.2 Nanoparticle Uptake

Uptake of NPs through barriers, for instance, the skin, blood–brain barrier, pulmonary mucosa, and placenta, can alter significantly with a decrease in size. Hence, toxicological data have to be acquired particularly for nanosized particles (Simko and Mattsson 2010; Schleh et al. 2012; Lehr et al. 2011). Reactivity boosts concurrently with the reduction of size and subsequently increased surface area. Surface area and composition robustly determine reactivity, dispersion, interaction with biological environments as well as cellular macromolecules, and as result toxicity of ENM (Warheit et al. 2008; Kunzmann et al. 2011; Dhawan and Sharma 2010). ENMs once taken have the potential to be deposited in any area of the body (Borm and Muller-Schulte 2006; Oberdörster et al. 2005b). This is mainly due to their unique factors: size and specific functionalization. NPs materialize in various shapes and also cover diverse modifications such as restricted transformations of the interface properties and modifying the dissolution and degradation by controlled changes of surface functionalization, routine stabilization in course of macromolecules absorption, utilization of oxygen, light or reducing agents from the particle surrounding in amendment of catalytic activity, and surface area enhancement for molecule adhesion by dissolution and recrystallization of reactive material (Nel et al. 2006).

14.7 Effect of Nanoparticles on Prokaryotes

Reports existing from the researchers show that NPs can conjugate with the biological species in nature, making the nanomaterials gain soluble properties that may have adverse effects on prokaryotic and other aquatic organisms. The interaction of

carbon NPs such as fullerenes and carbon nanotubes with a biological system is well familiar mainly with DNA, RNA, phospholipids, and proteins (Ke and Qiao 2007). Kang et al. (2007) were the first to give out the connection between the break of the bacterial cell membrane and cell death with purified single-wall carbon nanotubes (SWNTs) using antimicrobial activity. Similarly, studies on the toxicity of CNTS using *Staphylococcus aureus* and *S. warneri* illustrated antimicrobial activity, inhibition of microorganism connection, and biofilm arrangement (Narayan et al. 2005). The study of Ghafari et al. (2008) reported the inability of *T. thermophila* (protozoa) to swallow and digest their prey (bacterial species), permitting free movement of SWNTs in the food chain. From the statement, it is proved that CNTs have an adverse effect on the aquatic system that eventually leads to ecological imbalance. The consequence of nanoparticles on microbes is a lot more widespread and assorted than for the plants, invertebrates, and vertebrates (Oberdörster et al. 2007).

14.8 Prokaryotes as Model Organism

Model organisms rather known as non-human species have turn out to be essential in biological study processes by many researchers, with anticipation that the discoveries ended in the organism model might provide insight to understand the specific phenomenon of organisms and can be studied and used to gain knowledge of other organisms or other species within their own variety giving a central pose in evolutionary development. Model organisms are in vivo models with typical characteristics including generation time, easy manipulation, accessibility, genetics, possible economic advantage, and management of mechanisms (Ankeny and Leonelli 2011). Common model organisms in use are prokaryotes, plants, protists, fungi, and animals. On one hand, microbes especially bacteria constitute as a major domain of prokaryotic organisms, with an ability to stay alive in any extreme circumstances (i.e., from optimal to extremely high environmental conditions). On the other hand, the simplest bacteria have a significant competence either to mobilize or immobilize and also is capable of reducing metal ions at the nanometer scale (Sharma et al. 2018). Synthesis of NPs such as Au, Ag, Pt, Pd, CdS, TiO_2, Fe_3O_4, and so forth can be potentially synthesized by cell biomass and cell extracts of bacteria (Iravani 2014). Few microbes, namely, *magnetotactic* and *S- layer bacteria*, are capable of synthesizing inorganic materials. A choice of bacterial species, for instance, *Bacillus cereus, E. coli, B. subtilis,* and *P. aeruginosa* has been detailed in support of removing silver, cadmium, copper, and lanthanum from solution and also including a binding facility of metallic anions and cations (Mullen et al. 1989). The impact of nanotoxicity on microorganism remains in its infancy stage. Before testing the toxicity of nanomaterials on microorganisms, it is indeed crucial to understand the physiochemical properties of the so-called nano-object (Niazi and Gu 2009).

Different perspectives are in use for evaluating the nanotoxicity with prokaryotic cells. They are, namely, disk diffusion toxicity (Ruparelia et al. 2008), minimum inhibitory concentration (Qi et al. 2004), colony viable count, viability assay for

cells (Rodea-Palomares et al. 2009a, b, 2010), quantification (Cuahtecontzi-Delint et al. 2013), superoxide dismutase activity or luminescence quantification (Lyon et al. 2008; Dumas et al. 2009), and microarray hybridization assay for gene expression (Yang et al. 2009). The best and better conditions for nanomaterial determination and standardization for toxicity are pH; ion presents (cation/anion); micro- and macronutrients such as amino acids, vitamins, sugars, lipids, and nucleotides; bacterial species in use; and temperature variations. Both gram-positive and gram-negative bacteria were used as models for toxicity evaluation.

14.8.1 Prokaryotic Models in the Evaluation of Silver Nanotoxicity

The use of Ag in the form of silver nitrate ($AgNO_3$) as an antimicrobial agent has been recognized from centuries (Klasen 2000). Since then, the $AgNO_3$ has been exploited widely in many applications, for example, in medical and industrial products and also in domestic products such as cleaning agents, clothing, and cosmetics. Due to its varied application directly or indirectly in the living system and environment, it is mandated to determine the toxic levels. The antibacterial activity of Ag NPs depends purely on the physicochemical characterization of the substance. That is to say, Ag NPs that are more soluble are more toxic and thus are likely to release more silver ions to be bonded to sulfhydryl groups coupled with protein and low molecular weight antioxidants such as glutathione. However, by contrast, Ag NPs of less soluble also show a toxic effect by way of oxidative stress (Yang et al. 2011).

Aerobic conditions increase silver nanoparticle suspension as a result of nanomaterial oxidation (Liu and Hurt 2010; Molleman and Hiemstra 2015). This phenomenon enhances the antibacterial activity of AgNPs by the release of ionic silver (Xiu et al. 2012) and the development of ROS (reactive oxygen species) (Joshi et al. 2015). Other effects that enhance antimicrobial activity are the disruption of cell membranes due to NP membrane interaction; this, in turn, activates the uptake of silver ions freely (Taglietti et al. 2012; Bondarenko et al. 2013). Antibacterial susceptibility of silver nanomaterial is species specific (Morones et al. 2005; Tamboli and Lee 2013) with gram-negative bacteria more resistant than gram-positive microbes. Echavarri and his colleagues in their study recommended the use of natural marine microbes *Cellulophaga fucicola, Pseudoalteromonas aliena,* and *Streptomyces koyangensis* as model organisms for assessing nano-silver particle (Echavarri-Bravo et al. 2017).

A study by Bowman et al. (2012) using *E. coli*, a standard prokaryotic model, supported the statement that the toxicity of Ag NM is due to the suspension of Ag ions from the surface of the particles. Bowman et al. analyzed the toxicity of Ag NM in two different ways: one is the mortality curve based on mass concentration and total surface area of particles demonstrating the dose response, and the other way is surface area-based toxicity. The conclusion drawn from the first parameter in Bowman study showed (a) toxicity to bacteria is dependent purely on particle size, with toxicity increasing as the size of the particle decreases basing on mass

concentration analysis and (b) a diverge conclusion when assessed basing on total surface area, showing no or little variation in toxicity among particles of varied sizes and with same surface area. When the same species are tested for surface area-based toxicity, it sighted that the total exposed surface area of the particle is the source driving toxicity, implying that dissolution of Ag^+ from the surface is causing toxicity. This statement was explained and supported by Radniecki et al. (2011) with *Nitrosomonas europaea* (gram-negative bacteria) as a model organism.

Few other species apart from the above were reported as model organisms in evaluating silver nanoparticles. They are *Shewanella oneidensis* (Suresh et al. 2010), nitrifying bacteria (Choi and Hu 2008), and *P. putida* (Fabrega et al. 2009) with standard protocols such as live/dead viability assay using flow cytometry, cytotoxicity assay by spectrophotometer at 600 nm, and also accordingly by disk diffusion method.

14.8.2 Prokaryotic Models in the Evaluation of Inorganic Nanomaterials

Zinc oxide (ZnO) is one of the currently used compounds in the food and drug administration. It plays an important role in treating zinc deficiency (Lopes de Romana et al. 2002). Some studies concluded antibacterial activity of ZnO which characterizes ROS generation (Sawai 2003; Sawai and Yoshikawa 2004) and also is ably a strong component resisting microorganisms (Hirota et al. 2010). However, by contrast, ZnO as a nanoparticle has a toxic effect on living organisms. The antibacterial activity by microtiter plate method with E. coli, *P. aeruginosa,* and *S. aureus* is tested for ZnO nanotoxicity (Premanathan et al. 2011). ZnO toxicity assessment using *Salmonella typhimurium* as the model organism was reported by the use of the Ames test (Yoshida et al. 2009) and cytotoxicity assay (Wahab et al. 2010). Other microbial species, namely, *Streptococcus agalactiae* (Huang et al. 2008), *Vibrio fischeri* (Mortimer et al. 2008; Heinlaan et al. 2008), *Mycobacterium smegmatis, Shewanella oneidensis, Cyanothece* (Wu et al. 2010), *Thalassiosira pseudonana, Chaetoceros gracilis,* and *Klebsiella pneumonia* (Wahab et al. 2010), as model organisms were shown to be detailed in assessing the toxicity of ZnO nanomaterial via cytotoxicity assay, luminescence inhibition test, and growth inhibition assay.

Nano-objects titanium oxide (TiO_2) has also been used as an antibacterial agent despite its particle size, but this activity is enhanced when carried in nanoparticulate form. TiO_2 nanotoxicity using various methods such as cell viability assay, lipid peroxidation assay, cellular respiration determination test, cytotoxicity assay using spectrophotometer, and Ames test was studied using prokaryotic bacterial species specifically *E. coli* (Maness et al. 1999; Adams et al. 2006), *S. typhimurium* (Kumar et al. 2011), *S. aureus* (Mortimer et al. 2008), *B. subtilis* (Adams et al. 2006), and *Cupriavidus metallidurans* (Simon-Deckers et al. 2009).

E. coli and *S. aureus* as a model organisms were used in evaluating nanotoxicity for magnesium oxide using halo test and conductance assay for cytotoxicity (Sawai et al. 2000), standard plate count method, and also spectroscopic method (Jones

et al. 2008). Other inorganic nanomaterials evaluated for toxicity using prokaryotic bacterial species are SiO_2 (*E. coli* and *B. subtilis*), Al_2O_3 (*E. coli, C. metallidurans,* and *S. typhimurium*), and Co_3O_4 (*S. typhimurium*).

In case of CuO, both prokaryotic algae (*Microcystis aeruginosa*) and prokaryotic bacteria (*S. typhimurium* and *S. aureus*) (Wang et al. 2011; Pan et al. 2010; Jones et al. 2008) were used as models for toxicological evaluation for nanotoxicity.

14.8.3 Prokaryotic Models in the Evaluation of Carbon-Based Nanotoxicity

Carbon 60 (C60), also known as fullerene, has been reported to slow down the antimicrobial activity (Fortner et al. 2005). However, this statement remained insufficient to prove that all nanoparticles have an antimicrobial activity or all NPs are toxic to an organism in the environment. C60 NP toxicological evaluation by *B. subtilis* using spectroscopy method at 600 nm for cytotoxicity was studied by Lyon et al. (2006). The study concluded that C60 exhibited antimicrobial activity with a minimum inhibition concentration of 0.5 ± 0.13 mg/L.

Carbon nanotubes which are the most widely used carbon nanomaterials are actively engineered nanomaterials. The NM toxicity is evaluated via dead discrimination assay by flow cytometry using PI dye with *E. coli* species as a model organism. The study gives an outcome where different shapes of CNTs exhibit growth inhibition (Kang et al. 2008).

14.9 Conclusion

Though nanomaterials have been beneficial with its increased application in industrial and medical health, it is shown to have harmful effects on the environment and life forms. Over a decade, extensive exploration on nanomaterials and its consequence turned out to be a major challenge. Outlining the mechanism or the exact process of the nanoparticles causing toxicity is still unclear, and estimating the overall scenario remains difficult.

This chapter, in detail, illustrated an overview of the nanoparticle origin and classification, its toxicity and effect on the biological system, and the use of the prokaryotic model for assessing the toxicity. Prokaryotic models, especially bacteria, have been a significant module due to their unique properties such as low production time, simple structure, nonpathogenic nature, and functional organization. Many studies have evaluated the toxicity of nanoparticles with microorganisms as they are the first source in the food web of all known ecosystems and also could help to extrapolate the understanding of nanomaterials on the environment and higher organisms. In order to study more about the biological toxicology of NPs, the particle characterization, uptake, and different assays are to be well learned to help assess toxicity and synthesize many additional green nanoparticles for the betterment of the society and environment.

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Evaluation of Toxicity of Nanoparticles Using Cell Lines

15

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Abstract

Nanoparticles range from 1 to 100 nm in size, and though the size is in nanometers, its application is in broad areas such as biomedical, industry, food, and cosmetics. With increasing utilization, the toxicity of the nanoparticle has been a great concern to evaluate their potential. To use the nanoparticle effectively, it is necessary to know the toxicity of NP and different evaluation methods and characteristics. "Dosimetry: Too complicated to consider, too important to ignore" as stated by Dr. Philip Demokritou in the seventh International Nanotoxicology Congress; Dosimetry is one of the important factors besides the surface area and high reactivity to determine the toxicity nature of the NP. Every NP may not show the same toxicity; it varies with the material it is made up of, site of its action, and exposure routes. This chapter addresses the current knowledge of evaluation of nanosized particles toxicity using in vitro derived cell lines from different literature, as a primary step for screening their toxicological effects, which contributes to the further development and advancement of nanotechnology on a safe, unbiased level. The in vitro derived cell lines however does not ensure the same cell habitat as in the tissue, as nanoparticles interact with proteins and physiological barriers, immune response in the tissue has a more complex environment. Hence, these in vitro evaluation methods give us a base for further considering the nanoparticle potential and its toxicity.

Keywords

Toxicological effects · Biological systems · Reactive oxygen species · Genotoxicity · *In vitro* assays

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15.1 Introduction

A promising research interest in the delivery of biomolecules using particular delivery systems, which act as carriers for small and large molecules, especially for drugs is being carried on from the past few years. These delivery systems are not only found to be efficient for transporting drug molecules but also help in improving the pharmacokinetic and pharmacodynamic properties (Mohanraj and Chen 2006). Drug delivery is a procedure of administering a pharmaceutical compound to acquire a therapeutic effect in humans. The development of drugs is expensive, time consuming, and labor intensive (Tiwari et al. 2012). Various drug administration methods are introduced, likely with targeted, controlled, sustained delivery of pharmaceutical products (Tiwari et al. 2012). Among the different delivery systems, nanoparticles have played a promising role in accomplishing the need in every field of science. For the past 50 years, nanotechnology is into commerce, and many new directions are in progress. Within the past 15 years, nanoparticles revolutionized different delivery mechanisms. The first report of adverse effects of nanoparticles has been published within the last 10 years from in vivo and in vitro studies (Takenaka et al. 2001; Oberdörster et al. 2004; Bermudez et al. 2004; Lam et al. 2004; Geiser et al. 2005; Oberdörster et al. 2005a, b; Shvedova et al. 2005; Elder et al. 2006; Mercer et al. 2008). A knowledge gap between the technological progress and potential hazards of new developing nanotechnology creates a mystifying experience (Schulte et al. 2008). To resolve this, one needs to evaluate the toxicity of nanoparticle through different in vitro methods, and they are discussed in this chapter for a proper assessment of nanoparticle safety.

15.2 Nanoparticles

Nanoparticles are particulate dispersions or solid particles with a size range of 10–100 nm (Jeevanandam et al. 2018). The chemical synthesis of metallic nanoparticles dates back from fourteenth and thirteenth century BC, where the Mesopotamians and Egyptians started making glass using metals—beginning of nanoparticle metallic era (Schaming and Remita 2015). The Lycurgus cup is a fourth-century Roman glass cup made up of dichroic glass which displays various colors: green when light is passing from the front of the cup and red when passing from behind (Leonhardt 2007). These cups contain silver–gold alloy nanoparticles with a 7:3 ratio in addition to 10% of Cu (Jeevanandam et al. 2018). Clay minerals of few nanometers in thickness are best examples of natural nanomaterial usage since ancient times (Rytwo 2008). Michael Faraday reported the colloidal AuNP synthesis in 1857, which is known as the first scientific description report of nanoparticle preparation in the scientific arena (Jeevanandam et al. 2018).

An ancient history of nanoparticle and its benefits represents an active research area and a technoeconomic sector with full expansion in many domain applications. The Standard British Institution (Jeevanandam et al. 2018) has given the following definitions for scientific terms that have been used:

- Nanoscale: Measurement of approximately 1–1000 nm in size.
- Nanotechnology: Manipulation and control of matter on a nanoscale dimension by using scientific knowledge of various industrial and biomedical application.
- Nanomaterial: Material with any external or internal structures on nanoscale dimension.
- Nanoparticle: A nano-object with three external nanoscale dimensions. The terms nanorod or nanoplate are employed when the NP of the longest and shortest axes length of a nano-object is different.

The following nanoparticle types are obtained by a different method of preparation:

- Nanocapsules: A system in which drug is confined to the cavity, covered by a unique polymer membrane
- Nanospheres: A matrix system in which drug is physically and uniformly dispersed
- Nanoparticle: A system that is coated with a hydrophilic polymer-like PEG (polyethylene glycol)

Liposomes, being potential carriers with inherent problems such as low encapsulation efficiency, poor storage stability, and leakage of water-soluble drugs in the human environment led to a scope for designing nanoparticles as delivery system over liposomes. NPs are found to be the best fit in drug delivery systems with more flexible characteristics (Mohanraj and Chen 2006). Characteristics of nanoparticles in drug delivery systems include:

- Particle size and surface characteristics of nanoparticle can be easily manipulated.
- They control and sustain the release of drug at the site of localization, altering organ distribution of drug and subsequent clearance of drug to achieve high therapeutic efficacy and reduce the side effects.
- Site-specific targeting by attaching a ligand to the surface of particles or use of magnetic guidance.

Nanoparticles have gained prominence in technological advancements due to their physiochemical characteristics such as melting point, thermal conductivity, light absorption, catalytic activity, and scattering of light which assist in improving the performance of bulk counterparts (Jeevanandam et al. 2018).

Nanoparticles are prepared from a variety of materials such as protein, polysaccharides, and synthetic polymers. Nanoparticle has been prepared mostly by three common methods (Mohanraj and Chen 2006):

- Dispersion of preformed polymer
- Polymerization of monomer
- Ionic gelation or coacervation of hydrophilic polymer

Other methods such as supercritical fluid technology (Reverchon and Adami 2006) and PRINT

(particle replication in no wetting templates) are also used for nanoparticle preparation (Khan et al. 2017).

- Top-down synthesis is a destructive approach in which large molecules are broken down into smaller units and converted into nanoparticles.
- Bottom-up synthesis is a constructive approach in which the nanoparticles are obtained from smaller molecules.

15.2.1 Types of Nanoparticles

A nanoparticle is generally classified based on their material, size, morphology, and physicochemical properties. Based on their physicochemical properties, nanoparticles are classified as (Khan et al. 2017; Jeevanandam et al. 2018):

- **Carbon-based nanoparticle:** NPs made up of carbon, as hollow tubes, ellipsoids, or spheres such as fullerenes (C60) carbon nanotubes, carbon nanofibers, carbon black, graphene, and carbon onions. Laser ablation and chemical vapor deposition (CVD) are some of the important production methods for carbon-based nanoparticles.
- **Inorganic-based nanoparticle:** NPs are made up of metal and metal oxides. These NPs may be synthesized from different metals such as Au or Ag nanoparticles, metal oxides such as TiO_2 ZnO, and semiconductors such as silicon and ceramics. They have a unique optoelectrical property. The size, shape, and facet-controlled synthesis of metal nanoparticle are critical in the current cutting-edge material (Dreaden et al. 2012).
- **Organic-based nanoparticle:** Nanoparticles are made up of organic matter, excluding inorganic or carbon-based material. The noncovalent interaction for self-assembly transforms organic nanoparticles into structures such as dendrimers, liposomes, micelles, and polymer nanoparticles. Lipid-based nanoparticles are 10–1000 nm of diameter in range. Surfactants and emulsifier stabilized the external core of nanoparticles.
- **Composite-based nanoparticle:** Nanoparticle with multiphase, with one phase on nanoscale dimension that may combine with other NPs or with large-type materials such as hybrid nanofibers.

Based on the origin, the nanoparticles are classified (Jeevanandam et al. 2018) into:

- **Natural nanoparticle:** They are produced in nature by biological species or anthropogenic activities. These nanoparticles are naturally present throughout the earth's sphere (hydrosphere, atmosphere, lithosphere, and biosphere).
- **Synthetic (engineered) nanoparticle:** They are produced by engine exhaust and smoke synthesized by physical and chemical, biological, or hybrid methods. Various risk assessment strategies are highly helpful in forecasting the behavior of synthetic nanoparticles in various environmental media. New schemes have focused on synthesizing other semiconductors (SCs) to avoid toxic ion-generating elements such as Se, Cd, and As and also to avoid the low availability elements (e.g., Te, Ga, and In) (Thomas et al. 2011).

15.2.2 Applications of Nanoparticles

Nanoparticles are effectively utilized in multiple domains. The key properties of nanoparticles designated them as a vital delivery system, in medications, where a broad scope of research on mechanism of its action is necessary. The following are some of the important applications in various fields:

- **Drugs and medication.** In field of medicine, a high interest in nanoparticles to deliver drugs in low dosage, high therapeutic effects, and negligible side effects and to improve patient compliance (Alexis et al. 2008). Superparamagnetic iron oxide nanoparticles with surface chemistry was used for in vivo applications as MRI contrast enhancement, tissue repair, cell separation, and for many more applications (Khan et al. 2017).
- **Materials and manufacturing.** In material science, nanocrystalline acts as a good substance, as their properties deviate in a size-dependent manner. Resonance energy transfer (RET) consists of noble metal nanoparticles and organic dye molecules and is important in material science and biophotonics.
- **Environment.** Nanoparticles have increased their scope in environment protection owing to their its eco-friendly characteristics. They are widely used in sensors for environment prediction, remediation of materials, contaminated with hazardous substances, and photodegradation (Khan et al. 2017). Nanoparticles are involved in degradation process in fluorescence and optical fields (Rogozea et al. 2016; Olteanu et al. 2016a, b).
- **Energy harvesting and mechanical industries.** The nonrenewable resources such as fossil fuels, a typical issue is in the synthesis and storage of energy; using nanoparticles to generate energy is widely utilized in photoelectrochemical (PEC) and electrochemical water splitting method (Avasare et al. 2015; Ning et al. 2016). In energy storage, different applications to reserve energy in nanoscale as nanogenerators are available (Greeley and Markovic 2012; Liu et al. 2015). In mechanical industries, nanoparticles are involved in tribological

properties of materials, to enhance the mechanical strength of polymer matrix and metals. Nanoparticles are also involved in the lubrication, coating, and resistance of metals (Khan et al. 2017).

15.2.3 Toxicity of Nanoparticles

Besides tons of nanoparticles enter the environment, and very little is known about its possible interactions with biological systems, nanotoxicology has emerged as a new discipline to investigate the potential adverse effects of nanoparticles (Bakand et al. 2012). Besides many applications in different domains, several kinds of toxicities are associated with NPs (Khan et al. 2017; Khlebtsov and Dykman 2010, 2011). Different types of nanoparticles are available in the bioapplications with early acceptance and rapid progress of nanobiotechnology. Even then, severe health effects that occurred due to prolonged exposure of humans to nanoparticles have not been established yet (Khan et al. 2017). The environment exposure of nanoparticles seems to increase in the future and thus the toxicity. The state of nanoparticle dispersion will alter the ecotoxicity and many factors of abiotic influence such as salinity, presence of organic matters, and pH (Handy et al. 2008). Health hazards of nanoparticles are always a concern due to their extended use and discharges to the natural environment in order to make it more environment friendly and more reliable (Khan et al. 2017). It is necessary to gain basic knowledge about nanotoxicology to overcome their toxicity efficiently. The toxicity of nanoparticles is majorly associated with their physiochemical properties, affecting their behavior in biological systems (Seaton 2006).

Existing and possible toxicities of nanoparticles are associated with their different characteristics such as small size distribution, large surface area, surface characteristics, insolubility, and aggregation. Modification in these characteristics would allow a convenient, efficient, and safe method to employ in major domain applications.

Nanoparticle properties are unique characteristics which are related to their synthesis (Jeevanandam et al. 2016).

Different data from the literature for the toxicological studies reveal that nanomaterial toxicity depends on various other factors, namely:

- **Dose and exposure time effect** – The number of nanoparticles that penetrate cell directly varies with its molar concentration in the medium multiplied with its exposure time (Buzea et al. 2007).
- **Particle size and shape effect** – Nanoparticles exhibit a size- and shape-dependent different levels of toxicity at aspect ratio (Jeevanandam et al. 2018).
- **Surface area effect** – Toxicity of nanoparticles increases with decreasing particle size and increasing surface area (Jeevanandam et al. 2018).

- **Crystal structure effect** – Nanoparticles exhibit a different cellular uptake, subcellular localization, and oxidative mechanism based on crystal structure effect (Jeevanandam et al. 2018).
- **Surface functionalization effect** – This effect of nanoparticles has an effect on its translocation and its oxidation processes (Oberdörster et al. 2005a, b; Sayes et al. 2004).
- **Pre-exposure effect** – Considerable cellular phagocytic activity at lower nanoparticle exposure time (Buzea et al. 2007).

Besides these, there are other characteristics and properties which affect the toxicity nature of nanoparticle. These engineered nanoparticles are established by humans and are assumed to have a different effect. Each type of engineered nanoparticles (TiO_2 – titanium dioxide) has severe to minimal biological effects which are of great concern on the usage of nanoparticles (Schulte et al. 2008).

The above information reveals the cause of the toxic effect of nanoparticles. It is of prior importance to know the action of the nanoparticles in biological effect in order to estimate its potentiality. The nanosize of a nanoparticle itself causes several adverse effects as they are similar to the size of natural proteins and can get access to the nucleus (in vivo case) and transfer across the placental barrier of pregnant mice (Gu et al. 2009; Chu et al. 2010). In this manner, a nanoparticle can affect the homeostasis. Some of the patterns of nanoparticles toxicity include oxidative stress, inflammation, inhibition of cell death and cell division, age, and genetic damage (Thanh and Green 2010; Verma and Stellacci 2010; Mironava et al. 2010; Lanone et al. 2009). Among these mechanisms, few are discussed with some details:

- **Reactive Oxygen Species**
- The generation of reactive oxygen species (ROS) is found to be either harmful or protective biological interaction based on its levels and further effects. They are reactive species of molecular oxygen and are key signaling molecules for homeostasis and cell signaling. They are generated extrinsically and intrinsically within the cell. Different ROS molecules contain a pool of oxidative species such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and hydroxyl radical ($OH^{\cdot}$) (Manke et al. 2013). ROS generation happens regularly when a cell is under stress such as high temperature, pressure, and improper homeostasis leading to active oxygen-containing molecules. $O_2^{\cdot}$ is generated by molecular oxygen, which is a primary ROS by one electron reduction catalyzed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Multiple reductions of oxygen may lead to H_2O_2 or $OH^{\cdot}$ by dismutation and metal-catalyzed Fenton reaction, respectively (Vallyathan and Shi 1997; Thannickal and Fanburg 2000). The sources where the ROS can be generated are an inflammatory response, mitochondrial respiration, and peroxisomes, while engineered nanoparticles are known as the exogenous ROS inducers (Manke et al. 2013). Different types of nanoparticles inducing metal oxide particles induce ROS as the main mechanism of cytotoxicity (Risom et al. 2005). ROS influence further intracellular calcium concentrations and modulate

cytokine production by free radical generation (Li et al. 2010; Oberdörster et al. 2005a, b). Most of the cells can resist the ROS generation for a limit beyond the concentration with the increase of time of exposure which results in the cell damage (Soenen et al. 2011).

- The large surface area of nanoparticles and its surface molecules result in massive oxidizing capabilities. Nanoparticles generate ROS by different mechanisms (Pisanic et al. 2009):
  (a) ROS generation on exposure to an acidic environment – Nanoparticles in the acidic environment of lysosomes generates ROS by direct reactivity of the surface coating, and degradation of coating leads to the direct interaction of acidic media on metal surface or by degradation of whole nanoparticles resulting in synthesis of ions (Fe^{2+}, Cd^{2+}) inducing ROS generation (Stroh et al. 2004; Jain et al. 2018).
  (b) ROS generation on interaction with cellular organelles – Nanoparticles interacting with mitochondria result in the deregulation of electron transport chain of oxidative phosphorylation (Soto et al. 2007).
  (c) ROS generation by NADPH oxidase – Nanoparticles interact directly with NADPH oxidase, resulting in ROS generation in immune cells (Pisanic et al. 2009).
  (d) ROS generation on interaction with cell surface receptors – Nanoparticles interact with surface receptors leading to its activation triggering intracellular signaling cascades, resulting in stress response gene activation and thus ROS generation activation and change homeostasis of the cell (Pisanic et al. 2009).
- As ROS play a major role in the toxicology of nanoparticles, the evaluation of elevated ROS level is of prior importance in toxicity evaluation methods of nanoparticles.
- **Cytoskeleton and Cell Morphology Defects**
- Nanoparticles that occupy the cell lead to the alteration in its morphology or the structure of cytoskeleton (Soenen et al. 2009, 2010). Different effects in disorganization of a cell cytoskeleton are observed based on the coating of inorganic nanoparticles (Gupta and Gupta 2005). The actin and tubulin proteins of human umbilical vein endothelial cells (HUVECs) are disrupted considerably, which decreased the capacity of HUVEC for vascular network formation on nanoparticles exposure (Wu et al. 2010). More attention is focused on the effect of nanoparticles on cytoskeleton and cell morphology as it leads to inflammation, affecting the reliability of a nanoparticle. The effect of nanoparticles on cell decreases with its concentration, and therefore, no adverse effects are observed at low concentration; a variety of nanoparticles are to be tested to evaluate the maximum loading capacity without any adverse effect. It is also necessary to evaluate the secondary effects of cytoskeleton disruption and morphology by a variety of nanoparticles to use them efficiently (Soenen et al. 2011).
- **Genotoxicity**

- The size of the nanoparticles makes it more possible to enter the nucleus and interact with the nucleoproteins leading to adverse effects. Nanoparticles interfere with the cellular homeostasis resulting in a cascade of mechanisms such as:
  (a) High levels of ROS – ROS generation by nanoparticles induces point mutations leading to single- or double-strand breaks.
  (b) Perinuclear localization of nanoparticles – nanoparticle localized in perinuclear space by loaded lysosomes affecting the molecular processes of cell (transcription and translation involving with disruption in the protein synthesis and modifying gene expression).
  (c) Alteration in homeostasis – leaching of metal ion to cell cytoplasm through complexes (e.g., a divalent metal transporter) resulting in degradation of messenger RNA.
  (d) Interacting with cell surface receptors – activation of receptor and triggering signaling cascades as intracellularly, altering activation status.
  (e) Cellular stress induced by nanoparticles – ROS generation by nanoparticles induces stress indirectly affecting gene expression pattern and activation of repair genes (Soenen et al. 2011).
- Most of the nanoparticle genotoxic details are to be known. The genes involved in regulation and repair are to be evaluated for the nanoparticle toxicity for most of the biological applications. Nanoparticle can be transported to the cell interior, where it is active, and it should not induce any adverse toxic effects in the cell (Soenen et al. 2011).
- **Interaction with the Biological Molecules of Cell**
- The equivalent size of the proteins and nanoparticle seems to be an issue in interacting or misleading the cell more often, for nanoparticles as a cellular protein. When nanoparticles enter the cell, the surface charges favor the binding of available proteins, leading to protein corona (Cedervall et al. 2007). This resemblance of nanoparticle with the cellular protein affects its bioavailability by the attack of the immune system as foreign material to eliminate from the body. Besides proteins, the nanoparticles are found to interact with the lipid molecules based on the surface charge, creating a channel on cell membrane inducing a cytotoxic effect (Lin et al. 2010).

15.3 Evaluation of Toxicity

With the growing commercial interest of nanoparticle, minimal research interest is focused in evaluating the potential adverse effects of the engineered nanoparticles (Manke et al. 2013). The assessment of NP safety has been critical due to variations in:

- Types of nanoparticles (Soenen and Cuyper 2010)
- Stabilizing coating agents (Clift et al. 2009)
- Physiochemical parameters of nanoparticles (diameter, topography, surface charge) (Verma and Stellacci 2010)

- Incubation conditions such as concentration and time (Mironava et al. 2010)
- Type of assay used (Monteiro-Riviere et al. 2009)
- Type of cell used (Lanone et al. 2009)

Standardization of protocol is necessary to understand and compare the generated data from different literatures regarding the toxicity of nanoparticles. Cell viability is quite a good indication for the safety and efficacy of nanoparticle and is usually accomplished by different assays such as (Soenen et al. 2011) (1) MTT assay; (2) lactate dehydrogenase assay (LDH), trypan blue, propidium, iodine assay (to check cell membrane integrity); and (3) fluorescent annexin V (apoptosis indicator); many other assays are generally used to check the homeostasis of the cell. The results of one assay cannot be compared to another assay as each is performed on its standard parameter (Soenen and Cuyper 2009). Assays are to be performed with safety, precaution, and general controls to be included, as nanoparticles interact with components of the assay (Monteiro-Riviere et al. 2009). Animal assay, the test performed by using cell lines, is performed by routine test guidelines, and more knowledge is essential to know about the potential toxicity of vast nanoparticles and its associated complexity (Bakand et al. 2012). More business communities and research organizations continue to invest in nanoparticles, to develop an alternative test system to characterize the toxicity profile of nanoparticles (Bakand et al. 2012). Besides, in vitro models are expanding faster for evaluation in a simple, quick, and least expensive way; but the results cannot replace the in vivo studies of compound toxicity (Bakand et al. 2012). In vitro test using cultured cells generates more toxicity data than in vivo models, but high standardization is required (Blank et al. 2009). Hence, for toxicology studies, in vitro test systems with both human- and animal-based cellular needs are employed for a better evaluation. Toxicity is assessed by characterizing shape, size, and structure of nanoparticles, by high-resolution imaging techniques: transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Drobne 2007). Nanomaterial characteristics, such as high chemical bioactivity and reactivity, cellular as well as tissue and organ penetration ability, and bioavailability of nanoparticles, show both positive and negative effects on a biological environment. Thus, certain regulations are implemented by different government organizations to prevent the risk of using nanoparticles (Jeevanandam et al. 2018). There is no internationally approved protocol for manufacturing, handling, testing, and evaluating the impact of nanoparticles (Jeevanandam et al. 2018). The European Union and United States of America have regulatory and guideline legislations to control risks associated with nanomaterials. In the United States, the regulatory agencies such as Food and Drug Administration (FDA), The United States Environmental Protection Agency (USEPA), and Institute for Food and Agricultural Standards (IFAS) are associated with standard protocols to deal with the risks of nanoparticles (Jeevanandam et al. 2018). The European Medicines Agency (EMEA) and United States Food and Drug Administration (USFDA) regulate the medical usage of hazardous nanomaterials. These regulations help to control the usage of nanomaterials and nanoparticles and to determine the need for evaluation of toxicity of a nanoparticle.

15.4 Methods for Toxicity Evaluation of Nanoparticles Using Cell Lines

Cell culture studies are involved in awaking the knowledge of how nanoparticles react to the body. In comparison with in vivo methods, in vitro studies are less ethical, easy, fast, reliable, and less expensive to perform (Lewinski et al. 2008). Different assays are performed to check the toxicity of nanoparticles based on the characteristics such as the type of cell used in the assay and the type of toxicity/effect of the particle to be evaluated. As the nanoparticles are capable of absorbing dyes and remain in the redox state, a variety of in vitro assays are found to be efficient ways of testing nanoparticles toxicity using cell lines. Most of the test results or cell deaths are measured by the colorimetry (Lewinski et al. 2008).

Different cell lines are used for the in vitro assays. Typically, the cell cultures of human cell lines are grown in optimum conditions of 37 °C, 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Huo et al. 2015). A variety of human cell lines are used in in vitro assay for toxicity evaluation of nanoparticles such as human bronchial epithelial cells (HBE), human umbilical vein endothelial cells (HUVECs), human hepatocellular liver carcinoma cells (HepG2), human dermal fibroblasts (HDF), human monocyte–macrophages, human epidermal keratinocytes (HEK), and many more. Each cell type has a unique nature; and hence, all cell lines may not respond similarly to the same nanoparticle under similar optimal conditions. Eventhough the cell lines determine the in vivo environment as precise, the choice of the type of cell line for evaluating nanoparticles toxicity is critical.

Some of the in vitro assays are briefly discussed to get an overview of the types of toxicity evaluation methods:

- **Neutral Red Assay**
- Neutral red is a weak cationic dye which can diffuse the plasma membrane of the cell. It accumulates within the cell. If the integrity of the cell membrane is lost by the toxicity of nanoparticles, the uptake of dye decreases (Lewinski et al. 2008). Cytotoxicity of carbon nanotubes was assessed by neutral red assay (Flahaut et al. 2006). This assay helps in evaluating the cell membrane's permeability and its integrity in the cell lines used.
- **Trypan Blue Assay**
- A diazo dye is permeable to cells without membranes, and therefore the dead cell remains blue and live cells as colorless. The number or quantity of dead cells is evaluated by light microscopy (Lewinski et al. 2008). Gold nanoparticles and single-walled nanotubes were evaluated for the cytotoxicity by trypan blue assay (Bottini et al. 2006; Goodman et al. 2004).
- **TUNEL Assay**
- The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay detects the fragmented genomic DNA formed by endonucleases or caspases activation in apoptosis (Hengartner 2000). To count the number of cells in the tissue sample, DAPI (4′,6 diamino-2-phenylindole) was added before

mounting the coverslip and results in the staining of nuclei. Images are observed through a fluorescent microscope (Huo et al. 2015). Three different image areas of about 500 cells are counted by a microscope to know the apoptosis rate.

- **Hemolytic Assay**
- It is a colorimetric assay of detecting red colored cyanomethemoglobin in the solution. The nanoparticles are incubated in blood, and hemoglobin released by damaged cells is oxidized by methemoglobin in the presence of bicarbonate by ferricyanide. The cyanide converts methemoglobin to cyanomethemoglobin (Neun and Dobrovolskaia 2010). The cells are then centrifuged, and the undamaged erythrocytes producing cyanomethemoglobin is measured by spectrophotometry at 540 nm. The result of this assay evaluates the hemolytic properties of nanoparticles (Neun and Dobrovolskaia 2010).
- **3D Spheroid Culture-Based NP Toxicity Testing System**
- Human hepatocarcinoma (HepG2) cells are used in preparing 3D live tissue spheroid models, as the liver is the main organ for the nanoparticle uptake (Gao et al. 2004). The inverted colloidal crystal topology is used as a 3D cell growth substrate, prepared from transparent and cell repulsive polyacrylamide hydrogel (Lee et al. 2006). The spheroid formation of HepG2 cells enhances optimal prediction through the matrix. The toxic effects of cadmium telluride (CdTe) and gold (Au) NPs were tested using different approaches to evaluate the membrane integrity, metabolic activity, and comparison with 2D cell toxicity (Lee et al. 2009). The morphological changes are observed in scanning electron microscopy, whereas the live–dead assay assesses cell viability.
- **Live–dead Viability Assay**
- The assay determines if the cell is alive or dead with different absorbing capabilities of the live and dead cells; it includes two chemicals – calcein acetoxymethyl (calcein AM) and ethidium homodimer. The former is electrically neutral; an esterified molecule can enter cells by the diffusion process (Lewinski et al. 2008). Once the calcein AM enters the cell, it converts to calcein by esterases to a green fluorescent molecule. By contrast, the dead cells get stained by ethidium homodimer, a membrane impermeable molecule, and turn to fluorescent red if it binds to the nucleic acid. The fluorescence is emitted by calcein and ethidium homodimer at a wavelength of 515 nm and 635 nm, respectively (Lewinski et al. 2008).
- **MTT Assay**
- MTT assay is a colorimetric method to determine the mechanism of cell death. MTT 3-(4,5- dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide is a pale-yellow solution that produces dark blue or purple formazan by the live cells. This color formation is due to mitochondrial dehydrogenase enzyme present in living cells (Malich et al. 1997).
- The toxicity of silver nanoparticles is tested using MTT assay on human pulmonary cell lines: THP-1 and A549. MTT assay is found to be a more sensitive test and widely used assay for evaluating cell toxicity (Lanone et al. 2009). The potential cytotoxicity of silver nanoparticles was assessed by MTT assay, using human epidermal keratinocytes (HEKs), and found the AgNPs exposure, indicating a dose-dependent decrease in toxicity (Samberg et al. 2010). The cytotoxicity

of hematite nanoparticles is detected by MTT using MCF-7, A549, and Hep3B. After incubation with nanoparticles overnight at conditions of 37 °C and 5% CO_2, the supernatant is removed and the MTT solution is added. The formation of formazan is quantified by spectrophotometry at 545 nm (Rajendran et al. 2017).

