Chapter 15
Cyto-, Geno-, and Ecotoxicity of Copper Nanoparticles

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Abstract Nanotechnology has been playing a crucial role in twenty-first century in solving various problems particularly in the field of agriculture, medicine, and electronics. Nanotechnology is a broad and interdisciplinary area of research that has been growing explosively worldwide. Due to the small size, surface tailor ability, improved solubility, and multifunctionality of nanoparticles it may open up new research avenues. Nanoparticles are being viewed as fundamental building blocks of nanotechnology. The synthesis of nanoparticles is an important component of rapidly growing research efforts in nanoscale science and engineering. The synthesis of copper nanoparticles (CuNPs) by physical method involves the mechanical grinding of bulk metals. Subsequently the resulting nanoparticles are to be stabilized by the addition of protecting agents. Whereas in case of chemical methods of synthesis copper salts use to be reduced to copper nanoparticles. Moreover, biological method of synthesis make use of biomolecules for the reduction and stabilization of nanoparticles.

The increasing use of nanoparticles leads to the release and accumulation of these particles in soil, air, and aquatic environment. Therefore, evaluation of nanotoxicity to the ecosystem must be considered. Nanoparticles with their distinct properties require development of methods, which will assess the possible benefits to possible risks and health hazards associated with exposure to nanomaterials as they are used in manufacturing and medicine. Common methods for toxicity evaluation include MTT Assay, Neutral Red Assay, LDH Assay, Comet Assay, and ROS Assay.

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In the present chapter, we have briefly discussed the methods of synthesis of CuNPs, toxicity evaluation and harmful effects of CuNPs on plants, mice, fishes, and worms.

15.1 Introduction

Copper nanoparticles, due to their unique physical and chemical properties and the low cost of preparation, have been of great interest (Varshney et al. 2012). These are being used for many different industrial applications (Malathi and Balasubramanian 2012). Current usage includes lubricants, polymers, plastics, and metallic coatings and inks (Chen et al. 2006). Copper nanoparticles possess superior mending effects (Liu et al. 2004). One study showed that copper nanoparticles effectively decreased wear and friction and mended worn surfaces when used as oil additive (Tarasov et al. 2002). Copper nanoparticles have also been used as a bimetallic catalyst on activated carbon to effectively reduce elevated levels of nitrate in water (Barrabés et al. 2006). Chen and Hong (2005) reported that the addition of 15 or 30 nm copper nanoparticles greatly increased the ductility of diamond-like carbon (DLC) nanocomposite films. Copper nanoparticles have also been used in electrically conductive polymer composites as fillers (Zhang et al. 2007). Moreover, the multiple uses of copper nanoparticles extend beyond industrial applications.

Copper is already known as an effective antibacterial agent due to its ability to combine with the –SH enzyme group and lead to protein inactivation (Yoon et al. 2007). The authors further reported the use of copper nanoparticles (100 nm) as antibacterial agents against *Escherichia coli* and *Bacillus subtilis*. The latter demonstrated the greatest susceptibility when exposed to the copper nanoparticles. In addition, copper-fluoropolymer (Cu-CFx) nanocomposite film layers have been effectively utilized as a bioactive coating to inhibit the growth of microorganisms including *E. coli*, *Lysteria*, and *Staphylococcus aureus* (Cioffi et al. 2005).

Chatterjee et al. (2012) developed a method for preparation of CuNPs by reduction of $CuCl_2$ in the presence of gelatin as a stabilizer (50–60 nm in size). The bactericidal activity of this CuNPs on Gram-negative *Escherichia coli* was verified by the methods of agar plating, flow cytometry, and phase contrast microscopy. A low minimum inhibitory concentration showed that CuNPs are highly effective against *E. coli*. The same effect was also observed in Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, with values of inhibition similar to *E. coli*.

Despite the potential benefits of nanotechnology, the knowledge base of human health and environmental effects in the production and use of nanomaterials is insufficient. Human and environment can get exposed to nanoparticles via spillage during shipping and handling of nanobased products (Chen et al. 2006). Nanoparticle toxicity is associated with its smaller size, the efficiency with which it enters the cells, and its increased surface area (Dowling 2004). Like any other novel products, the acceptance of nanotechnology based products will also rely on promising benefits versus the potential risks associated with them (Tsuji et al. 2006). Even though different nanoparticles display similar properties, not all nanoparticles can be treated as equal and must be studied individually (Holsapple et al. 2005). In addition,

many nanomaterials present unique properties because of the type of surface coating applied on the material (Thomas and Sayre 2005).

The impact of nanoparticles on health has attracted considerable attention of the public and government worldwide. So far, most of the nanotoxicity research has been focused on different routes of exposures. Oberdorster et al. (2005) has defined nanotoxicology as the "science of engineered nanodevices and nanostructures that deals with their effects on living organisms." In regard to research on the health effects of nanomaterials, the inhalation (via the respiratory tract) exposure route has been extensively studied as compared to the ingestion (via the gastrointestinal tract) or skin absorption routes of exposure (Tsuji et al. 2006). More research is needed to determine if nanoparticles can penetrate the skin (Tsuji et al. 2006), because little information exists as to whether nanoparticles can be absorbed through the stratum corneum (Holsapple et al. 2005).

