Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.
DOI 10.1007/s40011-012-0072-7

REVIEW

Nanomaterials: Exposure, Effects and Toxicity Assessment

Vyom Sharma · Ashutosh Kumar · Alok Dhawan

Received: 18 November 2011 / Accepted: 4 August 2012

Abstract Nanotechnology is the understanding and control of matter at the nanoscale, at dimensions between approximately 1–100 nm, where unique phenomena such as improved physical, chemical, biological properties enable novel applications. The increasing use of engineered nanomaterials (ENMs) in consumer and industrial products has also aroused global concern regarding their fate in biological systems and resulting in a demand for parallel risk assessment. A variety of ENMs with different chemical compositions, synthesized through different methods, differing in size, shape, surface coatings, etc. have been shown to be genotoxic and cytotoxic in different organ specific cell lines (in vitro) and mouse. However there is a dilemma in the selection and validation of the test methods for the ENMs characterization, dose selection, cytotoxicity and genotoxicity assessment, because of the altered behaviour of ENMs as compared to the chemicals. A multidisciplinary team effort from material scientists, molecular biologists, toxicologists and physicists is necessary as it will facilitate the interlinking of different facets of nanotoxicology thus aiding in the understanding of cellular responses to nanomaterials exposure and mechanisms involved.

V. Sharma · A. Dhawan (✉)
Nanomaterial Toxicology Group, CSIR-Indian Institute of Toxicology Research, Council of Scientific and Industrial Research (CSIR), Mahatma Gandhi Marg, P.O. Box 80, Lucknow 226001, Uttar Pradesh, India
e-mail: alokdhawan@iitr.res.in; alok.dhawan@ahduni.edu.in

A. Kumar · A. Dhawan
Institute of Life Sciences, Ahmedabad University, 16/1 Vikram Sarabhai Marg, Opp. IIM, Vastrapur, Ahmedabad 380015, Gujarat, India
e-mail: ashutosh.kumar@ahduni.edu.in

Keywords Nanomaterials · Cytotoxicity · Genotoxicity · Nanoparticle exposure

Introduction

Nanotechnology holds great potential for creating new materials with enhanced properties. A number of nanotech based products are finding applications in industries like medical devices, imaging, sports, biosensing, electronics, drugs, environmental cleanup, cosmetics and sunscreens etc. [1, 2]. The global economy will be increasingly influenced by nanotechnology as more products containing nanomaterials move from research and development into production and commerce (Fig. 1).

A nanometer (nm) is one billionth of a meter (10^{-9} m)—about half the size of the diameter of DNA. The prefix nano is derived from the Greek word for 'dwarf'. The definition for the 'Nanoparticle' given in the new PAS71 document developed by the UK's National Standards Body—British Standards Institution (BSI) is: "a particle having one or more dimensions of the order of 100 nm or less" [3]. Nanoparticles are also called as ultrafine particles by some toxicologists [2], aitken mode and nucleation mode particles by atmospheric scientists [4, 5], and engineered nanoparticles by the materials scientists [6].

Different Types of Nanoparticles

Nanoparticles can be classified into two main categories: natural and anthropogenic. Natural nanoparticles existed in the environment long before the nanotechnology era started. Examples of natural nanoparticles include soil colloids, airborne nano-crystals of sea salts, fullerenes, carbon nanotubes and biogenic magnetite etc. [7, 8]. Soil contains