- **Cell Cycle Analysis**
- Pheochromocytoma cells (PC 12) were plated on a well plate in Dulbecco's minimal essential medium. The cells are then placed into the centrifuge tube on treating with glycerol monooleate nanoparticles. The cells are centrifuged at 1500 rpm for 5 min, and the pellet obtained was washed with PBS 1×, discarding the supernatant. The pellet obtained was washed frequently with saline, and samples were analyzed in cytofluorimeter to study the cell cycle. A standard optical filter at 585/542 nm was used to determine the number of cells in each phase of the cell cycle (Valente et al. 2018).
- **Analysis of Apoptotic Markers**
- PC 12 cell lines were treated with glycerol monooleate nanoparticles to evaluate proapoptotic cell stress response, by molecular mechanisms such as transcription and translation activation. Key apoptotic markers such as BCL-2 and Bax are evaluated by the real-time quantitative polymerase chain reaction (RqPCR) (Valente et al. 2018).
- **Micronucleus Assay**
- The presence of the micronucleus is detected to check the genotoxicity of the nanoparticles. The human hepatoma cell line (HepG2) was treated with nanosilver solution. Relaxin B solution is added to the sample, after 24 h of exposure, the cells are fixed with the solution (glacial acetic acid/methanol in 1:3 ratio), and then stained with Giemsa. The Type I and Type II micronucleus predicts the chromosome breakage and loss, respectively. Nuclear buds can predict gene amplification, and its change is observed on microscopy (Wang et al. 2019).
- **ROS Assay**
- The dichlorodihydrofluorescein diacetate (DCFH-DA) is an oxidative fluorogenic dye that measures the peroxyl, hydroxyl, and other ROS within the cell. A549 cells were used to evaluate the cytotoxic ROS generation by graphene oxide nanoparticles through ROS assay. The microplate reader monitors the fluorescence. The graphene oxide nanoparticles found to cause ROS generation even at low concentration (Chang et al. 2011).
- **SRB Assay**
- The sulforhodamine (SRB) detects the cytotoxicity of curcumin solid dispersions. SRB assay was performed on MCF-7 (breast cancer cell line) and NCIH 460 (non-small cell lung cancer cell lines). These cells are treated for 48 h, and toxicity was evaluated (Abreu et al. 2011).
- **TBARS Assay**
- Thiobarbituric acid reactive substance (TBARS) assay is used to predict the formation of malondialdehyde (MDH) and other reactive substance that is generated by lipid peroxidation. Porcine brain cells were utilized to evaluate curcumin

nanoparticle cytotoxicity and observed to reduce or discourage the TBARS level in the cell (Sá et al. 2019).

- **Lactate Dehydrogenase Assay**
- The enzyme lactate dehydrogenase oxidizes lactate to pyruvate and promotes the conversion of a tetrazolium salt into formazan with an absorbance at 490 nm. The amount of LDH released from cells is proportional to damaged cells (Lewinski et al. 2008). Human embryonic kidney cells (HEK)-293 on exposure to copper oxide nanoparticles cause the peroxidation of lipids (Reddy and Lonkala 2019).
- **Mitochondrial Membrane Potential (MMP) Assay**
- The silver nanoparticles are evaluated by MMP in BRL 3A cells by staining with rhodamine 123. On treatment, the qualitative effect on mitochondrial membrane potential was found to be affected by the silver nanoparticles, and the intensity of the fluorescent brightness is reduced (Hussain et al. 2005).
- **GSH Assay**
- Glutathione is a major antioxidant which is oxidized to glutathione disulfide (GSSG) in the presence of ROS (Lewinski et al. 2008). Reduced glutathione (GSH) maintains the oxidation–reduction homeostasis, and its alteration in GSH level indicates damage to the cell. In BRL 3A rat cells, the GSH level was decreased on treatment with silver nanoparticles, which is found to be significant (Hussain et al. 2005).
- **Clonogenic Assay**
- Clonogenic assay is a method to cell reproductive death. MCF-7 cells were trypsinized and seeded, followed by incubation in the presence of B26 organic nanoparticles. After incubation, for few days, the cells are fixed with methanol and crystal violet solutions. The cells are counted by an inverted microscope (Dhanwal et al. 2019).

The above discussed in vitro assays are typically performed to detect either cytotoxicity or genotoxicity. A new approach to evaluate or predict the toxicity of nanoparticles easily is by using computer nanotoxicology – QSAR (quantitative structure-activity relationship) – which is a quick, mostly accurate, and no resource-intensive test to detect toxicity of nanoparticles.

15.4.1 QSAR

A statistical model correlates a set of the structural parameter of a compound to its activity. The parameters are mostly based on electric and steric properties of a compound. Physiological measurements of biological assay data determine the biological activity of a compound. The QSAR workflow is as follows (Burello and Worth 2011):

QSAR is most widely used in drug discovery and is still having a limited application in the evaluation of nanoparticles. More research and analysis on the toxicity of nanoparticles will be done easily by the computer nanotoxicology in the near future,

along with a collaborated work among computational scientists and nanomaterials descriptions with toxicologists to develop new computational assays for evaluating nanoparticles toxicity.

15.5 Conclusion and Future Perspective

This chapter reviews the toxicity of nanoparticle and its evaluation methods using cell lines. The typical interactions between the nanoparticles and the biological systems are gaining more interest to evaluate the potentiality of nanoparticles, and still it seems to be challenging to get a conclusion of underlying mechanism of toxicity. This review outlines the importance to evaluate the toxicity of nanoparticles and how easy and reliable to use those different evaluation methods. Most of the assays were performed on engineered or human-made nanoparticles as the natural nanoparticles are found to be much more safe and efficient to use biologically. It is known that nanomaterials are not hazardous particles and many are nontoxic and have some healthy beneficial effects. However, risk assessment or evaluation helps one to determine the further actions needed to assess the effect of nanoparticles on human health and environment. The use of cancer cell types is to be minimized to evaluate the toxicity nature of nanoparticle, which is less susceptible to nanoparticle-induced cytotoxicity. The toxicity evaluation methods are found to be efficient, quick, and reliable to assess the toxicity nature of the nanoparticles.

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Saccharomyces cerevisiae: Model Organism to Evaluate Nanoparticle Toxicity

16

V. T. Anju, Busi Siddhardha, and Madhu Dyavaiah

Abstract

Emergence of nanotechnology field in biomedicine advised researchers to investigate the toxicity of nanosized particles. The interactions between metals and microorganisms are always a subject of interest in the last few decades owing to their possible transfer of stored metals to higher organisms. The toxicological impacts of nanoparticles on microbial metabolism and growth have always been the subject of interest for researchers. Several nanomaterials such as metal and metal oxide nanoparticles and carbon materials were screened for their potential applications in the biomedical field. Even though nanoparticles have widespread applications in the field of biology and medicine, there is a serious impact of nanomaterials on human health and environment. There are nanoparticles with huge toxicity as they can pass through biological membranes affecting the physiology of the cell. Potential hazards of nanoparticles are much more wide and diverse on plants, vertebrates, and invertebrates than microorganisms. Yeast, *Saccharomyces cerevisiae*, is a prominent and highly informative biological model among all in vitro models to evaluate the toxicity of different nanoparticles. It is also important to evaluate the toxicity of nanoparticles using model organism such as *S. cerevisiae* for the possible applications in different fields. Toxicological effects of nanoparticles vary depending on the size, chemical nature, and surface chemistry of particles. *S. cerevisiae* and its gene deletion mutant collections are successfully employed as a model to evaluate nanoparticle toxicity owing to their ability to reveal cellular toxicity and detoxification

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mechanism of nanoparticles. This will further add valuable information for testing the potential of emerging scope of nanoparticle world.

Keywords
Nanotechnology · Nanomaterials · Toxicity · *S. cerevisiae* · Gene deletion mutants

16.1 Introduction

Nanotechnology is considered as one of the most innovative technologies of the twenty-first century. Nanoscience and nanotechnology are emerging areas of science that deal with the objects of intermediate size between smaller and larger structures. Nanotechnology deals with the particles having dimension ranging from few nanometers to less than 1000 nm. Nanoscience in physics and electrical engineering is associated with the quantum behavior and behavior of electrons and photons in nanomaterials. There is a deep interest of nanotechnology in biology and biochemistry as most of the interesting components of the cell (DNA, subcellular organelles, etc.) can be considered as nanostructures (Whitesides 2005). The wide spread application of nanoparticle in different fields are due to their nanoscale size. A nanometer scale is one billion of meters which is 100,000 times lesser than the diameter of human hair. Humans are utilizing nanotechnology in their day-to-day life to produce lightweight components, cosmetics, electronic systems, medicines, high-performance battery storages, etc. This new technology has the potential to develop and modify our society and all sectors ranging from environmental remediation to disease diagnosis and treatment. Currently, our scientific society is engaged in the future development of nanotechnology in different fields (Rajak 2018).

Another term associated with nanoscience in life sciences is nanobiotechnology. Several nanobiotechnological methods to analyze signaling pathways may help to understand disease processes and also enable us to identify efficient biomarkers and mechanism of action of drugs. Ultimately, nanobiotechnology has provided a new scope for drug discovery with novel approaches (Jain 2009). As per European Union, nanotechnology is regarded as one of the key regulators empowering other technologies. New nanomaterials and nanotechnology are heading in all sectors from the production to consumption units. This technology unbolts novel applications in various divisions of animal husbandry, environment, industries, and food packaging (Mishra et al. 2019). Peculiar physical and chemical characteristics of nanomaterials transformed several sectors such as biotechnology, medicine, and pharmaceutical fields. In fact, nanoscience solved many of the unsolved issues associated with modern medicine that offered advantage to patients, healthcare workers, and society. Even though nanoparticle is having tremendous applications, there always emerges serious concern regarding the possible adverse impact on human health and environment (Leso et al. 2019). To address this shortcoming, greener nanoscience has been introduced. Green nanotechnology is focused toward the development of safer and effective alternative methods to reduce the toxic effect of

nanomaterials. Greener nanoscience integrates principles of green chemistry to design, produce, and use nanoparticles. Moreover, greener nanoscience focuses to eliminate the threats on the environment and human health through the redesign and process of nanoparticle production and optimization (Hutchison 2008). Several efforts are taken by scientists and academicians to balance the potential benefits and risks of nanoscience and nanotechnology. There are many in vitro and in vivo methods available to evaluate the toxic effect of nanoparticles for optimizing their usage in different sectors.

16.2 Recent Developments in Nanotechnology and its Applications

Abundant potential of nanotechnology on several sectors has revolutionized the field of biology and medicine. Recently, nanotechnology attracted intensive research and several development practices in the academic and industrial level due to their wide range of applications in agriculture (Kim et al. 2018) (Fig. 16.1). Nanotechnology is regarded as an integral part of food processing and production of conventional foods. This is due to the property of many foods to relay on nanosized components. However, the ability of nanoparticles to cause chronic health effects and environmental toxicity is always a serious concern for researchers (Abbas et al. 2009). Economic promises and opportunities contributed by nanotechnologies are

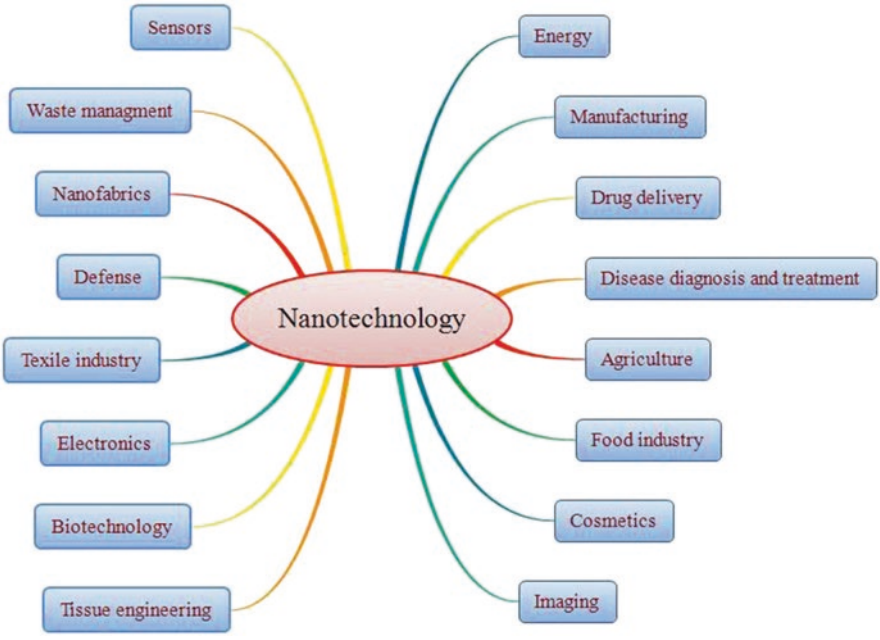

Fig. 16.1 Developments and applications of nanoparticles in different sectors

very significant for humans as it has a considerable impact on the public in relation to the quality and wealth creation. Future horizons of nanotechnological products in some sectors are discussed as biocompatible materials, nano-sensors, nano-medicine, nano-photonics, nanomaterials with new functional properties, nano-fluidics, drug delivery using nanoplatforms, plastic electronics, nano-catalysis, nano-optics, and hydrogen technology (Karaca and Öner 2015). Another interesting application of nanoscience involved in existing fire programs. It has been reported nanoscience and nanotechnology as the future of fire safety (Olawoyin 2018).

Nanoparticles have become alternative for the packaging materials. Nanoparticles that are used as packaging materials positively affected the quality, safety, security, and shelf life of food (Mihindukulasuriya and Lim 2014). Another wide application of nanoscience, the concept of nano-medicine, relies on the progress in nanomaterial research and nanoengineering. In nano-medicine, unique physical and chemical characteristics of nanoparticles enable them to be effectively used for the diagnosis and treatment of diseases at molecular level. Usually, nanoscience and nanotechnology in medicine integrate multidisciplinary fields for better results. For example, one of the potential advances in nano-medicine is the treatment and prevention of disease within the body. Nanoscience has revolutionized different medical and scientific fields such as prevention, diagnosis, and treatment of diseases, targeted delivery of drugs to cells, disease evolution control, protection, monitoring damaged tissues, tissue repair, and improvement of human biological systems and pain relief (Tekade et al. 2017). The quantum effect of nanomaterials is important when their size decreases to 100 nm or smaller. The high surface-to-volume ratio allow more promising interactions of nanomaterials with the surrounding structures (Yarlagadda et al. 2019). The essence of nanotechnology and nanoscience falls on the properties of materials which are quite different from that of bulk materials. It is known that when dimensions of materials are decreased below 100 nm, drastic modifications occur in their properties. Nanoparticle also exhibits an enhanced performance in the in vitro and in vivo conditions when used along with the bulk materials for similar applications (Nasrollahzadeh et al. 2019).

16.3 Nanotoxicology

Nanotechnology has attained the public interest owing to their social and economic impact. Evaluation of nanoparticle toxicity is compulsory in order to promote the complete understanding of health and environmental impact of nanomaterials. Even though we observed rapid advancements in nanotechnology, there is very minimal information available about nanotoxicology. Issues related to nanoparticle exposure to humans and environment are still not sufficiently explained and adequately solved. Hence, it is compulsory to develop methods to assess the risk related to it for the identification and elimination of potential damage (Kalantari 2013). Nanotoxicology is emerged as a budding research area under toxicology that deals with the evaluation of toxicological implications of nanomaterial causing a societal threat. Aquatic ecosystems are under the pressure of nanoparticle toxicity

accumulated in water sources. Extent and fate of nanoparticle toxicity among aquatic organisms are purely dependent on properties of nanoparticles and water chemistry. Major mechanisms of nanoparticle toxicity among inhabitant organisms are due to the oxidative stress-induced cytotoxicity, inflammatory responses, genotoxicity, DNA damage, and other effects (Walters et al. 2016).

16.4 Physicochemical Features of Nanoparticle Influencing the Toxicity

The behavior of nanoparticles in various environments is different and complex. Unique properties of nanoparticles such as surface area, charge, structure, size, surface coating, and solubility that are different from their conventional materials affect nanoparticle toxicity. Owing to their small size, nanoparticles can easily penetrate the cell membrane and other biological barriers causing cell damage. There are several studies revealing the enhanced damage caused by nanoparticles than their bulk particles (Lankveld et al. 2010). Toxicological studies indicate an inverse relationship between toxicity potency and size of nanoparticles. Nanoparticle will have a higher surface area-to-volume ratio on decreasing the size of particles which is explained by an excess of energy at their surface. This is in line with their thermodynamic instability and enhanced toxicity (Luyts et al. 2013).

Midander et al. (2009) described enhanced toxicity of micrometer-sized copper nanoparticles (2.7 μm) than smaller particles of 25 nm. These nanoparticles induced DNA damage and cell damage in A549 epithelial cells (Midander et al. 2009). One of the problems encountered frequently while preparing nanocomposites is their aggregation and dispersion. Agglomerated nanoparticles usually accumulate in different lung regions causing chronic effects. Larger aggregates especially of carbon nanotubes cannot be easily removed from the accumulated area (Luyts et al. 2013). Although magnetic nanoparticles are having enormous potentials, they cause toxicological effects on animal cells. Silver nanoparticles present in various consumer products release their ions to aquatic system, causing toxic effect on aquatic organisms such as algae, fish, bacteria, and daphnia (Navarro et al. 2008). The respiratory system is known as a unique target for the possible toxicity of nanoparticles. This also acts as the portal of entry of nanoparticles for the inhaled particles causing chronic effects (Ferreira et al. 2013). Another effect of nanoparticles is related to proteins and structural biomolecules. Due to the ability of nanoparticles to concentrate around proteins, they generate adverse effects on biological molecules such as loss of enzyme activity, thiol cross linking, unfolding of protein, and fibrillation. Another pattern of toxicity is through the release of toxic ions to the aqueous medium when the thermodynamic features favor their dissolution. They also form aggregates in sea water and hard water which is dependent on the organic matter present in water causing ecotoxicity (Pokhrel et al. 2009).

16.5 Methods for Risk Assessment of Nanoparticles

Despite all the beneficial roles of nanoparticles, it is important to evaluate their risks and harmful effects to the living system and environment. An effective assessment system and framework should be developed to identify, detect, and organize the potential threats caused by nanoparticles. Several nanoparticles are being synthesized and applied optimally, and their risk was compared with nontoxic effect to generate specified assessment methods. According to the type of nanoparticles, the nature of hazards also varies. For instance, nanoparticles with rod, tube, and fiber shapes are more persistent in biosystems and environment due to their high stability. Particles produced by combustion and operation of factories or by other human activities may cause respiratory and cardiovascular problems than those produced as fine particles. Toxicity produced by fine nanoparticles is because of their surface area and adsorption of poisonous agents on their reactive surface (Nasrollahzadeh and Sajadi 2019). Thus, the collection of data about toxicity mechanism and identification of processes involved in various nanoparticles is an important method of monitoring and assessing the risk associated with nanotechnology. According to the size, morphology, reactivity, penetrability, and stability of nanoparticles, nanotoxicity can be defined differently based on their impact on human and ecosystem. In order to improve the framework of nanotoxicity, different tests or methods are integrated such as assessing risks or damages occurred in human organs, cell or tissue uptake tests, and bio-persistence and bioaccumulation tests for nanoparticles (Baun et al. 2008; Barnes et al. 2008; Wang et al. 2008).

Ecotoxicity tests of nanoparticles are used to assess environmental hazard and intrinsic dangers of chemical substances released into the environment. Potential threat of adverse effects can be characterized when hazard and exposure assessment of nanoparticles are compared. Generally, a tiered approach is followed for hazard assessments. Acute tests or short-term tests are employed initially that observe the survival of organisms. Chronic tests or long-term effects are used when results of acute tests suggest a potential risk to environment. In chronic tests, sublethal effects on growth and reproduction of organism are measured (Crane et al. 2008). There are several in vitro and in vivo risk assessment models from whole animals to cell lines which are used for testing the toxicity of nanomaterial. Information obtained from both model provides knowledge about the toxicity and safer exposure levels of nanoparticles (Balbus et al. 2007). Structure, chemical composition, solubility, and size of nanoparticles have direct impact on biological effects of nanoparticles as these can modify the protein binding and cellular uptake (Rivière 2009). Specific biological and mechanistic pathways through which nanoparticles cause adverse effects can be studied through cellular assays which are possible with in vivo models. Various in vitro and in vivo models used for risk assessment of nanoparticles are shown in Fig. 16.2. Fundamental processes of nanoparticle toxicity at single-cell level are detected using spectroscopic and electro-optical methods (Xia et al. 2009; Chen et al. 2009). Nanoparticle interaction with cell is analyzed through different factors such as their particle size, specific chemical interactions, and hydrophobic and electrostatic interactions. Nanoparticles may alter cell membrane, decrease cell

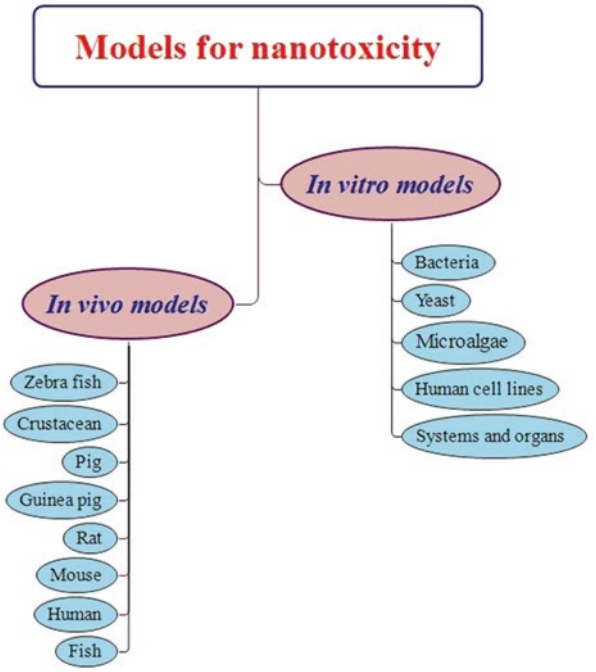

Fig. 16.2 Types of in vitro and in vivo models employed in the assessment of nanoparticle toxicity

viability, disrupt mitochondrial function by reactive oxygen species (ROS), and change gene expression (Andreescu et al. 2011).

Titanium oxide nanoparticles are thought to cause toxicity by membrane breakage, oxidative stress, and changes in the cell surface. Copper oxide nanoparticles cause glycolysis, fatty acid beta oxidation, and mitochondrial failure (Meng et al. 2007; Li et al. 2008). Iron oxide nanoparticles in moderate levels interfere neural cell functioning (Pisanic et al. 2007). Cadmium selenium quantum dots increase cytoplasmic calcium ion levels in cultures of rat hippocampal neuron (Tang et al. 2008). Electrostatic interactions between particles are important in protein adsorption and cellular uptake. Increased cellular uptake of negatively charged nanoparticles is associated with the nonspecific binding on cell membrane and synthesis of nanoparticle clusters (Tang et al. 2008). An in vivo model helps to understand the interaction of particles with target organs such as muscles, cartilage, digestive system and intestine, and also tissue response. Studies reported that in vitro cell culture models reveal only the minor responses of nanoparticle exposure at high doses (Warheit et al. 2009). In vivo studies were helpful in determining short-term cytotoxic or lung inflammatory responses. In vivo methods are more reliable methods to assess nanotoxicity as they are very simple and provide only readily available cytotoxic test. Scientists recommended for more developed, standardized, and validated in vitro cellular systems to provide useful information regarding nanoparticle

toxicity. Further refinement in cellular systems to mimic the in vivo conditions is required for better understanding of mechanisms of toxicity. Hazardous effect of nanoparticles on human health can be evaluated by the use of both in vivo and in vitro models (Sayes et al. 2007).

16.6 In Vitro and In Vivo Assessment of Nanoparticle Toxicity

Nanoparticles are investigated for evaluating their genotoxicity, immunotoxicity, and cell toxicity. Cell viability is used to measure through tetrazolium-based assays such as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and water-soluble tetrazolium salts (WST1). Cell inflammatory responses are measured through enzyme-linked immunosorbent assay (ELISA) by analyzing the level of biomarkers such as IL-8, IL-6, and tumor necrosis factor. Lactate dehydrogenase assay was employed to check the cell membrane integrity. There are various cell culture types including cancer cell cultures as in vitro models (Bahadar et al. 2016). In vitro toxicity assessment has several advantages over in vivo methods that include faster, minimum ethical concerns, and lower cost. Different assays used to measure the nanoparticle toxicity through in vitro methods include necrosis assay, oxidative stress assay, DNA damage assay, apoptosis assay, and proliferation assay. In vivo toxicity assessment is carried out using mice, rat, or other animal models. Different studies to evaluate toxicity in animal models include clearance, hematology, serum chemistry, biodistribution, and histopathology tests (Kumar et al. 2017).

16.7 Assessment of Nanotoxicology by Using *S. cerevisiae* and Their Gene Deletion Mutants

Yeast or *S. cerevisiae* is widely used as typical, unicellular eukaryotic model system for evaluating the toxicity of nanomaterial. *S. cerevisiae*, a known in vitro model, is used due to its simplest structure and functional organization that are similar to higher organisms. Even genetic information of yeast is fully available to study the mechanism of toxicity in yeast. These are frequently used in the evaluation of toxicity of heavy metal nanoparticles and engineered nanoparticles (Bao et al. 2015). *S. cerevisiae* is one of the widely used eukaryotic models in testing nanoparticle toxicity as they can be easily cultivated such as bacteria and require only short generation time. As it shares more similarities with higher organisms, this model organism provides links to understand the nanoparticle toxicity in higher organisms. It is widely employed to study apoptosis and oxidative stress response (Carmona-Gutierrez et al. 2010) (Fig. 16.3). Typical toxicity tests using yeast consists of determination of growth inhibition, cell viability, survival test, incubation of cells with different nanoparticle concentrations, calculation of growth rate or biomass yields, and determination of median inhibitory concentrations.

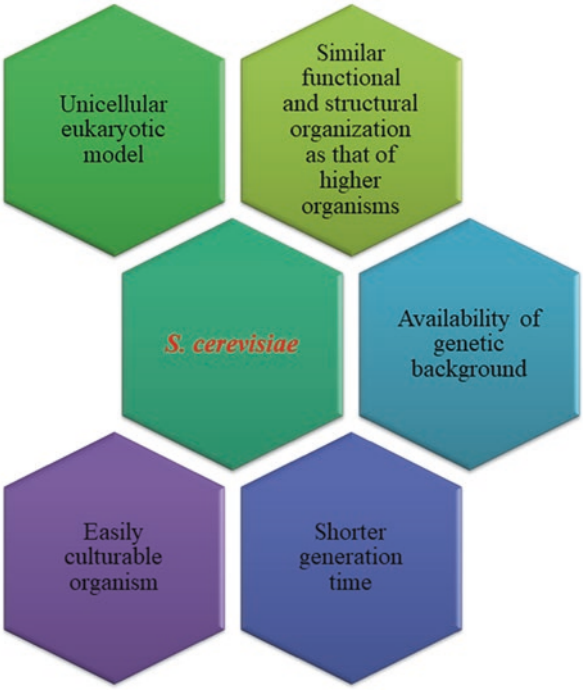

Fig. 16.3 Characteristics of yeast that make it a suitable in vitro model for evaluating nanoparticle toxicity

S. cerevisiae was first used in 2009 to assess the toxicity of zinc, copper, and titanium nanoparticles. In that study, bulk zinc and zinc nanoparticles were found to be toxic owing to their release of zinc ions, whereas copper ions were not found as toxic as that of copper nanoparticles (Kasemets et al. 2009). Toxicity mechanisms of several nanoparticles to yeast can result due to ROS-mediated oxidative stress, soluble ion release, or particle-related effects (Gunawan et al. 2011; Ivask et al. 2014). In another study, single-gene deletion mutants and copper vulnerable mutants of yeast were used to elucidate the role of copper and oxidative stress in toxicity to *S. cerevisiae*. A study revealed that copper vulnerable mutants were more sensitive (16-fold) to copper nanoparticle than wild type (Kasemets et al. 2013). It has been added that deficiency of a single gene may not cause the generation of desired phenotype owing to their compensatory mechanism in cells. Therefore, metal-induced toxicity can be studied elaborately in multiple gene deletion mutants of yeast rather than single-gene deletion mutants (Xiu et al. 2014). Zinc nanoparticles exhibited toxicity in budding yeast through the inhibition of growth by 80%. It was due to the release of zinc ions and further by oxidative stress (Kasemets et al. 2009). Another study revealed that silver nanoparticles showed toxicity on yeast cells that affects proteins, RNA, amino acids, and plasma membrane. Possible mechanisms of silver

nanoparticle toxicity are production of hydroxyl radicals and induction of apoptosis (Galván Márquez et al. 2018).

Functionalized polystyrene latex nanoparticles were found to be toxic to eukaryotic model yeast cells through adhesion and internalization to cells. Nanoparticles with negative charge failed to show toxicity in yeast, whereas amino-functionalized nanoparticles with positive charge showed toxicity. Amino-functionalized nanoparticles dissolved in 5 mM NaCl entirely adhered to the surface of cell and induced cell death through internalization or endocytosis. Negatively charged nanoparticles could not adhere and internalize to yeast. There was no toxicity observed in yeast due to the decreased electrostatic interactions between yeast and nanoparticles (Miyazaki et al. 2014). For testing the toxicity of nanoparticles in single-gene deletion mutants, mutant *yap1*Δ strain was used. *Yap1*Δ gene is a posttranslational factor which encodes genes of protective enzymes under oxidative stress. For multiple gene deletion mutants, *cwp1*Δ*:Cwp2*Δ:*snq2*Δ*:pdr5*Δ mutant strains (quadruple mutant) were used. *Cwp1* and *cwp2* encodes major mannoproteins present in cell

Table 16.1 Evaluation of nanoparticle toxicity using *S. cerevisiae* and their mechanisms of toxicity

Model systems	Nanoparticles	Mechanisms	References
S. cerevisiae gene deletion arrays	Zinc nanoparticles	Cell wall function Cell membrane integrity	Galván Márquez et al. (2018)
S. cerevisiae gene deletion arrays	Silver nanoparticles	Decreased transcription Reduced endocytosis Dysfunction of electrons transport system	Galván Márquez et al. (2018)
S. cerevisiae	Zinc nanoparticles	Altered physiological and metabolic processes	Kumar Babele (2019)
S. cerevisiae (wild type, single gene and multiple gene deletion mutants)	Copper nanoparticles	Oxidative stress through the production of ROS Soluble ions or particle-related effects	Bao et al. (2015)
S. cerevisiae gene deletion mutants	Zinc nanoparticles	Oxidative damage and mechanical damage	Zhang et al. (2016)
S. cerevisiae BY4741 single-gene mutants and copper vulnerable mutant	Copper nanoparticles	Oxidative stress	Kasemets et al. (2013)
S. cerevisiae	Yttrium oxide nanoparticles	Release of yttrium ions induces oxidative stress and protein denaturation	Moriyama et al. (2019)
S. cerevisiae	Silver nanoparticles with size of 20 nm	Cell membrane disruption and oxidative stress	Horstmann et al. (2019)
S. cerevisiae	Cadmium-based quantum dots	Cytotoxicity due to the release of cadmium ions leading to ROS production and mitochondrial damage	Han et al. (2019)

wall, and *snq2* which is highly homologous to *pdr*5 encodes an ATP-binding cassette transporter. Yeast with gene deletions showed higher sensitivity to nanoparticles because of the enhanced cell permeability (Bao et al. 2015) (Table 16.1).

Yeast is widely used to study toxicological studies of titanium, copper, aluminum, zinc, cerium, silica, and manganese oxide nanoparticles (Ly et al. 2003; Kasemets et al. 2009; García-Saucedo et al. 2011). Also, toxicity mechanism of multiwalled carbon nanotubes, iron oxide, and graphene oxide was studied on *S. cerevisiae* (Zhu et al. 2017a, b). Zhu et al. (2018) studied biocompatibility of oxidized single-walled carbon nanotubes in *S. cerevisiae*. A study reported that nanoparticles were internalized and distributed well in model system. Possible mechanism of nanoparticle toxicity was penetration of carbon nanotubes, endocytosis, and oxidative stress-induced apoptosis (Zhu et al. 2018). Zinc nanoparticles caused oxidative stress-induced toxicity in yeast cells and exhibited strong inhibition of growth. Analysis of oxidative stress markers in yeast cells showed a decrease in reduced glutathione levels and increase in enzyme activities (ascorbate peroxidase (APX), glutathione S-transferase (GST), and glutathione peroxidase (GPX)). Results suggested the application of *S. cerevisiae* as a suitable model for studying oxidative stress-mediated toxicity (Khebbeb et al. 2015).

S. cerevisiae is used to find out pathways that help eukaryotes to survive with the metal nanoparticles. A major mechanism of nickel toxicity and carcinogenicity in humans was found to be unprogrammed gene silencing using yeast model. Genomic phenotyping of knockout mutants is another method to understand the general functional genes involved in metal homeostasis in yeast. In one study, knockout mutants of haploid strain *S. cerevisiae* BY4741 was used to identify mutants resistant and sensitive to yttrium. Yttrium toxicity affected different cellular responses such as protein translation, vacuolar compartment, endocytosis, sphingolipid metabolism and signaling pathways (Grosjean et al. 2018). ROS accumulation and plasma membrane damage are the two well-known mechanisms of nanoparticle cytotoxicity. Interestingly, it was found that manganese nanoparticles caused nanoparticle toxicity through endoplasmic reticulum stress rather than ROS accumulation. ROS were produced as a by-product of manganese toxicity in the yeast cells. Endoplasmic reticulum stress led to decreased protein secretion and further resulted in decreased cell growth (Yi et al. 2017). Mechanisms of nickel toxicity were also elucidated in *S. cerevisiae*. Several mechanisms of nickel toxicity include the release of nickel ions and intracellular accumulation of ROS that cause cell death in yeast. Nickel oxide nanoparticles can adsorb to the cell wall but cannot be internalized by yeast, suggesting an indirect mechanism of nanoparticle toxicity. The study can be useful for the regulation of risks associated with nickel oxide nanoparticles (Sousa et al. 2018).

Silver nanoparticles exhibit different mechanisms of toxicity toward yeast *S. cerevisiae*. A transcriptome profile of silver nanoparticles-exposed S. *cerevisiae* showed upregulation of genes related to chemical stimuli. In case of copper and cadmium nanoparticle-exposed cells, most of the upregulated genes code for metalloproteins (CUP1-1 and CUP1-2). Previously described mechanism of silver

nanoparticle toxicity was oxidative stress and cell membrane damage leading to the leakage of cytoplasmic contents and endocytosis (Käosaar et al. 2016). Zinc nanoparticles displayed toxicity in yeast mutants but not in wild-type strain. Even though oxidative damage contributed to adverse toxicity effects in yeast strains, mechanical damage was the essential mechanism behind the zinc toxicity in yeast mutants. Also log Te (particle) and Te (ion) were calculated to assess the zinc nanotoxicity in mutants (Zhang et al. 2016).

In one study, toxicogenic analysis of quantum dots on yeast was revealed. Genetic basis of quantum dot toxicity was exploited through the screening of gene deletion mutant collections. DNA repair, abiotic stress response, mitochondrial organization, and transport were the possible mechanisms of toxicity (Libralato et al. 2017). Another study described mitochondrial organization as the leading mechanism for the genes affected by toxicity. There was a reduction in the mitochondrial membrane potential, cytochrome content, oxygen consumption, etc. It is also added that *S. cerevisiae* is an ideal facultative anaerobe to study the adverse effect of nanoparticle that shut off mitochondrial function (Pasquali et al. 2017). Babele et al. (2018) described zinc nanoparticle-induced toxicity mechanisms in yeast are cell wall stress, induction of ROS generation, and cell membrane damage. Also, cell wall stress and ROS activate dysfunction of organelles such as mitochondria and endoplasmic reticulum, causing imbalanced lipid metabolism. Cellular lipid imbalance, ROS production, and dysfunction of organelles lead to autophagy and eventually cell death. As yeast is the simplest model for toxicity assessment, studies on mechanisms of oxidative stress and apoptosis can provide new insights on nanoparticle toxicity on complex higher organisms.

16.8 Future Perspectives and Conclusions

Modern technology and science are developing rapidly, stimulating their benefits on the society. Fields that revolutionized modern science with its vast applications in several sectors are named nanoscience and nanotechnology. Recent developments of nanotechnology are boon to medicine and biology. Nanotechnology deals with the nanosized particles and manipulates the materials at their molecular, atomic, and macromolecular levels. Nanomaterials provide potential benefits to different fields such as food industry, agriculture sectors, medical field, targeted drug delivery, disease diagnosis, cosmetics, bioengineering, medications, waste management, textiles, electronics, information technology, etc. There are different types of nanoparticles with diverse mechanism of actions and applications. As the synthesis (chemical, physical methods), bulk production, and uses of nanoparticle increases, there is a huge challenge faced by the humans and environment. The toxicity of nanoparticles on human health and ecosystem is always a topic of research. They pose enormous threat to the Earth and living organisms. Metal and metal oxide nanoparticles, carbon nanomaterial, polymeric nanoparticles, etc., impose different kinds of toxicity on the environment. Generally, nanoparticles exert genotoxicity, immunotoxicity, and cytotoxicity on living systems. The assessment of nanotoxicity

integrates in vitro and in vivo models and organ systems. The study of nanoparticle exposure on model systems gives their effect on growth inhibition, gene expression, and reproduction. Among all in vitro models, *S. cerevisiae* is one of the best studied unicellular eukaryotic models for the evaluation of nanoparticle toxicity. The availability of lots of genomic data regarding yeast helps them to serve as excellent models for nanoparticle toxicity studies. Hence, toxicity studies using yeast will provide consistent results for the better understanding of nanotoxicity on higher organisms. Nanoparticles produced toxicity on yeast through ROS-mediated oxidative stress and apoptosis. Yeast serves as an ideal model for toxicity studies as they possess inherent features such as easy culturing conditions and short generation time. However, there are some gaps in deciphering the scientific mechanism behind nanoparticle toxicity on yeast correlated to higher organisms. Yeast deletion mutant's collections are developed recently to study their effect on some strains with deficient genes. Considering the potential benefits of nanoparticles in several fields, some expanded studies are required using in vitro and in vivo models to evaluate their beneficial and harmful effects. In the future, scientists will look on biased toxicological assessment using in vivo models that may provide more reliable results.

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Caenorhabditis elegans: Evaluation of Nanoparticle Toxicity

17

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Abstract

The relevance of *Caenorhabditis elegans* (*C. elegans*) as an in vivo model organism in the study of nanoparticle/biological interactions and nanotoxicology has gained popularity recently. This is attributed to its short life cycle, a high degree of homology with higher organisms, and cost-effective maintenance. The ability of worms to self-fertilize and generate large numbers of progeny aided with the presence of complex tissue systems is ideal for nanotoxicological multiple endpoint study both in terms of mechanistic and high-throughput screening approaches. Nanoparticle-mediated toxicity in *C. elegans* can be assessed using different standard methods and protocols. For example, assays that determine worm growth, mortality rate, reproductive capability, and locomotion changes can provide accurate measurements and predictability when applied to higher mammalian systems. The use of reporter gene analysis such as green fluorescence protein (GFP) in transgenic strains and microRNAs studies in *C. elegans* has led to the discovery of different biomarkers for toxicity studies. Thus, researches on *C. elegans* model have contributed immensely to our realms of knowledge in nanoparticle-based toxicity, and this has allowed for elucidation of alterations at the cellular and molecular levels. In this chapter, discussions are directed toward our general outlook of *C. elegans* as a model organism to study nanoparticle-mediated toxicity and the different approaches and assays employed regularly in the measurement of nanotoxicity. Special emphasis is taken considering significances of different biomarkers and molecular responses involved in the process (e.g., oxidative stress, DNA damage, and apoptosis, endoplasmic reticulum stress). Finally, based on recent evidence, the roles of common and important signaling pathways in regulations of nanotoxicity formation in *C. elegans* (p38

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MAPK signaling, insulin signaling, programmed cell death, and TGF-β signaling pathway) are discussed.

Keywords
Nanoparticles · *Caenorhabditis elegans* · Toxicity · Neurotoxicity · Apoptosis · Immunotoxicity · Insulin signaling

17.1 Introduction

Recent advancement in nanotechnology has encompassed across various disciplines as seen with its applications in different areas such as pharmaceutical, healthcare, transportation, and energy. Indeed, in our day-to-day life, we encounter many of the nanoparticle products ranging from gold nanoparticles (AuNPs) in our facial creams (Guix et al. 2008), silver nanoparticles in preservatives (Kokura et al. 2010), to titanium dioxide and zinc oxide nanoparticles in colorants and sunscreens for skin protection (Gulson et al. 2010). The bigger question is how safe regular exposure to nanoparticle is.

"Nanoparticles are generally defined as those microscopic particles which are having one structural dimension less than 100 nm, intentionally designed engineered nanoparticles having a similar physicochemical characteristic" (Gonzalez et al. 2008). Due to its characteristic physicochemical properties such as nano-size and large surface area per unit volume, it can elicit functions that can have wide applications. On the contrary, amidst its popularity and the wide spectrum of usability, the same inherent characteristic nature of nanoparticles or engineered nanomaterials (ENMs) predisposes biological systems such as biomolecules and organelles as an obvious target of unwanted and long-term toxicity. They can readily pass through the lipid bilayer of the cell membrane and other biological barriers, and this may lead to unwanted interactions that can alter homeostasis and normal cellular functions (Xia et al. 2008; Brar et al. 2010).

According to the latest studies, it is highlighted that human beings are in constant exposure to nanoparticles in their daily life through inhalation of airborne ultrafine/nanoparticles (respiratory tract), direct and indirect skin contact, ingestion through the oral route, and most significantly through injection by any means to blood circulation (Nel 2006). Nonetheless, physicochemical and biological interactions are poorly understood (Buzea et al. 2007). On the practical front, experimentations utilizing in vivo whole animal model are expected to throw lights to the unknown nanoparticle/biological interface. Similarly, in model organisms, the availability of defined cells, tissues, and organ systems as exposure routes of study may provide realistic cases on the different unknown effects of nanoparticles, and this can be correlated to that of a higher mammalian system such as a human. Fundamental insights obtained from these organisms can be adopted for the safety designs and development of future nanoparticles and in the judicious usage and control applications of the current notable toxic nano-based products.

17.2 Why Choose *C. elegans* for Nanotoxicology?

Experimental approaches utilizing various in vitro, cell-based, computational approaches and different in vivo models have undoubtedly enhanced our understanding of the process and precision requirements of toxicological science. Nowadays, a free-living *Caenorhabditis elegans* (*C. elegans*) model has become a trend for biosafety assessments of nanoparticles especially in ecotoxicological studies (Leung et al. 2008; Zhao et al. 2013; Qiao et al. 2014). Inherently similar to higher mammalian system, this model organism has provided useful information both in terms of the mechanistic and molecular basis of toxicity besides its scope in the field of modern predictive toxicology mentioned in Table 17.1. The answer to a bigger question of how these tiny little worms can contribute so much to

Table 17.1 General comparisons of *C. elegans* model in nanotoxicological studies

Advantages	Disadvantages
• Short life cycle—life span of 2–3 weeks multigeneration toxicity studies can be done in a couple of weeks rather than years • Easy, simple, and inexpensive cultivation with cooling incubator requirement • Small size and large number of offsprings. Can be cultured in 96 and 386 well plates • Transparent body allows for direct visualization, fluorescence monitoring, ontogenetic approach for different physiological studies • Whole genome sequence completed and availability of mutants or GFP transgenic strains for visualization at the tissue, cellular, and subcellular levels after nanoparticle exposure • Behavioral studies can be done through fully mapped body, and neuronal plans allow for rapid morphology and physiological and behavioral assessment • No restriction of use derived from bioethical regulations • *C. elegans* has well-functioning and characterized innate immune system for nanotoxicity studies • Conserved alimentary features and architectures make *C. elegans* a good oral nanotoxicity model • Accessible online resources and research community	• *C. elegans* lacks specific tissues such as the eyes, lungs, heart, kidney, and liver • Lack of circulatory system • Limited information about toxicity • Lack of adaptive immunity • Temperature-dependent development • *C. elegans* is not a good absorption model due to its tough cuticle • pH range is wide but still limited, and liquid culture testing requires soluble test compounds • Small changes in temperature, nutrient, salt can give inaccurate result

toxicological sciences and in particular to nanotoxicology is mentioned in the description below.