Both in vivo and in vitro studies have been conducted on the toxicology of metal nanoparticles including CuNPs found in environmental and occupational settings to include effects on the respiratory system and extra-pulmonary organs (Oberdorster et al. 2005). Many factors contribute to the pulmonary toxicity of nanoparticles to include size, dose to target organ or tissue, surface coating or treatment, degree of aggregates formed, surface charge, and shape (Tsuji et al. 2006). However, Colvin (2003) remarked in "the potential environmental impact of engineered nanomaterials," that, although research has been focused on the inhalation exposure route, the more common exposure route may be dermal absorption or oral injection exposure because many nanomaterials are prepared in liquids and agglomerate strongly, making it difficult for them to become respirable nanoparticles.

15.2 Synthesis of CuNPs

Nanotechnology is an enabling technology that deals with nano-sized materials in different field of science. Instead of availability of physical and chemical methods for synthesis of metal nanoparticles, biological synthesis is an important method for development of clean, nontoxic, and environmental-friendly procedure. Nanobiosynthesis by using various biological agents is a rapidly emerging method for synthesis of nanoparticles with well-defined shapes and controlled monodispersity.

15.2.1 Biological Synthesis

Extensive research has been performed by scientists all over the world in the field of biosynthesis of metal nanoparticles using different biological systems and the main focus is always on silver and gold nanoparticles. There are a few reports on the biosynthesis of copper and copper oxide nanoparticles (Thakkar et al. 2010). However, a thorough survey of literature on synthesis of CuNPs provides evidence that CuNPs can be easily synthesized in their oxide form instead of simple CuNPs

(Hasan et al. 2008; Singh et al. 2010) because copper is well known to be susceptible to oxidation. Therefore, synthesis of pure metallic CuNPs in aqueous phase is still an open challenge. However, there are few reports concerning biogenic synthesis of copper nanoparticles (Varshney et al. 2010, 2011; Valodkar et al. 2011; Majumber 2012; Harne et al. 2012; Ramanathan et al. 2013).

The mechanism for the synthesis of nanoparticles using biological systems has not been elucidated, so far. However, it is suggested that different biomolecules are responsible for the synthesis of nanoparticles. The use of fungi is potentially exciting since they secrete large amount of enzymes and are simple to deal within the laboratory (Mandal et al. 2006). But in extracellular biosynthesis of nanoparticles, especially in the case of fungi, several reducing and capping agents secreted are possibly involved which also effects the reducing agents on the shape and size of nanoparticles (Mohanpuria et al. 2008; Mukherjee et al. 2001). Lee et al. (2011) reported the biosynthesis of CuNPs using plant leaf extract *Magnolia* as a reducing agent. After treating aqueous solutions of $CuSO_4 \cdot 5H_2O$ with leaf extract, stable CuNPs were formed, which were ranged in size from 40 to 100 nm.

15.2.2 Chemical Synthesis

Many methods have been proposed for the chemical synthesis of CuNPs. Among these, aqueous reduction method is most commonly employed because of its benefits, like, simple operation, high yield and quality, limited equipment requirement, and simplicity of control. In the year 1997, CuNPs were prepared by the reduction of copper (II) acetate in water and 2-ethoxyethanol using hydrazine under reflux. The synthesized nanoparticles demonstrated different absorption peaks in the spectral range of 572–582 nm. Average size varies from 6.6 to 22.7 nm in ethoxyethanol and from 15.5 to 30.2 nm in water after addition of various amounts of a protective polymer, i.e., poly-(*N*-vinylpyrrolidone) (Huang et al. 1997). Different reducing agents have been tried and used for the synthesis of CuNPs to achieve a high degree of synthesis (i.e., higher yield) and to develop rapid, easy and eco-friendly method. Xiong et al. (2013) obtained water-soluble and stable dispersions of copper nanoparticles in the presence of dopamine as reductant and capping agent and copper chloride of around 2 nm of size.

Zhang et al. (2010) used potassium borohydride as reducing agent for the synthesis of high dispersive CuNPs by chemical reduction method. In addition, they studied the effect of different parameters like reactant ratio, concentration of copper sulfate, reaction temperature, and dispersant on the size of CuNPs and formation rate. The shape and size of nanoparticles were characterized by scanning electron microscopy. The average particles size of CuNPs with spherical shape was reported to be about 100 nm.