Published online: 06 September 2012

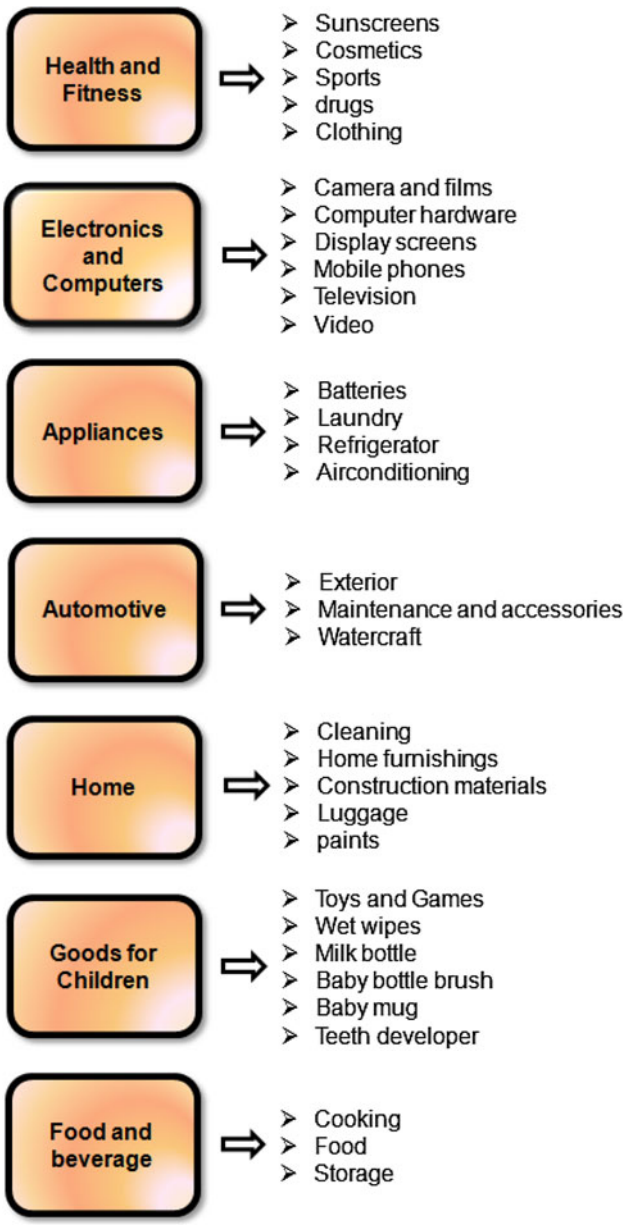

Fig. 1 Applications of engineered nanoparticles in the consumer products

many kinds of inorganic and organic nanoparticles, including clay minerals, metal oxides and hydroxides, humic substances, allophane, and imogolite [9]. Natural organic nanoparticles can also be found in plants [10]. Anthropogenic nanoparticles can be divided into two categories: incidental, which are nanoparticles produced unintentionally in manmade processes (e.g. carbon black, carbon nanotubes and fullerenes, platinum- and rhodium-containing nanoparticles from combustion by-products [8], and engineered/manufactured, which are nanoparticles that are produced intentionally for their specific properties.

A variety of engineered nanomaterials are already known and more novel materials are being engineered. The ways to produce engineered nanoparticles can be categorised in two different classes: "top-down" or "bottom-up". Top down techniques involve etching or milling down a block of material to desired shape whereas bottom up involves arranging smaller subunits into more complex assemblies [11].

Some of the most widely used nanomaterials are listed below.

Carbon-Based Nanomaterials

These nanomaterials are composed mostly of carbon, in the shape of hollow spheres or tubes. Spherical carbon nanomaterials are referred to as fullerenes, while cylindrical ones are called carbon nanotubes (CNT). There are two types of CNT-singlewalled (SWCNT) or multiwalled (MWCNT). Both of these are typically a few nanometer in diameter and several micrometers to centimeters long [11].

Metal/Metal Oxide Nanoparticles/Nanomaterials

Metal oxide nanoparticles have applications in cosmetics, textiles, fuels, drug delivery and paints. Examples include silver, gold, zinc oxide, aluminium oxide, cerium oxide and titanium dioxide nanoparticles.

Quantum Dots

Quantum dots (QD) are semiconductor nanocrystals used extensively in biomedical imaging. Fluorescent QDs can be conjugated with bioactive moieties (e.g. antibodies, receptor, ligands) to target specific biologic events and cellular structures [12].

Dendrimers

Dendrimers are spherical polymeric molecules, formed through a nanoscale hierarchical self-assembly process. Dendrimers can act as nanoscale carrier molecules and can be used in drug delivery [11].

Nanoparticle/Nanomaterials Toxicity

Nanomaterials have a high surface area to volume ratio compared to the same mass of material produced in larger form because the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size [13]. This leads to high surface reactivity which affects their strength and physical properties.

The small size, and subsequent larger surface area of nanoparticles, endows them with some highly useful and specific properties but, it also renders them biologically more active leading to unexpected and unanticipated

consequences on interaction with biological systems. Smaller size also imparts a different biokinetic behaviour and ability to reach more distal regions of the body [13]. The occupational exposure will also increase with the growing production and use of nanomaterials in society. Environmental contamination is yet another concern. These apprehensions have generated concerns about the potential adverse effects of engineered nanomaterials on human health and the environment.

Government/Regulatory Authorities and Environmental, Health and Safety (EHS) of Nanotechnology

Governments and scientific authorities all over the world are realizing the importance of nanomaterial risk assessment. The UK Government commissioned The Royal Society and The Royal Academy of Engineering in June 2003, to look into the ethical, health and safety issues related to nanotechnology. The Royal Society recommended in its report "Nanoscience and Nanotechnologies: Opportunities and Uncertainties" published in 2004, that "chemicals in the form of nanoparticles or nanotubes should be treated as new substances under the existing notification of new substances (NONS) regulations and in the registration, evaluation, authorisation and restriction of chemicals (REACH)" to trigger additional testing [11]. Committees on the Toxicity, Carcinogenicity and Mutagenicity of Chemicals in Food, Consumer Products and the Environment have also identified the risk assessment of nanomaterials as an area of interest in their 'Joint Statement on Nanomaterials Toxicology' [14]. United States Environmental Protection Agency (USEPA) while recognizing the potential benefits of nanotechnology has also stressed on the need for a responsible development of nanotechnology and a proactive approach. In its document—EPA 100/B-07/001 (Nanotechnology White Paper) published in 2007, it has stated "as the use of nanomaterials in society increases, it is reasonable to assume that their presence in environmental media will increase proportionately, with consequences for human and environmental exposure" [2]. The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has also reviewed the existing information/data and issues to be considered in conducting risk assessment on nanomaterials [15]. European Commission's Scientific Committee on Consumer Products (SCCP) issued a document titled "Opinion On Safety of Nanomaterials in Cosmetic Products" and raised a concern about large data gaps, inappropriateness of existing methodologies for nanoparticle risk assessment and inadequate information regarding nanoparticles skin absorption in both normal and abnormal (diseased) skins [16]. Guidance documents on safe handling of nanomaterials are also being drafted by researchers [17]. Non-governmental organisations like the Friends of the Earth warned against nanotechnology in cosmetic and sunscreen products, since they may produce a possible uptake of particles by human skin: *if nanoparticles penetrate the skin, they can join the bloodstream and circulate around the body with uptake by cells, tissues and organs* [18].