17.2.1 General Experimental Considerations, Parameters, and Techniques

C. elegans represents many fascinating characteristics as an in vivo model shown in Fig. 17.1. The first and the most significant characteristic of these nematodes is their short life cycle and self-propagation ability, which allow for large brood size progeny to be produced, whereby an individual animal can populate a plate. This unique short life cycle and its capacity to generate numbers of progeny in a short span of

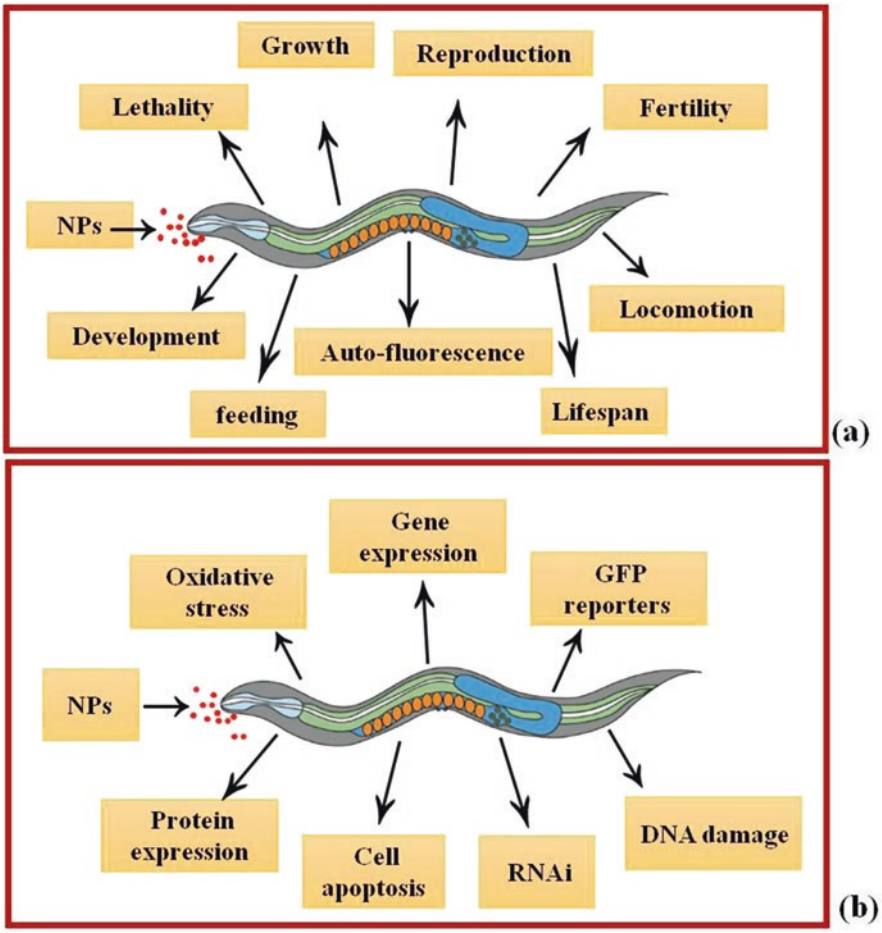

Fig. 17.1 *Caenorhabditis elegans* in nanotoxicity assessment and its endpoints (**a**) biological parameter and (**b**) molecular marker

time are ideal for experimenting with lots of animals as required for high-throughput screens without any ethical constraints (Fig. 17.1). The next important feature is ease of cultivation which requires a simple composition of nematode growth media (NGM) and a cooling incubator. Thus, it compensates for the financial expense and highly skilled professionals required. Third, because of its small size which is around 1 mm, it can be cultured easily on a Petri dish and 96 and 584 well plates, and it can be preserved for long-term at −80 °C. Worms can be synchronized easily using available standardized protocols, and this enables for uniform and age-dependent toxicological studies when needed.

With being simple, transparent, and multicellular, it can be used to elucidate the nanotoxicological process step by step such as uptake, translocation, distribution, and metabolism in certain targeted organs. *C. elegans* can also be useful for the assessment of different nanoparticles for sublethal endpoints. Its feasibility for high-throughput nanoparticle toxicity screening allows for the fast and easy identification of barren or toxic nanoparticles either engineered or synthesized. With whole-genome sequenced, *C. elegans* model is also ideal for different specialized and sensitivity studies. Different mutant strains can be generated easily through self-propagation with fast propagation time, and the feasibility of worms targeting gene inhibition by simple RNAi feed has further accelerated the process. These mutants allow easy assessment and identification of a particular gene or pathways which are involved in toxicity. The temperature-dependent growth of worms allows controlling the rate of growth, and temperature-sensitive strains can be used in toxicity studies. Another desirable feature which is not usually mentioned is that it is harmless to humans because worms cannot grow on the existing temperature of the human body (Table 17.1).

17.2.2 *C. elegans* Capacity for High-Throughput Nanotoxicity Screening

In terms of efficient productivity and predictivity that often involved in preclinical toxicity studies, evaluation in more than one mammalian model organisms is advantageous (Olson et al. 2000). Additionally, to minimize the unwanted cost and time involved in safety design and development of synthesized or engineered nanomaterials (ENMs), high-throughput-based toxicity screening is the current leading archetype. Previously, *C. elegans* toxicity assays were used only for small-scale validation studies to predict the harmful effects of compounds in genetic and chemical screens in mammalian species (Leung et al. 2008). Recently, Jung et al. (2015) presented with the first successful large-scale multi-endpoint and high-throughput screening design for nanomaterials. This method employed a whole animal *C. elegans* model system and has been applied to examine 20 ENMs including carbon-based ENMs. Most importantly, it utilizes and quantifies many important sublethal and lethal endpoints of *C. elegans* population including its growth, locomotion, fitness, and life span. Thus, it demonstrates the potential of *C. elegans* for high-throughput micro-techniques in different nanotoxicological studies. Its capacity in

generating cohesive dataset at high speed can have translational applicability for environmental and human health safety precautions prior to the applications of nanoparticles. Most significantly, this information is also publicly available at www.QuantWorm.org/nano (Jung et al. 2015).

17.2.3 Homologous Genes and Concordant Pathways

The reproducibility of *C. elegans* model in nanotoxicology relies heavily on the availability of homologs and orthologs genes, and this has been estimated for 60–80% of the human genes (Sonnhammer and Durbin 1997; Harris et al. 2004; Kim et al. 2017b). Similarly, counterparts for genes linked with many human diseases have been identified in *C. elegans* genomes (Kaletta and Hengartner 2006; Markaki and Tavernarakis 2010). Because of its well-conserved apoptotic pathway to human, the same fullerenol nanoparticles that are widely used in the medical field are found to enhance apoptotic cell death in Bristol N2 and mutant *C. elegans* strains as reported in different multicellular organisms (Vaux et al. 1992; Cha et al. 2012). Similarity is also observed in elements of the insulin and IGF-1 (IIS) signaling pathway with identical modes in regulations of metabolism, growth, and life span in *C. elegans* to that of mammals IGF-1 signaling pathway (Hunt et al. 2012). Zhao et al. (2016) highlighted the significance of IIS pathway in the control of graphene oxide (GO) toxicity in *C. elegans*, where mutations of *daf-2*, *akt-1*, *age-1*, or *akt-2* gene enhanced resistance of worms to GO toxicity. On the contrary, enhanced toxicity and susceptibility of worms that carry a mutation of the only *daf-16* gene to GO exposure was also reported. These findings provide evidence that GO can modulate the functions of *daf-2* (encodes the only homolog of IGF-1 mammalian receptor), AGE-1 (phosphatidylinositol 3-kinase), AKT-1, AKT-2-mediated kinase cascade, and DAF-16 (FOXO) transcription factor. These findings described on how different *C. elegans* strains can contribute to our knowledge of understanding on the roles of this signaling pathway in nanotoxicity formation in vivo (Zhao et al. 2016; Ma et al. 2009).

In terms of neurotoxicology, the nervous system of the worms represents another important target system where its neural fitness and neuromuscular defects in the forms of altered locomotion, reduced fecundity, and impaired olfaction can be used as parameters for nanotoxicity screening. Interestingly, most of the important human neurotransmitter systems employed for neuronal signaling and transmissions perform the same function in the worms (Kaletta and Hengartner 2006; Peterson et al. 2008). For example, serotonin and dopamine's significant roles in the movement are also required for the locomotion of worms (Vidal-Gadea et al. 2011). Worms have been pioneered as a model organism for various human pathologies including Alzheimer's disease (AD) (Levitan et al. 1996; Braungart et al. 2004), diabetes (Ogg et al. 1997), and human infections (Markaki and Tavernarakis 2010). Scharf et al. (2016) identified the neurotoxic effects of silica nanoparticles through widespread protein aggregations and activated amyloid fibrillation in *C. elegans* (Scharf et al. 2016).

17.2.4 *C. elegans* Mutant and Transgenic Strains in Nanotoxicology

The active involvement of researchers in the worm community aided with the output from *C. elegans* deletion mutant consortium has led to the creation of different mutant and transgenic strains. At present, more than 20,377 protein-coding genes in *C. elegans* and 6764 genes with associated molecular lesions through deletions or null mutations are available (WormBase WS220). Thus, mutants with predicted sensitivity for a distinct type of nanoparticles can be utilized to elucidate a distinct mechanism and signaling pathways involved in nanoparticle-mediated toxicity. One good example of such a mutation is in the genes involved in the antioxidant defense mechanism. Mutations of manganese/superoxide dismutase's encoding *sod-2* and *sod-3* genes heightened the sensitivity of worms to nanoparticles in comparison with wild-type N2 Bristol (Li et al. 2012). Wu et al. reported that exposure of *C. elegans* to DMSA-coated iron oxide nanoparticles, there were increased ROS productions with altered locomotion in *sod-2* and *sod-3* mutants in comparison with the wild-type worms, and this effect is much more pronounced in worms with double mutation of *sod-2* or *sod-3* genes (Wu et al. 2012). In another study, metallothionein-2 (MTL-2) protein that is encoded by *mtl-2* genes in *C. elegans* is observed to have protective effects against nanotoxicity by scavenging the enhanced ROS generation from metals exposure and also by binding with released metal ion nanoparticles (Table 17.3).

In another development, taking advantage of *C. elegans* transparent body combined with the availability of functional genetics tools, the upregulation and downregulation of target genes can be easily visualized using transgenic *C. elegans* which carries GFP reporter gene fused with DNA construct (Kaletta and Hengartner 2006). Thus, the gene of interest can be monitored and analyzed, and the fluorescence intensity quantified gives accurate information about the biological effect of a particular nanoparticle (Ma et al. 2009; Wu et al. 2012). For example, mutant strain CL2122, which carries *mtl* gene fused with the GFP reporter, has been used to detect the uptake of silver nanoparticle (Kim et al. 2017b). In another study, that demonstrated the similarities of toxicity mechanisms in *C. elegans*, both ZnO and $ZnCl_2$ nanoparticles could enhance the *mtl-2*::GFP expressions in transgenic *C. elegans*. This allows for speculations of the similarity in the process of intracellular biotransformation of both the nanoparticles to mediate the toxic effect observed (Ma et al. 2009).

Another commonly used markers for stress response genes are *daf-16* and *gcs-1* where GFP is fused to the either the C terminus of *daf-16* or promoter region of *gcs-1*. Responses to stress can be monitored by DAF16-GFP localization, which under optimal condition are located in the cytoplasm and translocation into the nuclei under different stressful conditions. In the case of GCS-1, GFP expression in worm intestine increases dramatically on exposure to toxic stress such as arsenic toxicity (Mohan et al. 2010). In monitoring different kinds of stresses, PMK-1, GST-4, and HSP-16.2 GFP carrying strains are often used to measure stress responses of nematodes exposed to different environmental toxicants or

Table 17.2 Examples of selected *C. elegans* transgenic strains commonly employed in nanotoxicology

Nanoparticles (NPs)	*C. elegans* transgene	Gene description	Findings	References
TiO_2 NPs	*sod-3::gfp*	Superoxide anion radical scavenger, protect against oxidative stress	Declined locomotion, intestinal ROS overproduction. Enhanced oxidative stress reporter in combination with nanopolystyrene particles	Dong et al. (2018)
MWCNTs	*let-7::gfp*	It is micro-RNA which exhibits mRNA 3′-UTR binding activity, involved in molting cycle, can regulate signaling macromolecule metabolic pathways	Decreased GFP expression in the body and intestine of a nematode. Dysregulation of development-timing transition, which is controlled by the let-7 gene, enhances intestinal ROS production and locomotion deficits	Zhao et al. (2017)
AgNPs	*mtl-2*::*gfp*	Gene encoding GFP fused with metallothionein 3 promoter. Gives protection against metal toxicity	The fluorescence signal of the AgNPs-exposed worms enhanced by fourfold in comparison with the nonexposed worms	Kim et al. (2017b)
Al_2O_3 NPs	*hsp16.2::gfp*	Encodes heat shock protein fused with GFP, it is involved in defensive response to heat, and localization is in the cytoplasm	Accumulation of intestinal lipofuscin in L1 larvae stage increased stress response and decreased in survival	Wu et al. (2011a, b)
CuO NPs	*hsp-16.2::gfp*	Gene-encoding GFP reporter driven by an hsp-16.2 promoter. Response to heat and unfolding protein function	Enhanced fluorescence intensity in hsp-16.2 transgenic strain upon nanoparticle exposure in comparison with untreated nematodes	Mashock et al. (2016)

(continued)

Table 17.2 (continued)

Nanoparticles (NPs)	*C. elegans* transgene	Gene description	Findings	References
ZnO NPs	*mtl-2::gfp*	Gene encoding GFP under the control of metallothionein 2 promoter, gives protection against metal toxicity	Nanoparticle exposure enhanced transgene expression in the mutant worms	Ma et al. (2009)
CeO_2 NPs	*gst-4::gfp*	It exhibits glutathione transferase activity	Enhanced ROS production levels, increased GST-4 fluorescence intensity on nanoparticle exposure	Rogers et al. (2015)
CeO_2 NPs	*hsp-4::gfp*	Orthologs of human HSP-70 have RNA polymerase II transcription factor binding activity, involved in ER stress response	Increased HSP-driven fluorescence intensity upon nanoparticle exposure, and this is dependent on dosage and exposure time	Rogers et al. (2015)
Fluorescent nano-diamond	*gcs-1::gfp*	Encodes GFP under the control of the *gcs-1* promoter, plays a role in resistance to arsenite and oxidative stress	Nanoparticle (0.5 mg/ml) exposure does not induce changes in GCS-1 and DAF-16 expressions	Mohan et al. (2010)
Ag NPs	*tph-1::DsRed*	Orthologs of human tryptophan hydroxylase 1; involved in axon regeneration, entry in dauer stage and adult life span regulation	Enhanced aggregations of tph-1::DsRed in ADF neurons, serotonergic neurons are a more sensitive target for Ag NPs	Piechulek and von Mikecz (2018)

nanoparticles (Wu et al. 2012). For example, exposure to Al_2O_3-nanoparticles was reported to have increased HSP-16.2 expression in nematodes (Yu et al. 2011) (Table 17.2). Overall, the analysis of gene expressions from transgenic worms can be easily reproducible with minimal variability unlike endpoints such as worm motility which measures lethality (Roh et al. 2006). Some examples of *C. elegans* mutant and transgenic strains commonly employed in nanotoxicology are mentioned in Tables 17.2 and 17.3.

Table 17.3 Examples of selected *C. elegans* mutant strains commonly employed in nanotoxicology

Nanoparticles (NPs)	*C. elegans* mutants	Findings	References
GO	*daf-16(mu86)*	Mutants strains exhibit reduced life span in comparison with wild-type nematodes	Zhao et al. (2016)
GO	*lys-1(ok2445)*	Enhanced the susceptibility to GO toxicity on the functions of both the primarily targeted organs and the secondary targeted organs	Ren et al. (2017)
GO	*daf-18(ok480)*	Worm carrying mutation exhibited susceptibility to GO toxicity as evidenced with defective in locomotion behavior and life span reduction	Zhao et al. (2016)
MWCNTs	*let-7(mg279)*	Worms carrying this mutation showed increased resistance to MWCNT toxicity upon exposure. Thus, the levels of intestinal ROS production and defective locomotion behavior are reduced in comparison with control	Zhao et al. (2017)
Ag-NPs	*pmk-1(km25)*	The increased levels of ROS formation and declining reproductivity observed were counteracted in comparison with wild type. Involvement of ROS and innate immune pathway PMK-1 p38 MAPK	Lim et al. (2012)
GO	*daf-2(e1370)*	Mutants showed resistance upon exposure to GO with enhanced head thrashing and the body bending capacity. Have a longer life span as compared with wild-type nematodes	Zhao et al. (2016)
MWCNTs	*hbl-1* or *lin-41*	Highly sensitive to MWCNTs, higher intestinal ROS production, and locomotion behavior is affected significantly	Zhao et al. (2017)
GO	*fat-5(tm420) nhr-49(nr2041)*	Mutant strain-dependent toxicity. Enhanced intestinal fat accumulation and shortened life span in *fat-5(tm420)* mutants. Enhanced life span in *nhr-49(nr2041)* mutants	Kim et al. (2019)
Ag-NPs	*pmk-1(km25)*	Ag-nanoparticle modulation of HIF-1, glutathione *S*-transferase (GST) enzyme activity, and reduced reproduction ability in wild type (N2) but not in a mutant strain of *C. elegans*	Lim et al. (2012)
Ag-NPs	*sod-3(gk235)*	Mutants exhibit dramatic enhancement in expression of different genes involved in MAPK signaling pathways as compared with the wild-type N2 worms	Roh et al. (2012)

17.2.5 *C. elegans* Lethal and Sublethal Endpoints

There are several endpoints which have been proposed for the assessment of nanomaterials using *C. elegans*, and these endpoints are classified under two categories: lethal endpoints and sublethal endpoints (Dhawan et al. 1999) as mentioned in Fig. 17.1. On the one hand, lethality is the basic endpoint for nanotoxicological

studies, and it can be measured by killing (mortality) assay, a manual method of scoring dead or live worms after exposing worms to nanoparticles. However, this manual counting increases the errors and can affect the accuracy in experimentation. Sublethal endpoints, on the other hand, are classified into morphological, behavioral, reproductive, developmental, and enzymatic endpoints (Jiang et al. 2016). In recent years, the importance of "3 Rs" (replacement, reduction, and refinement) is commonly referred for animal studies (Burden et al. 2015; Singh 2012), and *C. elegans* with its unique features has met with the 3R demands. Different toxicity ranking screens have repeatedly shown that *C. elegans* has predictive endpoints as that of the rat and mouse LD_{50} ranking by different researchers (Hunt et al. 2012). In some reports, there were suggestions that toxicity can also be easily assessed or equated by the survivability and mortality assays in *C. elegans* using its lethal endpoint. Williams and Dusenbery (1988) highlighted that mortality in adult *C. elegans* worms is comparable with that of higher mammals such as the rat and mouse LD_{50} ranking, and the same experiment in *C. elegans* can be done at a cheaper cost (Williams and Dusenbery 1988) (Fig. 17.1).

To monitor different sensitivities and on track changes, assessment of sublethal endpoints in worms has been used regularly for in vivo assessment and safety evaluation of different ENMs (Zhao et al. 2013; Charão et al. 2015). The credibility of these assessments has come from years of research on this model organism. Thus, evaluations of different sublethal endpoints can be conducted in a well-established and fairly systematic manner. These endpoints mainly include the rate of worm's survival and life span monitoring (Barsyte et al. 2001; Harada et al. 2007); growth inhibition and development (Anderson et al. 2001; Swain et al. 2004); increased cell death and germ-line apoptosis (Kim and Sharma 2004); changes in reproduction, progeny production, or phenotypes (Wang and Yang 2007; Wang et al. 2007); and changes in pharyngeal pumping rate, body motion, behavior, and feeding behavior (Wang and Xing 2009; Chen et al. 2013) (Fig. 17.1).

Based on its specificity and sensitivity, sublethal endpoints for different toxicants are manifested differently. Thus, morphological endpoints can be carried for a fast and more sensitive indicator of toxicity. Briefly, morphological endpoints can be assessed in terms of body width and length measurements of the nematodes. These measurements can be generally done manually with capturing images of worms by light microscope and its further analysis with image software. Different behavioral endpoints can also be assessed where changes in behavior can serve as an indirect measurement of internal physiological state or response to external stimuli. Thus, changes that affect the locomotion, body bend frequency (turning frequency), head thrash frequency, and pharyngeal pumping are often informative (Jiang et al. 2016). Scharf et al. observed that *C. elegans* on exposure to silica nanoparticles and its accumulation in the pharynx and vulva often resulted in an altered organ function, reduced pharyngeal pumping, and increased egg-laying capability (Scharf et al. 2013) (Fig. 17.1).

Sublethal endpoints are also associated with oxidative stress, and this is routinely monitored using transgenic/mutant strains fused with anti-oxidative enzyme and stress response genes (Li et al. 2012). Furthermore, parameters ranging from the

innate immune response, nervous system and neuronal functions, intestinal morphology, and epidermal vulnerability have been employed and raised (Wang and Wang 2008; Ma et al. 2009; Yang et al. 2015; Zhao et al. 2016a). For example, exposure to CeO_2 nanoparticle aggregates even at higher concentration is not linked with mortality/lethality endpoints but is associated with organism stress markers with sublethal endpoints in the form of higher levels of ROS, HSP-4, and declined fertility rate (Rogers et al. 2015). Using light microscopy and complex object parametric analyzer and sorter (COPAS), it was shown that cadmium can reduce the intestinal diameter and opacity of the worms. This provides a clue that identification of other similar intestinal toxicants using high-end optogenetic devices and high-throughput microfluidics techniques in *C. elegans* can make identification of classifications of various nano-toxins easier (Hunt et al. 2012) (Fig. 17.1).

17.2.6 Predictive Nanotoxicology Using *C. elegans*

Predictive toxicology forms a part of the modern pharmaceutical approach in drug discovery, it is proposed to have simplified the drug development process, and this is applicable too for fast and accurate assessment of nanoparticle toxicities in the near future. Recently, *C. elegans* in vivo assays have been used successfully in predictive toxicology testing (Hunt 2017). Interesting findings were also reported by Yang and his colleagues (2017) on the toxicity of zero-valent nanoparticles, wherein a toxicity-based-toxicokinetic (TBTK)/toxicodynamic (TD) modeling of different endpoints of *C. elegans* model has been formulated, a reiteration on the significance of this organism in the field of predictive toxicology. Empirical data obtained from this bioaccumulation experiments and nanotoxicity studies on fertility, locomotion, and development of *C. elegans* after Fe_0 nanoparticle exposure have been used to investigate environmental and health risks of Fe_0 nanoparticles and to regulate eco health with controlled applications of Fe_0NPs for environmental remediation and sustainability (Yang et al. 2017). Thus, *C. elegans* biomarker-based risk model, although in its early stage of development, is most likely to have a huge application in the field of modern predictive nanotoxicological sciences.

17.3 Biomarkers and Molecular Response to Nanoparticle Toxicity

At the cellular and molecular levels, nanoparticle toxicity can be initiated and responded by several mechanisms, and this is dictated directly by the physicochemical properties of nanoparticles and exposure conditions mentioned in Fig. 17.2. Many mechanistic studies on nanoparticle toxicity have highlighted the involvement of overwhelming oxidative stress level for nanoparticle-mediated toxic effects (Thomas et al. 2011; Handy et al. 2012) shown in Fig. 17.4. Its indirect involvement in the mechanism of nanoparticle toxicity has been proposed; for example, Hussain and his colleagues observed that Au-nanoparticle exposure leads to endoplasmic

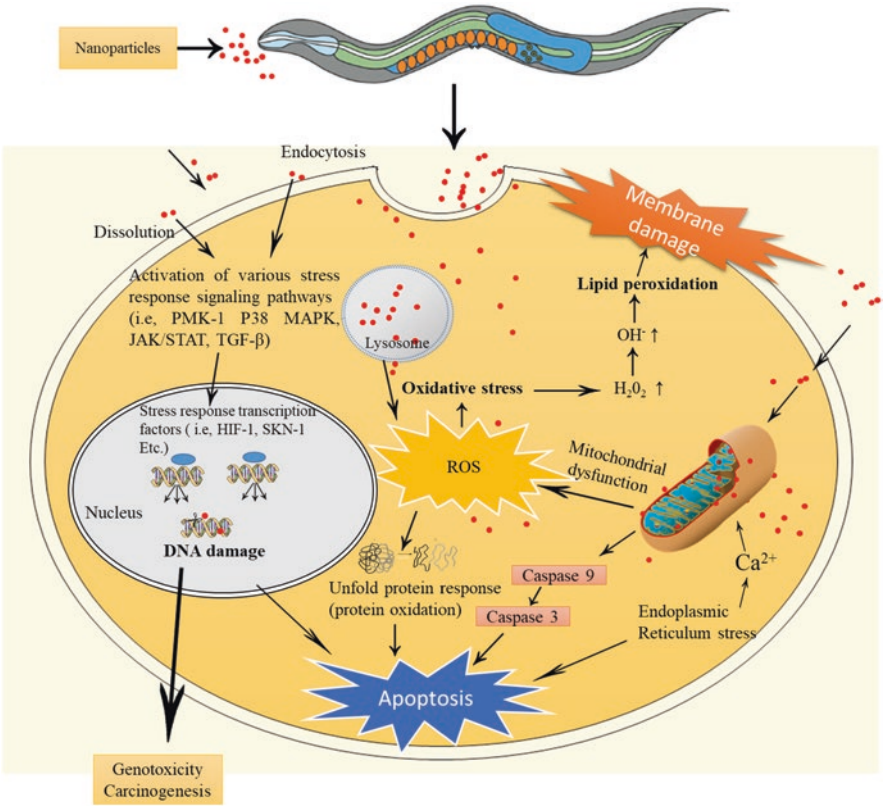

Fig. 17.2 Common mechanisms of nanoparticle-mediated toxicity in *Caenorhabditis elegans*

reticulum (ER) stress and an unfolded protein response (UPR) in *C. elegans* (Hussain et al. 2005) (Fig. 17.3).

17.3.1 Oxidative Stress

The high concentration of reactive oxygen species (ROS) or free radicals can exaggerate oxidative stress, and this is mainly due to imbalances between prooxidant and antioxidant enzyme level in an organism (Jat and Nahar 2010; Tan et al. 2018). ROS could be overproduced either directly by intrinsic ability (i.e., direct generation of ROS by nanoparticles due to acidic surrounding such as intestine or lysosomes either from leached ion or from the surface of the nanoparticles) or indirectly by nano-biological interactions (Choi et al. 2014) (Fig. 17.3). Chemically, it was proposed that on exposure to nanoparticles, an overproduction of ROS can occur due to the availability of an electronically active surface or photoactivation, transition metal impurities, and due to toxic metal ions (Ma 2010; Ludwig et al. 2007; Li et al. 2008; Damoiseaux et al. 2011). Wu and his colleagues have shown that TiO_2,

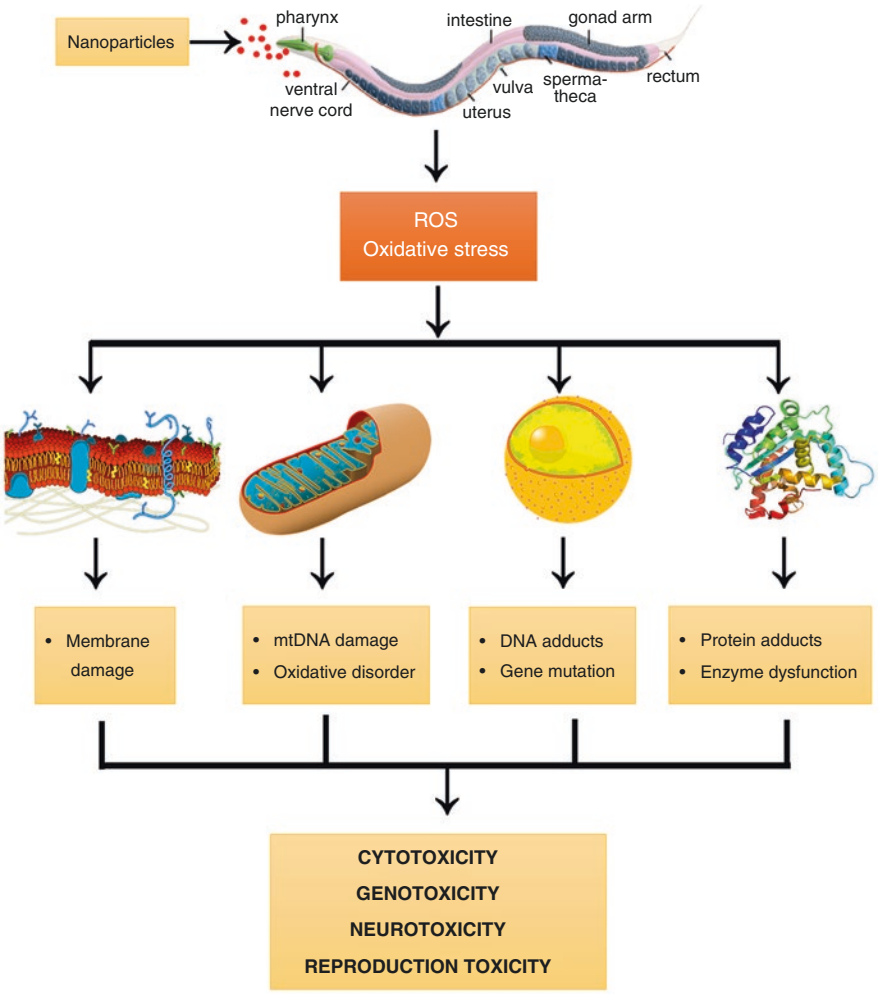

Fig. 17.3 Common cellular and molecular events of nanoparticle-based toxicity mediated through enhanced ROS generation and oxidative stress

ZnO, and SiO_2 nanoparticles enhance the production of ROS, and their toxicities can be correlated with different worms endpoints such as mortality, locomotion, development, and reproduction (Wu et al. 2013). Furthermore, among all the three nanoparticles studied, TiO_2 nanoparticles were reported to be more toxic as observed with significant decline in head thrash and body-bending movement in mutant strains of *sod-2*, *sod-3*, *mtl-2*, and *hsp-16* strains compared with Bristol N2 type (Wu et al. 2014).

The chemical properties of the nanoparticles directly dictate the levels of ROS that can be generated, and what kind of existing nano-biological interactions exists could orchestrate the whole process. Thus, it is possible that some

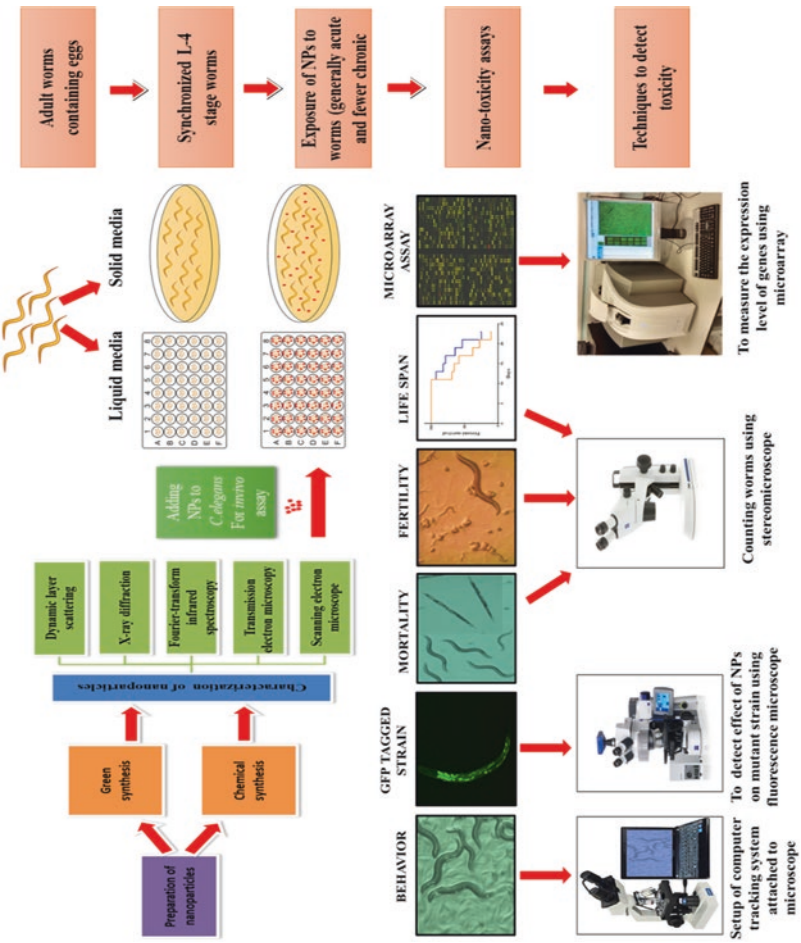

Fig. 17.4 Summarized and simplified diagram depicting the common utilities and methods involved in nanotoxicological studies using *C. elegans* model organism

nanoparticles which lack the intrinsic ability of ROS production can also generate ROS via interaction with the biological system and organelles. For example, nanoparticle direct interaction with organelle-like mitochondria could initially begin with disruption of cell cytosolic membrane, followed by changes in membrane potential and interrupted functions of the electron transport chain and oxidative phosphorylation. This will finally end up with overwhelming ROS levels (Xia et al. 2006; Meyer et al. 2013). Choi et al. have indicated that binding of nanoparticles to membrane receptor can activate the receptor present, and the amplification of intracellular cascades such as MAPK changes the expression levels of stress response genes that could influence ROS production (Choi et al. 2014). Other mechanism reported to have been involved in enhanced oxidative stress levels on nanoparticle exposure is the increased accumulation of high calcium level (Marano et al. 2011; Soenen et al. 2011).

Some types of nanoparticles can have a catalytic activity which is due to photoactivation. Kim et al. (2017a) examined the adverse effect of TiO_2 nanoparticles on the nematode *C. elegans* with or without UV activation, and they observed that UV-activated TiO_2 nanoparticles significantly reduced the reproduction potential of the worms via oxidative stress mechanism (Kim et al. 2017a). Exposure to TiO_2 nanoparticles is reported to enhance levels of intestinal ROS, brood size reduction, and retarded locomotory behavior in worms (Li et al. 2012; Wu et al. 2013). In another interesting study, Dong et al. (2018) have emphasized on the role-combined effects, wherein nano-polystyrene particles further exaggerate TiO_2 nanoparticle-mediated toxicity as seen with the impaired motor neuron and change in locomotion behavior in SOD-3 mutant worms. This enhanced toxicity which could not be produced by nano-polystyrene alone is linked to enhanced activation of intestinal ROS and accumulation of oxidative stress (Dong et al. 2018).

Among the nanoparticles, Ag-nanoparticles are the most studied in *C. elegans*, and this is based purely on their antimicrobial properties and different potential biomedical applications. On the contrary, there were reports suggesting that the release of Ag^+ ions from the Ag-nanoparticles could catalyze the production of free radicals (Chávez-Andrade et al. 2017). Lim et al. (2012) have shown that exposure of Ag-nanoparticles to wild-type *C. elegans* (N2) enhanced ROS formation through significant upregulation of PMK-1 of p38 MAPK pathway at both gene and protein levels. Additionally, Ag-nanoparticle modulation of HIF-1, glutathione *S*-transferase (GST) enzyme activity, and reduced reproduction ability in wild-type (N2) but not in a mutant strain of *C. elegans pmk-1* (km25) were also reported (Lim et al. 2012). Similarly, CeO_2-nanoparticle exposure was linked to enhanced oxidative stress, inflammation, and genetic damage, whereby the redox cycle and oxidation state between Ce^{3+} and Ce^{4+} are thought to magnify the production of free radicals. In *C. elegans*, CeO_2 nanoparticle exposure is linked to a short life span, inhibition of growth and development, decrease fertility, and thermo-intolerance (Rogers et al. 2015).

17.3.2 Genotoxic, Apoptosis, DNA Damage, and Repair

Apoptosis or programmed cell death which is under extreme regulations is part of normal animal development. Similarly, in *C. elegans*, the numbers of genes are linked with the apoptotic process during embryonic development, wherein apoptosis process is involved in the removal of 113 cells during the normal development process of adult hermaphrodite, while in the larval stages, the death of 18 cells occurred due to apoptosis (Sulston and Horvitz 1981; Sulston et al. 1983). In *C. elegans*, options for both developmental cell death (programmed cell death) and necrotic-like cell death during extensive cell injury have been reported too. Hence, to protect the somatic cells of adult *C. elegans* which are mainly post-mitotic in nature, it is crucial that the structures, numbers, and fidelity of DNA replication are at their best constantly.

At the cellular level, the process of replication of DNA and the different mechanisms required for repair is reported to be conserved between *C. elegans* and other higher mammals (Leung et al. 2008). Two accepted paradigms for nanoparticle toxicity arises because of their involvement in excess production of ROS and other proinflammatory markers. These excess markers which are interconnected at different levels in their targets can overwhelm the cell components, thereby causing irreparable damage if not properly controlled. Thus, with DNA considered to be susceptible to enhanced oxidative stress, the most accepted hypotheses to explain nanoparticle roles in DNA damage rely directly on ROS levels. Overproduction of ROS influenced by nanoparticles can oxidatively modify DNA, leading to strand breaks, unspecific base pairing, and formation of abasic sites which in return induces mutation, tumorigenesis, and aging-related diseases (Valko et al. 2006) (Fig. 17.4). Chatterjee et al. observed that Ag-nanoparticle exposure to *C. elegans* can produce oxidative modifications of DNA and strand break which triggers the *hus-1* components of DNA damage checkpoint pathway and finally programmed cell death. In the *pmk-1* mutant (homologue of p38 MAPK and function in apoptosis), the DNA damage level was reported to be higher, and instead of apoptosis, necrosis occurred in *pmk-1* mutant (Chatterjee et al. 2014). In another comparative and evidence-based toxicity study, it was found that Ag-nanoparticles and $AgNO_3$ nanoparticle exposure in *C. elegans* enhanced the levels of a specific biomarker of oxidative damage and mutagenesis, 8-OHdG (8-hydroxy-2′-deoxyguanosine), a clue for the cause toxic effect observed (Ahn et al. 2014).

The exposure of an organic-based nanoparticles and hydroxylated fullerene nanoparticles in *C. elegans* was also reported to have induced its programmed cell death pathway (PCD) (Cha et al. 2012). This is also linked with ROS exaggerations on nanoparticle exposure that ultimately proceeds with either necrosis, PCD, or both (Stergiou and Hengartner 2004; Lant and Derry 2013). Khare et al. (2014) provide evidence that ZnO nanoparticle apoptosis activation occurred in *C. elegans* with upregulated expressions of different proapoptotic genes such as *ced-3*, *cep-1*, *ced-13*, *ced-4*, and *egl-1* observed in ZnO nanoparticles exposed worms in comparison with untreated control. Similarly, based on the size, a 21 nm ZnO nanoparticles could downregulate important antiapoptotic gene *ced-9* (Khare et al. 2015).

According to nucleotide sequence homology, proteins which function in DNA repair mechanism are highly conserved between *C. elegans*, *Mus musculus*, and *Homo sapiens*. Similarly, the use of mutant strains deficient in DNA repair (e.g., *nth-1*, *xpa-1*) and germline DNA damage checkpoints mutant strains (e.g., *mrt-2*, *hus-1*, and *rad-5*) has been significant in understanding biologically relevant mechanisms involved in the toxicity of nanoparticles. This has wide applications with respect to DNA damage, genomic instability, and in testing the anticancer activities of different NPs (Stergiou and Hengartner 2004). Hunter et al. reported that efficiency of base excision repair (BER) in *C. elegans* found to be similar with that observed in mammals in repairing in vivo of alkylating and oxidatively modified mtDNA, nuclear DNA that is damaged after H_2O_2 pretreatment (Hunter et al. 2012). Thus, *C. elegans* has an enormous potential in the investigating field of DNA damage and repair processes and in understanding the roles of different nucleic acid toxicants.

17.3.3 ER Stress and Heat Shock Proteins

Endoplasmic reticulum (ER) is known as an organelle required for proper protein formation, leading into their native conformations besides its role as a storehouse of calcium. Normally, proteins are synthesized from ribosome which is bound to rough ER, and improperly folded proteins are detected and modified to their native conformations by molecular chaperones and enzymes which are present in the ER lumen. On the contrary, under numerous unwanted conditions, accumulation of misfolded/truncated proteins followed with disturbance in Ca^{2+} homeostasis predisposed to a cell to ER stress. It was reported that Au-nanoparticle exposure can lead to protein denaturation and improper folding (Nel et al. 2009). Under stressful conditions, ER loses its functions, and this causes further accumulation of misfolded proteins and protein denaturation. Added with changes in calcium homeostasis, this can lead to enhancing the activity of the unfolded protein response (UPR) pathway. This activation of UPR pathway is required for the degradation of protein via apoptosis (Lai et al. 2007; Nel et al. 2009; Xu and Park 2018). In *C. elegans*, the UPR pathway is triggered by both canonical and noncanonical pathway. The canonical pathway consists of upregulated molecular chaperones (heat shock protein). The noncanonical pathway comprises UPR response, and it includes 25 genes upregulated from abu/pqn families (Haskins et al. 2008).