Pure metallic CuNPs at a high concentration were synthesized by the reduction of cupric chloride with hydrazine in the aqueous cetyl trimethylammonium bromide (CTAB) solution (Wu and Chen 2004). They used ammonia solution for the adjustment of solution pH up to 10 and hydrazine as a reducing agent in a capped

reaction bottles which were found to be crucial for the synthesis of pure CuNPs. The synthesized nanoparticles were characterized by UV–Vis absorption spectrophotometry, transmission electron microscopy (TEM), electron diffraction pattern, energy dispersive X-ray analysis (EDX), X-ray diffraction (XRD), and X-ray photoelectron spectroscopy (XPS). The nanoparticles were found to be with diameter of 5.1 nm (Wu and Chen 2004).

Because of the strong reducing ability of $NaBH_4$, it is widely used as a reductant for this aqueous reduction process. Based on these findings, Liu et al. (2012) prepared CuNPs by reducing Cu^{2+} ions with $NaBH_4$ in alkaline solution. They also studied the effects of $NaBH_4$ concentration and dripping rate on the formation of CuNPs.

15.2.3 Physical Synthesis

For the preparation of CuNPs, several physical synthesis routes have been described, such as precipitation (Zhu et al. 2004), thermal decomposition (Kim et al. 2006), microemulsion (Lisiecki and Pileni 1993), surfactants solution (Wu and Chen 2004), microwave-assisted techniques (Nakamura et al. 2007b), and vacuum vapor deposition (Liu and Bando 2003).

In 2007, Nakamura and collaborators in their two different studies produced Ag core-Cu shell nanoparticles (denoted as Cu/Ag nanoparticles) having 10–40 nm in size by a microwave (MW)-assisted alcohol reduction process. And CuNPs were synthesized via a microwave-assisted alcohol reduction process with or without the surface plasmon absorption using copper (II) octanoate and copper (II) myristate. These nanoparticles were found to be in size range of 5–6 nm (with the surface plasmon absorption) and 2–3 nm (without the surface plasmon absorption) (Nakamura et al. 2007a, b).

In another similar study, Blosi et al. (2011) synthesized colloidal CuNPs using microwave heating by a polyol method that exploits the chelating and reducing power of a polidentate alcohol (diethylenglycol). The synthesis was performed by using eco-friendly additives, for example, ascorbic acid (reducing agent) and polyvinylpyrrolidone (chelating polymer) to improve the reduction kinetics and sols stability. The synthesized nanoparticles were reported to be stable for several months in spite of the high metal concentration.

15.3 Methods of Toxicity Evaluation

Apart from their potential use, these nanomaterials are striking both basic science and technology. The increasing human exposure to them together with their distinct properties requires development and validation of nanobased product along with their predictive toxicity, and hazard capabilities. The increasing human exposure to them together with their distinct properties requires development and validation of nanobased product along with their predictive toxicity, and hazard capabilities.

These methods must assess the possible benefits to possible risks and health hazards associated with exposure to nanomaterials as they are used in manufacturing and medicine. Most common methods of toxicity evaluation are MTT Assay, Neutral Red Assay, LDH Assay, Comet Assay, ROS Assay, Chlorophyll fluorescence, and AlgaToxKit assay.

15.3.1 MTT Assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, a Yellow Tetrazole]

The MTT tetrazolium salt colorimetric assay measures cytotoxicity and cell proliferation (Mosmann 1983). In this assay, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is taken up into the cells. It is then reduced to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The resulting formazan product accumulates in cells as it cannot pass through the plasma membrane. The MTT assay was tested for its validity in various cell lines (Mosmann 1983).

This method was further explored to embrace its application to the measurement of cell activation. The level of MTT cleavage by viable cells of several origins was found to be directly correlated to the number of viable cells. In this method, the cell are plated into the 96-well microplates and incubated in the presence and absence of the test compound for desired time period. Then, MTT is added and after a short time period the medium is removed from the well. It is then followed by addition of dimethyl sulfoxide (DMSO), which dissolves MTT. The amount of MTT dissolved is determined using spectrophotometer at 560 nm, and the results are presented as percentage of cell survival.

A new modification of this method is established in the form of XTT. It is 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide. XTT is metabolized to a water soluble formazan product and thus eliminates the solubilization step required for MTT assay.

Hernández-Ortiz et al. (2012) proved by MTT assay that as the cell exposure time to nanoparticle increases, cell viability decreases. The 2D cell cultures do not effectively represent the widespread cell–cell, cell–matrix interaction and also different transport circumstances. Hence testing cytotoxicity in 2D cell culture is not an accurate method to evaluate actual toxicity of nanoparticles. To attain more satisfactory and detailed information, Lee et al. (2009) introduced 3D-spheroid-culture-based NP toxicology testing system. They verified toxicity of CdTe and Au nanoparticles in both 2D and 3D spheroid cultures. In which it proved that toxic effects are significantly reduced in the 3D spheroid culture when compared to the 2D culture system.

Advantages:

1. It is relatively simple, rapid, sensitive, and cheaper assay.
2. The use of ELISA reader allows the processing of large number of samples.