Exposure to Nanoparticles

Exposure Sources

The exposure of general population to the nanoparticles can occur either directly or indirectly.

Occupational Exposure at Work Place

In the production processes there is a likelihood of exposure during synthesis and recovery phases. The nature and probability of the exposure would differ according to the specific stage of process. Nanoparticle synthesis and manufacturing can also contribute to environmental contamination from industrial effluents or spillage during shipping and handling.

Exposure Through Consumer Products

Nanoparticles are being used in personal care products such as cosmetics and sunscreens. This provides a direct source of exposure to humans and can also enter the environment from washing off of consumer products.

Exposure Through Unintentional Release

Nanoparticles being used in electronics, tyres, fuel additives and many other products may reach the environment either through accidental leakage or during their disposal [19].

Exposure Routes

Depending on the exposure source, nanoparticles can enter the organism through different routes and can have varied health effects. If nanoparticles contaminate the air, they will mainly enter the organism by inhalation and will interact with the respiratory system. In the case of dermal exposure, nanoparticles have to pass through the skin. When soils or waters are contaminated or nanoparticles are deliberately injected or swallowed, their absorption is mainly through the gastro-intestinal or circulatory route.

Inhalation Exposure

The most common route of exposure is through inhalation. This involves intake of airborne nanoparticles through the respiratory system. Inhalation exposure of airborne

particles may occur while processing and/or packaging the dry powder during nanomaterials synthesis.

Studies on rats have shown that nanometer particles are more potent than the micrometer particles in inducing pulmonary toxicity [13]. The inhaled nanoparticles can target all three regions of the human respiratory tract—the nasopharyngeal, tracheobronchial, and alveolar regions. Any foreign body in the respiratory tract may face several clearance mechanisms. The mucociliary escalator dominates the clearance from upper airways (nasopharyngeal and tracheobronchial region); while clearance from the deep lung is predominantly by macrophage phagocytosis. The alveolar macrophage is the most important defense mechanism in the alveolar region for fine and coarse particles, yet inhaled singlet nanoparticles are not efficiently phagocytized by alveolar macrophages [13].

A study based on the inhalation exposure of rats to ultrafine (20 nm) and fine (200 nm) titanium dioxide particles, demonstrated that the ultrafine particles cleared significantly slower, showing more translocation to interstitial sites and to regional lymph nodes than the fine titanium dioxide particles [20]. The nanoparticles can be introduced into the blood circulation from the pulmonary interstitial sites. From the blood circulation they can be distributed to other target organs like liver and spleen.

Dermal Exposure

Dermal exposure to nanomaterials has received much attention, perhaps due to concerns with occupational exposure and the introduction of nanomaterials in cosmetics [21]. Dermal exposure may also occur during the equipment cleaning and maintenance.

Skin is the largest organ of the body and structured in three layers: the epidermis, the dermis, and the subcutaneous layer. It functions as a strict barrier to exogenous toxicants and micron sized particles. The efficiency of dermal barrier against nanoparticles however, is still to be investigated completely. The possible routes for nanoparticle entry through the skin are: inter-cellular, trans-cellular and trans-appendageal [22, 23]. In the intercellular route—the lipid-soluble particles may move through lipid medium passing between skin cells whereas in the trans-cellular route the substance enters the skin cells. The trans-appendageal route is through the sweat glands and hair follicles which are scattered all over the skin in different densities and may become portals for nanoparticles to get through deeper parts of the skin [24].

Gastrointestinal Exposure

Nanoparticles can be ingested directly when used in food, food packaging, drug delivery and cosmetics. Workers can be exposed by unintentional hand-to-mouth transfer of materials. Furthermore, because of their use in a wide array of consumer products, they may enter the environment by a variety of routes such as discharge of industrial waste water and disposal of nanoparticles containing products. From the environment they can enter the human body through food chain. In addition, some of the nanoparticles can be swallowed into the gastrointestinal tract when they are expelled from the mucociliary system of the lungs after inhalation exposure [13]. Nanoparticles could be translocated from the lumen of the intestinal tract into different organs. Jani et al. [25] found a particle size-dependent uptake (6.6 % of the administered 50 nm particles, 5.8 % of the 100 nm particles, 0.8 % of 1 μm particles, and 0 % for 3 μm particles) of polystyrene particles by the gastrointestinal mucosa. The particles translocated from the Pyer's patches into the mesenteric lymph and then to systemic organs (i.e., liver, spleen, blood, bone marrow, and kidney). Bockmann et al. [26] also found the uptake of the TiO_2 nanoparticles from the gastrointestinal tract into the blood.

Assessing the Biological Responses to Nanomaterial Exposure

The concerns and awareness related to health and safety aspects of nanoparticles have culminated in a number of scientific publications concerning toxicological evaluation of nanomaterials. Some of the major biological effects exhibited by different categories of nanomaterials and the methods undertaken to study them have been reviewed in this section.