Tsyusko et al. (2012) reported that Au-NPs (4 nm) have the ability to gain access inside *C. elegans* cell through clathrin-mediated endocytosis, and this entry enhances the formation of improperly folded/unfolded proteins, followed by mediation of ER stress, and the commence activity of both canonical and noncanonical UPR pathways. Thus, irreversible and accelerated cell death will occur that is significant in the case of *C. elegans* postmitotic tissues. Additionally, Au-NPs exposure to worms is reported to have interfered with Ca signaling and amyloid processing pathways, that would result in the accumulation of Ca^{2+} intracellularly and the ultimate promotion of non-caspase proteases events and calpain/cathepsin axis activation leading to

cell necrosis and destruction (Tsyusko et al. 2012). The role of p38 MAPK in giving protection against nanopolystyrene particle toxicity by activating XBP-1-mediated ER-UPR in *C. elegans* intestine was also reported (Qu et al. 2019).

Molecular chaperones are a highly conserved and ubiquitous class of folding modulators expressed in all subcellular compartments that have a crucial role in preventing nonnative conformations and stabilization of various proteins. Molecular chaperones also known as heat shock proteins (HSPs) are classified according to their molecular mass and different families of genes. On exposure to Au-NPs, 26 pqn/abu genes of the noncanonical unfolded protein response (UPR) pathway are reported to be upregulated besides molecular chaperones (*hsp-16.1*, *hsp-70*, *hsp-3*, and *hsp-4*), and this further confirmed ER stress involvement. Additionally, the heightened sensitivity to Au-NPs in a mutant strain of worms (*pqn-5*) is an indication for the direct involvement of this pathway in amelioration of Au-NPs toxicity (Tsyusko et al. 2012). Furthermore, on chronic exposure of worms to CeO_2 nanoparticle, aggregation of proteins is linked to the enhancement of ROS generation and HSP-4 expression, but not mortality (Rogers et al. 2015).

17.4 *C. elegans*-Based Assays for Nanoparticle Toxicity Studies

17.4.1 Survival Assay

Among the different assays available in the worm model, the notable survival assay has been used regularly prior to in-depth identification of novel genetic factors, molecules, or signaling pathways involved (Hae-Eun et al. 2017). As part of its application, exact environmental conditions for different survival conditions (life span assays, abiotic stress resistance assays, and pathogen resistance assay) should be standardized first based on the sensitivity and features of a typical survival assay to be employed (Amrit et al. 2014; Keith et al. 2014). Survival assay can be carried out with synchronized isogenic populations in both solid and liquid media, fed with *E. coli* OP5O as their food source. With the exception of some temperature-sensitive mutants, worms can be grown at different temperatures (15–25 °C), with 20 °C considered to be ideal for survival assay. After nanoparticle exposure, counting of live and dead worms by simple light microscope can be done at regular intervals (e.g., hours or days). To facilitate counting and to differentiate between dead and live worms, fluorescent dyes, such as SYTOX, can be used to measure the viability of *C. elegans* cells differentiated with fluorescent signals of dead worms (Gill et al. 2003). Subsequently, for survival analysis, two widely used curves are simple survival curves/mortality rate with Kaplan/Meier survival plots to illustrate the percentage of animals alive at different time scales (Kaplan and Meier 1958). Additionally, log-rank test and Fisher's exact test and other statistical methods are also employed for analyzing survival curves (Fisher 1990; Mantel 1966).

17.4.2 Biochemical and Oxidative Stress Assays

Different standardized biochemical assays and protocols are available to evaluate changes in the biochemistry of *C. elegans* upon nanoparticle exposure. The metabolic activity can be measured by monitoring oxygen consumption level (polarographically using Clark-type electrodes), carbon dioxide generation (gas respirometry), and heat production (by microcalorimetry). Carbon dioxide generation is reported to reduce drastically by about 50% in 12 days in comparison with a 6-day-old worm; hence, proper references should be utilized (Braeckman et al. 2002; Van Voorhies and Ward 1999). Changes in the energy status of the worms after nanoparticle exposure can be done with ATP measurement of worm fractions using enzymatic or luciferase-based reactions. Biochemical changes in activity levels of different enzymes such as acetylcholinesterase (AChE), alkaline phosphatase (ALP), catalase (CAT), and SOD enzymes (Wang and Wang 2008; Roh and Choi 2008) and relevant molecular and genetic endpoints such as HSP and MTL are other markers that are routinely monitored (Swain et al. 2004; Shashikumar and Rajini 2010).

Emphasizing on the significance of enhanced ROS and oxidative levels in nanotoxicology, different sensitive methods and protocols are available for its measurement. Oxidative stress resistance assay is employed to measure the sensitivity and resistivity of the worms. These include exposure to t-BOOH, H_2O_2, and paraquat treatment (Castello et al. 2007; Keith et al. 2014). Most of these assays enhanced ROS production levels and can be used to check to the resistance of the worms prior to or after nanoparticle exposure. Detection of carbonylated proteins by DNPH and lipid peroxidation adduct measurements can be done spectrophotometrically to measure oxidative damage after exposure. Staining techniques commonly employed include fluorescent markers such as CM-H_2DCFDA (5-6,chloromethyl-2,7-dichlorodihydrofluorescein diacetate), MitoSOX, propidium iodide, and Sytox to measure cellular viability (Hunt et al. 2012; Roth et al. 1997). Nile red and BODIPY-labeled fatty acids stain for lipid (Ashrafi et al. 2003; Mak et al. 2006) can be performed to get a wholesome idea of nanoparticle toxicity whether in larvae or adult worms. Antibody-based histochemical stains and protein expression studies such as Western blot and Elisa (enzyme-linked immune-sorbent assay) with sensitive detection systems have also been applied with high accuracy in *C. elegans* (Duerr 2006). Other commonly used methods are transmission electron microscopy (TEM) for visualization of body morphology, the TUNEL (terminal transferase dUTP nick end labeling) for detection of DNA fragmentation and apoptosis, SYTO dyes, and 4,6-diamidino-2-phenylindole (DAPI) to assess nuclear morphology and dead cells. Numbers of apoptotic cells can be visualized using green fluorescent protein (GFP) transgenic nematodes strains fused with genes of different components of PCD pathway (Chalfie et al. 1994).

17.4.3 Toxicogenomic Studies and Genomic Assays

To monitor the effect of tested nanoparticles at the genetic level routinely, visualization of fluorescence intensity of *C. elegans* using the reporter genes of GFP and β-GAL (LacZ) by fluorescence microscopy is employed routinely (Fire et al. 1990). Characteristics of fragmented DNA of apoptotic cells or germline can be visualized using the fluorescent dye acridine orange (AO) dye (Kelly et al. 2000). Microarray, ChIP-seq with histone modification-specific antibodies and qRT-PCR are another recent and more sensitive techniques that have been proved to be useful for genetic studies in *C. elegans* (Hall et al. 2010). Genome-scale RNAi screen to elucidate target gene expression during exposure conditions has been reported too (Wang et al. 2009). To provide in-depth information into the activating functions of different genes within different tissues, determination of the spatiotemporal pattern of gene expressions in *C. elegans* has also been reported. Unique transcriptional expression patterns for the effect of a particular nanoparticle can be determined in *C. elegans* model on a large-scale by the application of serial analysis of gene expression techniques (SAGE) (Ruzanov and Riddle 2010; Velculescu et al. 1995).

17.4.4 Reproductive Assays

In *C. elegans*, the development of reproductive organs can be assessed with different assays to measure reproductive toxicity of a particular nanoparticle. These assays include brood size, the number of transgenerational progeny (beyond the egg stage), number of oocytes, embryonic lethality, and male formation assay (Zhao et al. 2016). The size of gonads can be a good prediction of reproductive toxicity potential of a tested nanoparticle (Wu et al. 2011a, b). Toxic effects can also be determined by the decline in egg-laying capacity, disturbed egg-laying pattern, and the decline in number of viable progenies that are reproduced at different time points (Gomez-Eyles et al. 2009; Smith et al. 2013). Multigeneration reproductive toxicity of nanoparticles can also be evaluated for different worm generations.

17.4.5 Nervous Tissues Toxicity Assay

In *C. elegans*, primary evaluation of the toxic effects of different nanoparticles on the nervous system can be carried out by simple tracking of the locomotion behavior and movement speed, an estimation of motor neurons functions in nematodes. Specific fluorescently tagged neurons can be visualized by microscopy. Example are DAergic neurons through dopamine transporter (*dat-1*::GFP reporter) (Helmcke et al. 2010; Vanduyn et al. 2010), serotonergic neurons through tryptophan hydroxylase (*tph-1*::GFP reporter) (Sze et al. 2000; Nass et al. 2002), GABAergic neurons through glutamic acid decarboxylase (GAD) (*unc-25*::GFP) (Cinar et al. 2005),

cholinergic neurons through *unc-1*::GFP (a close homolog of mammalian protein stomatin) (Winnier et al. 1999; Nass et al. 2002), and glutamatergic neuron through *eat-4*::GFP (Lee et al. 1999; Earls et al. 2010). Additionally, analysis of axonal degeneration and loss of neuronal contact, analysis of certain neurons types such as the AVL and the DVB neurons, thermotaxis learning assays, paralysis, neurotransmitter, and enzyme assays such as AChE levels are often carried out. Indeed, with an expanding *C. elegans* toolkit and the availability of new technologies in the optogenetics field, the transparent body and well-defined nervous system of worms are ideal for evaluation of nanoparticle toxicity.

17.4.6 Growth, Development, and Life Span Assays

Measuring the synchronized nematode body length is a common method used for determining the developmental effects of different nanoparticles. Using standard references, measurement of the length (dorsal/ventral; tip of head to tail) and width (ventral to posterior end of the vulva) can be done using simple microscope and image analyzer (Boyd et al. 2010; Cha et al. 2012; Rudel et al. 2013; Wu et al. 2013; Zhuang et al. 2014). Applications of high-tech equipment such as COPAS Biosort, microfluidic devices with automated sorting, counting devices, and morphological detection systems have made the process faster with accuracy. Dauer formation assay can also be used to investigate the possible effect of nanoparticles on development (Wang et al. 2010).

For life span assay, healthy synchronized L4 stage worms can be grown in the presence of 5-fluorodeoxyuridine (FUDR) to prevent progeny production, and the parent plate is transferred into a fresh plate every 3 days where counting of dead or live worms is scored (Shen et al. 2009; Wang et al. 2010; Zhuang et al. 2014). Lipofuscin measurement is an autofluorescent marker of oxidative degeneration of cellular components that usually increased in levels with the aging of organisms (Brunk and Terman 2002). Lipofuscin levels can be measured spectrofluorimetry using a band filter of 525 nm, and the intensity can be determined using the mean or net pixel of the whole body/intestine of each animal to determine the effect of a particular nanoparticle on the age of the worms. Other parameters that can be useful indicators of normal and accelerated aging include pharyngeal pumping rate, body movement, chemotaxis response, defecation pattern, lipids, and enzyme activity levels (Klass 1977; Garigan et al. 2002).

17.5 Major Signaling Pathways and Their Roles in Nanotoxicity in *C. elegans*

17.5.1 p38 MAPK Signaling Pathway Roles in Nanotoxicity

Stress-associated mitogen-activated protein kinase (MAPK) pathway responses to a variety of stressors, thereby serving a transducer role to convert extracellular

signals into various intracellular outputs. In *C. elegans*, the p38 MAPK signaling pathway is highly conserved with orthologs *pmk-1*, *sek-1*, and *nsy-1* available. On receiving signals from the extracellular cell surface, p38 isoforms transduce the signals into the nucleus to modulate the activities of numbers of transcription factors. For example, as part of its response and regulation to arsenite stress, *pmk-1* of MAPK transactivates transcription factor *skn-1* gene. The SKN-1 protein, in turn, can translocate into the nucleus which activates *aip-1* gene (which encodes for a protein carrying the ring finger domain) to protect cells from the overwhelming arsenite toxicity (Inoue et al. 2005). Inhibition of *pmk-1* gene by RNAi was also found to enhance the worm's susceptibility to pathogens, which suggests that PMK-1, NSY-1, and SEK-1 proteins are crucial players in the defense response of the worms (Kim et al. 2002).

On the one hand, Roh et al. reported that Ag-nanoparticle exposure in worms can upregulate genes involved in MAPK pathway in wild-type *C. elegans* as seen with activation of *sod-3* gene expression (Roh et al. 2012). On the other end, it was found that Ag-nanoparticle exposure increased ROS production in wild-type *C. elegans* which was rescued from *pmk-1* (km25) strain, a direct indication that Ag-nanoparticle modes of toxicity are through oxidative stress (Lim et al. 2012). In addition, SKN-1/Nrf is thought to play additional roles through phase II detoxification involving glutathione *S*-transferase 4 (GST-4) which is present in the pharynx, hypodermis, and intestine of the worms. Studies have shown that intestinal knockdown of GST-4 by RNAi enhanced the susceptibility of worms to GO toxicity manifested in the form of decreased life span and enhanced intestinal ROS levels.

17.5.2 Insulin Signaling Pathway Roles in Nanotoxicity

In *C. elegans*, the transcriptionally active DAF-2/IGF-1 signaling pathway regulates many genes which are important for *C. elegans* longevity, growth, and metabolism. The activation of this signaling pathway has been linked to various processes such as fat storage and immune and stress responses (Zhao et al. 2016b). Its significant roles required for maintaining the longevity of worm intestine and neurons have been reported based on tissue-specific activity assay (Libina et al. 2003). When a ligand molecule insulin binds to DAF-2/IGF-1 receptor, it transmits signals through tyrosine kinase domain of the receptor to stimulate several kinases that includes phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase (PDK-1/3), serine/threonine kinase (AKT-1/2/Akt/PKB), and serine or threonine-protein kinase (SGK-1) (Gami and Wolkow 2006). Further phosphorylation of AKT and SGK-1 leads to downregulation of DAF-16/FOXO transcription factor which inactivates its target gene such as *sod-3* (Gami and Wolkow 2006; Yang et al. 2015).

In normal physiological condition, DAF-2 activation and the ultimate DAF-16 phosphorylation are necessary to keep it sequestered in the cytoplasm for regulating the normal life span of the worm (Antebi 2007). On the contrary, inactivation of DAF-2 by different capable stimuli can free DAF-16 from phosphorylation, and thus, it can translocate to the nuclei to transactivate or initiate the expression of a

series of genes that include defense and stress-related genes (Baumeister et al. 2006). ZnO-nanoparticle exposure is reported to have modulatory roles in the insulin signaling pathway in a dose-dependent manner (Khare et al. 2015). On the one hand, multi-walled carbon nanotube (MWCNT)-mediated toxicity is reported to have repressed the expression levels of *daf-16* and *daf-18* genes (Zhao et al. 2016a). Nano-polystyrene, on the other hand, has been linked with downregulation, and *daf-2*, *age-1*, and *akt-1* transcriptional factors (Shao et al. 2019) of the insulin signaling pathway were also reported.

17.5.3 The Programmed Cell Death (PCD) and DNA Damage Pathway

The programmed cell death is the evolutionarily conserved program for self-destruction and is important for the development and homeostasis of the functional organ (Lettre and Hengartner 2006). The core PCD pathway in *C. elegans* is similar to the human pathway, and this includes *egl-1* (*BH3*-only gene), *ced-9* (*bcl-2*), *ced-4* (*apaf-1*), and the terminal *ced-3* (caspase) (Conradt and Horvitz 1998; Hengartner and Horvitz 1994; Hengartner et al. 1992). For the execution of apoptosis, CED-3 is required, and it requires autocatalytic cleavage which is initiated by *ced-4*, acting as an initiator caspase. In living cells, interactions of CED-9 are required for sequestration of CED-4, and hence its affinity for CED-3 protein to remain on the outer surface of mitochondria will inhibit the cells to undergo PCD (Chen et al. 2000). In cells that are destined to undergo PCD, conformational changes of CED-9 take place through interactions with EGL-1, and these changes relieve CED-4 from the regulation of CED-9 (Yan et al. 2004). Other candidates include EGL-1 (apoptosis upstream activator and checkpoint), CEP-1 (ortholog of human tumor suppressor p53), CLK-2 (ortholog of telomere length-regulating protein Tel2p), and checkpoint protein HUS-1.

In *C. elegans*, ZnO nanoparticle is reported to have modulatory activity on the expression level of *cep-1*, which causes germ-line cell death through cep-1/p53-dependent signaling pathway (O'Donnell et al. 2017). The CEP-1 activation triggers the activation of *egl-1* and *ced-13* genes. EGL-1 and CED-13 interact with CED-9 homolog of BCL-2 (antiapoptotic protein) that cause inhibition of its transcription, activates and induces the release of CED-4, and therefore activates CED-3 (caspase) which initiates apoptosis. Upregulation of retinoblastoma protein-coding gene *lin-35* which inhibits ced-9 has also been reported, and this can further promote physiological apoptosis. In addition, *efl-2* and *dpl-1* encode for transcription factor E2F, and the cooperative work of DPL-1, RB, and E2F increases the expression levels of caspases which promotes the germ cell apoptosis (O'Donnell et al. 2017). Recent reports have also shown that *C. elegans* carrying either mutation of *ced-3* or *ced-4* can reverse germline apoptosis that is activated by GO exposure; whereas, mutation of *ced-9* accelerates germline apoptosis upon GO prolonged exposure (Conradt and Horvitz 1999).

17.5.4 TGF-Beta Signaling Pathway

The intercellular signaling molecule, transforming growth factor-β ligands (TGF-β), plays a significant role in communication between cells in eukaryotic organisms. TGF-β ligand functional and molecular mechanisms are highly conserved, with regulations and components of the TGF-β signaling pathway in *C. elegans* found to be similar to higher organisms. Genes which encode for five members of TGF-β ligands (*dbl-1*, *daf-7*, *unc-129*, *tig-2*, and *tig-3*) have been identified in *C. elegans* genome. From these ligands, DBL-1 and DAF-7 interact and play their role by canonical receptor-*smad* signaling mechanism, while UNC-129 interacts and functions through the noncanonical signaling pathway. TIG-2 and TIG-3 members of TGF-β ligand function are not well described, and *daf-1*, *daf-3*, *daf-5*, *daf-8*, *daf-12*, and *daf-14* are the essential components in TGF-β pathway in *C. elegans* (Savage-Dunn and Padgett 2017). In 2017, Kim et al. described a comparative analysis in which *C. elegans* response was observed underexposure of TiO_2 nanoparticles and UV-activated TiO_2 nanoparticles using mutant strains which clearly showed that TiO_2 nanoparticles induce toxicity through JAK/STAT pathway while UV-activated TiO_2 nanoparticles could induce toxicity through TGF-β pathway. The activation of both JAK/STAT and TGF-β pathway across TiO_2NPs leads to phototoxicity and reproductive failure in *C. elegans* (Kim et al. 2017a).

17.5.5 Other Signaling Pathways

Levels of important molecules involved in the innate immune system of the worms change drastically on exposure to nanoparticles. There were instances of significantly increased and decreased expressions of different isoforms of lysozymes, saponin-like proteins, etc. (*lys-1*, *dod-6*, *F55G11.4*, *lys-8*, and spp-1), in *C. elegans*, which is presumed to be dependent on types of nanoparticles and exposure time. Similarly, mutations of any lysozyme genes (*lys-1* and *lys*-8) and RNAi knockdown of different antimicrobial genes could accelerate GO toxicity as observed with increased intestinal ROS production and declined locomotive behavior (Ren et al. 2017). These findings pinpoint to the fact that innate immune response signaling pathways have a defined role to play in the process of nanotoxicology.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway represent a signaling pathway that can integrate a multitude of signals for development and homeostasis in animals. Transcriptomic studies suggested that exposed TiO_2 nanoparticles cause deregulation of JAK/STAT pathway, a pathway known for its involvement in development and reproduction process. Also, TiO_2 NPs exposure can lead to downregulation of glutathione (GPx) which is an antioxidant enzyme required to counteract with enhanced oxidative stress levels generated from JAK/STAT signaling pathway (Kim et al. 2017a).

17.6 Systemic Approaches and Evidence of Nanotoxicity in *C. elegans*

17.6.1 Effect of Nanoparticles on *C. elegans* Nervous System (Neurotoxicity)

In *C. elegans*, the nervous system contains 302 neuronal cells along with 56 glial cells (Hobert 2010). These 302 neurons belong to two different subtypes of the nervous system, the major somatic system comprises of 282 neurons and the remaining 20 neurons belong to the small pharyngeal system. Within the nematode nervous system, different behaviors and sensory functions, with associative and non-associative learning, are observed. Worms use all major neurotransmitters (White et al. 1986; Susman et al. 2016). The locomotion behavior is controlled by serotonin, dopamine, and glutamate neurotransmitters in *C. elegans* (Yu et al. 2015). The neuronal network within this model organism is fully elucidated, and almost all genes necessary for transmission can be traced and found in *C. elegans*. For example, the neurotoxic effect of particular nanoparticles can be monitored utilizing eight dopaminergic (DAergic) neurons available in worms that express GFP driven by the dopamine transporter (*dat-1* gene) promoter. Thus, it is easy to understand neurological disorders such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease with worm locomotion behavior, and GFP assays can be a direct link to motor neuronal functions (Hu et al. 2018b) (Fig. 17.4).

Neurons are known for their sensitivity and are under constant exposure for the ill effects of nanoparticles and ENMs such as quantum dots (QDs). In the biomedical field, TiO_2 NPs are regularly used in various applications including cancer treatment and as therapies for antiparasitic and antimicrobial drugs (Nadeem et al. 2018). However, recent findings have shown that exposure of TiO_2 nanoparticles to *C. elegans* can have adverse impact on the worms, such as the decrease in their population, size, movement, offspring generation, fecundity, and pharyngeal pumping rate (Li et al. 2012; Zhao et al. 2013). Engineered nanoparticles such as TiO_2 NPs cause a reduction in locomotion behavior and decrease in premature pharyngeal pumping in Bristol-type N2 (Yu et al. 2015). In neurons, TiO_2 nanoparticles are reported to have reduced axon length, which is likely to obstruct the worm locomotion behavior. According to DNA microarray analysis, expression levels of metal-binding or detoxification gene change significantly. These changes elucidate that TiO_2 NPs are toxic to *C. elegans* nervous system (Hu et al. 2018a).

Piechulek and von Mikecz (2018) employed fluorescence reporter strains, with expressions of tryptophan hydroxylase-1::DsRed to find whether Ag-nanoparticles can mimic behavioral defects in worms. They correlated the fluorescence intensity levels with the rate of aggregations of axonal proteins and neurodegeneration of serotonergic and sensory neurons. Notably, they also found that serotonergic ADF neurons are more sensitive as targets for Ag-nanoparticle toxicity, whereas GABAergic neurons can withstand degeneration under the same condition (Piechulek and von Mikecz 2018). Gold NPs are also important components of the biomedical field

which is commonly used for bioimaging, biosensing, facial creams, and targeted therapeutic purposes. In *C. elegans*, the AuNPs with 11-mercaptoundecanoic acid (MUA) and without MUA are reported to have a toxic effect on worm body length, locomotion behavior, along with a change in axonal neuronal growth. Similarly, AuNP exposure reduces axons generation in cultured neurons of worms. In another gene expression study, it was observed that there is change in expression level, cellular defense gene (clec-174), body morphogenesis gene (cut-3), gene which is expressed in neurons of embryonic tissues (*dpy-14*), and gene (*mtl-1*) which is involved in metal detoxification and regulations (Hu et al. 2018b).

Some other studies have shown that exposure of Al_2O_3 NPs has significantly reduced the locomotion behavior in worms. Recently, Yu et al. (2015) have reported that exposure of Al_2O_3 nanoparticles enhanced neural disorders related to phenotype in worms, such as neural disorder in D-type GABAergic neuron manifested with an adverse effect on the thermotaxis behavior and thermotaxis perception. These changes suggested that Al_2O_3 nanoparticles are neurotoxic in nature (Yu et al. 2015). Nitric oxide plays a key role in neurotransmission, water and salt balance, growth, and immune function. Rogers et al. have shown that CeO_2 nanoparticle exposure can downregulate nitric oxide synthase (NOS) activity while diminishing the production of NOS and NO observed and impaired nervous system functioning (Rogers et al. 2015).

17.6.2 Effect of Nanoparticles on *C. elegans* Immune System (Immunotoxicity)

Immune toxicity is the new and emerging field which deals with interactions of nanoparticles with the immune cells. Nanoparticles can directly cause damage to immune cells either by apoptosis or necrosis or indirectly by deregulations of immune-specific signaling pathways. Nanoparticle interactions cause a change in an inflammatory response which either generates ROS or releases proinflammatory cytokines. These changes are measured by the expression level of surface markers, cytokine production, cell differentiation, and activation of immune cells and antimicrobial peptides (AMPs) (Hartung et al. 2013).

Antimicrobial peptides are important components of the innate immune response of *C. elegans*, and on nanoparticles, exposure alternations in expression levels of gene engaged in antimicrobial peptide formation can lead to immune toxicity. *C. elegans* requires the activation of the p38 MAPK pathway for generation of the innate immune response (Shakoor et al. 2016). In *C. elegans*, *lys-1* and *lys-8* genes encode lysozymes, *dod-6* gene encodes a protein downstream of DAF-16, F55G11.4 gene encodes a protein containing a CUB-like domain, and spp-1 gene encodes a caenopore. Similarly, in nematodes, it has been observed that mutation of *lys-1*, *lys-8*, or *spp-1* RNAi knockdown of the antimicrobial gene enhanced the susceptibility of *C. elegans* to GO-induced toxicity that is manifested with intestinal ROS production and decreasing locomotion behavior (Mallo et al. 2002; Ren et al. 2017).

17.6.3 Effect of Nanoparticles on Development and Reproductive System of *C. elegans*

The reproductive system represents an important system for nanotoxicity studies with adverse effects of nanoparticles observed on the development of offspring, along with sexual activity and fertility rate of organisms. Although the reproductive system is considered as a secondary target organ of NPs, it was suggested that nanoparticles can access reproductive system via two routes: through its movement from the pharynx to the intestinal system and through the vulva. For experimental purposes, nanoparticles can also be delivered to the reproductive system by microinjection to the gonads (Pluskota et al. 2009). Recently, a high-throughput complex object parametric analyzer and sorter (COPAS) assay optimization and methods for assessing the developmental and reproductive toxicity of ENMs using *C. elegans* model have been reported. This can aid in accelerating reproductive and developmental toxicity studies of different ENMs and in the assessment of dose- and size-specific response on the development of nanoparticles in the near future (Kim et al. 2019) (Fig. 17.4).

In some cases, the properties of nanoparticles such as their surface charge, size, and stability, for example, quantum dots, can also reach the reproductive system, and this is dependent on their surface charge (Qu et al. 2011; Choi et al. 2010). This is manifested in the form of defecting egg-laying capacity and shortened life span in comparison with the control (Hsu et al. 2012). Similarly, SiO_2 nanoparticles are reported to be toxic to *C. elegans* with the declined rate of progeny production, internal hatch (i.e., "bag of worms"), abnormalities of reproductive organs, and BOW phenotype observed.

On the contrary, it was observed that surface coating and modifications of nanoparticles can ameliorate the toxicity of some nanoparticles such as citrate Ag-nanoparticles. The observed reproductive defects that were obvious within 24-h exposure of young worms were not observed when worms are exposed to PVP-coated Ag NPs (Roh et al. 2009). Even among the nanoparticles derived from the same metal source, their reproductive toxicity levels might differ significantly. ZnO nanoparticles are reported to have more toxic effects with enhanced germ cell apoptosis in *C. elegans*, in comparison with $ZnCl_2$, and this has been linked to cep-1/p53-dependent pathway (O'Donnell et al. 2017). Multigenerational effects of gold nanoparticles (AuNPs) and induction of multigenerational *C. elegans* germ cell death on worms were also reported based on the exposure mode (Luo et al. 2016; Moon et al. 2016).

17.7 Conclusion

Nanoparticles have various biological applications in the field of biomedical research such as therapeutics, biosensors, and bio-imaging; hence, it is essential for researchers to learn more about the toxic effects mediated through regular exposure to different nanomaterials. In practical terms, the ease of use of *C. elegans* has

attracted itself as a model for high-throughput nanotoxicity studies. Various lethal and sublethal endpoints which are used for safety evaluations of nanoparticles in *C. elegans* have shown the accuracy of *C. elegans* in predicting toxicity levels as required for translational correlations to higher mammalian systems. *C. elegans* also enhances systemic approaches in measurements of toxicities via its well-defined nervous, immune, and reproductive systems. With the availability of genetic tools such as RNAi and the ease of generating transgenic and mutant strains, toxicogenomic approach and nano-biological interactions can also be easily carried out using this model. At the cellular and molecular level, fundamental insights from research on *C. elegans* have led to a better understanding of the roles of oxidative stress in nanoparticle-mediated toxicity and how ER stress and other significant pathways are involved. With the recent technical advances in *C. elegans* handling, culture, and phenotyping, it is now increasingly possible to conduct mass screens in whole, intact organisms for developmental nanotoxicity risk assessment assays in the near future.

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Zebrafish: A Laboratory Model to Evaluate Nanoparticle Toxicity

18

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Abstract

Presently, nanoparticles (NPs) technology is a booming business marked by a significantly fast growth rate that covers a wide range of industries. NPs demand increases and they have a potential market value. The nanotoxicity sector has grown significantly for the last 10–15 years, which will pose serious problems in the coming future. Nano-toxicology is an innovative area of toxicological study that assesses the toxicological assets of NPs to decide whether they constitute a risk or an ecological problem and to what degree. To assess the different NPs toxicity, numerous nanotoxicological studies were piloted using different methods. The vital mechanisms of nanomaterial toxicity were recently studied especially in aquatic wildlife. In recent years, nanoparticle toxicity evaluation amplified exponentially by using zebrafish as an animal prototypical system. Zebrafish has been tested as an established model system for experimental biological study and are evolving as a solid nanotoxicity prototype which is progressively used as an in vivo model. It is principally used as a platform for rapid testing and assortment of molecules in the object or phenotype techniques. It offers a number of advantages over other living prototypes by offering prospects to speedily screen nanoparticulate medicines beneath in-vivo environments, also

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an economical way to link the present gap among in vitro and vertebrate studies. Many researchers have summarized experimental parameters critically used by zebrafish as an animal model for biomedical tests such as sample size, organ, and type wild against transgenic lines. Current chapter will discuss considerable factors of experimentation, advantages, and usage of zebrafish in nanomedicine; different methods of evaluating the nanotoxicity such as hatching exploration; malformation of embryos and organs of development; genetically modified zebrafish by means of living biosensor; disturbance in the endocrine system, skin, and gill; reproductive toxicity; genotoxicity; neurotoxicity; immunetoxicity; and behavioral analysis. Furthermore, it will also discuss an overview of studies about investigation of the toxicity of silver, carbon nanotube, metal oxide, and quantum dots nanoparticles using zebrafish. At the end, future lookouts of zebrafish model are discussed. It is projected that this chapter will update study directed at emerging biocompatible nanoparticulates for a choice of uses and toxicity investigation.

Keywords
Nanomedicine · Nanotoxicity · Nanotoxicology · Nanoparticulate · Zebrafish · Nanoparticles · Toxicity · Prototype · Vertebrate · Embryos · Larvae

18.1 Introduction

Nanotechnology is dealing with drafting, development, interpretation, and application of nanomaterials (Thanh and Green 2010; Xu et al. 2012a, b). Administration of drugs using nanoparticles (NPs) (e.g., nanodynamics) is a successful tool for acceleration of drug absorptions, in particular on specified tissues or part, as to attain its optimum peak level and reduce adversative effects of nonspecific carriers (Wicki et al. 2015). This distinct pharmacodynamics of nanomedicine has been used effectively to optimize the physicochemical properties of the underlying NPs. Ideally, NPs-based drugs should have lower cytotoxicity, good constancy in biotic environs, precise circulation, and reproducible specificity of cell line/tissue half-life, besides in vivo efficiency/functionality. Therefore, the drug formulations in the NPs are planned and escalated, conferring toward practically adjustable factors. These take account of size, shape, chemical content, surface charge, or surface modification (Mitragotri et al. 2017).

The performance of nanomedicine strongly influences the biological properties imitated in specific experimental configurations (Dai et al. 2018). Cellular systems are highly sensitive to toxicity because nanomedicine may have a pitiable particle size dispersal and its incompetence to counterweigh for pressure generated by equilibrium balance (Gustafson et al. 2015). The lack of detection of rapid tools for in vivo study to demonstrate the effects of various parameters of drug synthesis in biological conditions prevents the real optimization of nanomedicine. Alternatively, vertebrate models available in large quantities are cheaper to

maintain and easier to handle and facilitate the choice of pharmaceutical formulations in NPs. Therefore, they have importance and interest in narrowing the disagreement between the in vitro and in vivo practical investigations (Paunovska et al. 2018; Witzigmann et al. 2018).

Currently, the variety of NPs in market is widely used by individuals for their everyday applications. As the demand for NPs increases and they have a potential market value, the nanotoxicity sector has grown significantly over last 10–15 years, which pose a serious problem in coming future. Nanotoxicity also reveals regulatory problems related to explosion of NPs technology. A significant increase in exposure and possible toxic effects of NPs happening in the system for life and the environment is also a significant problem that needs to be highlighted (Maynard et al. 2011).

In fact, there is a strong daily increase in the literature that corroborates the problem of the toxicity of various nanomaterials (Seaton et al. 2010). Some experimental tests/different models of organisms are used, such as honeycomb analysis, multicellular models for in vitro studies, and higher animal prototypes such as mouse and rabbit for in vivo study. The latter found and accepts as, advanced living animal models are greatly useful and more important than simple experimental models (Gambardella et al. 2014; Gad 2014).

Each year, the quantity of nanomaterials produced increases and the products continue to grow. Therefore, it is imperative to develop a representative animal model that can accurately identify nanotoxicity and detect the toxicity of NPs.

Several organisms have been tested and studied to understand the mechanisms and genetic disorders in numerous human diseases. Commonly these are mouse (Iguchi et al. 2015), monkey (Riccio et al. 2015), chicken embryos (Vargas et al. 2007), yeast (*Saccharomyces*) (Delorme-Axford et al. 2015), *Drosophila* (Foriel et al. 2015), frogs (*Xenopus laevis*), *Caenorhabditis elegans* (*C. elegans*) (Gonalez-Moragas et al. 2015), zebrafish (*Danio rerio*) (Zhao et al. 2013), and many more.

Zebrafish has been tested as an established model system for experimental biological study and is increasingly used as an in vivo model in bioscience, particularly as a platform for high-speed testing and selection of candidate molecules in the target or phenotype methods. Many researchers have summarized experimental parameters critically used by zebrafish as an animal model for biomedical tests such as sample size, organ, and type wild against transgenic lines (Hill et al. 2005; Rennekamp and Peterson 2015; MacRae and Peterson 2015).

Given the current empirical study of NP toxicity, zebrafish is used as an in vivo growing model at an early stage and readily available for the development of NPs. The zebrafish as an in vivo test model has aroused the interest of researchers. Zebrafish have particularly interesting properties for biomedical research (biological properties of conservation, availability of genetic tools, imaging models, and pathological patterns) (Campbell et al. 2018; Ali et al. 2011; Sieber et al. 2017).

18.2 Zebrafish: Investigational Animal Model for Nanomedical Research

Danio rerio (zebrafish) is an experimental modeling species that belongs to the family Cyprinidae, order Cypriniformes. It is a small freshwater fish and a tropical species that lives in the waters of India, North Pakistan, Nepal, Bhutan, and South Asia. The adult zebrafish is 4–5 cm long. A temperature range of 24.5–28.5 °C can be tolerated by zebrafish. Variation in temperature affects its growth rate.

The embryonic development of zebrafish has become known. During the first days of life, their embryos are transparent just as the chorus is transparent. Approximately 30–72 h after fertilization (haf), the embryo chroma starts to develop and fertilization activates the cytoplasmic movements. It appears in about 10 min. The first resection of the newborn egg after fertilization takes about 40 min. The cytoplasmic parts, meroblastic cytoplasmic divisions, and after, a blastodisc form. A blastocoel is not present, and stem cells are "stereoscopic." The blastocyst stage conforms at 2.250–5.250 h post fertilization (hpf) while the gastrula stage at 5.25–10.0 hpf at 28 °F temperature. This is the moment of morphological development of embryos in other vertebrates. They are at embryonic stage during the incubation period (48–72 h) and "larva" stage at the end of the third day, depending on whether or not they are born (Kimmel et al. 1995; Beasley et al. 2012).

The zebrafish has a high fertility rate, can create a huge number of eggs all year round, and produces a large number of embryos. For example, about 300 eggs a week in the last conditions are created by the orientation of the females. This can give per kilo of female fertility >300,000 eggs. In addition, in the aquarium, spawn can be reproduced in the test site by introducing vegetation and sand stones to the reservoir. It is noticed that the egg hatched quickly and that organogenesis was done very quickly. Therefore, in larvae, the foremost body part is developed at 5–6 days after fertilization (daf) (Hill et al. 2005; Belyaeva et al. 2009; Kari et al. 2007).

With an average of 350 days post fertilization (dpf), the females can reach a size of 3.8 cm and weight of 0.9 g, while the males can reach an upmost average size of 3.5 cm and weight of 0.6 g (Chakraborty et al. 2016). The details of the zebrafish are summarized in Table 18.1.

A female zebrafish can be distinguished from the male one via their lengthy belies plus nonexistence of roseate color sideways, the slivery long line mark as shown in Fig. 18.1.

The different developmental stages of the zebrafish and its relevance to the study of nanotoxicity are described in Fig. 18.2, whereas Fig. 18.3 shows an inverted microscopic view of the different stages of developmental zebrafish.

Table 18.1 Details about Zebra fish (*Danio rerio*) (Braunbeck et al. 2005)

Origin	Melaka, India, Sumatra, Burma
Sexual deviation	Eggs carrying females have extended belly Males are slimmer than female, amongst blue, long stripes, alos orange stain observed
Fish weight	Males with 0.50 ± 0.1 g; females with 0.65 ± 0.13 g
Ornamentation	Wide spectrum fluorescent bulbs of 540–1080 lux, 10–20 μE/M^2/s, at lab condition; 14 h light, 10 h dark
Quality of water	Hardness—20°dH, NO_3^-: ≤ 48 mg/l, NO_2^- and NH_3− < 0.010 mg/l, O_2 ≥ 8.50 mg/l, pH −7.80 ± 0.2
Water feature	Total organic carbon should be <2 mg/l, particulate matter must be <20 mg/l, ammonia unionized form <1 μg/l, residual chlorine should be <10 μg/l; total organic chlorine level <25 ng/l, total organochlorine pesticides, and polychlorinated biphenyls level <50 ng/l; organophosphorus pesticides should be <50 ng/l
Capacity or size of tank for maintenance	180 (maximum 200 species)
Water purification	Permanent (internal filter)
Male-to-female breeding ratio	4:2
Breeding tanks	Tanks fitted with external heating coil, steel grid bottom provided, and spawning stimulant (plant dummy)
Egg appearance and structure	Firm chorion, exceedingly transparent, non-tacky, having diameter ~0.08 cm
Growth of embryo (room temperature condition)	Development of somites at 18 h, tail detachment at 21 h, heart beat visible at 26 h, blood circulation start at 28 h, and hatching starts after 72 h
Key toxicological depot at 25 °C	Tail and somite develop at 24 h: Also heart beat visible at 48 h

18.3 Advantages of Zebrafish Model

The zebrafish as a modern experimental organism determines the general acceptance that it had developed gradually. This animal model is gaining popularity in both adult and fetal biomedical research and toxicology. This widely accepted reason has an excellent set of properties (Strahle et al. 2012; Chakraborty and Agoramoorthy 2010).

The reproduction of a zebrafish is low as compared to other rodents. It grows outside the mother and are in large quantities. The adult zebrafish is small (about 5 cm in length), therefore, there is no difficulty in handling. It also minimizes the area of the house along with the land expenditure. The test solution quantities requires are reduced because of small size of larvae and the adult and therefore the limited volumes of waste to be eliminated. Small embryos can be used to analyze reasonably sized samples (Lin et al. 2013; Karlsson et al. 2001). From the egg stage to yolk absorption, zebrafish embryos can survive for several days and can be visually assessed for malformations.

Fig. 18.1 Zebra fish; female species (upper individual) and male species (lower individual) (Braunbeck et al. 2005)

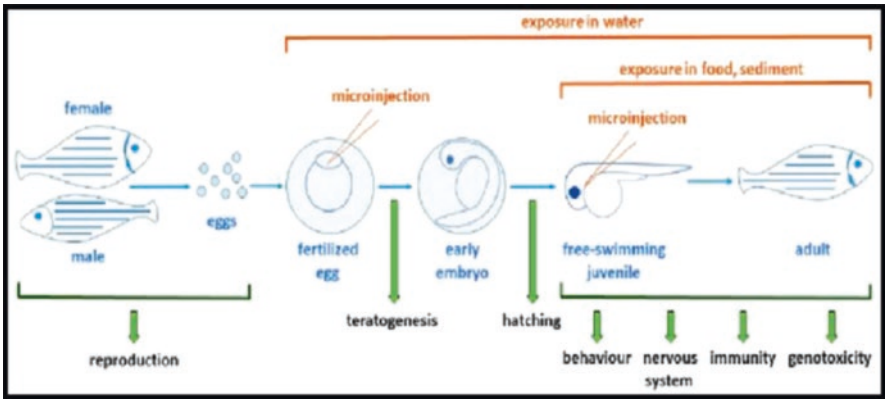

Fig. 18.2 Relationship between different stages of development of zebrafish and nanotoxicity studies (Haque and Ward 2018)

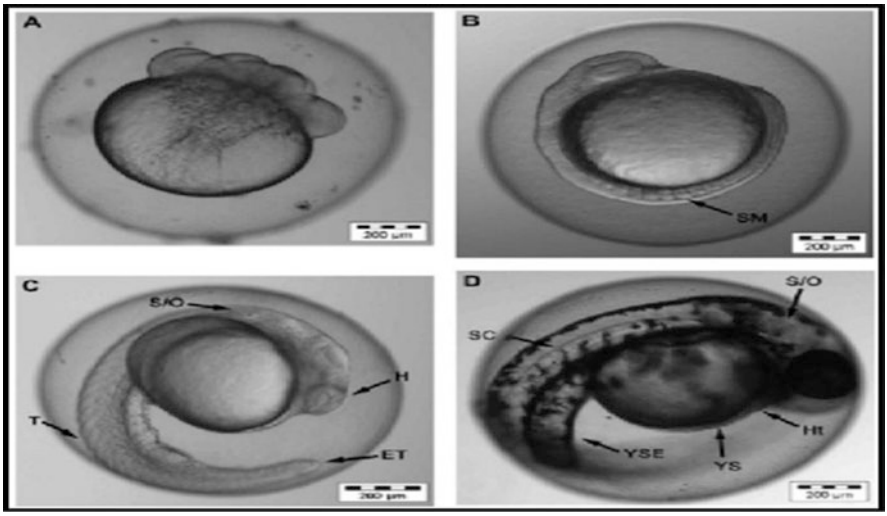

Fig. 18.3 Inverted microscopic view of different development stages of zebrafish. (**a**) 1 hpf—8 cells; (**b**) 12 hpf—somites; (**c**) 24 hpf—tail detached; (**d**) 48 hpf—pigmentation at 26 °C (Busquet et al. 2008). *ET* End tail, *Ht* Heart, *SC* Spinal cord, *S/O* Sacculi/otoliths, *SM* Somites, *T* Tail, *YS* Yolk sac, *YSC* Yolk sac extension

They have very high reproducibility, high fertility, fast growth and maturation, which facilitates testing of transgenic reproductive parameters. In eggs hatch quick and rapid organogenesis occurs. One day post fertilization (dpf), rapid growth of zebrafish embryos results in the formation of ears, eyes, internal organs and union of the brain. Their eggs are transparent from fertilization to tissue density, and staining begins about 30–72 h post fertilization (hpf). This makes it possible to clearly observe the main morphological changes up to pharyngulation (Lin et al. 2013; Sieber et al. 2019).