3. It could be used to monitor cell activation independently of proliferative activity (Mosmann 1983).

Disadvantages:

1. The results are sensitive to environmental conditions.
2. The assay depends on cell metabolism for the formation of formazan. The background use to be variable due to protein precipitation on adding an organic solvent like DMSO to dissolve the blue formazan product.
3. The resulting product has low solubility (Denizot and Lang 1986).
4. In particular copper nanoparticles interfere the results of MTT assay (Jose et al. 2011).

15.3.2 Neutral Red Assay

The Neutral Red Assay is also used to measure cell viability. It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes (Fautz et al. 1991) and other cell lines (Morgan et al. 1991). The Neutral Red Assay is based on the protocol described by Borenfreund and Puerner (1984). It determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. Neutral red is the marker of cell viability. It has been shown to be selectively retained by the lysosomes of living cells because of the differential pH between the inside of the lysosome and the surrounding cytoplasm. The amount of neutral red taken up by the population of cells is directly proportional to the number of viable cells in the culture (Barstad et al. 1991).

In this method, the cells are seeded in 96-well plates and are treated by the compound for appropriate time period. Then plates are incubated for 2 h with neutral red containing medium. The cells are subsequently washed and the dye is extracted in each well. The absorbance of neutral red extract is to be measured at 540 nm (Repetto et al. 2008).

The test material-induced cytotoxicity is measured over a wide range of concentrations, and the concentration yielding a 50 % reduction in neutral red uptake is used as the measure for comparison between the test materials. The "percentage of untreated control" values versus the test agent concentrations are plotted, and determine the 50 % inhibition of neutral red uptake by the cells.

Advantage:

1. This assay is cheaper and more sensitive than other cytotoxicity tests.

Disadvantages:

1. Prolonged exposure of the cells to the fixative can result in discharge of the dye into the solution.
2. They may get interfered by precipitation of neutral red reaction medium.

3. The test materials that have absorbance maxima near 540 nm may interfere the assay design and results (Johnson et al. 2009).

15.3.3 Lactate Dehydrogenase (LDH) Assay

The LDH leakage assay is based on the measurement of lactate dehydrogenases activity in the extracellular medium. In this assay lactate is converted into pyruvate in the presence of LDH with parallel reduction of nicotinamide adenine dinucleotide (NAD) to NADH. This reaction thus results in a change in absorbance at 340 nm. Thus, LDH released into the media acts as a marker of dead cells. Reliability, speed, and simple evaluation are some of the characteristics of this assay (Decker and Lohmann-Matthes 1988). It is widely accepted method as a marker of cell death, however, it should be noted that this test signifies only the cell membrane integrity (Hillegass et al. 2010).

Advantage:

1. It is among the most sensitive nondestructive assay, mainly used for short-term exposure studies (Bopp and Lettieri 2008).

Disadvantage:

1. The uncertainties in this assay increase at lower compound concentrations (Bopp and Lettieri 2008).

15.3.4 Comet Assay

Comet assay is commonly used to assess the genotoxic potential of chemicals and environmental contaminants. This method is also called as single cell gel assay (SCG) and microgel electrophoresis (MGE). It was firstly established by Ostling and Johanson (1984) as a technique to directly visualize DNA damage in individual cells. In this method, individual cells are encapsulated in a thin layer of low melting point agarose gel on a microscope slide. These cells are then lysed followed by the electrophoresis of DNA. Under the electric charge, intact, unbroken DNA travels modestly due to its large size, but if present, small DNA fragments migrate much further resulting in a comet shape with an extended tail, containing damaged DNA towards the anode. The DNA is detected by staining ethidium bromide or propidium iodide staining. Here, the length and fluorescence intensity of the comet tail is directly proportional to the amount of DNA damage. It is known as a simple, sensitive, and rapid method for measuring single/double strand breaks in DNA in each cell.

Advantages:

1. The comet assay is a sensitive and rapid method for DNA strand break detection in individual cells (Fairbairn et al. 1995).
2. Small amount of sample is required for the assay.
3. The assay requires relatively short time period (Tice et al. 2000).

Disadvantages:

1. The comet assay requires individual cells. Some cell types like blood cells can be directly used for the study. However, for the other tissues, cells need to be individualized (Cotelle and Ferard 1999).
2. The assay requires viable single-cell suspension. However, if the sample contains the necrotic or apoptotic cells, then it will mislead the results (Olive and Banáth 2006).

15.3.5 Reactive Oxygen Species Assay

A key mechanism thought to be responsible for the genotoxic effects exerted by nanomaterials which comprise oxidative stress, referring to a redox imbalance intracellularly (Singh et al. 2009). It occurs usually as a result of increased intracellular reactive oxygen species (ROS) and decreased antioxidants species. ROS are highly reactive molecules that contain the oxygen atom. They are highly reactive due to the presence of unpaired valence shell electrons. They can disturb the homeostasis of the intracellular environment by reacting unfavorably. During any environmental stress, ROS levels can increase dramatically leading to significant damage to cell structures. The resulting situation is known as oxidative stress. ROS are also produced by exogenous sources such as ionizing radiation.