Nanoparticle-Induced DNA Damage

Genotoxicity is a central element of risk assessment for any chemical compound to which humans may be routinely exposed to (e.g. in foods or personal care products). Information about genotoxicity is vital as DNA damage can not only initiate cancer development, but can also have an impact upon fertility and the health of subsequent generations if disturbances arise in reproductive cells [27]. Nanoparticles have been demonstrated to possess genotoxic potential [28] which may be attributed to following main reasons.

Direct Interactions with the DNA

Nanoparticles may gain direct access to DNA after transport into the nucleus [29]. The entry of nanoparticles into the nucleus has been demonstrated by several studies [30, 31]. The nanoparticles are unable to cross the nuclear

membrane but accumulate in the cytoplasm, can gain access to the nucleus during mitosis when the nuclear membrane breaks down [27]. The direct interaction of nanoparticles with the DNA and DNA-related protein may lead to physical damage to the genetic material. Interference with the structure or function of DNA repair enzymes in the nucleus might be another reason for DNA damage.

Oxidative DNA Damage

The nanomaterials due to their high surface area to volume ratio are known to produce reactive oxidative species (ROS). A previous study showed that the perinuclear distribution of TiO_2 nanoparticles in BEAS-2B cells correlated with the induction of ROS in the same region as visualized by a fluorescence dye. ROS can induce DNA damage in the form of single and double stranded DNA breaks, base modifications and DNA cross-links [32]. They may also produce ROS by interacting with the cellular organelles. It can be by particle-induced disturbance of the mitochondrial electron transport chain function [33]. The ROS can also be generated during particle elicited inflammation. Inflammation from activated phagocytes (macrophages, neutrophils) during the inflammation produce bursts of ROS in order to destroy invading pathogens [34]. These ROS can damage the DNA of invaded cells, and also nearby host cells that were not invaded.

The single cell gel electrophoresis, also known as Comet assay is a well established, simple, rapid, visual, sensitive procedure. It is an extensively used biomarker to assess DNA damage quantitatively as well as qualitatively in individual cell population [35]. It has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies [36], genotoxicity testing [37] and population biomonitoring [38].

The assay requires small number of cells per sample ($\sim$10,000). It can also be used to detect specific classes of DNA adducts (e.g. thymidine dimmers and oxidative damage) by using lesion specific antibodies or specific DNA repair enzymes. The collection of the data at the level of the individual cells allows robust types of statistical analysis. Over 60 studies in different cells have investigated the genotoxic effects of manufactured nanoparticles by the Comet assay and the majority of them found positive results in the form of DNA damaging potential [39–44]. Lin et al. [45] have shown the genotoxic potential of ZnO nanoparticles in the Comet assay in A549 cell line after 24 h exposure at concentrations which were significantly cytotoxic. However, the Comet assay guidelines by Tice et al. [46] suggests that in conducting in vitro Comet studies, care should be taken to avoid conditions that would lead to positive results that do not reflect genotoxicity but arise from DNA damage (i.e., double strand breaks) associated with cytotoxicity.

The micronucleus assay detects the chromosomal damage representing possible clastogenic or aneugenic activity of the test agent. When used with some actin inhibitor (e.g. cytochalasin B), it can differentiate between mononuclear and binucleated cells to give information about the background micronuclei levels. The use of some centromere specific probe in the micronucleus assay can further help in establishing the identity of micronucleus as aneugenic or clastogenic event.

The cytochalasin B which is an actin inhibitor may affect the process of endocytosis which is needed to for the cellular uptake of nanoparticles. Moreover, the potential interactions of nanoparticles with the cytochalasin B, therefore also need consideration while performing micronucleus assay with nanoparticles. When assessing the mutagenic effects of nanoparticles at high concentrations, the presence of nanoparticles on the prepared slides may hinder the visualization of micronucleus and thus disrupting the counting process [47].

The genotoxic effects of nanoparticles are not only confined to the human species but also extend to other species like bacteria. Therefore, the effects of nanomaterials on microbial systems also have been explored as they form an important component of the ecosystem and play a vital role in ecological balance. Ames test which is also known as salmonella reverse mutation assay is a well recognized genotoxicity test which has been applied to different nanoparticles [48–52]. Ames test detects mutation in single gene (point mutations) in prokaryotic cells with and without S9 fraction. The Ames test has been used for assessing the ecotoxicity of TiO_2 and ZnO nanoparticles in the *Salmonella typhimurium* and *Escherichia coli*. These nanoparticles demonstrated a weak mutagenic potential *in S. typhimurium* strains TA98, TA1537 and *E. coli* [49, 50, 53]. Some other studies have reported negative results while assessing the mutagenic potential of nanoparticles by this test [51]. However, different sources of nanoparticles, their physico-chemical characteristics, methods of nanoparticle suspension preparation (concentration, sonication etc.) may give rise to these differences.

The testing of mutagenic potential of nanoparticles by Ames test has posed several questions. The structure of bacterial and mammalian cell membrane is different and the endocytosis is supposed to play an important role in the process of nanoparticles uptake which can subsequently cause mutations. The conventional Ames test strain were made for testing the chemical mutagenesis and hence do not account the physical interactions of nanoparticles with DNA.