Zebrafish larval visual lucidity may be delayed for numerous days by chemical treatment to restrain melanogenesis [e.g., the use of 1-phenyl-2-thiourea (PTU)], which provides a sharp resolution imaging of precise biological result (Karlsson et al. 2001; Lee et al. 2017). The transparent larvae of the zebrafish, luminous reference lines, and enlightened imaging methods are key factors that provide a macromolecular level for studying NP's pharmacological behavior in vivo (Kari et al. 2007).

A number of molecular biology tools that are available can be used for genetically modified zebrafish lines creation, which are of particular interest for the development of drug-based NPs. They are summarized in Table 18.2.

The zebrafish information network (ZFIN) (zfin.org), as an information network, collective through the circumstance that, embryos and iced-up spermatozoa can easily grow. It is transported from one laboratory to the other and is speedy and stress-free on specific transgenic lines (Karlsson et al. 2001). The zebrafish physiology and anatomy well describe its organ systems and physiologic factors with specific attention on nanoparticulate toxicity. The main advantage is that the composition of

Table 18.2 Summary of zebrafish strains using precise significance designed for the in vivo explication of nanomaterial medicines (Sieber et al. 2019)

Particular features	Promising diligence	Zebrafish strains
Luminescence white corpuscle	Immune carrier systems reciprocation	*Tg(zmpo:GFP)*
Fluorescence histiocyte	Immune systems reciprocity	*Tg(mpeg1:mCherry)*
Small-density lipoprotein Receptor inadequacy	Biodistribution of LDLR dependent, hepatocyte, or brain directing	*LDLR mutant*
Apolipoprotein defeat of utility	Apoc2 reliant biodistribution	*Apoc2 mutant*
Luminescence lymphatic system	Lymphatic use and circulation	*Tg(lyve1:EGFP)*
Fluorescence M1 histiocyte	Immune systems reciprocity	*Tg(tnfa:EGFP-F)*
Translucent mature one	Prolonged stint cancer prototypes, mature zebrafish luminescence imaging	Casper
Luminescence vasculature	Blood passage enactment	*Tg(flk1:EGFP)*

blood, nervous, cardiovascular, digestive, immune, lymphatic systems, blood-brain barrier and liver of the zebrafish is similar to that of mammals. They also share many important physiological homologies with mammals (Hsu et al. 2007).

Extremely preserved signaling pathways of humans and zebrafish have very elevated degree of gene order similarity (Beliaeva et al. 2010). Therefore, genetic evaluation of function of a particular gene can also be achieved by the development and production of transgenic zebrafish experiments by simple modulation (Varshney et al. 2013). Zebrafish has also successfully implemented a genetic map that presents 400 different genes and more than 2000 microdirectional markers (Chakraborty et al. 2009). On the genetic extent, 76% of humanoid genomes (roughly 82% of ailment-associated genetic factor) possess orthologs within the zebrafish. There is a high degree of similarity between the human genome and the zebra genetic order, creating a genuine animal prototype meant for analytical experiment of NPs.

Therefore, the scientific interest on zebrafish by means of an in vivo organic model is fascinating due to its unique properties

18.4 Considerate Factors of Zebrafish Experimentations

The successful outcome and reliability of NP studies in zebrafish are simulated by numerous investigational factors (Fig. 18.4).

Proper selection of the zebrafish development phase is necessary and the duration of the experiment should be cautiously determined in designing a zebrafish experiment. Embryonic growth phases and the presence of other important animal organ structures are soundly defined and informal to forecast in zebrafish (Kimmel et al. 1995). In a developing zebrafish, progressive defeat of lucidity, organ growth,

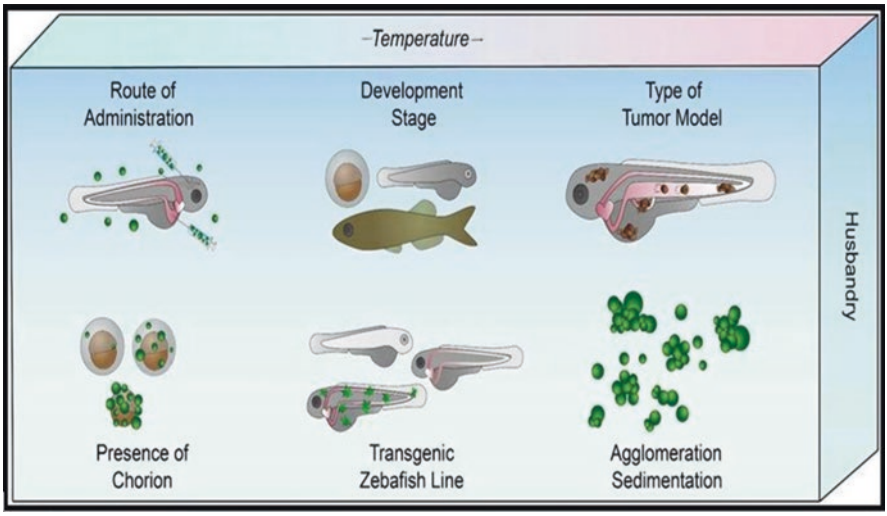

Fig. 18.4 Experimental critical parameters that affect the results of zebrafish nanomedicine studies (Sieber et al. 2019)

and immune system advancement occur over a relatively less period of time, which may affect experimental data. Depending on the purpose and nature of the study, it is recommended to start with the different stages of zebrafish development. For example, the production of genetically modified zebrafish lines is carried out with mutagenic agents, such as N-ethyl nitrosourea (ENU), in an adult zebrafish (Sassen and Koster 2015).

The stages of zebrafish development and the accuracy of the required dose helps to determine the best and appropriate route of administration. Several NP administration pathways were studied and used in the zebrafish. The injection of NPs into the blood is usually done through the readily available channel. Alternatively, in central nervous system (CNS) (i.e., the brain chamber), the intraperitoneal or retro-orbital injections were used. Direct injection into the blood consists of terrestrial tissue, also used in embryonic zebrafish (Sieber et al. 2019).

One more vital factor is investigational temperature. The gestation temperature affects the growth rate and natural immune response of zebrafish larvae at the beginning of zebra growth. As a general rule, when areas with high water temperatures are selected, zebrafish are under stress (exposure to chemicals, pain). Physiological processes are also depend on temperature, as immune and/or behavioral response which may affect test results. It is noted that increasing temperature over extended times may influence the physiologic mechanisms initiated due to presence of heat-shock proteins in zebrafish (Rabergh et al. 2000). Therefore, the investigational temperature requisite remains sensibly selected and studied under controlled, evaluated, and standardized accurate conditions, especially when immune-related processes are analyzed in zebrafish (Zhang et al. 2018; Keller et al. 2008; Rabergh et al. 2000).

Many factors such as pH, nutrition, fish density, control of water conduction through reproduction and maintenance of zebrafish should be considered, verified, and determined in accordance with experimental requirements to guarantee coherent experimental conditions.

18.5 Use of Zebrafish for the Development of Nanomedicine

The benefits of zebrafish over rodents are increasingly used for the design, development, optimization, and evaluation of NPs. The biological distribution, systemic circulation, efficacy, stability, functionality, and toxicity of nanoparticulate drugs have been successfully evaluated in complex biological systems, in vitro in zebrafish larvae as shown in Fig. 18.5 (Sieber et al. 2019).

18.6 Methods for Evaluating the Nanotoxicity in Zebrafish

The zebrafish has many advantages as it is a unique model in terms of EHS (environment and human safety). To govern the hazard of nanomaterials and products created by nanotechnology, data received deriving out of Nano EHS experiments can be beneficial in the nearby forthcoming. The statistics can likewise assist in preparing operational guiding principles for protective actions, design approaches, and quality controls to improve the nanomaterial system and minimize its toxicity (Chakraborty et al. 2016). Using the zebrafish organism model, some precise procedures were used and intended for the finding of toxicity such as the following.

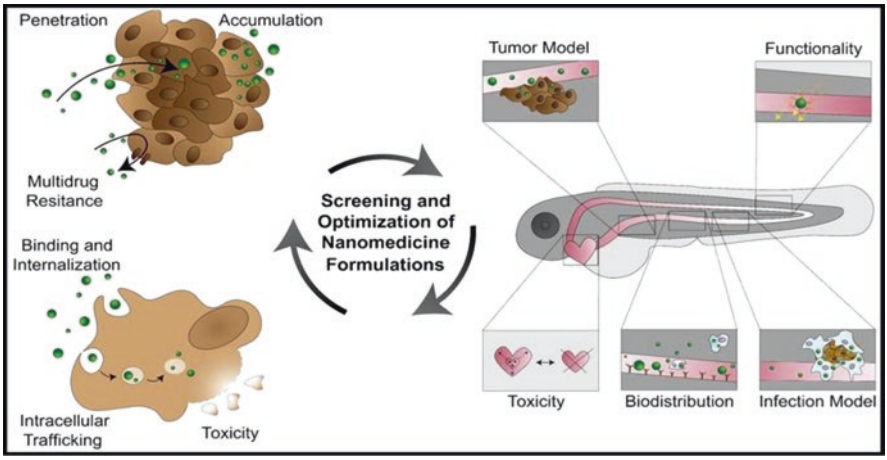

Fig. 18.5 Complementary application of experimental configurations of zebrafish and in vitro models for the design and optimization of nanomedicine formulations (Sieber et al. 2019)

18.6.1 Hatching Exploration

The parameters related to the hatching can be one of the important end parameters and can be underestimated in different studies. Variable results were obtained at the endpoint of the same species and in different species (Paterson et al. 2011; Xu et al. 2012a, b), the results of which are difficult to analyze and interpret.

For investigators to know the toxicity of chemicals and NPs, the hatching of zebrafish is one of the utmost important events. Several studies have shown in detail the stage of embryonic developmental stages.

Villamizar et al. (2012) studied this phenomenon of hatching event associated with a rhythmical model equivalent to the light stage. They showed that the utmost number of eggs was produced at about 2 dpf besides the respite at about 3 dpf. Therefore, the correlation between hatch productivity and fetus toxicity is the foremost factor for nanotoxicity studies (Villamizar et al. 2012).

In addition, researchers used TiO_2 NPs to study hatching phenomenon and fetal toxicity. They assessed the relationship between the success rate of the hatching and the number of hours after exposure. An experimental study has shown that premature dose-related hatching in zebrafish embryos may be due to TiO_2 NPs (Samaee et al. 2015).

Ong et al. designed NPs using cadmium selenide, silicon, silver, zinc, and carbon nanotubes to evaluate the effects of NP on the hatching process of zebrafish. They studied complete retardation of hatching and fetal death in chorea after exposure to the NPs, while the NPs link through conceiving enzymes. Therefore, NPs remain accountable for toxicity and not for ionic or dissolved metal components (Ong et al. 2014).

18.6.2 Analysis of Malformation of Embryos and Organs of Development: Zebrafish Embryo Toxicity Test (ZFET)

Dysplasia of embryos and organs is another parameter that provides access to the detection of toxicity. Defects associated with development of incomplete body parts, such as the head and tail; incomplete organ development, such as eyes, malformations of some parts of the body such as the spinal cord and fin deformity lack staining (Chakraborty et al. 2016).

The zebrafish embryo test (ZFET) is an alternative modern non-animal test approach to the acute toxicity test because it has the same sensitivity, accuracy, and specificity. It has a profitable, simplified, and faster execution. The European Community proposed ZFET to diminish the load of trial tests going on live mammals (Embry et al. 2010; Pecoraro et al. 2017a, b; Roper and Tanguay 2018).

The fish embryo toxicity test is incorporated with the Environmental Protection Agency (EPA), ICH, and FDA regulations designed for pharmaceuticals, also with the Organization for Economic Cooperation and Development (OECD) for chemicals and drugs used to perform the toxicity test (OECD 2013).

The fish embryo larva is an identification and evaluation tool for testing more NPs. ZFET is not sufficient evidence to study and evaluate developmental abnormalities 96 h post fertilization (hpf). For example, malformations in skeletal, since calcification system in zebrafish begins on the seventh day of growth (Pecoraro et al. 2017a, b; George et al. 2011; Xu et al. 2017).

Igartua et al. tested zebrafish larvae and embryos by using magnetic nanotheranostics (MNP) and folic acid (FA) for the administration of doxorubicin in the treatment of cancer. They used zebrafish embryos (0–3 dpf) to analyze the developmental toxicity of doxorubicin, FA-FA-MNP, and MNP containing doxorubicin. To various concentrations of doxorubicin (3.12–50 μg/ml), FA-MNP containing doxorubicin (3.12–50 μg/ml doxorubicin in 70.75 mg/ml of FA-MNP) or FA-MNP (70.75–1132 μg/ml) zebrafish embryos were exposed. They studied hatching events and morphological changes. Also, larvae, vitality, neurotoxicity, cardiotoxicity, and abnormalities in morphology were analyzed as shown in Fig. 18.6.

They observed that doxorubicin exhibited concentration-dependent toxicity in zebrafish embryos and its larvae. With 50 μg/ml doxorubicin for 48 h, the test resulted in 30% larval mortality and important morphological abnormalities. In addition, doxorubicin containing FA-MNPs reduces cardiotoxicity and

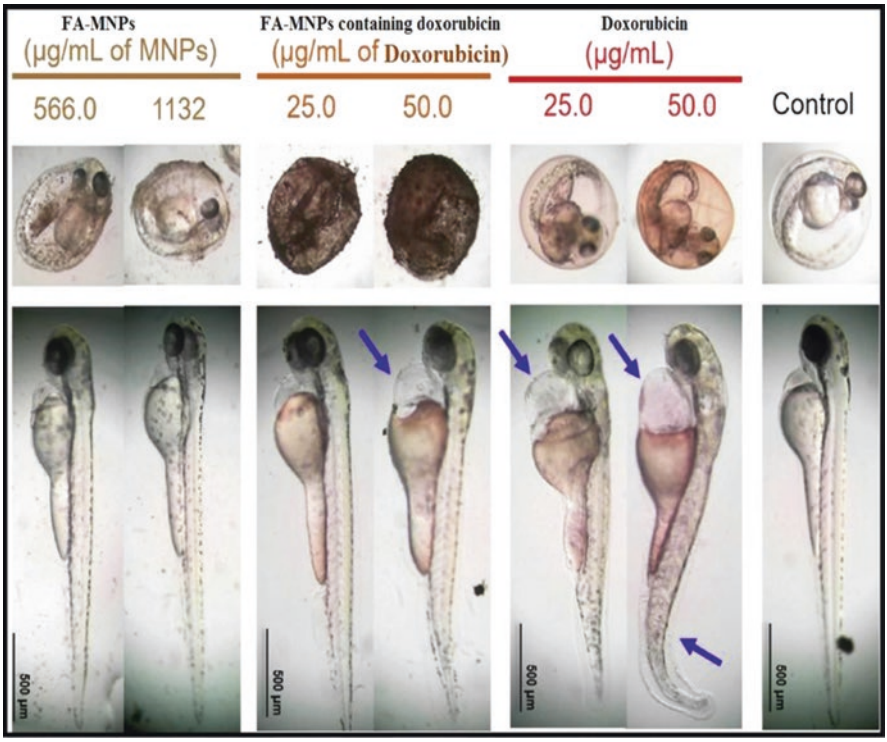

Fig. 18.6 Morphological abnormalities of zebrafish embryo exposed to doxorubicin, FA-MNPs, and FA-MNPs containing doxorubicin (Igartua et al. 2018)

promotes faster and more significant uptake of doxorubicin in zebrafish larvae (Igartua et al. 2018).

Liegertova et al. synthesized carbosilane glucose glycodendrimers (glyco-DDM), and their toxicological and teratogenic effects in embryos of zebrafish were studied using the modified fish embryo test (FET). Carbosilane glyco-DDM toxicity in the range of 0.01–100 μg were tested and determined after 96 h during normal exposure. For intact embryos, no significant toxic effects on fetal development were observed at any of the tested concentrations (i.e., without embryo coagulation or deformed body parts). They also found that exposure at a concentration of 10 and 100 μg was sufficient to cause mortality of 65–80% (mainly by coagulation) at 24 hpf. They concluded that chorion is an important factor of toxicity. The negative effects of DDM on fetal development are evident after chorion eradication with an LD_{50} calculated on exposure (LD_{50} = 2.78 μg at 96 hpf) (Liegertova et al. 2018).

Kalishwaralal et al. studied selenium-based NPs (SeNPs) with a new method and evaluated their toxicity in zebrafish embryos. At altered concentrations of SeNPs, embryos were treated for 24 h. In cardiovascular abnormality prevention, a lesser application of selenium may perform a key part because it does not change the heart rate, does not induce cardiac dysplasia, or is undergoing treatment. They noted that at all the times, there was no mortality at lesser concentrations (i.e., 5–10 μg/ml) and there were no significant defects such as pericardial edema and no defects in the tail. Zebrafish embryos were exposed to various developmental abnormalities, pericardial edema, uremic malformations, and decreased heart rate, observed at 96 hpf. In addition, they showed that the direct influence of heart rate was also significantly decreased in zebrafish embryos concerning greater concentrations of SeNP (Fig. 18.7). Approximately 20–25 μg/ml SeNP showed moderate cardiac congestion and pericardial edema, as well as early onset of cardiac dysfunction (Fig. 18.8b, c). Exposure of these embryos to greater concentrations of SeNP (20 and 25 μg/ml) brings about noteworthy mortality related to the controlled set 96 hpf.

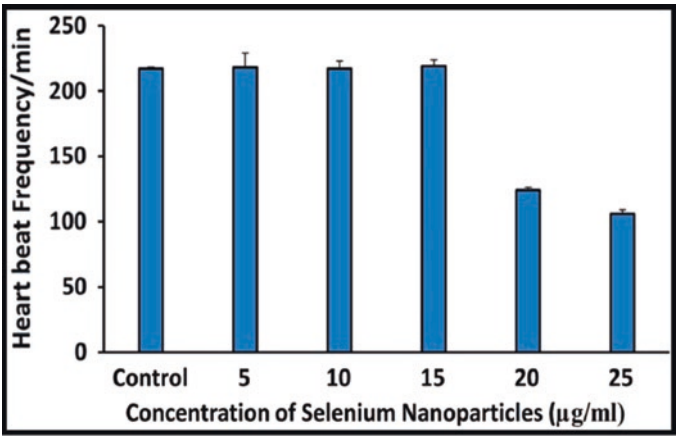

Fig. 18.7 Effects of selenium nanoparticles on heart rates of zebrafish embryos at 96 hpf (Kalishwaralal et al. 2016)

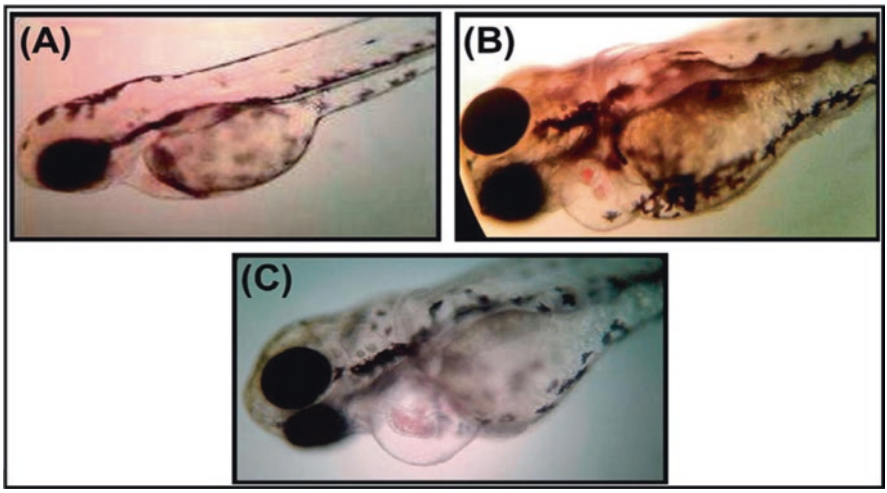

Fig. 18.8 SeNPs effects at 96 hpf on malformations (e.g., pericardial edema). (**a**) Controlled embryo; (**b**) 20 μg/ml SeNPs; (**c**) 25 μg/ml SeNPs (Kalishwaralal et al. 2016)

They concluded as, especially SeNP low concentration might be beneficial as remedial molecules designed for cardiovascular avoidance. The results show that at 5–10 μg/ml SeNP can be a cost-effective remedial choice in favor of cardiovascular ailments and concentration-induced malformations (Kalishwaralal et al. 2016).

Therefore, the zebrafish model may be useful in selecting the immune-toxicity profile due to its rapid assessment.

18.6.3 Transgenic (Genetically Modified) Zebrafish by Means of Living Biosensor

The genetically modified model of zebrafish is used to evaluate the toxic effect as a living biosensor as well as the toxicological potential and for ongoing research (Table 18.3). A remarkable change has been observed in the morphology of zebrafish following exposure to toxic chemicals (Chakraborty et al. 2016).

Hung et al. identified the level of toxicity of water using polychlorinated biphenyls (PCBs) as a transgenic biosensor and a green fluorescent chimeric CYP protein (CYP-GFP) for in vivo imaging. They observed several morphological changes related to zebrafish exposure with polychlorinated biphenyls (PCBs) and suggested that CYP-GFP might be helpful as an in vivo biosensor (Hung et al. 2012).

Lee et al. reported a series of transgenically modified zebrafish embryos called "huORFZ" that have been developed and evaluated and noted to be capable of accurately detecting different types of infectious agents and compounds that can be used as a hazard detection system for hazardous waters. They demonstrated the importance of cytochrome P450 (CYP) as an important biomarker, such as

Table 18.3 Various forms of zebrafish transgenic series were utilized for the study of the toxicity of chemicals/NPs (Chakraborty et al. 2016)

To know the NPs toxicity; genetically modified zebrafish type is used	NPs/chemicals utilized	Observation
Genetically modified FLI-1 zebrafish larvae	Mercaptopropionic acid (MPA)-coated cadmium selenium (CdSe) quantum dots (QDs)	In genetically modified zebrafish larva, anomalous vascularization revealed
Genetically modified zebrafish embryos (fli1a:EGFP)	Nonorganic nano rods	Exhausting nonorganic nano rods in transgenic zebrafish embryo ROS facilitated angiogenesis
Zebrafish embryos gene manifestation	$C_{60}(OH)_{24}$ (hydroxylated fullerenes), TiO_2	NPs deregulate the circadian rhythm genes
Genetically modified zebrafish (fli1:EGFP/ nacre)	CuO, SiO_2, TiO_2	CuO NPs inhibit vasculogenesis when transgenic fish treated with a concentration of 0.01, 1, and 100 μg/ml of CuO, SiO_2, and TiO_2 particles
Green luminous protein expressing transgenic embryos in myocardium	Smaller molecules	Heart rate of transgenic embryos altered by smaller molecule

polycyclic aromatic hydrocarbons (PAHs), for the detection of carcinogenic factors (HC Lee et al. 2014).

18.6.4 Disturbance of the Endocrine System, Skin, and Gill

Toxicity, such as disruption of skin, gill, and endocrine lesions, as well as the complex mechanisms of toxicity of NPs, is one more factor to know and evaluate the toxicity caused by NPs. The gill is the most important target system for NPs transported through water (e.g., immunotoxicity, genotoxicity, neurotoxicity, or reproductive toxicity).

Insoluble forms of copper-NP suspensions showed highly toxic in zebrafish as well as can harm the gill lamella as revealed by Griffitt et al. They also indicate that NPs are necrobiosis toxic to the zebrafish skin (Griffitt et al. 2007).

Asharani et al. observed deposition of nanoparticle of Ag-BSA (Silver-Bovine Serum Albumin) and were studied in the zebrafish skin throughout exposure. They noted that NPs invade the fetal skin continuously by diffusion or endocytosis. In addition, they gathered in the epidermal layer part of zebrafish larvae and cause pleasant malformation due to necrobiosis (Asharani et al. 2008a, b).

Zebrafish endocrine interference may be caused by chemical exposure. Tu et al. studied change in the endocrine of zebrafish with chemicals. They observed an increase in the expression of estrogen-sensitive Vtgl genes. Their results also

revealed that there was no effect of chemical exposure on ERα gene expression (Tu et al. 2013).

18.6.5 Reproductive Toxicity

In the NP toxicity, the toxicity measurement for the reproduction is also part of important parameters. In evaluating reproductive toxicity, zebrafish prototype is the superior model because of its high reproductive rate. The literature reports that NPs affect the reproductive capacity and fetal development of male and female zebrafish (Braydich-Stolle et al. 2005).

Wang et al. evaluated the effect of chronic exposure to TiO_2 NPs in zebrafish and observed changes in zebrafish reproduction. Even after exposure to NPs of TiO_2, after 13 weeks, the total number of zebra eggs decreased by 9.5% (Wang et al. 2011).

18.6.6 Genotoxicity

Damage to genomic intelligence in a cell result from the presence of biochemical agents inducing chromosomal damage, genetic mutations, and DNA damage described in genotoxicity. For long-standing toxic consequences, such as carcinogens, genotoxicity is a significant risk factor (Bolognesi 2003). The zebrafish prototype has suggested to examine the genotoxicity caused by biochemical representatives using several methods.

Geffroy et al. assessed genotoxicity. They studied zebrafish and the effects of gold NPs on the genotoxicity using genotoxicity tests as RAPD-PCR (random amplified polymorphic deoxyribonucleic acid analysis-polymerase chain reaction). Very low doses of gold NPs revealed a substantial change in genetic configuration (Geffroy et al. 2012). Dedeh et al. also examined the genotoxic effects of gold NPs in zebrafish using a RAPD-based system after exposure to a gold NPs (Dedeh et al. 2015).

Cambier et al. investigated the effects of cadmium on zebrafish by random amplified polymorphic-DNA (RAPD) and reverse transcription polymerase chain reaction (RT-PCR) on genotoxicity (Cambier et al. 2010). But so far, there are fewer reports available for genotoxic evaluation of zebrafish with the NPs system and, therefore, most powerful and expensive studies are required in this area to explore the effect genotoxic of several nanomaterials.

18.6.7 Neurotoxicity

Neurotoxicity observed in contact with dangerous toxic substances and nerve tissue has been destroyed, leading to significant abnormalities of the nervous system. Neurotoxins are toxic to neurons.

Neurotoxicity of NPs has been studied and discussed from time to time, and it should be taken into account that NPs can reach the brain and cause neurodegeneration (Sheng et al. 2014). Combustion NPs are neurotoxic and appear in in vitro and in vivo experiments due to the presence of aggregation of NPs (Morimoto et al. 2010).

The zebrafish model is used to determine the neurotoxicity of dendrofuller as protective radiation NPs (DF-1). In the zebrafish embryo, to study neurotoxicity, C_{60} fullerene derivative was evaluated. Experimental results shown a level of dose-limiting toxicity (Daroczi et al. 2006).

Sheng et al. also assessed TiO_2 NP neurotoxicity in zebrafish model. They observed that the expression of genes (BDNF, C-fos, and C-jun) is significantly activated by the induction of TiO_2 NPs. In addition, the expression of genes, such as p38, NGF, and CRE, is eliminated with a TiO_2 NPs, responsible for brain damage of zebrafish (Sheng et al. 2014). These reports require additional studies to evaluate the neurotoxicity of the NPs.

18.6.8 Immune Toxicity

Immune toxicity is the noxious results of xenobiotics by direct or indirect methods of normal functioning of the immune system. Immune suppression that results in reduced resistance to various diseases (such as cancer) is due to direct immunotoxicity. Several studies have shown that NPs (including NPs of metal oxides) produce cytokines, regulated by the production of free radicals. Allergic sensitization is associated with some NPs and may increase the risk of asthma (Lankveld et al. 2010; Di Gioacchino et al. 2011).

Jin and Zheng have studied and described that a noxious harmful chemical such as cypermethrin induces cell death and immune toxicity in zebrafish. They suggested that zebrafish have potential application in immune-toxicity studies (Jin et al. 2011).

Xu et al. designed and investigated the immunotoxic effects of chemical substances such as dibutyl phthalate using genetically modified AB lines or albino on living zebrafish embryos (Xu et al. 2015). Many publications have proposed the application of zebrafish embryo to analyze and evaluate the immunotoxic properties of various chemicals (Zhuang et al. 2015).

However, further research is needed on nanoparticulate in zebrafish to evaluate immunotoxicity.

18.6.9 Behavioral Analysis

Nowadays, the behavioral response becomes very important parameter in assessing the level of toxicity. The behavioral response of zebrafish is a sensitive indicator of abnormal changes due to toxicity (MacPhail et al. 2011). The kinetics of swimming is the most accepted and highly studied behavioral response parameter. Significantly,

rotational speed and swimming depth have been modified due to chemical toxicity (Huang et al. 2014).

The influence of TiO_2 NPs on swimming parameters of zebrafish larvae, including speed and activity levels, was studied by Chen et al. (2011). Kokel et al. assessed chemical toxicity and created a "bar code" for behavior (Kokel et al. 2010). Truong et al. evaluated the effect of gold NP exposure at 122 dpf on behavioral disorders such as surprise abnormalities, behavior after a stimulus attack (Truong et al. 2012).

18.7 Measurement of the Toxicity of Nanoparticle-Based Drug Delivery Systems Using Zebrafish

Nanotoxicology is a new area of toxicological research that assesses the toxicological properties of NPs to determine whether they constitute a hazard or an environmental problem, and to what extent. It is an interdisciplinary field that brings together or interferes with topics such as NPs medicinal toxicology, biology, physics, chemistry, and their interactions with biological systems (Weiss and Diabate 2011).

The fate, behavior, transport, and harmfulness of NPs are influenced by their specific assets and also environmental factors. To assess the different NPs toxicity, several nanotoxicological studies were conducted using different methods. However, much remains to be done to determine if NPs can pose a threat to the environment. In fact, a growing figure of experiments have assessed the toxicity of numerous NPs such as graphite, metal-oxide NPs, fullerenes, metal NPs, amorphous materials, and nanoscale polymers.

The quantity of nanomaterials/drugs and/or their products is escalating every year, which requires a representative model to study nanotoxicity with precision and fidelity (Kari et al. 2007).

Based on diversity of anatomy and physiology in different animals, and animal models, some research teams have carried toxicological experiment on animal models (in vivo). The application of zebrafish as an animal prototype to examine the harmfulness of NPs in recent years has increased exponentially and has attracted more scientific interest (Cela et al. 2014).

The toxicological evaluation of NPs-based drugs has been one of the first applications of nanomaterial and zebrafish. In general, nanotoxicity studies on zebrafish embryos to NPs include exposure or by adding to the media substrate. This methodology is doubtful to some extent in relation to test and dose precision, definite revelation, media, and stability of the NPs. In case of general nanomedicine products toxicity, experimental results may be affected by the properties of the encapsulated drug. Depending on the different logP values, drugs of different shapes enter the skin of zebrafish by different methods, a feature that expressively affects the capacity to regulate plus systematize the dose as well as exposure (MacRae and Peterson 2015).

Studies in a controlled fashion and relatively rapid manner can measure toxicology, for example, abnormalities or existence. However, intensity of the abnormalities and degree is commonly individual. Recently developed nanotoxicity measurements that focus on the zebrafish larvae behavioral characteristics

Table 18.4 Zebrafish model used in nanoparticulate medicine study to explore toxicity (Sieber et al. 2019)

Zebrafish strain	Growth phase (in hpf)	Investigational Indicators
(AB)wild-type	2	Anatomy, growth, lethality
(AB)wild-type	0–2	Anatomy, lethality
Tg(cmlc2:EGFP), (AB) wild-type	6–30	Anatomy, growth, programmed cell death, heart functionality
Wild-type	Adult	Gill damage, kidney toxicity, biochemical liver and lethality
(AB)wild-type	2	Mobility, anatomy, body weight, hatching, thyroid level, lethality
Tg(lsl1:EGFP) (AB) wild-type	48–96	Mobility, anatomy, neuron cell volume, lethality
Tg(kdrl:GFP)	48	Blood circulation activities and NP effusion
Tg(kdrl:EGFP s843)	6–24	Tissue permeation blood stream, lethality,

For every experiment, investigated visual data are embodied. Genetically modified used zebrafish strains and their growth phase during study were mentioned. *hpf* hours post fertilization, *NPs* nanoparticles

demonstrated more mechanization and a sampling capacitor. These contain mechanized methods to study movement characteristics, such as depth and swiftness of swimming (King Heiden et al. 2007; Bar-Ilan et al. 2009; Jang et al. 2014).

Toxicity of nanomedicine is visible in the embryo/larva. Other toxicological endpoints such as the progression of glandular, cutaneous, and endocrine mechanisms, in addition to multifaceted phenomenon of toxicity (neurotoxicity, genotoxicity, reproduction, immune toxicity), was studied. Table 18.4 summarizes the study of zebrafish nanotoxicity (Chakraborty et al. 2016; Sieber et al. 2019).

The currently available toxicity data for several NPs studied using the zebrafish model are summarized as follows.

18.7.1 Nanoparticle of Silver

Silver NPs are the utmost investigated and evaluated NPs. They are extensively used as biosensors, in cosmetics, as therapeutic and antimicrobial agents, as well as in drug delivery systems. Dose toxicity of silver-NPs (AgNP) was revealed and detected that the dimensional NPs size is the foremost features of its toxicity outline (Chakraborty et al. 2016).

Surface defect in the NPs toxicity is one more consideration. George et al. studied effect and superficial toxic deficiencies caused by AgNPs in zebrafish embryo and fish cell strains have been studied and evaluated (George et al. 2011).

The transfer and toxicity of early-growing zebrafish embryos were studied and evaluated using peptide-stimulated NPs. In the neurological development of zebrafish, the effect of AgNPs has been studied by Xin et al., and they observed that

AgNPs could affect neurodevelopment and cause a small head, posterior brain hypoplasia, small eyes, and even heart failure (Xin et al. 2015).

Kannan et al. studied evaluation of toxicity of antimicrobial silver NPs (AgNP) in embryonic zebrafish. They discovered numerous biogenic malformations such as heart failure, swelling of the head and eyes, curvature of the tail, and deformity of the developing organ of embryos 1–5 days after fertilization, which was found at 14–20 ng/ml for in vitro pathogens and 10 ng/ml in embryos. It was revealed that biosynthesized nanoparticle was at 22 ng/ml in all the developmental stages of the embryos which disrupts the normal organogenesis(Kannan et al. 2011).

18.7.2 Carbon Nanotubes (CNTs)

Characteristic physical and chemical properties of carbon nanotubes makes it equal to the biological macromolecules such as enzymes plasmid DNA, enzymes, antibodies, etc. This is due to the large surface area of the CNT, which can be associated with a wide range of therapeutic molecules (Liu et al. 2009).

At present, however, CNT toxicity is a significant challenge (Madani et al. 2013). The toxicity ratios are inadequate because they show the potential of CNTs for human health and environmental impact. The toxicological study of CNTs in zebrafish and physicochemical fish characterization methods are presented in Table 18.5.

Sun and Cheng have tested and evaluated the long-standing consequence of multi-walled carbon nanotubes (MWCNTs). In early stages of transformed zebrafish embryo, they observed immune response with accumulation of white blood corpuscular circulating in the shaft area were originally bacterial cells in the initial phase and second generation produced later. Second-generation larvae survival was lesser than that of controlled groups, signifying a destructive outcome on reproductive prospective. These outcomes indicate and emphasize that functionalization procedures and broad refinement can support to develop the biologically compatible CNTs. They also illustrated that refined CNTs when administered long-term in the body may exert toxic effects (Sun and Cheng 2009).

Ali-Boucetta et al. examined conventionally used subject of in vitro prototypes in toxicology and stressed the need for a systematic model of greater consistent

Table 18.5 Carbon nanotube nanotoxicity studies using zebrafish and physicochemical characterization methods (Bohnsack et al. 2012)

NPs	Notes	Dosing statistics	Avg. size ($d \times l$)	Analysis mode
Carboxyl acid-SWCNT	Added doxorubicin	48 hpf micro-injection	4–5 nm × 500–1500 nm	TEM
SWCNT	Raw	4 hpf aquatic	11 nm × 0.5–100 μm	EDS
BSA-MWCNT	Both	Micro-injection	19.9 nm × 0.8 μm	S-TEM

SWCNT single-wall carbon nanotube, *BSA-MWCNT* bovine serum albumin multiwalled carbon nanotubes, *TEM* transmission electron microscopy, *EDS* energy dispersive X-ray spectroscopy, *S-TEM* Scanning transmission electron microscopy

methods. They similarly executed LDH tests and MTT to understand the relations among cell cultures and CNT and evaluated CNTs cytotoxicity (Ali-Boucetta et al. 2011).

18.7.3 Nanoparticle of Metal Oxides

In several areas of development, such as energy storage, information technology, medicine, and catalytic metal, nanomaterials are used commercially. Various types of NPs of metal oxides in the industrialized commercial production of NPs carried out, for example, ZnO, Al_2O_3, TiO_2, CrO_3, and Fe_3O_4 (Franke et al. 2006; Fernandez-Garcia and Rodriguez 2007). The metal-oxide NPs toxicity using the zebrafish prototype was also studied.

Kovriznych et al. (2014) studied long-term toxic effect of NiO NPs on a couple of adult zebrafish. They assessed and observed that the acute toxicity of NPs NiO was low, but prolonged contact with NiO compounds could lead to tissue damage due to accumulation, increasing toxicity, and death (Kovriznych et al. 2014).

Thomas et al. studied and evaluated the toxicity of magnesium oxide and magnesium NPs on zebrafish. The magnesium oxide masses and the NPs were prepared by sonication. Different concentrations of 10–200 ppm of magnesium oxide in bulk and in NPs have been determined. They noticed that there was a significant decrease in the protein. Progressive and sporadic increase in catalase activity was also noted. In addition, the increase in magnesium NPs concentration significantly increases the specific activity of the GST enzyme. As a result it leads to more accumulation of magnesium NPs. They also showed that most magnesium oxides were more toxic than the NPs (Thomas et al. 2014).

Zhao et al. (2013) studied nano-ZnO toxicity on the embryo and accessed the oxidative trauma, growth toxicity, and DNA impairment in the fetal zebrafish larva. They found that nano-ZnO exhibited acute dose-reliant toxicity, decreased the rate of hatching, induced malformations in zebrafish embryo, and was more as compared to the dissolved Zn^{2+} solution (Zhao et al. 2013).

18.7.4 Quantum Dots

Quantum dots (QDs) are commonly used in the fields of photovoltaics, electronics, biomedical imaging, and diagnostics. It contains a semiconductor core (e.g., CdS) and is generally included in a package (e.g., ZnS) to enrich photosensitive and electric-electronic assets in addition to biotechnology. The physicochemical properties of QD affect toxicity, size and net charge, core composition and coverage, interaction of metal leachate from QDs, and interaction of protein. QDs containing calcium carbonates are generally produced, and heavy metals are dangerous for living organisms. Because nanoscale things that create them further are freely accessible, they release toxic metal ions. QD has been shown to catalyze Reactive oxygen species (ROS), which can lead to oxidative stress. It has been shown that

QD is added in vivo and is toxic when the number of quantum dots is greater than 10^8 (Bohnsack et al. 2012).

King-Heiden et al. (2009) studied the nanotoxicity of quantum dots using zebrafish embryos. They exposed embryos to Cd/Zn QD-functionalized aqueous suspensions. They found that visible evidence of Cd toxicity was shown by zebrafish larvae and were affected by the QD coating. In subsets of concentrations, various preparations caused Cd toxicity, showed characteristic signs (decreased growth, yolk sac, cardiac pocket, swollen sac, and swollen submandibular sac), and increased curvature of the spine. Although they observed that QD now accumulates dissolved Cd^{2+} in larvae and is more potent in causing mortality. An equivalent amount of CD^{2+} larvae were exposed to the Qd CdSen core/ZnS shell PEG 5000 assay for the toxic section of the Cd criterion, including apparent necrosis, yolk sac malformations, and malformed tail (King-Heiden et al. 2009).

18.8 Limitations of the Zebrafish Model in Nanotoxicology

In addition to the beneficial characteristics, there are practical restrictions of the zebrafish prototype, as mainly limited extent of the investigational scheme. It is difficult to obtain blood from zebrafish larvae and in some adult zebrafish. Only small quantities of biological molecules, for example, proteins, remain obtainable for additional exploration because of the tiny dimension of the zebra larva or the corresponding benign growth load. These tasks repeatedly necessitate the aggregation of a number of zebrafish larvae for exploration. In addition, there are existing methodical limits for in vitro prototypes. Procedures that encompass the reticence and fluorescence of definite cell capture besides operating tools remain primarily intended for in vitro environments and are still needed to optimize routine application in zebrafish (Lee et al. 2017; Hofmann et al. 2014; Faklaris et al. 2009).

The immunotoxicity test based on nanomaterials has not yet been found. In addition, because of the rapid growth phases of zebrafish, it is very difficult to obtain nanotoxicity during the routine test. Numerous nanoparticulates such as drug delivery and antimicrobial therapy are used for beneficial remedial resolution. Hence, this one is necessary to realize the pharmacokinetic properties of NPs. Pharmacokinetic test is indistinct in the zebrafish model after the administration of nanodrugs (Chakraborty et al. 2016).