The expansion of results from in vitro experiments to predict the in vivo toxicity is problematic. It is due to the fact that in vitro exposure conditions usually feature much higher concentration and exposure times than found in the cellular environment in vivo (Marquis et al. 2009).

ROS assay involves various types of assays. Most important of them involves the use of dichlorofluorescin diacetate (DCFDA). It is a widely used fluorescence-based probe for in vitro and in vivo measurement of ROS. This method involves the deacetylation of DCFDA by endogenous esterases to dichlorofluorescein (DCFH), which later reacts with ROS to form the DCF, a fluorophore (Wang and Joseph 1999).

15.3.6 Chlorophyll Fluorescence Assay

The absorbed light energy that is not used for photochemistry of Photosystem II (PSII) can be dissipated by chlorophyll a fluorescence of PSII. As the fluorescence

can be used to study the electron transport and energy transfer in photosynthesis, it can also be used to study the effects of pollutants and environmental conditions on photosynthesis. The use of chlorophyll fluorescence at 685 nm as an indicator of toxicity of any product is carried out with an algae (*Scenedesmus obliquus*). This bioassay could be used to detect environmental stress (Caux et al. 1992; Willemsen et al. 1995).

Advantages: This is a simple assay and fast method to measured toxicity products (Zemri et al. 2012).

15.3.7 AlgaToxKit-F

Blaise (1991) analyzed many different test protocols with bacteria, protozoa, microalgae, invertebrates, and fish cell lines and on base five criteria (availability in kitformat, portability, maintenance-free bioindicator, performance in microplates, minimaltraining, and equipment requirement); he concluded that the Toxkit tests are the only types of bioassays that fulfil all these five features. In a special kit called the Algaltox kit F contains all the materials, including the test species *Selenastrum capricornutum* and presently as *Pseudokirchneriella subcapitata* immobilized in algal beads, to perform algal growth inhibition assays, by internationally accepted standard methods using absorbance at 670 nm of chlorophyll for its growth inhibition (http://www.biohidrica.cl/assay_algaltoxkit.htm).

Advantage: All costs associated with maintaining algal cultures are eliminated as the algae are provided in the kit as algal beads. These algal beads can be stored for several months and therefore there no investment is required in culturing facilities and equipment.

15.4 Toxicity of CuNPs

15.4.1 Toxic Effects of CuNPs Administrated In Vitro and In Vivo

Due to many unexpected harmful effects of nanomaterials observed globally, now-a-days the nanoparticle toxicity is considered a major concern. The growing use of nanoparticles leads to the release and accumulation of those particles in soil, air, and aquatic environment. Therefore, evaluation of nanotoxicity to the ecosystem is a challenging task. There are many studies currently in process; however, the present knowledge about their ecotoxicity is still inadequate. Consequently, like other nanoparticles, CuNPs also have the toxic effects on different environmental systems. It is necessary to study the toxic effects of CuNPs on different organisms

of various habitats. Therefore, in the next section, a briefly discussion about the harmful effects of CuNPs on plants, mice, fishes, and worms have been incorporated.

15.4.2 Toxicity of CuNPs to Plants

Copper is a well-known essential micronutrient for virtually all plants. Therefore, it has been widely used for such purpose. After its entry into the environment, it can eventually get oxidized to form copper oxide (Lide 1994). Otherwise, the CuNPs can be formed naturally at the plant root–soil interface (Manceau et al. 2008). In fact, a condition may arise where CuNPs concentration will rise over certain toxic level. Higher plants interact strongly with their surrounding environment and hence they have the risk to get affected by the exposure of nanoparticles. Although, there has been growing amount of research based on the toxicity of nanoparticles to animal and bacteria, very limited study has been performed by using higher plants. The nanotoxicological studies on plants will therefore help us to understand the flow of nanoparticles leading to their accumulation in food chain.

CuNPs are insoluble in water and therefore, there are certain limitations in their use for toxicity testing assays as those assays require different reagents made in aqueous base. In order to study the toxic effects and bioaccumulation of CuNPs on plant seedling, a plant agar test can be performed for homogenous exposure of CuNPs onto the plant *Phaseolus radiatus* (mung bean) and *Triticum aestivum* (wheat) (Lee et al. 2008). The plant agar is soft gel which permits the dispersion and thereby avoiding precipitation of nanoparticles. The study reported the toxicity of CuNPs at 335 and 570 mg/L, respectively, against *P. radiatus* and *T. aestivum*. These effects were only due to the CuNPs and the contribution of copper ions to the toxicity was negligible. The authors concluded that the phytotoxicity and bioaccumulation of CuNPs in these experimental plant species was dose-dependent. CuNPs reduce the length of root emerging from plant seeds (Stampoulis et al. 2009). This study was performed on a terrestrial plant *Cucurbita pepo* (zucchini) grown in hydroponic solution.