Cytotoxicity of Nanoparticles

Different categories of nanoparticles have been reported to cause a decrease in the cell viability [54–59]. The in vitro

test systems and assays being easy, time and cost effective are usually performed to check the effects on cell viability. Although different assays have been used to assess the cytotoxic potential of nanoparticles, however, the most commonly employed are mitochondrial dehydrogenase activity, neutral red uptake and lactate dehydrogenase (LDH) release assay.

The activity of mitochondrial dehydrogenase can be measured by a tetrazolium dye called 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide or simply MTT dye. The mitochondrial dehydrogenase activity of viable cells cleaves the tetrazolium ring, yielding purple colored crystals insoluble in aqueous solvents. After dissolving the crystals in acidified isopropanol or DMSO, the absorbance is measured by spectrophotometer [60]. The lysosomal membrane damage can be assessed by neutral red uptake assay in which the vital dye neutral red is actively transported into the lysosmes of viable cells. However in the non-viable cells the dye cannot be retained in the lysosomes and is therefore washed away [61]. The LDH release assay is based on the measurement of LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage.

The nanoparticles display several unique physicochemical properties due to which they interfere with normal test systems and this has been well documented in the literature [62–65]. Examples of such properties include: high surface area, leading to increased adsorption capacity; different optical properties that interfere with fluorescence or visible light absorption detection systems; increased catalytic activity due to enhanced surface energy and magnetic properties that make them redox active and thus interfere with methods based on redox reactions [62]. A recent study by Zaquat et al. [66] have demonstrated that TiO_2 nanoparticles bind to LDH, and consequently, TiO_2 nanoparticle-induced toxicity could be underestimated by the LDH activity assay.

Nanoparticle Internalization in Biological Systems

It has been shown that size plays a critical role in cellular uptake of nano-structures [67]. A thorough understanding of the mechanisms of nanoparticles entering and leaving the cells could lead to a better understanding of NP toxicity as well as improvement in their bio-medical applications.

Earlier studies have demonstrated that when nanoparticles are exposed to cells at 4 °C or in ATP-depleted conditions, their entry into the cells is inhibited [67, 68]. Endocytosis, therefore has been proposed as the inferred pathway for cellular uptake of nanoparticles rather than the free diffusion. It can be clathrin mediated endocytosis [69], caveolae mediated endocytosis or clathrin and caveolae independent endocytosis [70].

Accumulation of nanoparticles in the perinuclear region or internalization via caveolae-mediated endocytosis to reach endoplasmic reticulum may facilitate physical contact of nanoparticles with nuclear pore complexes and subsequent transport into the nucleus [71]. The phagocytosis is unlikely to contribute to the nanoparticles uptake into the cells. This is evident from the fact that even non-phagocytic cells can efficiently internalize nanoparticles and the lower size cut-off described for phagocytosis is 500 nm [72].

Tracking nanoparticle internalization in cellular systems is of utmost importance for understanding and correlating the biological effects elicited by these nanoparticles.

Transmission electron microscopy provides a detailed view of the interaction of nanoparticles with cell structures. Due to its high resolution, transmission electron microscopy enables the imaging of membrane invaginations, vesicle formation, and organelles [73] which helps in understanding the mode of nanoparticle uptake. It also aids in understanding the ultrastructural changes that occur in cells subsequent to nanoparticle uptake [74, 75]. However, transmission electron microscopy is only a qualitative tool for assessing nanoparticle uptake, and is usually confined to imaging a few cells due to the complicated sample preparation and image analysis involved [76]. Further, the staining procedures generally used for electron microscopic preparations can introduce electron-dense artefacts that may be mistaken for nanoparticles.

Inductively coupled plasma mass spectroscopy (ICPMS) can be used as a sensitive and quantitative tool for the determination of trace amounts of nanoparticles. ICPMS becomes especially important in the context of an in vivo scenario, where it identifies the target organs for nanoparticles [77]. Using this technique, even trace amounts of nanoparticles that enter through a different route can be detected in various body organs.

Flow cytometry is a simple, easy and sensitive method used to study nanoparticle uptake in bacterial and mammalian cells [78–82]. It can be used for the detection of fluorescent as well as non-fluorescent nanoparticles inside the cells.

In Vivo Toxicity of Nanoparticles

The toxicity assessment of any novel entity in vivo is always desirable. This is due to the complex cell–cell, cell–matrix interactions, diversity of cell types, hormonal effects that are hard to mimic in the in vitro systems. In addition, studying long term chronic effects of the test

compound is possible only by in vivo experiments. The importance of the in vivo studies in nanomaterial toxicology has been highlighted [83].

There are no existing guidelines and standard methodologies for risk assessment of nanomaterials. However, the "committees on toxicity (COT), mutagenicity (COM) and carcinogenicity (COC) of chemicals in food, consumer products and the environment, UK" have suggested extrapolating the in vitro nanotoxicity findings to in vivo experiments and confirm the results [14]. The same committees (COT, COM and COC) have also advised to consider appropriate route of exposure in the in vivo experiments.