In general, zebrafish-established experimental methods need entitle cautiously authenticated against standard practices. Ultimately, this will proliferate approval in the scientific and technical communal and expedite the practice for the preclinical detection using zebrafish prototype for NPs systems.

18.9 Discussion

Many studies have explained the eventual importance of zebrafish animal prototype for the medical improvement of NPs. Zebrafish larvae are particularly unique in associating the gap concerning in vitro models and live vertebrate experiments because they have high availability, reproduction costs, experimental configurations, visual pellucidity, and accessibility of countless transgenic fluorescence strains. These properties simplify the speedy and economical study of nanoparticulate drugs beneath in vivo environments and lead to higher levels.

Zebrafish as an investigational prototype can be used to select a design and manufacturing exhausting up-to-date experimental setups and conventional imaging techniques. The study is capable of per day about 20 nanomaterials per investigator per optical microscope. However, in achieving a great detection capacity, it is necessary to develop fully automated injection, imaging, analysis, and evaluation protocols.

The zebrafish model is not a well-thought-out typical model for NPs study. More revisions and experiments are needed to obtain a fully developed model, so as to estimate prospective uses and determine its analytical significance designed for further animal study. The exact characterization of the physiological properties requires for the prognosis of pharmacokinetics as well as the therapeutic effect. In case of investigational factors, NPs delivery route and zebra growth stage are fundamental. NPs in the zebrafish larvae must always be given the same command as those used in the developed order of vertebrate which is also finally in subject. Given the developmental phase, it is important to understand that the development of zebrafish is extremely fast, principally in the primary phases of larval growth. Numerous cells and organ-tissue structures during the initial growth period can change in a few hours and maturation takes place. So careful measurement is needed, mainly with regard to NP delivery time and the capture of investigational images for several days.

Most research on NPs in zebrafish take place in the zebrafish larvae. This is mainly because it is easily accessible and the properties of the image (size and transparency) are favorable. These benefits are reduced in the later stages of development. Zebrafish larvae are consequently the model for the evaluation of interactions between nanosystem and biological system that occur quickly. This includes specific cellular binding, the functionality of innovative nanosystems (i.e., the administration of an enzyme/gene), and the elimination of definite immunologic system cells.

The toxicology of technical nanomaterials is a moderately innovative and growing sector. While their use claims are growing, their effects on the environment and health pose many problems (Asharani et al. 2008a, b).

Zebrafish as an innate prototype is endorsed in many studies for the reasons that it is economical, simple and fast to evaluate the NPs toxicity, and can offer countless toxicology research benefits (Zon and Peterson 2005; Fako and Furgeson 2009; Sieber et al. 2017). Specifically, ZFET is an substitute to severe toxicity control and vital for reducing the influence of investigational trials on live wildlife proposed by the European Community. Therefore, the use of a zebrafish model to detect the

nanoparticulate materials toxicity profile and for speedy response can be recommended.

18.10 Future Lookout

Massive capacity of zebrafish demonstrated itself as an in vivo prototype in determining the NPs toxicity profile. The use of different genomic biologics, macromolecular methods, and transgenic strains of zebrafish model were developed. Today, many important zebrafish genomic properties and microarrays are offered to assess nanotoxicity profile. In the near future, entirely these progressive properties create zebrafish an exceptionally flexible system of toxicological readings on NPs. Studies on the immune response, proteins, and gene expression of the zebrafish model and chronic long-term toxicity have enormous potential in detecting the toxicity of NPs that are still under discussion. Although high-performance probes using zebrafish larval stages are now being used in NPs toxicology, in nearby it has quiet considerable capability for studying the NPs toxicity.

18.11 Conclusion

NPs have intrinsic complexity and several physicochemical properties. The selection of appropriate biological models during the preclinical development of nanoparticulate drugs is essential. Several factors influence this option, including the size, time, cost, ability to analyze the correct number of test samples, operator regulator over investigational factors, and imitation of complex biological conditions. Currently, zebrafish has become a smart model for vertebrates for nanotoxicological testing. The zebrafish model is much cheaper, easier to handle, faster-growing, close to 75% in the animal model, and more effective for more than 10 years. This model is used for toxicological tests on NPs. The results obtained from the zebrafish experiment are relatively inexpensive because this mammal is available in less time and at a very low cost. Using modern and advanced technologies, the zebrafish animal model can be an important alternative to other mammalian models for NPs toxicology testing in the years to come.

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Evaluation of Toxicity of Nanoparticles Using Brine Shrimp

19

Sairengpuii Hnamte, Kasinathan Kaviyarasu, and Busi Siddhardha

Abstract

Nanoparticles (NPs) have a momentous role in disease healing and drug delivery system that leads to the development of a new field known as nanopharmacology. Nanoparticles are generally coated with polymers, metal ions, chemical surfactants, etc., owing to their properties, i.e., with the decrease in size they tend to evoke toxicity that is predominantly triggered by the environmental and human health risk. Therefore, it is imperative to evaluate the toxicity of nanoparticles using model systems. The purpose of the present chapter is to estimate the toxicity of nanoparticles against brine shrimp (*Artemia*). They are the essential part in the process of energy discharge of the food web in aquatic surroundings. Latterly, researchers have focal point on brine shrimp due to their accessibility, inexpensive and expeditious screening procedure. It is convenient to exemplify the toxicological impacts of nanoparticles toward brine shrimp, their mechanism, strategy and future prospective. The toxicity assay of NPs in *Artemia* are of low cost, continuously accessible, simple and steady. Researchers employed various types of nanoparticles to elucidate the toxicity and safety effects on brine shrimps. The green methods of synthesis have been attracted by scientists due to its low cost, ease of characterisation and capability to reduce NPs toxicity. In addition, researchers also used brine shrimp toxicity assay to evaluate the lethal effect of

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chemically synthesized nanoparticles. It also discusses the toxicological evaluation of NPs by in vitro and in vivo assessment and brief details on biology of brine shrimp.

Keywords
Toxicity · Nanoparticles · Brine shrimp · *A. salina*

19.1 Introduction

Nanotechnology is an emanate domain in various fields which cope with nanoparticles (NPs) at the nanoscale, which is approximately 1–100 nm in size and are of tubular, irregular or spherical shape (Taghavi et al. 2013; Muhammad et al. 2019). NPs are generally classified into different categories depending on their size, morphology and chemical properties. NP synthesis can be accomplished through different methods such as biological, chemical and physical approaches. The chemical and physical techniques are toxic and time consuming, whereas biological method is considered to be a nontoxic and safe approach (Muhammad et al. 2019). Furthermore, NP's synthesis can be broadly categorized into two different groups such as bottom-up and top-down approach (Bali et al. 2016). The bottom-up technique involves a biological and chemical approach that depends on manipulation of atoms, clusters or molecules to fabricate NPs of uniform size and shape. Some of the techniques used in bottom-up approach are microemulsion procedure, electrochemical, hydrothermal, chemical reduction and nonchemical reduction. The biological approach for NPs depend on living matters like plants, microorganisms and enzymes. The top-down method involves physical approaches such as grinding, cutting and milling in order to break the bulk material to nano-size dimensions (Muhammad et al. 2019). The schematical representation of different methods used for the synthesis of NPs is displayed in Fig. 19.1.

Due to the compelling potential of this technology, investment in the use of nanotechnology is a thriving trend worldwide (Taghavi et al. 2013). NPs are being widely investigated in the area of biology, catalysis, engineering, medicine, optics and pharmaceutical sciences considering their unique chemical and physical properties (Sumitha et al. 2018). NPs are presently used in various commercial purposes in different fields such as medicine, technology and industry (Gambardella et al. 2014). They are of distinct interest considering their remarkable physiochemical properties enabling them greatly relevant and attractive for customer products and industrial technologies (Montes et al. 2012). Due to their small size, comparatively huge proportions of atoms and molecules formulating up the particles are exposed to the surface of the particle correlated to greater fragment. This structural disparity

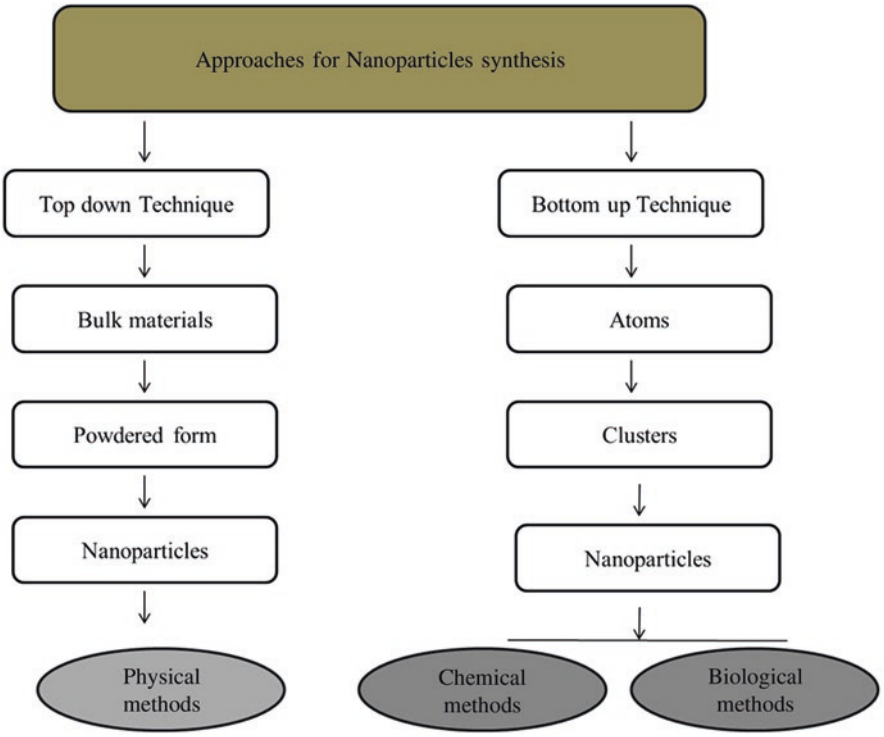

Fig. 19.1 Various methods employed for NPs synthesis

copulates with the comparatively large surface area enabling them to display properties that vary from bulk materials, accomplishing them advantageous in a broad variance of applications (Radhika et al. 2010).

NPs are able to intrude in the marine ecosystem in a greatly influential means which relies on the marine environmental surroundings and are exposed to aggregate and diffuse forms (Khoshnood et al. 2016). Various environmental determinants such as pH, ionic strength and dissolved oxygen content have influenced the detachment of nanoparticles and aggregation (Adam et al. 2015). NPs have gathered much interest owing to their lethal encroach in the surroundings during production and dumping of customer output (Kumar et al. 2017a, b). Therefore, their potential for generating harmful effects eventually turns into concern for researchers (Taghavi et al. 2013). The intention of this book chapter is to discuss the toxicological evaluation of NPs toward brine shrimp. It discusses the toxicological evaluation of NPs by in vitro and in vivo assessment and brief details on biology of brine shrimp. It also presents elaborative and detailed discussion on the toxicity imposed by assessment of toxicity of chemical and green synthesis of NPs against brine shrimp.

19.2 Toxicology Evaluation of Nanoparticles

Toxicology is the science that analytically prospects the nature, incidence mechanisms, occurrence and risk factors for the unfavourable effects of toxic substances (Yu and Lu 2018). It pursuits to examine all the associated hazards qualitatively and quantitatively and determine the exposure surroundings under which those toxicities are induced. Nanotoxicity is associated with different fields such as pathology and biology use of nano devices and nanomaterials for diagnostic and remedial prospect. Therefore, the main aim for a toxicologist is to analyse the in vitro and in vivo assays precisely reflecting the ability of NPs to induce the effects in the humans and in the environment (Rajabi et al. 2015). Further, standardized analysis for in vitro and in vivo evaluation are acquired to establish and surpass expeditious screening techniques and to forecast toxicity (Dhawan and Sharma 2010; Maccormack et al. 2012). Reactive oxygen species (ROS) and free radical production are the fundamental mechanisms of nanotoxicity; it may lead to oxidative stress, swelling and subsequent destruction to membranes, proteins and DNA (Nel et al. 2006). Different techniques are feasible for evaluating the toxicity promulgated by NPs on the organisms. The approach for toxicity evaluation is divided as in vitro and in vivo (Kumar et al. 2017a, b). Figure 19.2 depicts the different experimental techniques and toxicity effects imposed by NPs.

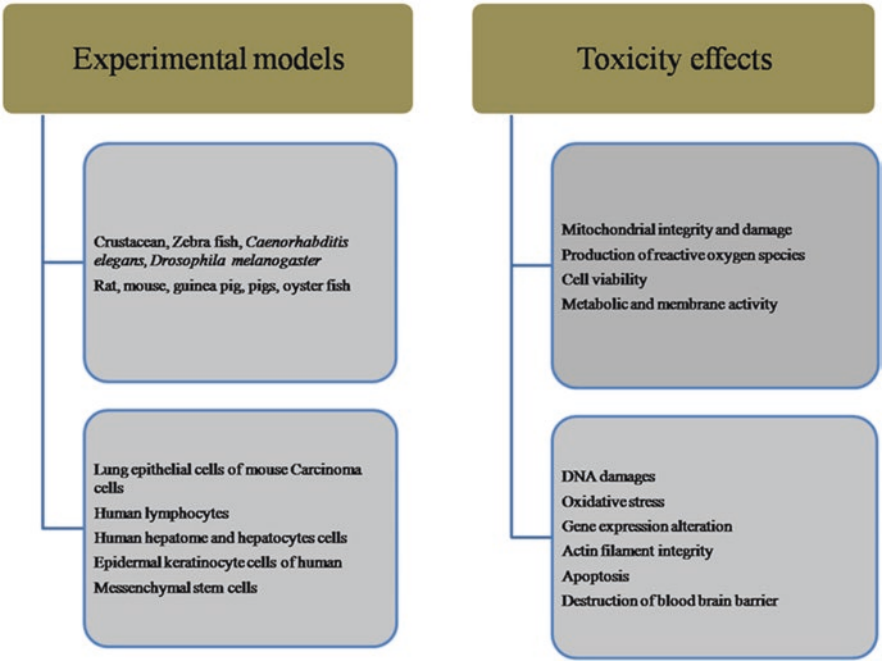

Fig. 19.2 Experimental technique toxicity effects of NPs

19.2.1 In Vitro Assessment

In vitro estimation of NPs toxicity is among the essential techniques which are rapid, at low cost and with minimum ethical concerns. The methods are subcategorized into apoptosis, necrosis, DNA damage, proliferation and oxidative stress assay (Kumar et al. 2017a, b). The brief methods of in vitro assessment are discussed below.

One of the main markers examined in the in vitro evaluation of NP's lethality is apoptosis. The production of extravagant free radical is deliberated to be responsible for DNA destruction and apoptosis (Kumar et al. 2017a, b). Various methods are feasible for evaluating apoptosis such as comet assay, inspection of morphological changes and Annexin-V assay (Mo and Lim 2005). The apoptosis induced by nanoparticles have been documented by different researchers. Ahamed et al. (2008) studied silver nanoparticles (AgNPs) that result in apoptosis of mouse embryonic cells. The findings suggested that distinct surface chemistry of AgNPs reduce various response of DNA damage.

Disclosure of nanoparticles resulted in the production of reactive nitrogen species and reactive ROS (Magder 2006). The reaction of 2,2,6,6-tetramethylpiperidine is intricated in the detection of ROS with O_2^- stable radical and could be identified using X-band electron paramagnetic resonance (Kumar et al. 2017a, b). An alternative and cost-effective method has emerged known as fluorescent probe. But there are drawbacks with fluorescent probes as they are incompetent owing the capability to react with different reactive species. This property results in inaccurate outcome frequently (Halliwell and Whiteman 2004). The most generally employed tetrazolium salt for in vitro toxicity evaluation of NPs is 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This method is advantageous due to their capability to yield rapidly, minimal manipulation of the model cells and reproducible outcome (Kumar et al. 2017a, b).

19.2.2 In Vivo Assessment

The evaluation of in vivo toxicity is generally executed on animal models including rat and mice. The estimation techniques for in vivo toxicity are haematology, histopathology, serum chemistry clearance and biodistribution. NPs are identified through radiolabels in the live or killed animals, and removal of nanoparticles is implemented by the investigation of elimination and metabolism of NPs at different intervals after exposure (Kim et al. 2001; Li et al. 2001). The histopathological evaluation has been adopted to nanoparticles exposed tissues like the eyes, liver, lung, brain, heart, spleen and kidneys (Baker et al. 2008; Zhu et al. 2008). Another technique in assessing the in vivo toxicity is examining the variance in the serum chemistry and types of cell after exposure of NPs (Li et al. 2001). The development of lethality evaluation involves the use of micro-electrochemistry and microfluidics (Kumar et al. 2017a, b). Nanotechnology experiments involved the use of animals with the approval from regulative bodies such as the Institutional Animal Care and Use Committee to warrant ethical treatment of animals (Suh et al. 2009).

19.3 Biology of Brine Shrimp

Artemia (brine shrimp) is an aquatic crustacean and distinguished by its short life span, small size, levels of salinity, production of large offspring and high adjustability to various temperature (Balalakshmi et al. 2017). Artemia has the inherent property of vigorous adjustability to hypersaline surroundings including coastal lagoons, constant salt lakes and artificial salt pans (Arulvasu et al. 2014). It is a hyper- or hypo-osmotic regulator that is able to keep up haemolymph ion concentration within restricted limits over an outside salinity range from 0.26% NaCl to supersaturated brines (Kumar et al. 2017a, b). *A. salina* is comprehended as among the test species for examining acute toxicity by the US Environmental Protection Agency (EPA 2002) (Gambardella et al. 2014). Adult *Artemia* are of 8 mm in length, and under nutritional supply and optimum environmental conditions, they can reach up to 20 mm. All stages in the *Artemia* life cycle are appropriate for testing toxicity; nauplii after 48 h of hatching are convenient for bioassay (Selvi 2016). *Artemia* is among the most relevant test organisms feasible for ecotoxicity test in marine environment as it is broadly adopted as a nourishing live food source to the larvae of different aquatic organisms. Therefore, procreate them the most appropriate, minimal labour-thorough life food accessible for aquaculture (Radhika et al. 2010).

There are six sexual species which are mostly geographically confined in salty lakes in distinct parts in Eurasia (regional endemism) at, or close to, the Mediterranean area where *Artemia* species deviated from the ancestral species 80 million years ago (Baxevanis et al. 2006). They are given as follows:

- *A. urmiana*
- *A. salina*
- *A. sinica*
- *A. tibetiana*
- *Artemia* sp.

Artemia usage in toxicology provided acceptable answerable questions such as relevance of ecology, systematic use, culture and acquisition of cyst, practical scrutiny of laboratory culture, sustainability and maintenance of laboratory conditions in the animal model. Thus, creating a sustainable development of *Artemia*-based bioassays (Arulvasu et al. 2014). *Artemia* species has a short life cycle and has been widely endorsed in testing ecotoxicology due to their adjustability to a great range of salinity (5–300 g/l) and temperatures (6–40 °C) in consideration of their growth and mortality as the essential end points (Libralato 2014). Various stages of their life cycle are vigorously convoluted in the reproduction and survival under environmental circumstances (Gajardo and Beardmore 2012). The life cycle of different stages of *Artemia* are displayed in Fig. 19.3.

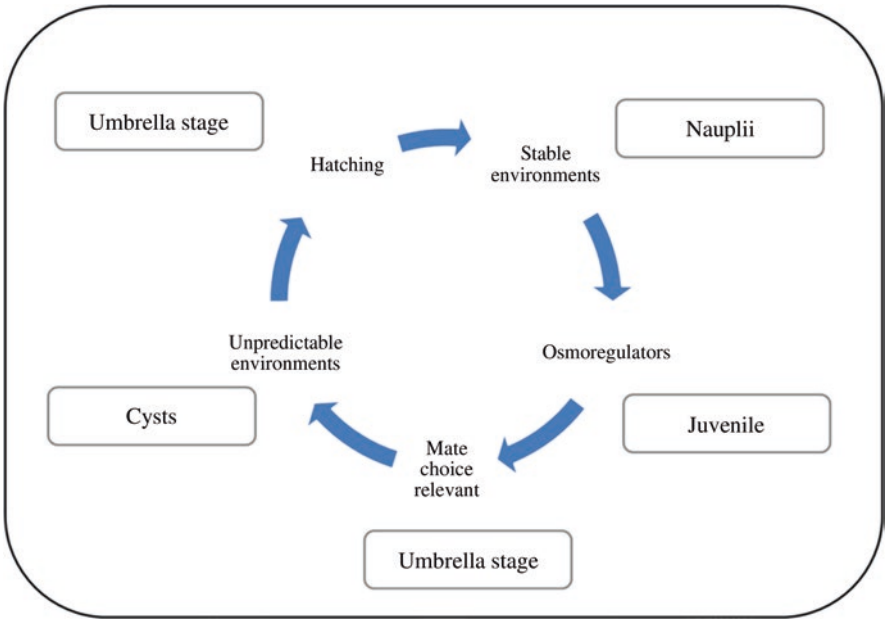

Fig. 19.3 Different stages of life cycle in *Artemia* sp.

19.4 Brine Shrimp Lethality Assay

Brine shrimp lethality assay is generally employed to examine the toxic effect of bioactive components (Sarah et al. 2017). It is an alternate approach to screen the lethality of plant extracts, nanoparticles, metal ions and heavy metal toxicity, cytotoxicity of dental materials and cyanobacteria and including screening of aquatic natural products (Hamidi et al. 2014). Michael et al. (1956) established the brine shrimp lethality assay and later established by others. It has been propitiously used as a bioassay guide for antitumour and active cytotoxicity in 1982 (Meyer et al. 1982). It is inexpensive, simple, rapid and effective and utilizes an enormous number of organisms for statistical approval and moreover, no aseptic method and tiny amount of sample is required to run the experiment (Sarah et al. 2017). Brine shrimp assay is largely used nowadays in applied toxicology and research. *A. salina* assay has been used to evaluate the toxicity and screen a huge number of extracts in the discovery of drugs in medicinal plants (Rajabi et al. 2015).

19.4.1 Advantages of Brine Shrimp Assay

The advantages of brine shrimp assay are as follows:

- Artemia has various advantages that make it excellent for toxicity approach such as extensive geographical distributions and ability to use distinct nutrient sources (Rajabi et al. 2015).
- This assay is appropriate because it is expeditious, cost-effective, efficient and uncomplicated.
- The eggs are promptly feasible at reasonable cost and endure viability for years in dry repository.
- It conveniently entertains an enormous number of nauplii for statistical demonstration.
- This assay does not desire animal agglutinin and therefore avoids the use of animals in scientific research.
- Good perception of ecological and biological perception.
- Small-size acquiesces for accessible laboratory operation including its well-developed adaptability to varied experimental conditions (Yu and Lu 2018).

19.4.2 Disadvantages of Brine Shrimp Assay

The limitations of brine shrimp assay are listed below:

- Consistent experimental conditions in temperature, salinity, aeration, light and pH.
- Similar geographical area of the cysts.
- Identical age of *Artemia nauplii* at the beginning of each test.
- In order to analyse the sensitivity of the larvae, positive and negative controls are a crucial part of the assay and to maintain conformity with standard.

19.5 Green Synthesis of NPs and Cytotoxicity Towards Brine Shrimp

It is reported that 70–80% of the population in the world depends on unconventional medicines predominantly in plant sources in the primary health insurance. Plants are perceived for their capability to yield abundance of secondary metabolites; majority of these natural products exhibited interesting pharmacological and biological properties which could aid as the outset in the advancement of current medicines. Various research investigations have latterly spotlight on both pharmacology and lethality of medicinal plants employed by human beings. This is of great concern passable to acquire an intact remedy with plant products (Parra 2001).

Researchers employed various types of NPs to elucidate the toxicity and safety effects on brine shrimps. Scientists focus on contemplating towards green synthesis of nanoparticles because of its eco-friendly approach (Muhammad et al. 2019).

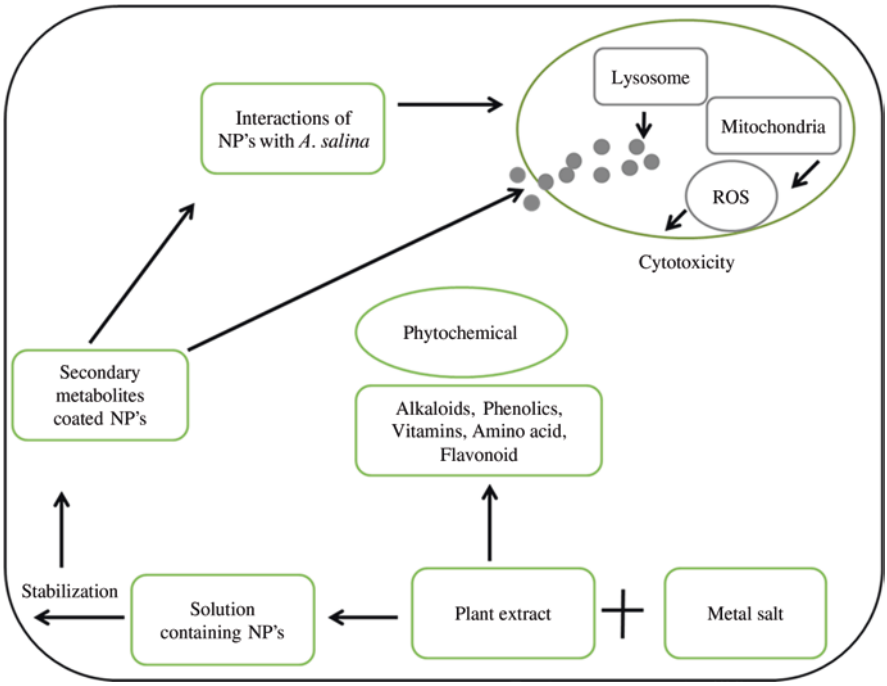

Fig. 19.4 Schematical presentation of green synthesized NPs cytotoxicity towards brine shrimp

Plants have become promising sources for the synthesis of metallic nanoparticles recently. Green synthesis of metallic nanoparticles is an appealing avenue, and various research workers have studied on the synthesis of metallic NPs such as gold, silver, copper oxide, titanium oxide and tungsten oxide with different plants (Supraja et al. 2018). Furthermore, in vitro methods of brine shrimp are employed to analyse the potential chemical constituent in the synthesized NPs (Muhammad et al. 2019). Brine shrimp assay is being generally used to detect the lethality of various plants products during the past 30 years. Brine shrimp (*A. salina*) is the most widely used among the *Artemia* species (Hamidi et al. 2014). The schematical presentation of green-synthesized NPs cytotoxicity towards brine shrimp is given in Fig. 19.4.

Anand et al. (2016) evaluated the biosynthesis of palladium NPs using an aqueous extract of *Moringa oleifera*. The study illustrates the in vitro one-step hasty assembly of palladium NPs with *M. oleifera* and their stability, morphology and toxicity assessment towards brine shrimp. The results suggest the hidden efficacy of the biosynthesized NPs as an asset for the disclosure of applications like antimicrobial, antioxidant, anticancer agent, biocatalytic property and safety approach. Green synthesis of AgNPs from crude extract of *Bergenia ciliata* and their biological assessment was investigated by Phull et al. (2016). The findings highlighted that the NPs were spherical in shape with particle size of 35 nm. Green-synthesized NPs exhibited embellish antioxidant activity correlated to the crude extract. The NPs also showed cytotoxicity effects towards *A. salina* with 33.92 µg/ml LD_{50}. The impact of green synthesis of gold nanoparticles with *Sphaeranthus indicus* extract was exemplified

by Balalakshmi et al. (2017). The extract of *S. indicus* responded as a reducing and capping agent during synthesis of gold. Gold NPs were evaluated as 0, 1, 3, 5, 7 and 10% doses in pollen germination, mitotic cell division assay and in vivo toxicity assay towards *A. nauplii*. Their findings explicated that Gold NPs did not exhibit any toxic effect on *A. nauplii* and plant cells. All the experimental animals showed 100% survivability. Hence, they concluded that Gold NPs have prominent applications in the advancement of plant tissue culture and germination seeds.

Balkrishna et al. (2017) elucidated Ag NPs using *Bryonia laciniosa* seed extract. The characterisation of synthesized NPs was executed through X-ray diffraction (XRD), transmission electron microscope (TEM), etc. TEM disclosed homogenous spherical shape and XRD disclosed the size of nanoparticles of ~15 nm. The biological investigation determined that both *B. laciniosa* seed extract and NPs lack antimicrobial property. Nonetheless, the NPs had preferably greater antioxidant activity and cytotoxicity. The outcome of the results recommended that *B. laciniosa* seed is secure to be used as medication and the production of NP's has further fortified the biological mobility, energy absorption and chemical reactivity. The synthesis of zinc oxide NPs using *Murraya koenigii* berry extract was evaluated by Yazhiniprabha et al. (2019). The formation of *M. Koenigii* berry extract-mediated zinc oxide NPs were characterized. The results of the in vivo toxicity assay exposed the lethal concentration of *A. nauplii* to be LC_{50}-78.73 µg/ml and LC_{90}-130.03 µg/ml. Their findings unveiled the plausible bacteriostatic effect and mosquito larvae regulating efficiency of *M. Koenigii* berry extract-mediated zinc oxide NPs. The various recent reports of green synthesis of nanoparticles against brine shrimp are given in Table 19.1.

19.6 Assessment of Toxicity of Chemical-Synthesized Nanoparticles Using Brine Shrimp

Various biological applications of metal NPs have been reported by researchers: gold and silver nanoparticles for the treatment of leishmaniasis, copper nanoparticles in the remedy for angiogenesis, and zinc oxide and titanium oxide nanoparticles also reported for their biomedical activities (Yazhiniprabha et al. 2019). The various

Table 19.1 List of validated green-synthesized nanoparticles towards brine shrimp

Plant	Synthesized NPs	Size and morphology	References
Moringa oleifera	Palladium (Pd)	10 nm, spherical	Anand et al. (2016)
Bergenia ciliata	Silver (Ag)	35 nm, spherical	Phull et al. (2016)
Bryonia laciniosa	Silver (Ag)	~10 nm, homogenous Spherical	Balkrishna et al. (2017)
Sphaeranthus indicus	Gold (Au)	25 nm, spherical	Balalakshmi et al. (2017)
Alstonia scholaris	Zinc oxide (ZnO)	20 nm, crystalline	Supraja et al. (2018)
Durio zibethinus	Silver (Ag)	20 and 75 nm, spherical and rod shaped	Sumitha et al. (2018)

Table 19.2 List of chemical-synthesized NPs and their potential against brine shrimps

Chemical-synthesized NPs	Dose	*Artemia* species	References
Au and AgNPs	Several concentrations	Brine shrimp	Maurer-Jones et al. (2013)
AgNPs	2–12 nM	*A. nauplii*	Arulvasu et al. (2014)
Cerium(IV) oxide (CeO_2) and Iron(II, III) oxide (Fe_3O_4)	0.01–1.0 mg/ml	*A. salina*	Gambardella et al. (2014)
AgNPs	1000–2500 mg/ml	*A. salina*	Kanchenton et al. (2018)
Zn and ZnO NPs	10, 50 and 100 mg/l	*A. salina*	Ates et al. (2013)
ZnO-TiO_2	0.1–1.0 mg/l	*A. salina*	Daglioglu et al. (2016)
TiO_2, ZnO and copper oxide NPs	1–200 mg/l	*A. franciscana*	Khoshnood et al. (2016)
TiO_2 and $AgTiO_2$ NPs	1 and 10 mg/l	*A. salina nauplii*	Ozkan et al. (2015)
Zero-valent iron nanoparticles	1, 10 and 100 mg/l	*A. salina*	Kumar et al. (2017a, b)

reports of evaluation of toxicity of chemical-synthesized nanoparticles towards brine shrimp are given in Table 19.2.

19.6.1 Gold Nanoparticles

Gold nanoparticles (AuNPs) have fascinated researchers in the areas of different fields. They have been extensively used for different applications such as antimicrobials, antioxidants, drug delivery, anticancer drugs, agriculture, larvicides and catalysis (Balalakshmi et al. 2017). Due to its exclusive properties, AuNPs have been used as a component in different drugs and are designated as passive medicines.

19.6.2 Silver Nanoparticles

Silver nanoparticles (AgNPs) have attained much concern owing to their antimicrobial activities (Arulvasu et al. 2014). They have impressive functions as virucidal compounds and a broad spectrum of bactericides that are beneficial in the treatment of infectious diseases. AgNPs are one of the significant nanomaterials that are found in several products such as cosmetics, food, textiles, medicines and electronic devices (Kanchenton et al. 2018). The most significant technique that executes the mobility and stability of AgNPs in the marine surroundings are dispersion, dissolution, sedimentation, aggregation and agglomeration. The method relies on the particle physiochemical properties that are successively persuaded by environmental factors like

temperature, ionic strength, pH and ubiquity of natural organic matter. Various reports on the lethality of chemically synthesized NPs are documented. Most of the described NPs system techniques are endowed to be correlated with discrete obstacles such as particle aggregation, lack of stability and crystal growth (Sumitha et al. 2018).

Arulvasu et al. (2014) determined the lethal effects of silver NPs in *A. nauplii* and assessed their hatching percentage, genotoxic effect and rate of mortality in *A. nauplii* cysts. The results of their study indicate that NPs at the concentration of above 12 mM used as a formulation in commercial purpose will lead to DNA damage at high levels of *Artemia*, owing to their toxicological effects. The toxicity of AgNPs was investigated using brine shrimp *A. salina* by Kanchenton et al. (2018). Histopathological study was executed using brine shrimps with 25% of LC_{50} of AgNPs concentration after 24 h of incubation. Their findings signify that AgNP's effects and their negative impact on marine animals might cause destruction of ecosystem. In conclusion, AgNPs are capable of inducing cytotoxicity to the tissue of the organism. Consequently, the appropriate amount of AgNPs in the consumer products for environment conservation should be of much concern.

19.6.3 Zinc Oxide Nanoparticles

Zinc oxide (ZnO) NPs are extensively employed in cosmetic products, sunscreens, paints, pigments, textiles, water disinfection, polishers, semiconductors, additives in food and catalysts (Khoshnood et al. 2016). ZnO NPs synthesis has been accomplished through physiochemical techniques such as simple precipitation, sol-gel, ultrasonication, inert condensation and electrochemical. Researchers also pursue the biosynthesis of nanoparticles widely from plants, animals and microorganisms (Yazhiniprabha et al. 2019). ZnO NPs are among the well-known nanomaterials fabricated in factory scale, and due to their broad applications in regard to the environment and well-being, the toxicological effects of ZnO NPs have been investigated through crustaceans, zebrafish embryo, nematodes, algae, bacteria and protozoa (Ates et al. 2013). The correlative evaluation of Zn and ZnO NPs towards brine shrimp (*A. salina*) larvae was studied by Ates et al. (2013). To assess their toxicity in aquatic ecosystems, Zn and ZnO NPs were exposed to various sizes. The results indicated that Zn and ZnO NPs were not meticulously lethal to *Artemia* at environmentally appropriate levels. Despite, prolonged disclosure to the similar suspensions induced oxidative stress and toxicity which ultimately leads in the increase of lipid peroxidation levels.

19.6.4 Titanium Dioxide Nanoparticles

Titanium dioxide nanoparticles (TiO_2 NPs) are adopted worldwide in different fields such as medicines, food additives, sunblock, cosmetics, construction materials and paints (Ozkan et al. 2015). Khoshnood and his co-workers studied the toxic effects and aquatic stability of TiO_2, ZnO and copper oxide NPs against *A. franciscana*.

Various concentrations of NPs (24, 48, 72 and 96 h) were used to conduct the acute exposures in sea water. *A. franciscana* mortality rate rises with the increasing concentration and exposure time of all the NPs. Based on their experimental findings, they concluded that all the NPs employed in this work may have acute dosage-dependent ecotoxicological effects towards *A. franciscana* (Khoshnood et al. 2016). Ozkan et al. (2015) also determined the lethal effects of TiO_2 and $AgTiO_2$ NPs towards *A. salina nauplii*. TiO_2 was found to be less toxic to nauplii than $AgTiO_2$. The results highlight that since the lethality of $AgTiO_2$ was greater than TiO_2, the discharge of $AgTiO_2$ into the aquatic environment can lead to ecological risks and may have unfavourable impact in saltwater ecosystem.

19.7 Conclusion

Although nanomaterials have been valuable with its increased application in industrial and medical health, it exhibited to pose harmful effects on the environment and life form. Over a decade, extensive expedition on nanomaterials and its consequence turned out to be a major challenge. Depicting the mechanism or the exact process of the nanoparticles causing toxicity is still imprecise and is problematic to estimate the overall scenario. This chapter, in detail, illustrated an overview of evaluating the toxicity of nanoparticles using brine shrimp. In vitro brine shrimp lethality assay is appropriate and convenient for researchers to a huge extent as they are cost-effective, reliable and easily scale up. They are used extensively to assess the toxicity of nanostructures. Keeping in view the noxious substances screening of the nanomedicines, which play the potential role in various diseases, this assay possesses a pivotal role. Therefore, brine shrimp lethally assay needs to be utilized for the detection to eradicate the harmful nature of the pharmaceutical drugs being synthesized in the industries on large scale.

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Drosophila Model to Decipher the Toxicity of Nanoparticles

20

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Abstract

In the twenty-first century, there is a significant advancement in the development of high-throughput therapeutic strategies and biotechnological upgradation in the fight against chronic microbial infections and infectious diseases. However, the increased incidence of antibiotic resistance and the solubility issues associated with naturally derived therapeutic drugs have propelled the scientific community to quest for novel platforms for the delivery of drug moieties that bypass the resistance mechanisms. In this context, the emergence of nanotechnological interventions could provide novel avenues for the controlled and site-specific delivery of drug candidates at the target sites by counteracting the resistance phenomenon. However, irrational and indiscriminate use of nanomaterials without any regulatory guidelines for the purpose of widespread applications could lead to severe toxicological implications to human and animal health, as well as have a profound impact on environmental sustainability. Hence, it is imperative to assess the toxicological profile of engineered nanomaterials before being considered for widespread applications across agriculture, biomedical and pharmaceutical sectors. The risk assessment of nanotoxicity could be implemented through both in vitro and in vivo model systems. Though, the in vitro systems seem to be easy and cost-effective, its reliability issues suggested to look for

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more reliable in vivo model system to assess the risk associated with the use of nanomaterials. The in vivo model system could provide ample research avenues for the biodistribution and bioaccumulation profile of administered nanomaterials in the in vivo model systems. Among the different in vivo model systems, *Drosophila melanogaster* has exhibited promising aspects such as short life span, ease of rearing, large progeny size, ease of handling, cost-effective experimentation, genetic tractability, genetic homology with human genetic system and less genetic redundancy for its candidature as promising model system for drug development, developmental biology and toxicity assessment. In this chapter, the advantages to use *D. melanogaster* as promising in vivo model system for the risk assessment of engineered nanomaterials have been discussed. The chapter also emphasizes the development of international surveillance system as well as regulatory bodies for the synthesis of different nanomaterials before being considered for its application and exposure to the environment.

Keywords
Nanotechnology · Toxicological profile · Model system · *Drosophila melanogaster*

20.1 Introduction

20.1.1 Drug Resistance, Disease Progression and Health Consequences

In the twentieth century, the emergence of antimicrobial therapy in the form of antibiotics development has revolutionized the biomedical and healthcare sectors in the fight against life-threatening infectious and non-infectious diseases. The development of antibiotics to treat various microbial infections and other infectious diseases has readily transformed the modern medicines in preventing high rate of mortality and morbidity due to chronic microbial infections (Friedman et al. 2016). However, irrational and indiscriminate use of antibiotics against various microbial infections, poor infection control practices and lack of proper awareness in the use of antibiotics have led to the generation of severe antibiotic resistance. The inherent spreading of drug resistance phenomenon against the conventional antibiotics in the healthcare settings becomes a serious public health issue (Rather et al. 2017). The pathogenic microorganisms especially *Mycobacterium tuberculosis*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* have already been reported for their inherent ability to show multidrug resistance (MDR), extensive drug resistance (XDR) and pandrug resistance (PDR) to the available antibiotics. The increased incidence of drug resistance phenomenon poses serious global health crisis both at individual and community levels, causing severe socio-economic burden for both developing and developed countries (Rather et al. 2017).

Apart from the irrational use of antibiotics in the treatment of microbial infection, the emergence of drug resistance phenomenon by pathogenic microorganisms to the

conventional antibiotics could also be attributed to different mechanisms followed by microorganisms such as mutational upregulation, modification of the antibiotic target, changes in cell permeability and efflux system and horizontal acquisition of resistance genes (Cox and Wright 2013; Rodriguez-Rojas et al. 2013; Brooks and Brooks 2014). The intrinsic approach of resistance pattern and their persistence significantly affect the conventional therapeutic efficacy. The increased incidence of resistance draws considerable attention owing to its detrimental consequences not only to the vulnerable hosts but also significantly affecting the hospital settings (Laxminarayan et al. 2013). Hence, the persistence of the resistance pattern shown by pathogenic microorganisms has generated an uphill task for the scientific community to develop new arsenal to fight against chronic infections and associated resistance phenomenon (Cox and Wright 2013; Courvalin 2016). The task of mitigating the drug resistance phenomenon and associated health consequences could be achieved through the development of potent therapeutic programmes, modification of the conventional therapeutics, high-throughput legislation, socio-economic awareness and surveillance on the optimal and specific use of antibiotics and therapeutic drugs in the fight against chronic infections and life-threatening disease progression (Frieri et al. 2017).

20.1.2 Conventional Therapeutic Strategies

From the last few decades, it has been observed that the impending threat posed by the emergence of antibiotic resistance in a global platform irrespective of developing and developed countries has a significant impact on socio-economic progress of any country. Apart from the emergence of resistance phenomenon, the development of new drugs in the fight against infectious diseases also gradually decreased, resulting in further increase in the magnitude of resistance-associated health impacts. In this context, it is important to develop new drug candidates which effectively target the microbial infections and also counteract the resistance mechanism (Chaudhary 2016). In the pursuit of developing novel therapeutic strategies and new drug candidates against microbial infections-associated health risks, it is important to consider three considerable aspects such as protecting the natural microflora, preventing the resistance phenomenon and localized targeting of therapeutic drug moieties by bypassing the resistance mechanisms (Brooks and Brooks 2014).