15.4.3 Toxicity of CuNPs to Animals

15.4.3.1 CuNPs Toxicity to Mice

Exposure of nanoparticles through gastrointestinal tract can lead to various harmful effects (Jani et al. 1994). However, there are very limited reports based on such aspect of copper nanoparticle toxicity. Mice orally exposed to copper nanoparticles showed acute toxicological effects and heavy injuries in kidney, liver, and spleen (Chen et al. 2006). Furthermore, these effects were sex-dependent, where a male

mouse has more toxic effects compared to female mice. Similar results were obtained in mice exposed through nasal route by CuNPs. Inhalation exposure of CuNPs (23.5 nm) at very high dose was reported to get translocate to metabolically active body organs like liver and kidney. Inhalation of high dose (40 mg/kg) of CuNPs by mice caused a hydropic degeneration around the central vein, spotty necrosis of hepatocytes, swelling in glomerulus, and severe lesions in olfactory system. It further resulted into the reduced body weight (Liu et al. 2009). This study demonstrates that the high dose of CuNPs could cause harmful effects to the viscera of the exposed mice.

From these studies it is imperative to understand the reasons why CuNPs induce toxic effects to liver and kidney. The answer lies within the activity of these organs. The homeostasis of copper ions is retained in vivo (Jesse and Mary 2004). They are metabolized in the liver where they are transferred to reduced glutathione (GSH). Whenever there is excess of copper in the body, the GSH depletion consequently gives the enhanced toxicity (Freedman et al. 1989; Steinebach and Wolterbeek 1994). Copper ions in excess are removed from the body through kidney (Turnlund et al. 1997). Since the CuNPs have strong ionization potential, increase in their amount leads to excess release of their ions. These copper ions then cause renal inflammation, which results into the reduced glomerular filtration rate. In another study, Meng et al. (2007) correlated the ultrahigh reactivity of nanocopper with the particle size/specific surface area. They showed that as compared to microcopper (17 μm), nanocopper (23.5 nm) interacts rapidly with artificial gastric acid juice. It leads to the formation of ionic copper with ultrahigh reactivity. Additionally, the study also reported the metabolic alkalosis and accumulation of copper in mice kidneys after oral exposure of CuNPs.

15.4.3.2 CuNPs Toxicity to Fish

CuNPs after its dissolution in water, probably release its particles in aqueous environment. Therefore, its dissolution in water is a major concern. As some dissolution of metal particles in water will occur, it is essential to discriminate that whether the toxic effects are due to nanoparticles themselves or if they are due to dissolved metals. Owing to this problem, Griffitt et al. (2007) have designed their experiment by studying the acute toxicity of soluble copper and 80 nm CuNP suspensions on *Danio rerio* (Zebrafish) embryo. The study concluded that the CuNP has acute toxic effect at 1.5 mg/L. The study reported that soluble copper as well as nanocopper induced drastic changes in the gill morphology (see Fig. 15.1). The activity of Na^+/K^+ pump, found in gill region, was prevented by the dissolved copper (Li et al. 1998). Therefore, the gills of zebrafish are prime targets of CuNPs, which result in severe injury to gills. These results were further confirmed by fact that CuNPs significantly induced the hypoxia-inducible factor 1 (HIF-1), heat shock protein 70 (HSP-70), and copper transport regulatory protein (CTR) genes. Exposure of embryonic zebrafish by CuNPs causes the morphological

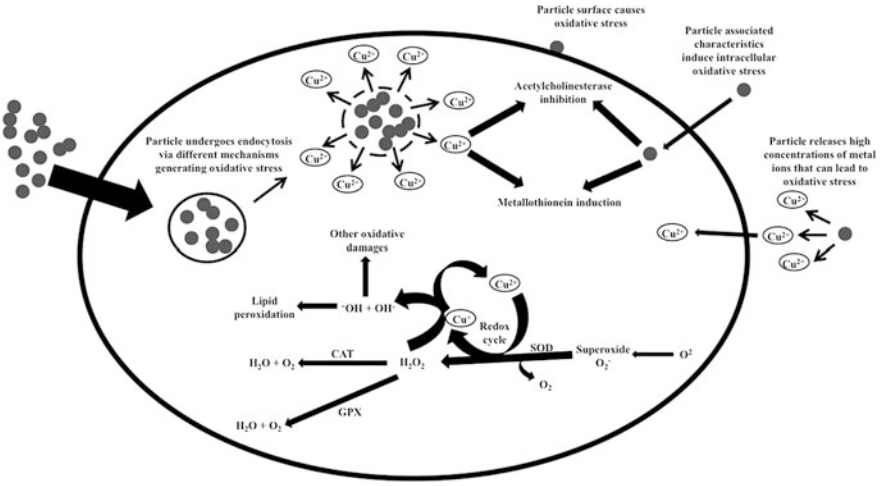

Fig. 15.1 Schematic representation showing the various pathways by which copper nanoparticles can induce oxidative stress on the cell (reprinted with permission from Gomes et al. 2011, copyright (2011) American Chemical Society)

malformation of the larvae (Bai et al. 2010). At some elevated concentrations (0.1 mg/L), the CuNPs even kill the zebrafish embryos in their gastrula stage.