Many investigations have studied the in vivo effects of nanoparticles by suitable systems and reported accumulation in organs like liver, adverse effects on DNA, immune system and neurobehavioral patterns [84–87].

In vivo studies with nanomaterials, unlike studies involving chemicals/compounds, are interlaced with many challenges. The in vivo dose used for experiments should be derived from the quantity of nanoparticles exposed to in the actual scenario. However, determining the quantity of nanoparticles in air, water, soil or any consumer product is a technical challenge due to their tiny size and the small quantity present. Even if the dose of nanoparticles is known, exceeding a certain dose in experiments is not advisable due to increased agglomeration of nanoparticles. The biodosimetry or biodistribution of nonfluorescent, nonradioactive, nonmagnetic nanoparticles is almost impossible to investigate with the presently available techniques.

Conclusion

The safety/toxicity aspects of nanomaterials have lagged far behind the rate at which they are being produced. A multidisciplinary team effort from material scientists, molecular biologists, toxicologists and physicists is necessary as it will facilitate the interlinking of different facets of nanotoxicology thus aiding in the understanding of cellular responses to nanomaterials exposure and mechanisms involved.

There is also a need for more comprehensive studies to fully understand and address the potential risks of engineered nanomaterials to human health and the environment. This will help in creating environment friendly and biologically safe nanoparticles.

References

1. NCPI (Nanotechnology Consumer Products Inventory) (2011) Available at: http://www.nanotechproject.org
2. USEPA (2007) Nanotechnology white paper. Science Policy Council, United States Environmental Protection Agency. EPA 100/B-07/001
3. PAS71 (Publicly Available Specification) (2005) Vocabulary-nanoparticles. British Standards Institution (BSI)
4. NRC (National Research Counil) (1983) Risk assessment in the Federal Government: managing the process. National Academy Press, Washington, DC
5. Kulmala M (2004) Formation and growth rates of ultrafine atmospheric particles: a review of observations. J Aerosol Sci 35:143–176
6. NNI (National Nanotechnology Initiative) (2004) What is nanotechnology? Available at: http://www.nano.gov/html/facts/whatIsNano.html
7. Buffle J (2006) The key role of environmental colloids/nanoparticles for the sustainability of life. Environ Chem 3:155–158
8. Nowack B, Bucheli TD (2007) Occurrence, behavior and effects of nanoparticles in the environment. Environ Pollut 150:5–22
9. Theng BKG, Yuan G (2008) Nanoparticles in the soil environment. Elements 4:395–399
10. Xia L, Lenaghan SC, Zhang M, Zhang Z, Li Q (2010) Naturally occurring nanoparticles from English ivy: an alternative to metal-based nanoparticles for UV protection. J Nanobiotechnol 8:12
11. RS/RAE (The Royal Society and the Royal Academy of Engineering) (2004) Nanoscience and nanotechnologies: opportunities and uncertainties. Royal Society Policy Document 19/04
12. Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ Health Perspect 114:165–172
13. Oberdorster G, Oberdorster E, Oberdorster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839
14. COT, COM, COC (2005) Joint statement on nanomaterial toxicology. http://cot.food.gov.uk/pdfs/cotstatements2005nanomats.pdf
15. SCENIHR (European Commission Scientific Committee on Emerging and Newly Identified Health Risks) (2009) Risk assessment of products of nanotechnologies. European Commission
16. SCCP (Scientific Committee on Consumer Products) (2007) Opinion on safety of nanomaterials in cosmetic products. European Commission, SCCP/1147/07
17. Dhawan A, Shanker R, Das M, Gupta KC (2011) Guidance for safe handling of nanomaterials. J Biomed Nanotechnol 7:218–224
18. FOE (2006) Nanomaterials, sunscreens and cosmetics: small ingredients, big risks. Friends of the Earth, United States. Available at: http://www.foe.org
19. Simonelli A, Miraglia N, Basilicata P, Pieri M, Loredana C, Genovese G, Guadagni R, Acampora A, Sannolo N (2007) Interactions between nanoparticles and living organisms: mechanisms and health effects. In: Zhao Y, Nalwa HS (eds) Nanotoxicology. American Scientific, USA
20. Oberdorster G, Ferin J, Lehnert BE (1994) Correlation between particle size, in vivo particle persistence, and lung injury. Environ Health Perspect 102(Suppl 5):173–179
21. Wiesenthal A, Hunter L, Wang S, Wickliffe J, Wilkerson M (2011) Nanoparticles: small and mighty. Int J Dermatol 50:247–254
22. Baroli B, Ennas MG, Loffredo F, Isola M, Pinna R, Lopez-Quintela MA (2007) Penetration of metallic nanoparticles in human full-thickness skin. J Invest Dermatol 127:1701–1712
23. Crosera M, Bovenzi M, Maina G, Adami G, Zanette C, Florio C, Filon Larese F (2009) Nanoparticle dermal absorption and toxicity: a review of the literature. Int Arch Occup Environ Health 82:1043–1055
24. Zhao Y, Meng H, Chen Z, Zhao F, Chai Z (2007) Chapter 1: biological activities of nanomaterials/nanoparticles. In: Zhao Y, Nalwa HS (eds) Nanotoxicology. American Scientific, USA