20.1.3 Non-conventional Therapeutic Strategies

Though the current understanding of drug resistance phenomenon has urged the scientific community to develop new drug candidates to counteract the drug resistance-related health issues, the antibiotic discovery is not keeping pace with the alarming rate of drug resistance. In this context, novel and alternative therapeutic strategies beyond the conventional antimicrobials development could be promising in the present scenario. The development of smart and novel therapeutics generally involves the emergence of innovative combinatorial therapeutics such as quorum sensing

inhibitors, biofilm inhibitors, quorum quenching enzymes, antimicrobial peptides and anti-biofilm peptides (Nigam et al. 2014; Pletzer et al. 2016). The development of these smart therapeutic strategies does not impose life or death selective pressure on pathogenic microorganisms but instead alters the microbial communication system-mediated virulence profile and biofilm development, thereby improving the therapeutic strategies in circumventing the majority of the known resistance mechanisms and related healthcare issues (Brooks and Brooks 2014).

It is evident from the current understanding of quorum sensing research that the complex regulatory network of quorum sensing regulates the formation and development of highly persistent biofilm dynamics. As the biofilm-forming microorganisms are thousand times more tolerant to their planktonic counterparts, targeting quorum sensing-mediated biofilm formation could be of promising aspect in the drug discovery programmes (Kalia et al. 2019). The development of novel anti-virulence or anti-infective strategies focuses upon the discovery of novel drug candidates targeting microbial virulence mechanisms, thereby bypassing the selective pressure associated with drug resistance without affecting the beneficial microbiota (Maura et al. 2016; Kahler et al. 2018). In recent times, the development of antibiotic adjuvants has gained considerable attention in attenuating the drug resistance profile of pathogenic microorganisms. The antibiotic adjuvants are administered in combination with conventional drugs and function in two important aspects such as disarming the microbial resistance profile and enhancing the antimicrobial action of the co-administered drugs by complementing the co-administered antibiotics. Based upon the role of antibiotic adjuvants in controlling antibiotic resistance mechanisms, they are being considered as ‘resistance breakers’ or ‘antibiotic potentiators’ (Wright 2016; Gonzalez-Bello 2017).

In addition, the advent of antimicrobial peptides also observed to be influential in effective clearance of drug resistance phenomenon by exhibiting antimicrobial and anti-biofilm properties (Chung and Khanum 2017). Though from the last few years a series of alternative therapeutic strategies have been developed in controlling the menace associated with antibiotic resistance, drug permeability issues and non-targeting of drug at preferred sites have drew certain limitations. In this context, it is imperative to develop smart therapeutic strategies such as development of novel drug delivery systems for localized and targeted delivery of administered drugs for efficient clearance of resistance phenomenon. Among the different drug delivery systems developed for smart delivery of drug moieties at the target sites, the introduction of nanotechnology has gained considerable interest among the scientific community based upon their advantages and widespread applications.

20.2 Nanotechnology as Smart Therapeutics

From the last few decades, the field of nanotechnology which constitutes an agglomeration of physics, chemistry, material science and biological sciences has been developed to complement the conventional therapeutics. Nanoparticles are solid, colloidal particles with the size ranging from 10 nm to <1000 nm. However, for biomedical and pharmaceutical applications and drug delivery strategies, the preferential size should

be less than 200 nm (Biswas et al. 2014). The advent of nanotechnology has a special impact on various fields such as tissue engineering, agriculture, energy, environmental, biomedical and pharmaceutical sectors (Kuppusamy et al. 2016; Patil and Kumbhar 2017). Based upon the unique physicochemical properties, thermal stability, optical characteristics and widespread biological activities, the nanotechnological intervention have significantly revolutionized the current knowledge of conventional pharmaceutical and biomedical settings with improved efficacy (Zhu et al. 2014).

The advent of nanotechnology into the field of biology and biotechnology has presented unprecedented opportunities and widespread applications in the pharmaceutical and biomedical sectors. The emergence of nanotechnology has gained significant attribution in last few years as smart drug delivery systems in the pursuit of overcoming the limitations associated with the conventional therapeutic strategies. The development of different nanomaterials such as nanoparticles, nanocomposites, nanorods, nanoemulsions and other nanomaterials could greatly improve the efficacy of the conventional antibiotics and traditional drug moieties (Jiang et al. 2017). One of the most important aspects of the use of nanotechnology is the smart and localized delivery of various drug moieties irrespective of their solubility issues at the target sites, thereby improving the therapeutic efficacy (Rizvi and Saleh 2018). The characteristic features such as controlled drug delivery, slow and sustained release profile, large surface area and high drug payload significantly contributed to the emergence of nanotechnological platforms as promising and smart therapeutic strategies as an arsenal against drug resistance-related health issues (Safari and Zarnegar 2014; Jurj et al. 2017). From the beginning of nanotechnological research, a number of different nanomaterials with unique and specific physicochemical characteristics have been developed. The characteristic optical properties of different nanomaterials served as promising attributes in the field of biomedicine by acting as promising biosensing, bioimaging and therapeutic agents. Hence, the emergence of nanotechnology provides a greater platform for the development of smart arsenal against infectious diseases in the post-antibiotic era (Rosen et al. 2011; Zhu et al. 2014).

20.2.1 Potential Drawbacks: Toxicity Profile of Nanoparticles

Though the advent of nanotechnological platforms has significant attribution to the welfare of the society owing to their widespread applications in the field of agriculture, pharmaceuticals and biomedicines, the inadvertent and irrational synthesis and applications draw considerable limitations. The inadvertent commercialization of engineered nanomaterials and frequent and uncontrolled exposure to human beings and environment resulted in the emergence of intense reactivity, thereby promoting nanotechnological toxicity (Kroll et al. 2009). The concept of nanotoxicity came into existence for the first time in 2004 which critically refers to the in-depth assessment of the potential toxic impacts of engineered nanomaterials on biological and ecological systems (Love et al. 2012). One of the important limitations associated with the synthesis and use of nanomaterials is the use of non-biodegradable compounds as reducing agents in the synthesis of nanoparticles. The use of these

non-biodegradable materials as reducing agents proves to be potentially hazardous to the environment as well as the well-being of biological system (Phull et al. 2016). As per the trend in the use of variety of nanomaterials, it is evident that the systemically administered nanomaterials are generally taken up by the liver and spleen. Apart from the liver and spleen, traces of nanomaterials are also destined to be accumulated in the lung, kidney, heart and brain after single administration. Hence, the applications of nanomaterials constantly raised questions over the bioaccumulation profile, biodistribution pattern and systemic toxicity after repeated administration (Lasagna-Reeves et al. 2010).

20.3 Toxicological Aspect of Nanoparticles

While nanotechnology and the production of nanoparticles are growing exponentially, research into the toxicological impact and possible hazards of nanoparticles to human health and the environment is still in its infancy (Elsaesser and Howard 2012). In the pursuit of technological advancement through nanotechnological intervention in the biomedical and pharmaceutical sectors, it is imperative to assess the toxicological aspects of the synthesized nanomaterials towards human health and environmental setup. A number of intrinsic and extrinsic factors are destined to be influential in determination of toxicological impacts of the synthesized nanomaterials (Fig. 20.1). The intrinsic factors such as chemical composition, size, shape, surface properties, crystalline nature and agglomeration propensity are established as influential in toxicological assessment of synthesized nanomaterials (Caballero-Diaz and Cases 2016). The surface area of the engineered nanomaterials also has a linear correlation with the toxicological profile of the synthesized nanomaterials (Donaldson and Poland 2013). It is also important to develop stringent approach to assess the toxicological impacts of different synthesized nanomaterials both in vitro and in vivo (Fig. 20.1).

Human beings are constantly being exposed to the inadvertent use of different engineered nanomaterials. The exposure of these nanomaterials occurs through different routes such as inhalation (respiratory tract), skin contact, ingestion and injection. Besides, the nano-sized engineered nanomaterials have the inherent ability to pass through biological membranes and other physiological barriers and accumulated in the cellular and subcellular spaces. The frequent accumulation of traces of these tiny-sized nanomaterials in the cellular and subcellular sites results in cellular dysfunction. Though the physicochemical properties such as high surface-to-volume ratio greatly improves the biological applications of the engineered nanomaterials, being highly reactive or catalytic in nature, they seem to be potentially toxic (Fu et al. 2014). The constant exposure of nanomaterials into human beings has gained considerable interest owing to their widespread health consequences. The irrational intake and exposure of nanomaterials irrespective of their route of entry has dreadful impact on the lung health, gastrointestinal dysfunction, myocardial infarction, skin irritations, cellular dysfunction and nervous system impairment (Agarwal et al. 2013).

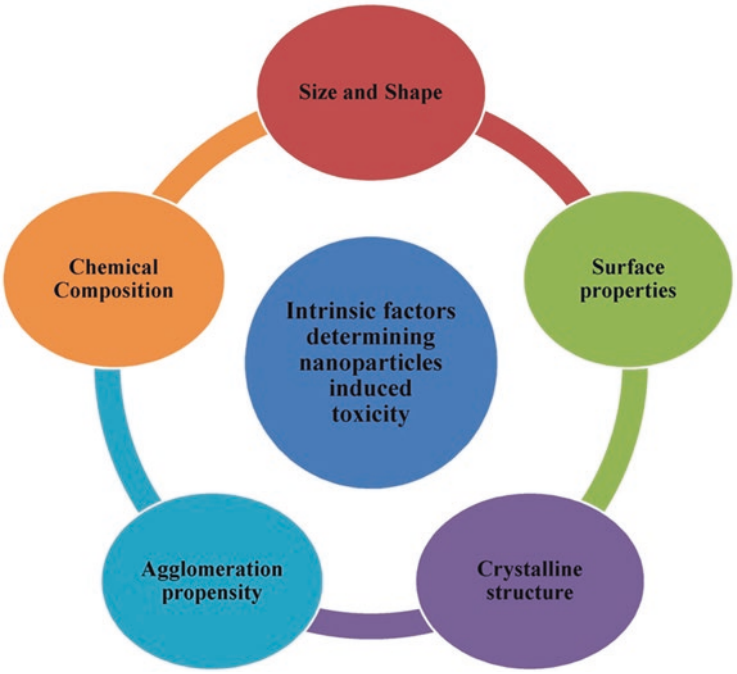

Fig. 20.1 Schematic illustration of different intrinsic factors that determine nanoparticle-induced toxicity

The engineered nanomaterials have profound impact on physiological health conditions of human beings through different mechanisms. Firstly, the unique physicochemical properties of different nanomaterials interact with the physicochemical system in the body and interferes the normal physiological processes of the embryos, growing animals as well as adults. In particular, the engineered nanomaterials have significant lethal impact on the embryos by critically disrupting the normal developmental processes. Apart from physicochemical properties, the tiny size of nanomaterials could easily penetrate across the cell membrane into the cellular and subcellular spaces and altering the cellular metabolism, thereby provoking cell death. Besides, the engineered nanomaterials also tend to interfere with the biological membranes, thereby altering the normal physiological ion transport or signal transduction processes (Exbrayat et al. 2015). From earlier evidence, it was observed that the effect of engineered nanomaterials includes thrombosis by activating the platelet aggregation and inflammation of respiratory tracts. It is also important to carefully regulate the synthesis of nanomaterials as the tiny-sized nanomaterials have the inherent ability to enter into cellular organelles, thereby drastically altering the cellular metabolic processes causing severe DNA lesions, genetic mutations and apoptosis of cell (Sukhanova et al. 2018).

20.3.1 In Vitro Models to Test the Toxicological Profile of Nanoparticles

The advent of nanotechnological platforms has both positive and negative impacts. On one hand, the nanotechnological interventions in the field of biomedicines and pharmaceuticals have gained considerable attention in the fight against microbial infections and infectious diseases. On the other hand, the irrational use of engineered nanomaterials also draws considerable limitations in the form of toxicity and associated health consequences and environmental hazard. Hence, it is imperative to assess the toxicological profile of the engineered nanomaterials before being considered for biomedical, pharmacological and environmental applications. Among the different assessment approaches, in vitro toxicity assessment proved to be simple, fast and cost-effective as compared to the in vivo toxicity assessment. The in vitro toxicity assessment of engineered nanomaterials includes cell viability or proliferation assay, apoptosis assay, necrosis assay, oxidative stress response, DNA damage assay and inflammatory response analysis (Fig. 20.2) (Kroll et al. 2009).

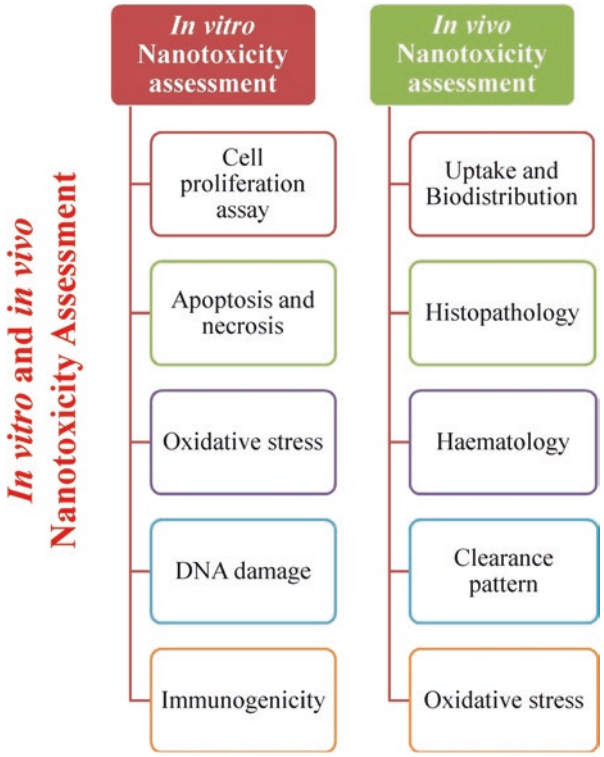

Fig. 20.2 Schematic representation of different in vitro and in vivo assessment strategies to determine the risk associated with the engineered nanomaterials

20.3.2 In Vivo Model Systems as Prolific Model for Nanotoxicity Assessment

Though the in vitro risk assessment of engineered nanomaterials proved to be simpler and cost-effective, the reliability issues limit their widespread use. In this context, in vivo toxicity assessment is normally preferred over in vitro risk assessment based upon their higher reliability. The in vivo risk assessment methods generally include biodistribution, effective clearance pattern, haematological profile, serum chemistry and histopathological profile. Among these methods, biodistribution and bioaccumulation studies proved to be essential in determining the localized distribution of nanomaterials within the biological tissues or cells (Kumar et al. 2017). The utilization of in vivo systems as platforms for the assessment of risks associated with the engineered nanomaterials are important in understanding the complex interactions of the nanostructures with the biological macromolecules such as proteins and cells. The in vivo study on interactions between the engineered nanomaterials and biological macromolecules could provide the pathway of nanomaterials distribution in the human body, effect on metabolic activity and effective clearance from the body. This strategy has also provided a promising platform for the design and development of novel nanomaterials with widespread biomedical applications and without any toxicological effect on human health and environmental sustainability (Fischer and Chan 2007). The in vivo risk assessment of nanomaterials toxicity involves the use of different model systems starting from invertebrates such as nematodes and arthropods to vertebrates including zebrafish and murine models.

20.4 Model Systems for Deciphering Nanoparticles Toxicity

In the pursuit of deciding the in vivo model system for deciphering the toxicity assessment in human health and environmental sustainability, both invertebrate and vertebrate model systems are being considered based upon their respective advantages. Among the invertebrate model systems, *Caenorhabditis elegans* and *D. melanogaster* showed promising properties in determining the toxicity profile of different nanomaterials. Meanwhile, among the vertebrate models, the zebrafish and mammalian model showed promising properties in determining the toxicity profile of different nanomaterials (Fig. 20.3). Among the different in vivo model systems used to study the risks associated with nanoparticles toxicity, *C. elegans* has been considered as a promising model system. The typical features such as simplicity, ease of maintenance, small size, prolific life cycle, invariant developmental trajectory, conserved and well-annotated genome and ease in genetic manipulation render *C. elegans* as a convenient in vivo model organism to assess the toxicological impacts of therapeutic drugs as well as engineered nanomaterials (Gonzalez-Moragas et al. 2017; Hu et al. 2018).

In the pursuit of developing promising in vivo model systems for the toxicity assessment of engineered nanomaterials, zebrafish (*Danio rerio*) proved to be a promising model system. The use of zebrafish as model system for in vivo toxicity assessment was not only based upon its close homology with the human genome but also

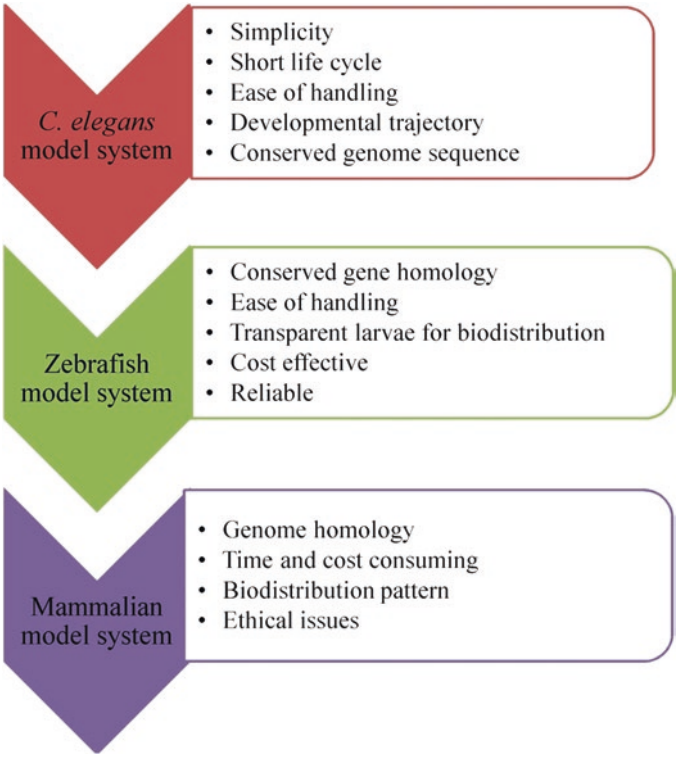

Fig. 20.3 Schematic representation of different in vivo model systems and their characteristic features

because it was found to be promising than the mammalian counterparts in terms of associated cost, reliability, biodistribution efficacy and time consumption. Apart from these aspects, the advent of zebrafish as model system also proved to be influential, being considered as promising and alternative model system (Fako and Furgeson 2009). Zebrafish could also be used for studies depicting the toxicological aspects of engineered nanomaterials for the purpose of environmental monitoring based upon the use of toxic heavy metals and organic pollutants for the synthesis purpose (Dai et al. 2014). Though the zebrafish was served as a promising in vivo model system to study the toxicological implications of engineered nanomaterials, determination of nanomaterials associated immunotoxicity in the zebrafish is still not well established. In addition, due to rapid developmental stages in zebrafish, it is very difficult to perform systematic embryo-based nanotoxicity assays (Chakraborty et al. 2016).

The use of mice and rat models is found to be an appropriate model system not only to study the physiological responses of administered drugs but also critically determine the toxicity profile of the drug candidates as well as drugs-embedded engineered nanomaterials. The advantage of using mammalian model in the risk assessment of nanomaterials is their explicit genome homology, with human beings suggesting a better

understanding of interaction of administered nanomaterials with the biological machinery and their subsequent distribution and clearance from the body (Yang et al. 2017). However, associated technological and ethical issues hinder the use of mammalian model systems.

20.4.1 *Drosophila melanogaster* as Model System to Study Nanotoxicity

Among the different in vivo model systems used for the assessment of risks associated with the use of different nanomaterials, the use of the fruit fly *D. melanogaster* as promising model system holds clinical importance. From the early part of the twentieth century, *D. melanogaster* has been regarded as a promising tool for geneticists, owing to its short and vital life cycle, large progeny, simpler genetics, less genetic redundancy and conserved genetic pathways. The popularity of *Drosophila* as an experimental organism ensures that its genome sequence will be a valuable resource for understanding the genetic programmes and drug discovery programmes with an aim to develop new medicines (Pasini et al. 2010). It is evident from earlier studies that the fruit fly *D. melanogaster* has been popularly being used as a genetic model system to understand the human diseases especially Parkinson's disease, endocrine neoplasia and serious metabolic disorders (Hughes et al. 2012).

20.5 *D. melanogaster* as a Promising Model System to Study Toxicity of Nanoparticles

20.5.1 Importance of *D. melanogaster* as Promising Model System

D. melanogaster has been used as a promising model system in understanding the molecular mechanisms associated with diseases and other metabolic disorders. One of the most important aspects of *D. melanogaster* as promising model system is the presence of highly conserved genetic pathways between the *D. melanogaster* and human being, thereby providing promising source to study different molecular mechanisms associated with human biological systems. Apart from the highly conserved genetic pathways, *D. melanogaster* also possesses several other important characteristics such as simpler genetics which allow the scientific community to understand the desired biological processes during the disease progression and hence could develop novel therapeutic strategies to counteract the disease-related health consequences. In addition to simpler genetics with overall less genetic redundancy, *D. melanogaster* could also be genetically modified and propagated as per the requirement during the development of novel therapeutics (Hughes et al. 2012). The intrinsic attributes such as low cost, rapid generation time, large progeny, ease of rearing and simple genetics tools significantly contribute to the candidature of *D. melanogaster* as promising model system for understanding the characteristic

features of developmental biology (Fig. 20.4) (Markow 2015; Tolwinski 2017). In recent times, *D. melanogaster* also exhibited promising aspects in understanding the biology underlying the tissue regeneration process. Understanding the mechanisms of regenerative biology using *D. melanogaster* model system will pave the way for the discovery of regenerative medicines (Jennings 2011). The typical features such as short life cycle, ease of rearing procedures, cost-effective experimentation, genetical homology and large group of progeny provided the strong candidature of fruit fly as a model organism. However, the most important aspect lies within its genes which are highly conserved for different genetic and signalling pathways despite being diverged from the vertebrate lineage approximately 700 million years ago.

20.5.2 Advantages of *D. melanogaster* as Promising Model System

The life cycle of *D. melanogaster* has four discrete stages such as embryonic, larval, pupa and adult. One of the most important characteristics of *D. melanogaster* as model organism is the involvement of each life cycle stages poses unique opportunities and platforms to assess the toxicity associated with drug candidates or engineered nanomaterials. Secondly, as compared to rodents or zebrafish model system, *D. melanogaster* exhibited less redundancy in the genome, thereby facilitating the rapid analysis of gene functions of particular interest. Another major advantage of

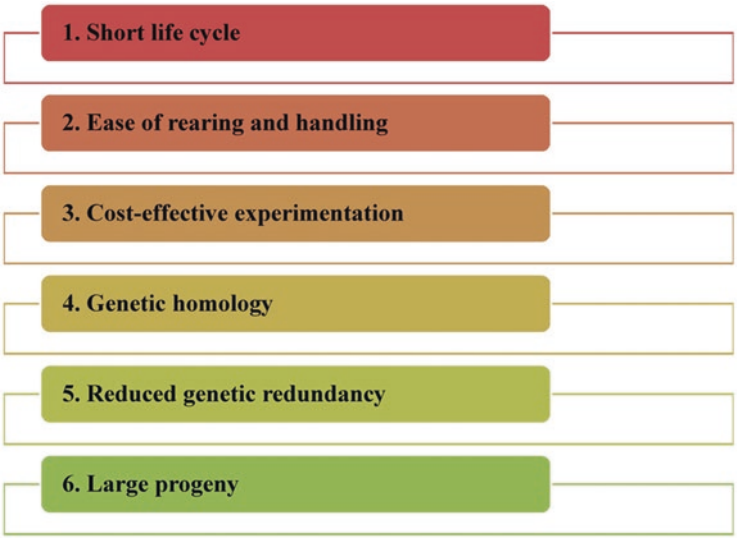

Fig. 20.4 Schematic overview of the different advantages of *Drosophila melanogaster* as prospective in vivo model system for studying reproductive biology, developmental biology, drug discovery and toxicology assessment of nanoparticles

D. melanogaster as model organism is that it can be manipulated experimentally much more readily as compared to their vertebrate counterparts due to both ethical and technical issues.

20.5.3 Applications of *D. melanogaster* as Promising Model System

Since the early phase of the twentieth century, *D. melanogaster* has proven to be a powerful model in understanding the human genetics and developmental biology and in studying various immunogenic responses (Table 20.1) (Buchon et al. 2014). Apart from genetics and developmental biology, *D. melanogaster* has also emerged as promising model system in understanding the nonpathogenic host-microbe interactions owing to its genetic and experimental traceability (Chandler et al. 2011). From the day of inception as promising model system, *D. melanogaster* is widely used as a model organism in understanding the genetics tools, mechanism of biochemical processes and patterns of cellular biology as well as understanding the pattern of developmental biology in particular the regenerative biology. Apart from these multitudinal aspects, from the last couple of years, *D. melanogaster* is also considered to be influential in determining the toxicological profile of therapeutic drugs before

Table 20.1 Schematic overview of the use of *Drosophila melanogaster* as promising in vivo model system to study disease progression, toxicity assessment and drug discovery

Sl no.	Experimental systems used	Target	Involvement in human disease	References
1.	*Drosophila* cell culture	Dengue virus	Dengue and haemorrhagic fever	Mukherjee and Hanley (2010)
2.	*Drosophila* cell culture (infected) Live *Drosophila*	West Nile virus	West Nile fever, encephalitis	Chotkowski et al. (2008), Brackney et al. (2010), Glaser and Meola (2010)
3.	Live *Drosophila*	*Mycobacterium abscessus*	Pulmonary infections	Oh et al. (2014)
4.	*D. melanogaster* (larvae, adult fly)	Epithelial cancer cell lines	Cancer progression and cell death	Mirzoyan et al. (2019)
5.	*D. melanogaster*	Insulin signalling pathways	Type I and Type II diabetes	Graham and Pick (2017)
6.	*D. melanogaster*	Neurotoxicity	Neurological disorder (Parkinson's disease)	Soares et al. (2017)
7.	*D. melanogaster* (larvae and adult)	Inflammatory responses	Immunological responses	Pandey and Nichols (2011)
8.	*D. melanogaster* embryo	Developmental toxicity	Developmental process (embryogenesis)	Rand et al. (2014)
9.	*D. melanogaster*	Nrf2 signalling pathway	Oxidative stress and ROS generation	da Cunha et al. (2015)
10.	*D. melanogaster*	Bacterial sensing	Bacterial sensing	Capo et al. (2016)

being administered in the fight against various human diseases. In addition, *D. melanogaster* also proved its candidature as promising tool in the assessment of toxicological profile of engineered nanomaterials (Abolaji et al. 2013). Based on the short life span and simpler genetics of *D. melanogaster*, chronic nanotoxicity studies could be carried out using specific tissues or organs of subsequent filial generations to examine the effects of different nanomaterials on the genome stability, development progression, reproduction ability and viability (Ong et al. 2015).

20.5.4 Deciphering the Nanoparticles Toxicity Using *D. melanogaster* Model System

The similarity between mode of nanoparticle response, behaviour and gene response in *D. melanogaster* and mammalian systems, combined with the power of *Drosophila* genetics, has recently made the candidature of *D. melanogaster* a very attractive model system to study nanoparticle toxicity. Despite the aforementioned advantages of the *D. melanogaster* model system across a wide spectrum of research fields including genetics, developmental biology, biochemistry and drug discovery, its use as a tool to assess the toxicological aspects of therapeutic drugs or engineered nanomaterials remain obscured. The reason behind it is the use of traditional model systems such as rodents or zebrafish as in vivo animal models in risk assessment and has almost overlooked the candidature of *D. melanogaster* model system in toxicity assessment.

Based upon the simpler genetics, short life cycle, ease of rearing procedures, close homology in genetic pathways and large progeny generation as shown by *D. melanogaster* model system, it is being considered for understanding the mechanism of immunological responses and developmental biology. In this context, it could be of promising platform in the assessment of toxicity associated with the engineered nanoparticles. The constant exposure of gold nanoparticles (AuNPs) in *D. melanogaster* resulted in severe genotoxic effects with altered phenotypic characteristics spread over subsequent generations of fruit flies. The phenotypic alterations associated with mutagenic effect of constant exposure to AuNPs suggested the hazardous effect of AuNPs not only to the current generation of flies but extended the toxic effect to the subsequent generation (Vecchio et al. 2012).

D. melanogaster has also been utilized in several studies aiming to demonstrate the severity of AgNP-induced adverse effects. The exposure of AgNPs to the *D. melanogaster* resulted in critical adverse effects such as alterations in the developmental prospects, mating success and survivorship. The adverse effect of AgNPs on *D. melanogaster* larvae is crucially dependent upon intrinsic factors such as administered dose of nanomaterials, size of the engineered nanoparticles and surface coating properties (Posgai et al. 2011; Alaraby et al. 2016). The size of AgNPs has a special influence on developmental cycles of *D. melanogaster*. The size of AgNPs has been inversely proportional to the toxicity of nanomaterials to the developmental programmes of *D. melanogaster*. It suggested that the smaller the size of AgNPs, the greater the toxicological profile of AgNPs on the developmental cycles of *D. melanogaster* (Gorth et al. 2011). The administration of engineered AgNPs into *D.*

melanogaster adult stage for short and long durations significantly affects the egg-laying capability with an impaired growth of ovary. The constant dietary intake of AgNPs in the larval stage also has profound impact on survival rate, longevity, size of the ovary for the egg production and egg-laying ability, suggesting their increased susceptibility towards the effect of AgNPs. One of the interesting aspects of AgNPs on *D. melanogaster* is the generation of the trans-generational effect, thereby suggesting its toxicological impact spread over generation to generation. The administered dose of AgNPs also significantly controlled the level of toxicity as at a higher dose, engineered AgNPs proved to be detrimental in reproductive health and survival of *D. melanogaster*. At a concentration of 20 mg/L, engineered AgNPs significantly affected the development of *D. melanogaster* by specifically targeting the developmental cycles and thereby influenced the toxic effect across several filial generations (Panacek et al. 2011; Raj et al. 2017).

Apart from metallic nanoparticles, metal oxide nanoparticles have also been regarded as promising for immense biomedical and pharmaceutical applications. Among the different metal oxide nanoparticles engineered, zinc oxide nanoparticles (ZnO NPs) are being highly considered for widespread applications. However, it is also evident that the use of ZnO NPs significantly contributed to toxicological impact on human health. From the in vivo study on the toxicological assessment of ZnO NPs on *D. melanogaster*, significant toxicity was observed in F1 progenies with a significant decrease in the egg-to-adult viability of *D. melanogaster*. The decreased viability on treatment with ZnO NPs is generally correlated with the activation of ROS generation by complementing the function of nuclear factor E2-related factor 2 (Nrf2) of *D. melanogaster* (Ng et al. 2017). *D. melanogaster* has been regarded as promising model system in deciphering the biological effects of metal oxide nanoparticles. In this regard, engineered cerium oxide nanoparticles (CeO_2 NPs) were evaluated for their prospective genotoxic effect on *D. melanogaster*. Though CeO_2 NPs were internalized through intestinal barrier with increased expression of heat shock protein, CeO_2 NPs do not have any genotoxic effect (Alaraby et al. 2015b). The engineered nanoparticles such as titanium dioxide (TiO_2 NPs), zirconium oxide (ZrO_2 NPs) and aluminium oxide (Al_2O_3 NPs) were screened for their prospective genotoxic potential using *D. melanogaster* model system. From the study, no genotoxic effect of the screened nanomaterials was observed. However, further studies are required to successfully establish the toxicological profile of the screened nanomaterials (Demir et al. 2013).

Owing to the unique physicochemical properties and electrical and mechanical characteristics, carbon-based nanomaterials like carbon nanotubes (CNTs) have gained considerable interest in diverse fields including electronics, optics, physics, material sciences, biomedicines and pharmaceutical sciences. Both single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs) are known for their widespread biological applications. However, the inadvertent use of CNTs also attracted widespread attention as it can lead to severe environmental concern as well as serious human health consequences. The irrational use of CNTs can cause cellular and tissue damage by stimulating inflammation and necrosis due to increased production of reactive oxygen species (ROS). Among the different CNTs, SWCNTs tend

to be more toxic as compared to MWCNTs. The toxicological profile of CNTs is also regulated by the shape, length and chemical modification (Liu et al. 2014). In recent years, the emergence of semiconductors quantum dots (QDs) has gained considerable attention owing to their interesting properties with widespread biomedical and pharmaceutical applications. However, the potential toxic effect of QDs remains a serious problem. The toxic properties of QDs depend on several parameters such as composition, size, surface coating, charge, period and route of exposure. In particular, CdSe QDs have two well-known toxic elements, cadmium and selenium, that can produce harmful effects to many cell types. In this context, *D. melanogaster* was considered for prospective in vivo model system to establish the toxic effect of CdSe/ZnS QDs (Alaraby et al. 2015a).

20.6 Current Trends and Future Perspectives

The advent of synthesized nanomaterials holds significant contribution in enhancing the quality of biomedical therapeutics with considerable aspects in biomedical imaging, biosensing, diagnostics and targeted delivery of therapeutic drug candidates. However, it is highly important to critically assess the biocompatibility, biodistribution and biodegradation pattern before being applied in a clinical setting or environmental exposure (Kunzmann et al. 2011). The emergence of toxicological aspects of nanotechnology has gained serious concern in the last few years, owing to its hazardous impact on human and animal health, environmental setup and aquatic organisms. The complexity of nanotoxicology significantly attributed in in vivo models as compared to in vitro models. However, the identification and strategies to counteract the toxicological impacts remain obscured and an uphill task expects for the scientific community to develop salient strategies. Though the route of entry of nanomaterials and their prospective pathways to the site of accumulation have been identified, it is imperative to work on understanding the current scenario and develop novel strategies to counteract the toxicological impacts on society and environment (Elsaesser and Howard 2012).

In the fight against toxicological impact of different nanomaterials, it is imperative to understand the synthesis route of nanomaterials, their species-specific physicochemical properties and their interactions with the biological systems. However, understanding the physicochemical properties that drive toxicological outcomes of nanomaterials remains a formidable challenge as considerable differences in the characteristic features, mechanism of action and types of biological interactions remain diversified for different nanomaterials. In this context, smart and species-specific surveillance strategies should be designed and developed with an aim to understand the mechanism of toxicity for the welfare of society and environment (Bhattacharya et al. 2013). From the onset of toxicological aspects of engineered nanomaterials way back in the early twenty-first century, the most important attribute is to design and develop systematic and regulatory guidelines for the synthesis of nanomaterials. The regulatory guidelines should be strictly followed starting from the synthesis to assess the physicochemical properties and most importantly

their prospective effect on cellular viability, health consequences and environmental sustainability. The regulatory bodies should determine the risk assessment associated with the engineered nanomaterials before being exposed to the society and environment for biomedical and pharmaceutical applications (Love et al. 2012).

20.7 Conclusion

From the early days of the twentieth century, *D. melanogaster* has been regarded as a powerful tool in genetic, behavioural and molecular biology research programmes. The unique features such as short life cycle, ease of rearing, ease of handling, cost-effective experimentation, genetic homology, genetic tractability and reduced genetic redundancy have provided the strong candidature of *D. melanogaster* as promising in vivo model system in drug discovery programmes as well as in the assessment of risks and health consequences of the irrational use of nanomaterials. Based on the highly conserved physiological and biological pathways in *D. melanogaster* as compared with the human beings, it has also been used in recent years in disease-oriented drug screenings for the treatment of various infectious and noninfectious diseases and disorders such as cancer, neurological disorders, endocrine disorders, renal disorders and cardiac diseases (Avanesian et al. 2009). Though the use of *D. melanogaster* model system exhibited promising results in understanding the toxicological profile of engineered nanomaterials, it is important to strictly follow stringent regulatory guidelines for the synthesis and applications of nanomaterials. Apart from that, new international surveillance systems should be strictly followed for efficient use of engineered nanomaterials for the well-being of the society and environment.

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Murine Model to Understand the Toxicity of Nanoparticles

21

Himani Meena and Busi Siddhardha

Abstract

Nanotechnology is a modern bloom in science and engineering arenas, i.e. biomedical, bioremediation, bioimaging, cosmetics, biofilm and mechanical engineering. Nanoparticles can cause direct or indirect threat in human life due to their biological activities in routine exposure through inhalation, minute particle penetration into dermal layer and ingestion. Till date, interaction with nanoparticles have been reported with toxicological profile involved in the cytotoxicity, respiratory system, immune system, cardiovascular system, hematic system, renal or kidney failure and lymphatic system. Examination of nanoparticle before releasing to routine applications for their toxicological properties is necessary to overcome the situation. Murine model should be used for better understanding of the mechanism involved in developmental toxicity, phenotypic abnormalities, and epigenetic potential. Nanotoxicological profile in vivo highlights the possible mechanism of nanoparticle toxicity based on their physical properties (size, shape, and surface modification) and chemical (cation or anion) and biological properties in different organs (hepatic, renal, pulmonary, haematological, cardiovascular, immune systems). Finally, scientists should focus on the biosafety and risk analysis to provide detailed information about nanoparticle usage to avoid the adverse effects.

Keywords

Murine model · Toxicological studies · Nanotoxicity · Immunomodulators

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21.1 Introduction

Nanotechnology has evolved as a new arena with advantages and can be used in various fields such as optical, biomedical, cosmetics, X-rays, bioremediation, coatings and nanostructure engineering. Nanoparticles are tiny particles with a 1–100 nm size that spreads easily in the human body as well as in the environment (Buzea et al. 2007). The physiochemical properties along with the biological function of nanoparticles points out the problem related to nanoparticle toxicity. *In vitro* studies mainly focuses on cellular damage, i.e. oxidative stress, reactive oxygen species (ROS) generation, cell proliferation, degranulation of cell cytoplasm, DNA damage and cell death (necrosis and apoptosis). *In vivo* evaluation of nanoparticle toxicity studies requires various parameters to establish toxicity profile such as biocompatibility, cellular metabolism, and metabolic clearance through the general body system (Fig. 21.1).

Nanoparticles have detrimental health effects on the human body, affecting tissues and organs based on their nature. Nanoparticles can penetrate the skin barrier and circulate within the blood system and reach to various body organs, resulting in cellular damage and organellar structure damage (Yah et al. 2011). Nanoparticles uptake via respiratory route compromise the primary immune line and causes delayed macrophage clearance, perturbation of cell proliferation in the lungs, and infiltration of inflammatory elements. If the nanoparticles remain in the system, it may induce protein denaturation and release of excessive pro-inflammatory cytokines that may act as self-destructive elements for own macrophages (Jeevanandam et al. 2018). Nanoparticles affect the cellular metabolism, interfering with early cell

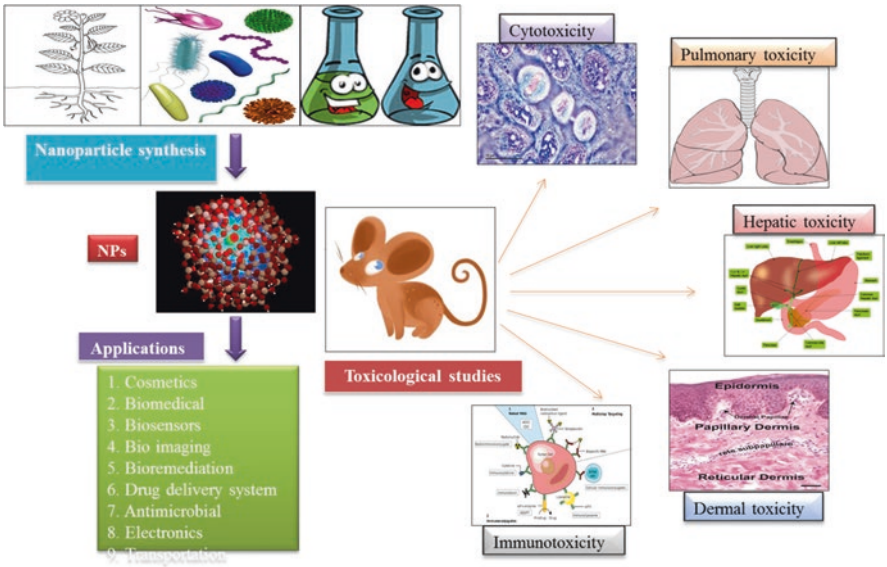

Fig. 21.1 Graphical representation of nanoparticle synthesis, their application in various fields and toxicological profile in murine model

apoptosis and obstinate cell proliferation. Ingestion of nanoparticle may lead to mitochondrial destruction, enlarged nephritic organs (spleen and lung), limited flow of bile liquid and kidney failure.

21.2 Significance of Murine Model to Understand Nanotoxicity

Nanotoxicity analysis using *in vitro* and *ex vivo* studies provides limited information about the toxicity profile of particular nanoparticles and narrow down the correct information. *In vivo*, murine model is designed to ensure the true interpretation to determine the safe dose for drugs, antibody or nanoparticles to reduce the risk of adverse effects (Adamcakova-Dodd et al. 2014; Yoshida et al. 2013; Hernandez-Adame et al. 2019). Murine model acts as replica for human anatomy and physiochemical parameters which helps scientists to understand the specificity of cellular metabolic mechanism, blood circulatory system, cardiac function, pulmonary system, hepatic system and immune response (Almeida et al. 2011). Murine model mimics human body system and are able to monitor changes upon treatment with nanoparticles which can be directly correlated with human physiochemical parameters. *In vivo* analysis involves two main mechanisms: primarily, morphological changes in organs system which includes tissue structural modification, programmed cell death and infiltration of pro-immunomodulatory elements and secondarily, participation of specific cells leading to bio-distribution of the nanoparticle through the system via hepatic sinusoid (liver) (Cengiz et al. 2015) and Kupffer cells (renal) (Jamshidzadeh et al. 2015) for toxicant clearance.