15.4.3.3 CuNPs Toxicity to Worms

There are very few studies that reported toxic effects of CuNPs to the terrestrial environment. Unrine et al. (2010) studied the correlation between harmful effects of CuNPs on the earthworm *Eisenia fetida*. They exposed the worm to CuNPs, in a series of concentrations, and studied its effect on growth, mortality, reproduction, and expression of various genes associated with metal homeostasis, general stress, and oxidative stress. The study reported that the CuNPs up to 65 mg/kg caused no adverse effects on the ecologically relevant endpoints. The study further extends that copper ions and CuNPs were taken up by the earthworms. Therefore, this study suggests that CuNPs may enter the food chains from soil; however, it would be harmful to earthworm only at higher concentrations. In another report, Amorim and Scott-Fordsmand (2012) studied the survival, reproductive output, and avoidance behavior of CuNPs on *Enchytraeus albidus*. The study indicated that CuNPs cause a reduced reproductive output, as well as high avoidance.

15.5 Size and Concentration-Dependent Toxicity

As nanoparticles have the greater surface area, they have distinctive physical and chemical characteristics. These characteristics, therefore, influence their behavior effect on biological systems (Zhao et al. 2007). Studies on animals suggest that copper exerts toxicity depending on its size. As the size reduces, copper toxicity increases abruptly. The study led by Chen et al. (2006) reported the toxic dose of ionic, nano-, and microcopper to be 110, 413, and 500 mg/kg, respectively. Additionally, this study also demonstrated that CuNPs release the toxic cupric ions to the surrounding medium. The toxicity of CuNPs is thought to be due to release of copper ion in the relevant media. This is because nano-sized copper releases more copper ions as compared to its bulk counterpart. This effect was confirmed by higher degree of DNA damage and significant cell death in the cells exposed to nanocopper, in comparison with microCuNPs (Midander et al. 2009).

Prabhu et al. (2010) compared the toxic effects of CuNPs on neuron of dorsal root ganglion (DRG), at concentrations from 10 to 100 μM. The study reported that exposure of CuNPs at 10–20 μM concentrations did not cause any morphological changes in the neuron, whereas the concentration in the range from 40 to 100 μM showed the harmful effect like shrinkage of cell, generation of vacuoles in cell cytoplasm.

Owing to the smaller size, compared to microsized CuNPs (17 μm), nanosized (23.5 nm) CuNPs interact fast with artificial gastric acid juice and thereby release cupric ion, having a very high activity, causing the metabolic alkalosis (Chen et al. 2006). CuNPs also get accumulated in the plant system as consequence of exposure to them. Their bioaccumulation was reported to increase with the increase in exposure concentration (Lee et al. 2008). Similar results were obtained by Kim et al. (2011), where the authors demonstrated that increasing doses of instilled CuNPs produced an increasing concentration of copper, measured in Bronchoalveolar lavage (BAL) fluids of mice lung. All these studies therefore, suggest that the concentration and dose of CuNPs play an important role in determining their level of toxicity. Higher CuNPs concentrations, higher would be their toxic effect. Moreover, smaller size CuNPs exert greater harmful effects to the exposed biological system.

15.6 Mechanism of Copper Nanoparticle Toxicity

As that of any nanomaterial, CuNPs have potential effects on plants, animals, and even microbes. However, very limited studies have been performed till date on deciphering the mechanisms of copper nanoparticle toxicity. Understanding the mechanisms of causing such toxicity is very important in order to develop nanoparticles for a targeted application. This scientific literature is a primary source of information for understanding the mechanisms of copper nanoparticle toxicity.

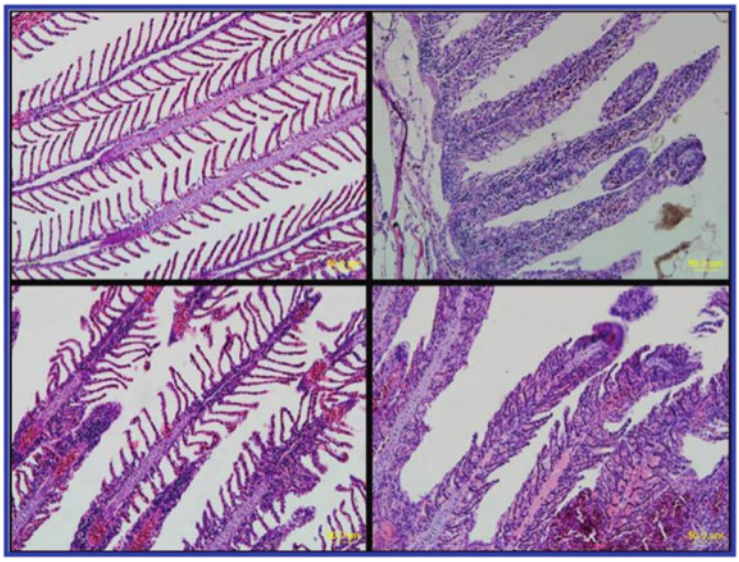

Fig. 15.2 Micrographs showing gill injury induced by 48 h copper exposure. Soluble copper and nanocopper induced dramatic changes in gill morphology. Clockwise from *top left*: control, 0.25 mg/L soluble Cu^{2+}, 1.5 mg/L nanocopper, 0.25 mg/L nanocopper (reprinted with permission from Griffitt et al. 2007)