25. Jani P, Halbert GW, Langridge J, Florence AT (1990) Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J Pharm Pharmacol 42:821–826
26. Bockmann J, Lahl H, Eckert T, Unterhalt B (2000) Blood titanium levels before and after oral administration titanium dioxide. Pharmazie 55:140–143
27. Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffeis TG, Wright CJ, Doak SH (2009) NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30:3891–3914
28. Karlsson HL (2010) The comet assay in nanotoxicology research. Anal Bioanal Chem 398:651–666
29. Chen M, Mikecz AV (2005) Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. Exp Cell Res 305:51–62
30. Alkilany AM, Murphy CJ (2010) Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? J Nanopart Res 12:2313–2333
31. Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S, Dhawan A (2011) ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicol In Vitro 25:231–241
32. Toyokuni S (1998) Oxidative stress and cancer: the role of redox regulation. Biotherapy 11:147–154
33. Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J, Nel A (2003) Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environ Health Perspect 111:455–460
34. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC (2007) TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 26:2166–2176
35. Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26:249–261
36. Speit G, Hartmann A (2006) The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol 314:275–286
37. Moller P (2005) Genotoxicity of environmental agents assessed by the alkaline comet assay. Basic Clin Pharmacol Toxicol 96(Suppl 1):1–42
38. Garcia-Leston J, Roma-Torres J, Vilares M, Pinto R, Cunha LM, Prista J, Teixeira JP, Mayan O, Pasaro E, Mendez J, Laffon B (2011) Biomonitoring of a population of Portuguese workers exposed to lead. Mutat Res 721:81–88
39. Sharma V, Shukla RK, Saxena N, Parmar D, Das M, Dhawan A (2009) DNA damaging potential of zinc oxide nanoparticles in human epidermal cells. Toxicol Lett 185:211–218
40. Vallabani NV, Mittal S, Shukla RK, Pandey AK, Dhakate SR, Pasricha R, Dhawan A (2011) Toxicity of graphene in normal human lung cells (BEAS-2B). J Biomed Nanotechnol 7:106–107
41. Sharma V, Anderson D, Dhawan A (2011) Zinc oxide nanoparticles induce oxidative stress and genotoxicity in human liver cells (HepG2). J Biomed Nanotechnol 7:98–99
42. Hackenberg S, Scherzed A, Technau A, Kessler M, Froelich K, Ginzkey C, Koehler C, Burghartz M, Hagen R, Kleinsasser N (2011) Cytotoxic, genotoxic and pro-inflammatory effects of zinc oxide nanoparticles in human nasal mucosa cells in vitro. Toxicol In Vitro 25:657–663
43. Kim HR, Kim MJ, Lee SY, Oh SM, Chung KH (2011) Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells. Mutat Res 726:129–135
44. Nabeshi H, Yoshikawa T, Matsuyama K, Nakazato Y, Tochigi S, Kondoh S, Hirai T, Akase T, Nagano K, Abe Y, Yoshioka Y, Kamada H, Itoh N, Tsunoda S, Tsutsumi Y (2011) Amorphous nanosilica induce endocytosis-dependent ROS generation and DNA damage in human keratinocytes. Part Fibre Toxicol 8:1
45. Lin W, Xu Y, Huang C, Ma Y, Shannon KB, Chen D, Huang Y (2009) Toxicity of nano- and micro-sized ZnO particles in human lung epithelial cells. J Nanopart Res 11:25–39
46. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221
47. Gonzalez L, Sanderson BJ, Kirsch-Volders M (2011) Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis 26:185–191
48. Balasubramanyam A, Sailaja N, Mahboob M, Rahman MF, Hussain SM, Grover P (2010) In vitro mutagenicity assessment of aluminium oxide nanomaterials using the *Salmonella*/microsome assay. Toxicol In Vitro 24:1871–1876
49. Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. Chemosphere 83:1124–1132
50. Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) Cellular response to metal oxide nanoparticles in bacteria. J Biomed Nanotechnol 7:102–103
51. Yoshida R, Kitamura D, Maenosono S (2009) Mutagenicity of water-soluble ZnO nanoparticles in Ames test. J Toxicol Sci 34:119–122
52. Shinohara N, Matsumoto K, Endoh S, Maru J, Nakanishi J (2009) In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicol Lett 191:289–296
53. Kumar A, Dhawan A, Shanker R (2011) The need for novel approaches in ecotoxicity of engineered nanomaterials. J Biomed Nanotechnol 7:79–80
54. Albers CE, Hofstetter W, Siebenrock KA, Landmann R, Klenke FM (2011) In vitro cytotoxicity of silver nanoparticles on osteoblasts and osteoclasts at antibacterial concentrations. Nanotoxicology. doi:10.3109/17435390.2011.626538
55. Kang SJ, Ryoo IG, Lee YJ, Kwak MK (2012) Role of the Nrf2-heme oxygenase-1 pathway in silver nanoparticle-mediated cytotoxicity. Toxicol Appl Pharmacol 258:89–98
56. Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) Engineered ZnO and TiO(2) nanoparticles induce oxidative stress and DNA damage leading to reduced viability of Escherichia coli. Free Radic Biol Med 51:1872–1881
57. Mittal S, Sharma V, Vallabani NV, Kulshrestha S, Dhawan A, Pandey AK (2011) Toxicity evaluation of carbon nanotubes in normal human bronchial epithelial cells. J Biomed Nanotechnol 7:108–109
58. Sharma V, Singh SK, Anderson D, Tobin DJ, Dhawan A (2011) Zinc oxide nanoparticle induced genotoxicity in primary human epidermal keratinocytes. J Nanosci Nanotechnol 11:3782–3788
59. Shukla RK, Kumar A, Pandey AK, Singh SS, Dhawan A (2011) Titanium dioxide nanoparticles induce oxidative stress-mediated apoptosis in human keratinocyte cells. J Biomed Nanotechnol 7:100–101
60. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
61. Borenfreund E, Puerner JA (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett 24:119–124
62. Kroll A, Pillukat MH, Hahn D, Schnekenburger J (2009) Current in vitro methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm 72:370–377
63. Monteiro-Riviere NA, Inman AO (2006) Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon 44:1070–1078