21.3 Physiochemical Properties Contribute to Nanoparticle Toxicity

21.3.1 Dose/Concentration/Exposure Time

Dose and exposure time refers to penetration of specific concentration of nanoparticles into the cells and their exposure which affects the toxicity profile of the nanoparticles (Fadeel and Garcia-Bennett 2010). Adamcakova-Dodd et al. (2014) examined efficacy of zinc-oxide nanoparticles (ZnONPs) to induce pulmonary toxicity in mice (C57Bl/6). Mice were exposed to ZnONPs for 13 weeks, with a cumulative dose of 10.9 mg/kg, and biochemical and histopathological changes were observed. At 2 weeks of time, subacute toxicity was observed with increased macrophages in BAL fluid and IL-12(p40) and MIP-1α after treatment with ZnONPs. Prolonged exposure to ZnONPs have enhanced the lung cellularity and variation in total protein amount, increased lactate dehydrogenase activity with pro-inflammatory cytokines in bronchoalveolar lavage (BAL) fluid. The study compared the changes occurred during 2 weeks to 13 weeks and proved ZnONPs dose and exposure time is responsible for severe subacute and subchronic toxicity. Another experiment on

female C57BL/6JBomTac (C57) mice by Jacobsen et al. (2015) suggested that ZnONPs toxicity occurs in a dose-dependent manner and can cause severe pulmonary dysfunction. Histopathological examination showed occurrence of slight vacuolization of hepatocyte cytoplasm with bronchiole epithelium desquamation at the concentration of 2 μg/ml ZnONPs. At a concentration of 6 μg/ml of ZnONPs, pulmonary system displayed congestion, oedema and enlargement of hepatocytes. At the highest concentration of 18 μg/ml ZnONPs, severe morphological changes were observed in the mice lung tissue including congestion, lymphocytes infiltration in circulatory system, bronchiole epithelium desquamation and enlargement of hepatocytes and necrosis of the single hepatocytes. In an inhalation study by Saptarshi et al. (2015) mice were exposed to 30 nm pristine and surfactant-dispersed ZnONPs with a concentration of 5 mg/kg. After 24 h of exposure, mice were euthanized; lungs tissues and blood samples were collected for further analysis. Lung tissue were stained with H&E staining and examined for morphological changes in the tissue sample. Mild neutrophilic inflammation, crucial pulmonary fibrosis and activation of macrophages were observed in the treated mice lung.

21.3.2 Particle Size/Shape/Surface Area

NPs display a size and shape-dependent toxicity which possess different toxicity profile levels at different aspect ratios (Sharifi et al. 2012). Spherical and rod-shaped titanium shows morphological changes in the lung tissues in murine models. This hypothesis was confirmed by Oosthuizen et al. (2012) using Balb/c mice. Lung tissues stained with toluidine blue dye demonstrated morphological changes and migration of inflammatory cells and fibrin molecule into the inter-alveolar space. Both spherical and rod shaped displayed infiltration of blood vessel in the tissue with difference in the feasibility of penetration into the cells.

Yoshida et al. (2013) exposed mice via intranasal exposure nanosilica particles (nSP30, nSP70 or nSP100) and conventional microscale silica particles (mSP300 or mSP1000) with diameter of their assigned numerical at 500 μg/mouse for 7 days. Throughout the histological examination of liver tissue, inflammatory responses with minor pathological abnormalities were found. Compared to large-diameter nanoparticles (nSP300 and nSP1000), small nanoparticles displayed drastic change in the platelet count in coagulation analysis. These nanoparticles showed activation of partial thromboplastin leading to initialization of coagulation cascade (by activating coagulation factor XII) with further recruitment of inflammatory cytokines and interferons.

21.3.3 Surface Functionalization/Coating/Crystal Structure

The toxicity profile of nanoparticle depends on surface properties which drastically affects movement of nanoparticle within the host system in in vivo model and crucial oxidation processes. Surface coating is required to change the surface properties of nanoparticles to enhance the nanoparticle shifting and magnify their

biological functions too. TiO_2 occurs in three different morphological forms known as rutile, anatase, and brookite. Experiment conducted by Rossi et al. (2010) using silica-coated TiO_2 further examined for pulmonary toxicity in Balb/C mice. The report concluded that silica-coated TiO_2 nanoparticle specifically affected the pulmonary system and have adverse effect on the immune-modulatory profile. It induces production of pro-inflammatory molecules and congestion in lung tissue with the production of cytokines and interferons.

21.4 Murine Model: Nanoparticle Toxicity Studies

Even a huge demand of nanoparticles or nanomaterial exists in various fields, i.e. chemistry field, food industry, bioremediation, biosensor, drug delivery and medical imaging; they possess limitations due to the toxicity to human as well as to the environment. Reactive oxygen species (ROS) generation is one of the main processes reported for the toxicity in human cell lines and assessed further to investigate the toxicity profile. It has been reported that tiny nanoparticle penetrates the cell membrane and manipulates the cell system which leads to changes in mitochondrial respiration process, rapid ROS generation and enzyme imbalance in NADPH-dependent system. The book chapter is a detailed report on the evaluation of nanoparticles toxicity in murine model such as cytotoxicity, hepatotoxicity, nephrotoxicity, inhalation toxicity, dermal toxicity, immunotoxicity, gene expression (genotoxicity), organ response, cardiotoxicity and interactive toxicity (Kumar et al. 2017).

21.4.1 Cytotoxicity

Han et al. (2016) investigated cytotoxicity of synthesized ZnONPs *in vitro* and *in vivo* using Leydig cells (LC) and Sertoli cells (SC) and CD1 mice, respectively. ZnONPs internalized into LC and SC and showed typical symptoms of toxicity on the cellular metabolism of the cells such loss of membrane integrity, DNA damage (nuclear and mitochondrial), loosen-up mitochondrial membrane, elevated level of ROS generation and apoptosis in a dose-dependent manner ranging from 5 to 20 μg/ml concentration. In vivo study was conducted on the testes of CD1 male mice with a single dose, and it displayed toxicity on the sperm cells. Changes in sperm morphology and function were recorded based on the observation, i.e. increased tissue oxidative stress, changes in testicular enzyme activities and suppression of spermatogenesis. Structural transformation in thickness of seminiferous epithelium and diameter of the seminiferous tubules were observed with abnormalities in sperm morphology. Surface modification of synthesized nanoparticles may enhance the biological properties, i.e. biocompatibility and their effects on the host system. Hernandez-Adame et al. (2019) biologically synthesized gold nanoparticles (AuNPs) using β-D-glucans isolated from the yeast *Yarrowia lipolytica* D1. β-D-glucans are used as reducing agent and help stabilize the nanoparticle structure in the host system. This study evaluated the cytotoxic effects on mouse spleen leukocytes and

reported that the AuNPs or β-D-glucans alone did not affect leukocytes but the AuNPs-β-D-glucans complex does. They found that AuNPs-β-D-glucans complexes with the size of 10–50 nM have increased oxidative stress by stimulating the hydrogen peroxide production in mouse splenocytes. Increased production of pro-inflammatory cytokines and nitric oxide was noted on exposure to AuNPs-β-D-glucan and supported the incompatibility of synthesized AuNPs in biomedical field due to cytotoxicity. Nabeshi et al. (2011) investigated the role of surface modification of sliver nanoparticle and its effect on murine macrophage cell line (RAW264.7). In this study, silver nanoparticles (nSP70) modified with amine (N) group, nSP70-N and carboxyl (C) group, nSP70-C were prepared and conducted cytotoxicity study to monitor the cellular changes at different concentration. At lower concentration of 121.5 μg/ml, nSP70 showed higher cytotoxicity compared to the modified nanoparticles, nSP70-N and nSP70-C at the concentration of 1000 μg/ml. Unmodified nSP70 showed higher cellular damage, i.e. inhibition of DNA synthesis, liposomal destabilization, apoptosis and irregularities in mitochondrial membrane. This report suggested the surface modification of silver nanoparticles may enhance the nanoparticles stability and minimizes the toxic effects.

21.4.2 Hepatotoxicity

The liver is a vital body organ that helps in clearance of toxic substance from the body and detoxifies the system for proper function. Hepatic sinusoid with Kupffer cells plays an important role in metabolism and changes in cellular metabolism can be used as marker due to sensitivity of liver cells towards toxic substances (Cengiz et al. 2015). Hepatotoxicity is a phenomenon coordinates with liver damage which can be detected by using immunohistochemistry and serum enzymology analysis. Liver inflammation and tissue damage are common symptoms for hepato-toxicity in a rodent model. Enzymes present in the serum such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and C-glutamyl transferase (C-GT) can be used as markers for liver dysfunction in the murine model (Yang et al. 2017). Heydrnejad et al. (2015) assessed the localization of silver nanoparticle (Au-NP) in male and female mice (*Mus musculus*) and examined the toxicity using biochemical tests and histopathological parameters. In the study, the biochemical report does not show any significant changes in the complete blood count, i.e. red blood cells count, haematocrit and white blood cell count. An elevated level of biomarkers in serum, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) supported the toxicity of Ag-NPs. Histopathological report displayed morphological changes such as hepatic cells granulation, granular degeneration and inflammation in hepatic sinusoids with hepatocyte necrosis in Au-NPs-treated mice compared to the healthy mice (control) irrespective of their gender.

The endoplasmic reticulum (ER) is an important cellular organelle unit that regulates protein folding, protein assembly, post-translational modification, micronutrient trafficking in vesicles and calcium channel throughout the body organ. Hepatocytes possess ample number of ER responsible for excretion of toxic elements during detoxification process. Dysfunctional ER due to elevated ROS generation and

oxidative stress can cause cellular imbalance and leads to death signals in liver hepatic cell with acute liver damage. Yang et al. (2015) reported liver damage in male C57BL/6 mice after exposure to zinc-oxide nanoparticles for a period of 90 days. Hepatocytes necrosis, liver tissue damage with escalated serum of ALT and AST, was found in the exposed mice with elevated levels of ER stress-associated genes (*grp78*, *grp94*, *xbp-1*, *pdi-3*) and ER stress-related protein (p-PERK, PERK, p-eIF2α, eIF2α) compared to the control. Electron microscope displayed critical sign of liver tissue damage with ER swelling and ribosomal degranulation. Chen et al. (2018) reported hepatotoxicity in mice upon exposure to rare-earth nanoparticles (RENP) for 7 days. RENP found to be localized into liver tissue and causes hepatic cell necrosis and inflammation with increased production of ALT (64.20 ± 15.50 U/l), AST and creatinine (27.80 ± 3.56 μmol/l). Patlolla et al. (2019) conducted a study to examine poly-ethylene-glycol-coated (PEG-coated) and uncoated gold nanoparticle (uncoated GNPs)-induced hepatotoxicity and oxidative stress in Sprague-Dawley rats. Both PEG-coated and uncoated GNPs showed liver injury due to oxidative stress, and excessive production of biomarkers ALT, AST and ALP was observed. PEG-coated displayed less toxicity than the uncoated GNPs which suggest PEGylation of nanoparticle to reduce the toxicity and to increase biocompatibility.

21.4.3 Nephrotoxicity

Nephrotoxicity refers to renal or kidney failure caused by foreign material or toxic substance confirmed by the histopathological changes in renal morphology such as renal glomerulus degeneration. Glomerulosclerosis and collagenous tubulointerstitial matrix indicate pathological changes in renal function which can be determined via biomarkers, i.e. transforming growth factor-β1, interferon-6, type I collagen, fibronectin and vimentin, quantitatively (Yang et al. 2017). Hong et al. (2015) introduced nano-TiO_2 via intragastric route to mice and evaluated the biomarker secretion level in the urine (kidney injury molecule-1, clusterin, osteopontin, β2-microglobulin and cystatin) as well as in the blood serum (uric acid, blood urea nitrogen, creatinine and urinary protein). The report suggests the nano-TiO_2 have increased the production of urinary and blood biomarker level and also showed the renal fibrosis and swelling in the renal tubules in microscopic observation. Jamshidzadeh et al. (2015) introduced male Swiss albino mice with gentamicin and gentamicin nanoparticles and recorded functional and morphological changes. Aminoglycoside antibiotic has adverse effect on the host body when used for prolonged time duration. An increased production of blood urine nitrogen and creatinine with obstructed kidney structure in microscopic analysis was observed.

21.4.4 Dermal Toxicity

Nanoparticles can penetrate the skin and cause dermal toxicity via biochemical parameters production of cytokines, anti-oxidant enzymes, catalase (CAT),

superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and protein involved in cellular metabolism such as lipid peroxidation, lactate dehydrogenase (LDH) activity and malondialdehyde (MDH). Alteration in dermal structure can be refined as dermal toxicity causing accumulation of neutrophils at dermis and epidermis layers of the skin, reflecting well-organized cutaneous architecture upon exposure to the nanoparticle. Unnithan et al. (2011) performed an experiment on Wistar rats and tested toxicity profile of TiO_2 nanoparticles in a dose-dependent manner. The toxicity profile was evaluated based on histopathological examination supported by biochemical analysis. A significant decrease in the production of serum enzymes, CAT, SOD and GST was observed in the TiO_2-treated rats compared to the control rats. An elevated level of LDH activity and MDH formation was noticed in the experimental group showing the changes in the dermal layer microenvironment. Morphological changes in the dermis, epidermis and cutaneous layers were remarked with bulk movement of neutrophils to the epidermis layer and skin inflammation. In another study, Murray et al. (2013) studied the effect of superparamagnetic iron oxide nanoparticles on human epidermal keratinocytes (HEK) and murine epidermal cells (JB6 P^+). NF-*k*B is a redox-sensitive transcription factor that controls production of inflammatory cytokines production during cell damage and can be used as standard biomarker. JB6 P^+ cells post exposed with UVB treatment showed cellular damage; oxidative stress with increased production of inflammatory cytokines IL-6, MCP-1, IFN-c, TNF-a, and IL-12; and decrease in the LDH activity.

21.4.5 Immunotoxicity

Immunotoxicity refers to functional and morphological changes in the level of immune response determinants (cytokines and transcriptional regulators), immune organ system (spleen, liver and lungs) and circulatory system. Wang et al. (2016) examined the toxicity of synthesized CdSe/ZnS quantum dots (QDs) in macrophages, lymphocytes and Balb/C mice. Macrophages treated with CdSe/ZnS quantum dots showed an escalated level of ROS generation and augmented cell apoptosis. Macrophages showed decreased cell viability, reduced phagocytic activity towards nanoparticle and diminished TNF-α and IL-6 production. Lymphocytes exposed to nanoparticles displayed aggravated cell viability with increased production of TNF-α and IL-6. In Balb/C mice, decreased cell viability, reduced production of CD3ε+-T lymphocytes and escalated release of CD19+-B lymphocytes and immune response molecules, IL-6 and TNF-α was observed. Mishra et al. (2017) administered bismuth selenide (Bi_2Se_3) nanoparticles in intratracheal space in mice and noted their effects on systemic and circulatory system. Nanoparticles showed partial effects on both systems; it has elevated the production of ROS elements that promotes apoptotic cell death and release of pro-inflammatory cytokines. Increased levels of immune response elements, IL-1β, MIP-2, IL-6 and IL-8 enhanced the chances of neutrophils infiltration and oxidative stress that defines immunotoxicity caused by Bi_2Se_3. Hong et al. (2016) challenged mouse testis with TiO_2 and kept under observation to examine the activation or inhibition of TAM/TLR-mediated

signal pathway in mouse. The study puts light on adverse effect of TiO_2 that includes migration of pro-inflammatory cell into circulatory system, cell death system in spermatocytes and Sertoli cells of male mice.

21.4.6 Cardiovascular Toxicity

Misconduct of medicines and adverse effect of heavy metals can cause cardiac toxicity that is basically electrophysiological dysfunction in the heart. Electrophysiological dysfunction comprises of myocardial necrosis, venous thrombosis, cardiac apoptosis and damaged haemostatic system supporting cardiac failure. Nanoparticle exposure to the murine causes escalation in serum-dominating factors, troponin T, myoglobin, TNF-α, IL-6, NO, IgG, VEGF, serum glucose and calcium and C-reactive protein and reactive oxygen species (ROS) generation with extreme effects on cellular metabolism of cardiac cell. Cardiac dysfunction leads to blockage of calcium channel, truncated cell length and alteration in mitochondrial membrane structure (Sangomla et al. 2018). Du et al. (2019) installed silica nanoparticles in tracheal space of male Wistar rats to evaluate the adverse effect on the cardiac function and myocardial tissue. In this study, they concluded that the application of silica nanoparticles on Wistar rats have increased the chances of cardiac myocytes apoptosis via the mitochondrial pathway. Elevated level of Bax, Bcl-2 and caspase-3 protein expression in that cardiomyocytes was observed. Physiological changes were noticed in the cardiomyocytes in the treated rats, and electron microscopic observation showed enlarged intercellular space, fragmented cardiac muscle fibres and dilation of cardiomyocytes membrane.

21.5 Conclusion

The development of nanotechnology has an impact on human daily life with wide applications in biomedical field, i.e. drug delivery system, bio-imaging, luminescent biomarker, cosmetics and engineering. With increased use of nanomaterials, humans have faced typical health problem. Nanomaterials should be monitored with systematically biosafety valuation to reduce risk of nanotoxicity in humans. Evaluation of nanoparticle toxicity using an in vitro approach is an easy method at lab scale and an in vivo approach provides detailed information about nanoparticle localization and responsive nature of a particular organ system to monitor changes in specific organs physiology, biochemistry and morphology. This chapter delivers comprehensive information about nanoparticle and their effects on body system (heart, kidney, liver, blood cells and inflammatory response elements) in murine model. Murine model reflects human body systems which provide advantages to the researcher for validation of their scientific work.

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Challenges and Future Perspectives of Nanotoxicology

22

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Abstract

Nanotoxicology is a branch of toxicology that is related to potential effects of nanoparticles of diameter less than 100 nm. Due to relatively small size, they are reported to enter through biological tissue barriers and cellular membranes leading to toxic effects. Release of nanoparticles on the target surface also induces high level of toxicity in target cells. The nanoparticles are usually cationic and

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are easily attracted to the anionic biological membrane, resulting in the destruction of the membrane and interaction with proteins, DNA, and enzymes of the host cell. The carcinogenicity of some multiwall carbon nanotubes and nanoparticles are also reported in recent researches. Various concerns about the usage of nanoparticles including systemic translocation, direct effects on the central nervous system, intestinal tract involvement, biocompatibility, deposition, and clearing are reported till date. In this book chapter, we will review the potent role of nanomaterials to confer their toxicity at cellular and subcellular levels. Efforts have been made to summarize the new aspects of interactions with other toxicants either by reducing or enhancing health risks and the potent negative effects associated with nanomaterial pollution.

Keywords
Nanotoxicology · Nanoparticles · Toxicants · Target cells

22.1 Introduction

Increasing demand for high-quality water fit for consumption calls for effective strategies to treat wastewater (Rajasulochana and Preethy 2016). The growing use of pesticides and heavy metals pollutes the water bodies (Ayangbenro and Babalola 2017). The use of nanoparticles can help to solve this problem and would address the consequences of pesticides and heavy metals present in water (Cicek and Nadaroglu 2015). However, despite the progress made, use of these emerging sustainable technologies has been limited, largely due to limitation of the material's properties, including cost (Lim 2017).

Nanoparticles possess useful characteristics such as direct band gap, high optical absorption coefficient, layered structure, and tunable band edges for optimized catalysis (Khan et al. 2017). Conversion of single-component nanomaterials to hybrid materials such as nanocomposites involves integration of synergistically different components in a controlled fashion (Camargo et al. 2009). Hybrid nanostructures have many advantages over single component nanomaterials such as multi-functionality, highly efficient charge separation at the interface and tunable band gap (Li et al. 2016). The use of nanoparticles for photocatalytic degradation will result in appreciable reduction in the pesticide amount in the water (Das et al. 2017). The combination of nanoparticles with bio-adsorbents to form nanocomposites is expected to show improved performance in terms of high efficiency of photo-induced charge separation and photostability (Hasija et al. 2019). The surface modification of the nanoparticles will facilitate the interaction of heavy metal ions with the particle's surface and hence would result in better adsorption and improved performance of the photocatalyst (Upadhyay et al. 2014). Use of hybrid nanostructures is also expected to be advantageous over the single-component and pure systems. Better performance in terms of material stability, efficiency, and cost is expected over the existing systems (Sanchez et al. 2011). Recent advancement in

nanotechnology industry has shown remarkable revolution over the last few decades, which progressively and hopefully will continue in future. Nanotechnology has shown significant contribution for the future of health science and medicine care (Fakruddin et al. 2012). In gene delivery, immunotherapy, and drug delivery systems, the ideal nanomaterials can achieve biocompatibility, high payload, low immunogenicity, efficient penetration and selective targeting to get timely arrival at tissues of interest (Singh and Lillard Jr 2009). Regular exponential growth in nanotechnology has led to consider new challenges to manage, predict, and understand the potential negative health effects followed by exposure (Setyawati et al. 2015). Different nanomaterials of different surface topographies, sizes, and compositions and various other properties need to be scrutinized to build the safety and efficacy for their use in human population (Jeevanandam et al. 2018). Nanotoxicology basically deals with the toxic nature of nanoparticles and elucidating their toxic effect on living systems (Taghavi et al. 2013). Most of the inert element becomes more active at nanoscale dimensions. Most of the nanoparticles are benign, and they may distribute throughout the body causing inflammation, oxidative stress, and other serious adverse effects (Buzea et al. 2007). High doses of nanoparticles represent realistic exposure and should be interpreted with caution which might result in toxico-kinetics and exposure assessment (Laux et al. 2018).

Multiwall carbon nanoparticles are discovered to cause asbestos-related serious health effects which prompted nanotoxicologists to cautiously check the release of nanoparticles at drug delivery sites (Yildirimer et al. 2011). Adverse effects of nanoparticles are evidenced in epidemiological, in vitro, and in vivo studies. However, data related to low dose exposures and chronic abnormalities still need to be explored (Gwinn and Vallyathan 2006). In most of the cases, these emerging engineered nanoparticles are directly linked to adverse health risks. New areas in toxicology includes the binding of nanoparticles with other contaminants either by reducing or enhancing various health issues and various adverse environmental effects related to nanomaterials pollution (Gupta and Xie 2018).

The purpose of this book chapter was to review the potential harmful effects of nanoparticles on the immune system with new approaches in nano-science. Efforts also have been made to scale up various biomarkers to monitor toxicity of nanoparticles at cell system.

22.2 Properties and Application of Nanomaterials

Nanomaterials exhibit various properties such as electronic, chemical, magnetic, optical, physical, thermal, and elastic properties. The nanoparticles find their application in a variety of fields such as medical field for drug delivery in vivo and in vitro, agriculture, and treatment of wastewater (Singh et al. 2019a, b; Kumar et al. 2019a, b), pesticide degradation (Singh et al. 2019c; Bhati et al. 2019; Kapoor et al. 2019), solar sensitizers, nanosensors, and photocatalysis because of their small size and physicochemical properties (size, shape, surface area, phase, and composition) (Sidhu et al. 2019; Kumar et al. 2019c).

Nanomaterials found their vast applications in different fields such as nanoscale carriers, nano-herbicides, nano-fertilizers, nano-pesticides, nanosensors, veterinary care, etc. (Kumar and Singh 2018a, b). Murphy (2008) and Tarafdar (2015) developed clay nanotubes (Halloysite) to reduce the concentration of pesticides by more than 70%, hence reducing its effectiveness impact on water streams. Panyam and Labhasetwar (2003) developed poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles for localized/targeted delivery of different agents including peptides, plasmid DNA, and proteins.

Recently, a titanium-based nanomaterial was found to have numerous applications such as in water splitting and degradation of organic compounds and as solar sensitizers. Titanium dioxide (TiO_2) has various features such as polymorphs, low cost, good stability, environmentally friendly, and having good optical and electronic properties. Li et al. (2018) investigated that core–shell-structured TiO_2 composites show tunable optical and electrical properties, even new functions, which are originated from the unique core–shell structures. The small size of Fe_2O_3 nanoparticles, changes their magnetic properties from paramagnetic to ferromagnetic and superparamagnetic and are used as contrast agents in intravenously injectable *T*2 MRI (Lee et al. 2014). The effective photocatalyst derived from TiO_2 nanoparticles are also reported to enhance photocatalytic degradation of triazine pesticides such as atrazine (Yola et al. 2014). Chitosan-based zinc oxide nanoparticles (CZNP) are spherical in shape and are used in the treatment of cervical cancer cells (Wu and Zhang 2018). Gold nanoparticles (GNPs) along with TiO_2 nanoparticles are used for fabricating conformal nanocomposite (NC) films of TiO_2–Au (Chander et al. 2014). Yuan et al. (2010) investigated the synthesis of ZnO quantum dots (QDs) combined with chitosan (*N*-acetylglucosamine) for its effectiveness against tumor-targeted drug delivery. It was observed that stability of the ZnO quantum dots is dependent on chitosan due to its cationic charge and hydrophilicity. Qiu et al. (2014) have developed a composite having core shell structure of ZnO interlayer and magnetic Fe_3O_4 core. Based on its properties, it has been shown to be effective against targeted delivery of anticancer drugs.

22.3 Hazardous Effect of Nanomaterials

The nanomaterials have a small size, i.e., few nanometers, and possess high reactivity to interact with organisms. They pose potential human health and environmental hazards when released directly into the environment and gets interacted with water, air, and soil (Elsaesser and Howard 2012). When the dust and air pollution consist of ultrafine particles of size <100 nm, it indicates possible long-term hazardous effects of man-made nanoparticles on humans. They can enter via oral, pulmonary (lungs), nasal, intraocular, and various other routes. Nanomaterials are found in aquatic and terrestrial environments by runoff and eventually reach into the food chain and accumulate in the body and other metabolic pathways. They are somehow toxic to various species including invertebrates, algae, bacteria, crustaceans, nematodes, mammals, fishes, rats, etc. (Landa et al. 2012; Exbrayat et al. 2015). Warheit et al. (2008) assessed the hazardous effects of several fine or nanoparticle types such

as carbonyl iron, amorphous silica, crystalline silica, and nano zinc oxide in rats. They observed that silica nanoparticles sustain cytotoxic and inflammation effects.

Karimi et al. (2018) used colloidal nanoparticles of fumed silica (f-SiO_2), silica (c-SiO_2), alumina (Al_2O_3), and ceria (CeO_2) as corrode in chemical and mechanical planarization (CMP) processes. The CMP slurries of CeO_2 and Al_2O_3 reduced reproduction in *Daphnia magna* upon chronic exposure which have negative consequences to water bodies. Jeng & Swanson (2006) investigated the effect of metal oxide nanoparticles ZnO, Al_2O_3, Fe_3O_4, TiO_2, and CrO_3 on apoptosis, cellular morphology, membrane leakage of lactate dehydrogenase (LDH mitochondrial function), and permeability of the plasma membrane, out of which ZnO nanoparticles were highly toxic, Al_2O_3 nanoparticles were moderately toxic, and TiO_2 and Fe_3O_4 exhibited low toxicity. It also results in the decreased mitochondrial function in the cells treated with ZnO nanoparticles ranging from 50 to 100 μg/mL.

Ghodake et al. (2011) reported the phytotoxicity of zinc and cobalt oxide NPs by *Allium cepa* test using onion bulbs as an indicator organism to check their effects on cell morphology, root elongation, adsorption potential, and root morphology of a plant. Zinc oxide NPs accumulate in the chromosomal and cellular modules, thus causing phytotoxic damage. Landa et al. (2012) studied the effect of titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles using microarrays on gene expression in roots of *Arabidopsis thaliana*. ZnO nanoparticles elicit stress response in phenotype and gene expression of *A. thaliana*.

22.4 Effects of Nano-based Products on the Immune System

Any alteration in the properties of nanoparticles transforms them either to a valuable or hazardous product (Jeevanandam et al. 2018). The deposition of nanoparticles in the human system acts as a foreign material that led to the emergence of a new branch, i.e., nanotoxicology (Suh et al. 2009). This field aims to cross verify the negative and harmful effects of nanoparticles on the environment as well as on human health (Table 22.1) (Singh 2009). This will aid in understanding how these nanoparticles cross the different barriers and enter into the blood system as well as interact with other tissues. Moreover, it will provide an insight into how the aggregation of these nanoparticles affects the normal functioning of the organ and induce ailments like fibrosis, inflammation, etc. (Barua and Mitragotri 2014). Nanoparticles induce biological toxicity by various possible routes in the human body via endocytosis and penetration into cell membrane and through the cell membrane channel (Manke et al. 2013). Most of the nanoparticles produces oxygen radicals and induces apoptosis and mitochondrial perturbation followed by toxicity (Behzadi et al. 2017). Nanoparticles react with biological fluids and body proteins and results in the generation of oxidative stress (Dayem et al. 2017). Nanoparticles such as silver NPs (AgNPs), titanium dioxide (TiO_2), NPs, and gold NPs (AuNPs) result in various immune-related disorders in mononuclear phagocytic system cells of the spleen and liver (Giannakou et al. 2016). Most of the immune cells such as macrophages, dendritic cells, leukocytes, platelets, monocytes, etc. recognize and uptake nanoparticles

Table 22.1 Immunotoxic effects of various nanoparticles in vitro and in vivo testing

S. No.	Nanomaterials	Size	Adverse side effects	References
1.	C60 fullerene	0.7 nm (diameter)	No effects	Fujita et al. (2009)
2.	Carbon black	<100 nm	Exaggeration of arthrosclerosis and induction of C-reactive proteins MCP-1, IL-6, and CCL2	Niwa et al. (2008)
3.	Carbon black	14 nm	Induction of MHC class II and CD80 expression Significant expression of DEC205 and CD86	Koike et al. (2008)
4.	Carbon black	14 nm	ROS production	Kroll et al. (2011)
5.	Citrate-stabilized AuNPs	10 nm	Induction of NF-κB-regulated luciferase reporter	Sharma et al. (2013)
6.	Fe_2O_3		Induction of TH0 cytokine (IL-2), pro-inflammatory cytokines (IL-6, TNF-α, IL-1), TH1-type cytokine TGF-α (IL-12), and IgE and TH2-type cytokines (IL-4, IL-5)	Park et al. (2010a)
7.	Fe_2O_3		Cell viability decreases and ferritin expression increases IL-1α expression and lactate dehydrogenase activity	Zhong et al. (2010)
8.	Gold	13 nm	Inflammation in the liver, induction of apoptosis, and nanoparticles localization in Kupffer cells of liver and macrophages in spleen	Cho et al. (2009)
9.	Gold	2, 40 nm	Internalization by primary hippocampal neurons and microglial cells and upregulation of TLR-2, olfactory bulb, and IL-1α	Hutter et al. (2010)
10.	Gold	0.8–15 nm	Oxidative stress induction	Brandenberger et al. (2010)
11.	Latex nanomaterial	25, 50, and 100 nm	Induction of fibrinogen	Inoue et al. (2009)
12.	Multiwalled carbon nanotubes	10–30 nm (diameter) 30–50 (length)	Induction of fibrosis	Ryman-Rasmussen et al. (2009)
13.	Multiwalled carbon nanotubes	20–40 nm (diameter) 5–30 μm (length)	ROS generation, induction of inflammatory cytokines, and activation of NF-κB in BEAS-2B or A549 cells	Ye et al. (2009)
14.	Nonporous silica nanoparticles	15 nm	ROS production in rats	Chen et al. (2013)

(continued)

Table 22.1 (continued)

S. No.	Nanomaterials	Size	Adverse side effects	References
15.	Polystyrene	60 nm	Highly toxic to human endothelial cells, BEAS-2B cells, macrophages hepatoma cells, and microvascular endothelial cells	Xia et al. (2008a)
16.	Polystyrene	20, 500, and 1000 nm	Migration of dendritic cells	Manolova et al. (2008)
17.	Silica	70, 300, and 1000 nm	Induction of inflammatory cytokines and liver damage	Nishimori et al. (2009)
18.	Silica particles	12 nm	Induction in mRNA expressions of COX-2, IL-1, iNOS, TNF-α, and IL-6	Li et al. (2009)
19.	Silicon	–	No changes or effects in HaCaT keratinocytes	Park et al. (2010b)
20.	Single-walled carbon nanotubes	1–4 nm (diameter)	ROS meditation via neutrophil myeloperoxidase in humans	Kagan et al. (2010)
21.	Single-walled carbon nanotubes	1–2 nm (diameter) 20 nm–several μm (length)	ROS generation, induction of inflammatory cytokines, apoptosis-related genesis macrophages	Chou et al. (2008)
22.	Single-walled carbon nanotubes	800 nm length	Inhibits production of MCP-1, TNF-α, and IL-8, 6	Herzog et al. (2009)
23.	Single-walled carbon nanotubes	50–200 nm (length) 1–5 nm (diameter)	Accumulation of SWNT in the kidney and liver for several months	Schipper et al. (2008)
24.	TiO_2	0.02–0.03 μm	ROS induction	Müller et al. (2010)
25.	TiO_2	4–6 nm	Lung inflammation, systemic inflammation cardiac edema, and induction of monocytes	Nemmar et al. (2008)
26.	TiO_2	20 nm	–	Geiser et al. (2008)
27.	TiO_2	15, 50, and 100 nm	Release of histamine	Yanagisawa et al. (2009)
28.	TiO_2	Less than 100 nm	Necrosis apoptosis in macrophage cells	Morishige et al. (2010)
29.	TiO_2	7–10 nm	Inflammatory responses via IL-1beta pathway ROS, inflammasome, etc.	Schanen et al. (2013)
30.	Zinc oxide, cerium oxide	11 nm, 8 nm	Oxidative stress induction	Xia et al. (2008b)

when they are in the tissue or in circulation process (Lameijer et al. 2013). Immune cells uptake nanoparticles from the bloodstream by adsorption process through opsonization. They remain in the body for a long term and cause various exposures. They also enhance intense manifestations that cause several disorders such as activation of complement system and acute inflammation (Look et al. 2010). It also has adverse effects on innate and specific immune responses. Acute inflammation is induced by activation of NF-κB pathway which results in enhanced production of chemokines and cytokines (Liu et al. 2017). Innate immune system results in the generation of ROS after exposure to metal oxide particles. Further, ROS lead to alterations in DNA and proteins which further causes inflammatory damage (Fu et al. 2014).

Gold nanoparticles are reported to induce various immunomodulatory effects by secreting inflammatory cytokines (IL-8 and TNFα) which activate NF-κB pathway when THP1 cells were exposed to AuNPs coated with negatively charged poly(acrylic acid) (Deng et al. 2011). In a similar study, Sharma et al. (2013) also confirmed that when B-lymphocytes were exposed to AuNPs stabilized with citrate, it induces NF-κB pathway and structural changes in cellular function of cells are registered. Another example of immunomodulatory effects by single and multiwall carbon nanotubes on various cell types was also reported in which they induce unregulated antigen-presenting cell maturation (He et al. 2013). CNT is also testified to enhance ROS production which causes alterations in fibrosis in lungs of rats and neoplastic damage. They also increased high risk against cardiopulmonary diseases in lungs by generating pro-oxidant and pro-inflammatory milieu (Dong and Ma 2016).

22.5 Mechanism of Toxicity of Nanomaterials

Recent studies have revealed that reactivity of the nanoparticles triggers the formation of ROS (especially, hydroxyl radicals and superoxide radical anions) due to activation of oxidative enzymes leading to the formation of oxidative stress (Kim et al. 2015). There are various reasons for the initiation of oxidative stress, such as (1) nanoparticles have the property to trigger the ROS production as the cellular response, (2) transition metal-based nanoparticles serve as the catalyst during the formation of non-metal nanoparticles, (3) formation of reactive molecules on the surface of nanoparticles, and (4) induction or activation of redox groups on nanoparticles (Fu et al. 2014).

Moreover, particle size is also considered to be the factor responsible for cellular cytotoxicity. As small particles provide the large surface area, it increases the chances of the interaction of nanoparticles with cellular components like carbohydrates, fatty acids, nucleic acids, and proteins (Wang et al. 2017). Further, nanosized particles have additional benefits as it readily enters the cell and leads to cellular damage (Wang and Wang 2013). Apart from this, the surface charge of particle also contributes to cytotoxicity as it controls the cellular uptake of particles and interaction among the biomolecules and cell organelles. This phenomenon can be understood by the context that positively charged nanoparticles interact with DNA (negatively charged), resulting in DNA damage (Fröhlich 2012). Additionally, the shape of nanoparticles has been considered to affect the toxicity level (Sukhanova

et al. 2018). Although the TiO_2 (amorphous) is known to have surface defects, this serves as evidence that active site stimulates the ROS production (Cheng et al. 2018). Besides, Fe_2O_3 nanoparticles (rod-shaped) were found to trigger high cytotoxic responses in comparison to Fe_2O_3 nanoparticles (sphere-shaped) in macrophage cell lines of RAW 264.7 of murine (Lee et al. 2014). Hence, it has become essential to understand the cellular as well as the molecular mechanism of nanoparticle toxicity and their effect on the biological system to develop a safe and precise assay of engineered nanoparticles for risk evaluation.

22.6 Biomarkers to Monitor Nanotoxicology

The advent of nanoparticles has gained significant attention in short period of time due to its widespread functionality in different fields. But the biggest challenge remains the same, i.e., their effect on the biological system (Riehemann et al. 2009). The outmost reasons are their applicability of nanotechnology in different industries and increase in the number of nanomaterials for different purposes in industries, increasing their chances of interaction with our body (Dowling 2004). Nowadays, researchers are focusing on understanding the potent effects of these nanoparticles on cells and tissues on the basic route, which can be due to dermal penetration, ingestion, injection, or inhalation. Moreover, studies have also been conducted to discover biomarkers involved during bio-interfaces, facilitating in creating the biomarkers database to monitor nanotoxicity (Della Rocca et al. 2011).

Biomarkers are stated to be characteristic which measure as well as work as an indicator to assess the biological process, pharmacologic response, or pathogenic process. Hence, it can be anything which can measure the change in antigens, cytokine concentration, genes, and even proteins (Wagner and Atkinson Jr 2015). Because of a wide range of biomarkers, we are focusing on the two groups of biomarkers pro-oxidative and pro-inflammatory because the primary responses induced by toxic nanoparticles in various tissues and cells are oxidative stresses and inflammation (Khanna et al. 2015). The outcomes of these two responses are impairment of tissue function and cell damage. Therefore, these biomarkers can serve as primary detection tool to measure the effect of nanoparticles on health and can also be used for early detection of the adverse effects (Iavicoli et al. 2012).

Pro-inflammatory biomarkers are commonly used to assess the variation in responses due to inflammation and oxidative stress in particular organs like the cardiovascular, immune, and respiratory systems (Bergamaschi 2012). Inflammatory immunological biomarkers are used to define any change in the immune system on the introduction of nanomaterial in the biological system which elicits inflammation. In these antigens, antibodies, chemokines, cytokines, and phagocyte congregation are measured and interrelated with the inflammation response (Xu et al. 2016). These biomarkers are effective in diagnosis of various diseases, but during nanotoxicological studies, its efficacy decreases. Hence, extensive care is taken while identifying the cause of inflammatory response via nanoparticles (Gendelman et al. 2015). This supports and provides evidence as to why the immune system

synthesizes different types of antibodies, cytokines, and chemokines, after encountering with pathogen or external agent causing stress (Gamucci et al. 2014). The major advantage of nanoparticles is its size, which allows them to penetrate directly through the cell wall, accumulate protein on their surface, and even translocate themselves through blood–brain barrier (Sonvico et al. 2018). The mobile nature of nanoparticles and their ability to aggregate themselves in various tissues elicit the immune response and make correlation between the immune response and presence of nanoparticles, which form the basis of biomarker analysis (Dobrovolskaia et al. 2016). At this point of time, major researchers are focusing on determining the toxic dosage which triggers immune response and how to prevent the toxic exposure of nanoparticles. Till date, numerous biomolecules have been identified which play a key role in inflammation (Elsabahy and Wooley 2013).

Numerous studies have highlighted metal oxide nanoparticles like iron oxide (Fe_3O_4), as it elicits immunogenic response in cell and can be used for biomarker studies for assessing potential toxicity (Arias et al. 2018). Joo with his colleague (2013) investigated the adverse effect of Fe_3O_4 on rodents. The results obtained were quite similar with Srinivas et al. (2012), as there was an increase in level of pro-inflammatory cytokines such as transforming growth factor beta (TGF-β TNF-α), interleukin-1 (IL-1,2,4,6,12), and immunoglobulin-E (IgE) which can serve as the biomarker for detecting various ailments (Srinivas et al. 2012). Additionally, tissue damage and inflammation have also reported to increase the expression of few genes encoding for different proteins like tissue-inhibiting metalloproteinase, serum amyloid A (SAA), and heat shock protein. The gene SAA is usually expressed in the liver which elicits the synthesis of TNF-α IL-1 and IL-6 which are also produced as a response to metal oxide nanoparticles (Skovgaard et al. 2009). The discussed biomarkers have recorded to involve in various situation when cell experiences stress. Moreover, they are also reported to be produced by the body in response to cold (Buzea et al. 2007). Biomarkers also serve as parameter for analysis in experimental design and aid in interpreting the result of biomarker assessment. Hence, studies focusing on the assessment of nanomaterial only triggering the inflammatory response enable us to discover the true biomarkers of nanotoxicity (Oberdörster 2010).

On the other hand, pro-oxidative biomarkers are the ones having response to various metal oxide nanoparticles, generally by generating the ROS stress. Therefore, it is essential to observe the ROS level induced by interaction of nanoparticles as ROS generation has been linked with different cardiovascular and respiratory ailments like atherosclerosis, asthma exacerbation, thrombosis, and inflammation (Fu et al. 2014). CuO (copper oxide), TiO_2 (titanium oxide), ZnO (zinc oxide), and Fe_3O_4 (iron oxide) are the metal oxide nanoparticles which have shown to cause the overproduction of ROS, as they allow the propagation of free radicals on their surface during their interaction with enzymes, oligomers, and proteins (Karlsson et al. 2008). Due to distinctive electrical surface properties, these nanoparticles generate substantial amount of ROS, which can be used as nanotoxicity biomarker. These are the two important types of biomarkers that are employed for nanotoxicological assessment.

22.7 Conclusion

There cannot be a second opinion that nano-sized materials have widespread applications in various fields of science and technology. However, there are numerous reports that depict the side effects of the nanomaterials on biological systems and cellular levels. Although they are relatively small sized, yet they have an enormous effect on human life and ecosystem. The elevated use of nanotechnology poses a risk not only to consumers but also firsthand to the workers. Their physicochemical parameters in addition to production of toxic ions, generation of free radical species, and high surface charge ratio result in cytotoxicity by nanoparticles which may include quantum dots, gold and silver nanoparticles, titanium dioxides, CNTs, etc. Both in vivo and in vitro assays require a better knowledge of toxicity mechanism so as to avoid side effects and exploit the benefits that nanotechnology has to offer. The information will further help to formulate the measures able to reduce the potential hazards of nanomaterials. Nanomaterials causing oxidative stress could be replaced with nanomaterials that are relatively less harmful. Further proper administration of antioxidants and other therapies to the occupational workers should also be taken into consideration to check their immune-related disorders. Also, the incorporation of nanomaterials should be considered effectively because the method of incorporation of nanomaterials in a product strongly influences its release in the environment. Thus, knowledge of pathogenic mechanisms of the nanomaterials is very crucial.

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