Exposing cells with CuNPs also increased the level of prostaglandin. Some proinflammatory mediators have impact on the restrictive characteristics of blood–brain barrier. After exposure to CuNPs, cells also respond by elevated production of these proinflammatory mediators like TNF-α and IL-1β (Trickler et al. 2012). Furthermore, like any other metal nanoparticles, CuNPs interact with cell membranes. This interaction can damage plasma membranes (Minocha and Mumper 2012). The copper nanoparticles might interact with the –SH groups of the protein thereby leading to the denaturation of the protein contents of cell membranes. Additionally, it is also reported that the exposure of cells to transition metals results in the disturbance of intracellular redox status or alteration of protein conformation and thereby leading to the inhibition of protein function (Kawata and Suzuki 1983; Li et al. 1994).

Apoptosis includes the series of molecular mechanisms which can lead to death of aged or diseased cell. This process includes various biochemical changes like caspase activation, DNA fragmentation, etc. (Shimizu et al. 2004). The apoptosis can be executed by various pathways and can be induced by any kind of stress (Rastogi et al. 2009). In this context, Sarkar et al. (2011) reported that CuNPs alter the levels of oxidative stress by increasing the production of ROS and reactive nitrogen species (RNS). Several studies have suggested that oxidative stress can induce the lipid peroxidation of the mitochondrial membrane, which can ultimately result into the decoupling of oxidative phosphorylation, interference of electron

transport, and a reduction in mitochondrial membrane potential (Freedman et al. 1989; Saris and Skulskii 1991; Mattie and Freedman 2001). Further studies also revealed that the nanocopper exposure induces ROS through various mechanisms as shown in Fig. 15.2 (Gomes et al. 2011). It also affects the mitochondrial membrane potential following the release of cytochrome c from mitochondria to cytosol. The exposure finally results in the induction of apoptosis through the activation of caspase 3 pathway, caspase 8, Fas, and tBid (Sarkar et al. 2011). Earlier to this study, Prabhu et al. (2010) while studying the effect of CuNPs on somatosensory neuron of rat, showed the reduction of mitochondrial activity by interfering with the activity of reductase enzyme presented in mitochondria.

15.7 Conclusion

Nanoparticles exhibit novel properties and functions that differ distinctly from those observed in the corresponding bulk counterpart, mainly because of their small size, large surface area, solubility, shape, and aggregation. There are concerns that the same properties that make nanoparticles so unique could also be responsible for their potential toxicity.

In view of increasing uses of CuNPs, the potential for adverse health effects due to prolonged exposure at various concentration levels in biotic components (plants, mice, fishes, and worms) and abiotic components of the ecosystem (air, water, and soil) have not yet been established. Moreover, the environmental impact of CuNPs is expected to increase considerably in the coming years. The behavior of the CuNPs inside the cells is still a mystery and metabolic pathway interference or immunological responses induced by the CuNPs are yet to be understood completely. Cytotoxic and genotoxic studies will take up this challenge to decipher the molecular events that could regulate bioaccumulation of CuNPs by prying the normal metabolic pathway. The importance of metal nanoparticles to our well-being is beyond argument; however, their potential adverse impacts need to be studied in more detail. Nanotoxicology as a new discipline will make significant contribution to the development of a sustainable and safe nanotechnology.

15.8 Future Perspective

Although considerable progress has been made in recent years in terms of studies based on cytotoxic and genotoxic effects of CuNPs, some key questions are yet to be answered like:

- In what extent the ions released from the nanoparticles are responsible for toxic effects?

- What are the effects of size, shape, and agglomeration of CuNPs on their toxicity?
- What are the accumulation studies and interference with normal metabolic activity?
- The necessity to develop in vitro test for risk assessment, etc.

The expanding uses of CuNPs in commercial products and their increase in consumption, makes obligatory for the understanding of genotoxic and cytotoxic studies of CuNPs. To address this issue, there is a need for concerted efforts from industry, academia, government, and public areas. Their combined efforts for initiating research and public communication at the same time for improving risk perception of the public towards the CuNPs would be helpful. In this context, academia can focus on basic research whereas government and industry can provide monetary and infrastructure support. The findings of such research will be later communicated to the public to make them aware about the cytotoxic and genotoxic effects of CuNPs in the product if any.

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