64. Monteiro-Riviere NA, Inman AO, Zhang LW (2009) Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 234:222–235
65. Pulskamp K, Diabate S, Krug HF (2007) Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol Lett 168:58–74
66. Zaqout MS, Sumizawa T, Igisu H, Wilson D, Myojo T, Ueno S (2012) Binding of titanium dioxide nanoparticles to lactate dehydrogenase. Environ Health Prev Med 17:341–345
67. Chithrani BD, Chan WC (2007) Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Lett 7:1542–1550
68. Torchilin VP, Rammohan R, Weissig V, Levchenko TS (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc Natl Acad Sci USA 98:8786–8791
69. Panyam J, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V (2002) Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J 16:1217–1226
70. Rejman J, Oberle V, Zuhorn IS, Hoekstra D (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 377:159–169
71. Chen M, Mikecz AV (2007) Uptake and cytotoxicity of nanoparticles. In: Zhao Y, Nalwa HS (eds) Nanotoxicology. American Scientific, USA
72. Rupper A, Cardelli J (2001) Regulation of phagocytosis and endo-phagosomal trafficking pathways in *Dictyostelium discoideum*. Biochim Biophys Acta 1525:205–216
73. Motskin M, Wright DM, Muller K, Kyle N, Gard TG, Porter AE, Skepper JN (2009) Hydroxyapatite nano and microparticles: correlation of particle properties with cytotoxicity and biostability. Biomaterials 30:3307–3317
74. Song MM, Song WJ, Bi H, Wang J, Wu WL, Sun J, Yu M (2010) Cytotoxicity and cellular uptake of iron nanowires. Biomaterials 31:1509–1517
75. Xie G, Sun J, Zhong G, Shi L, Zhang D (2009) Biodistribution and toxicity of intravenously administered silica nanoparticles in mice. Arch Toxicol 84:183–190
76. Dhawan A, Sharma V (2010) Toxicity assessment of nanomaterials: methods and challenges. Anal Bioanal Chem 398:589–605
77. Tang J, Xiong L, Wang S, Wang J, Liu L, Li J, Yuan F, Xi T (2009) Distribution, translocation and accumulation of silver nanoparticles in rats. J Nanosci Nanotechnol 9:4924–4932
78. Missirlis D, Hubbell JA (2009) In vitro uptake of amphiphilic, hydrogel nanoparticles by J774A.1 cells. J Biomed Mater Res A. doi:10.1002/jbm.a.32648
79. Suzuki H, Toyooka T, Ibuki Y (2007) Simple and easy method to evaluate uptake potential of nanoparticles in mammalian cells using a flow cytometric light scatter analysis. Environ Sci Technol 41:3018–3024
80. Wang Y, Wu W (2006) In situ evading of phagocytic uptake of stealth solid lipid nanoparticles by mouse peritoneal macrophages. Drug Deliv 13:189–192
81. Xu A, Chai Y, Nohmi T, Hei TK (2009) Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. Part Fibre Toxicol 6:3
82. Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A (2011) A flow cytometric method to assess nanoparticle uptake in bacteria. Cytometry A 79:707–712
83. Fischer HC, Chan WC (2007) Nanotoxicity: the growing need for in vivo study. Curr Opin Biotechnol 18:565–571
84. Abdelhalim MA, Mady MM (2011) Liver uptake of gold nanoparticles after intraperitoneal administration in vivo: a fluorescence study. Lipids Health Dis 10:195
85. Pasupuleti S, Alapati S, Ganapathy S, Anumolu G, Pully NR, Prakhya BM (2012) Toxicity of zinc oxide nanoparticles through oral route. Toxicol Ind Health 28:675–686
86. Zhang QL, Li MQ, Ji JW, Gao FP, Bai R, Chen CY, Wang ZW, Zhang C, Niu Q (2011) In vivo toxicity of nano-alumina on mice neurobehavioral profiles and the potential mechanisms. Int J Immunopathol Pharmacol 24:23S–29S
87. Zhang XD, Wu D, Shen X, Liu PX, Yang N, Zhao B, Zhang H, Sun YM, Zhang LA, Fan FY (2011) Size-dependent in vivo toxicity of PEG-coated gold nanoparticles. Int J Nanomedicine 6:2071–2081