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Review

Nanotoxicology and *in vitro* studies: The need of the hour

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ABSTRACT

Nanotechnology is considered as one of the key technologies of the 21st century and promises revolution in our world. Objects at nano scale, take on novel properties and functions that differ markedly from those seen in the corresponding bulk counterpart primarily because of their small size and large surface area. Studies have revealed that the same properties that make nanoparticles so unique could also be responsible for their potential toxicity. Nanotechnology is rapidly advancing, with more than 1000 nanoproducts already on the market. Considering the fact that intended as well as unintended exposure to nanomaterials is increasing and presently no clear regulatory guideline(s) on the testing/evaluation of nanoparticulate materials are available, the *in vitro* toxicological studies become extremely relevant and important. This review presents a summary of nanotoxicology and a concise account of the *in vitro* toxicity data on nanomaterials. For nanomaterials to move into the applications arena, it is important that nanotoxicology research uncovers and understands how these multiple factors influence their toxicity so that the ensuing undesirable effects can be avoided.

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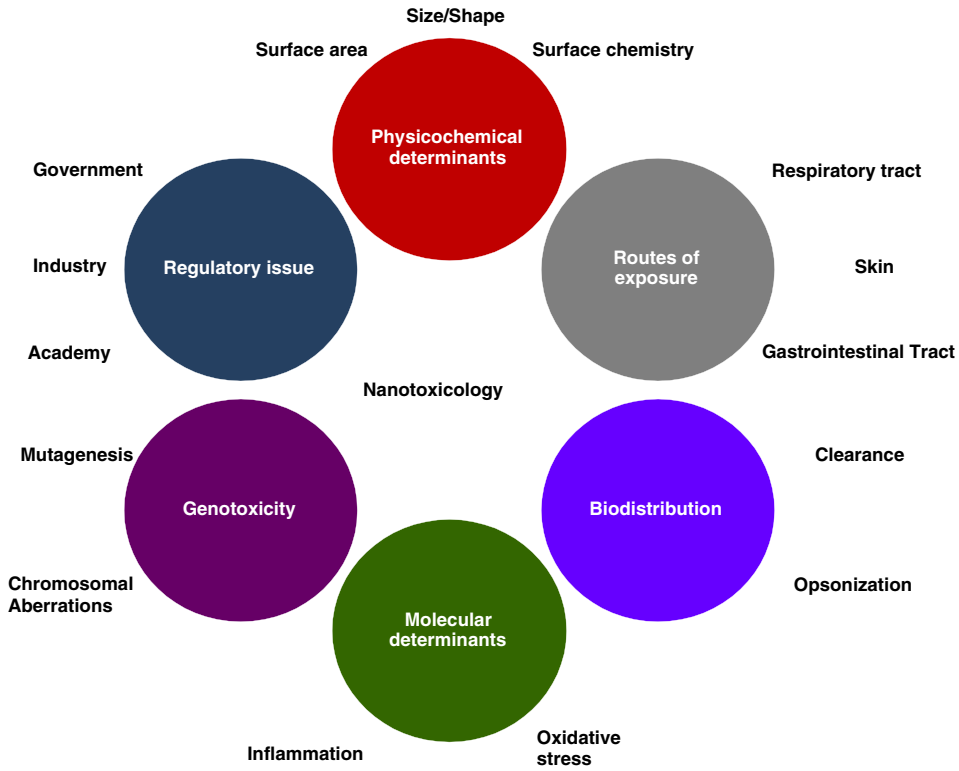

Fig. 1. Complex array of issues surrounding toxicity of nanoparticles.

Introduction

The prefix "nano" is derived from the Greek word "nanos" meaning "dwarf". Nanotechnology involves the manipulation and application of engineered particles or systems that have at least one dimension less than 100 nanometers (nm) in length (Hoyt and Mason, 2008). The term "nanoparticles" applies only to engineered particles (such as metal oxides, carbon nanotubes, fullerenes etc.) and does not apply to particles under 100 nm that occur naturally or are by-products of other processes such as welding fumes, fire smoke, or carbon black (Hoyt and Mason, 2008).

Growing exploration of nanotechnology has resulted in the identification of many unique properties of nanomaterials such as enhanced magnetic, catalytic, optical, electrical, and mechanical properties when compared to conventional formulations of the same material (Ferrari, 2005; Qin et al., 1999; Vasir et al., 2005; Webster et al., 1999, 2000). These materials are increasingly being used for commercial purposes such as fillers, opacifiers, catalysts, water filtration, semiconductors, cosmetics, microelectronics etc. leading to direct and indirect exposure in humans (Nel et al., 2006). Apart from the use of nanomaterials in consumer products, numerous applications are being reported in the biomedical field, especially as drug-delivery agents, biosensors or imaging contrast agents (Ferrari, 2005; Vasir et al., 2005). The applications pertaining to medicine involve deliberate direct ingestion or injection of nanoparticles into the body. Nanomaterials for imaging and drug delivery are often intentionally coated with biomolecules such as DNA, proteins, and monoclonal antibodies to target specific cells (Lewinski et al., 2008). Materials in this size range may approach the length scale at which some specific physical or chemical interactions with their environment can occur (Oberdorster et al., 2005a). Apart from this, due to their extremely small size, nanomaterials possess extremely high surface area to volume ratio which renders them highly reactive. High reactivity potentially could lead to toxicity due to harmful interactions of nanomaterials with biological systems and the environment (Oberdorster et al., 2005b).

Any *in vivo* use of nanoparticles entails thorough understanding of the kinetics and toxicology of the particles (Lewinski et al., 2008), establishment of principles and test procedures to ensure safe manufacture and usage of nanomaterials (Nel et al., 2006), and comprehensive information about their safety and potential hazard (Nel et al., 2006; Oberdorster et al., 2005b).

Nanotoxicology

Nanotoxicology was proposed as a new branch of toxicology to address the gaps in knowledge and to specifically address the adverse health effects likely to be caused by nanomaterials (Donaldson et al., 2004). In the original article on nanotoxicology, Donaldson et al. (2004) quoted, "discipline of nanotoxicology would make an important contribution to the development of a sustainable and safe nanotechnology".

Nanotoxicology encompasses the physicochemical determinants, routes of exposure, biodistribution, molecular determinants, genotoxicity, and regulatory aspects (Fig. 1). In addition, nanotoxicology is involved in proposing reliable, robust, and data-assured test protocols for nanomaterials in human and environmental risk assessment (Donaldson et al., 2004; Lewinski et al., 2008).

Physicochemical properties of nanomaterials: biological effects

The unusual physicochemical properties of engineered nanomaterials are attributable to their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings etc.), solubility, shape and aggregation. Actually, the very same properties that lead to the technical advantages of nanotechnology also lead to unique biological effects (Nel et al., 2006). In a review by Nel et al. (2006) a question, "Do nanomaterials properties necessitate a new toxicological science?" was raised. It was argued that the main characteristic of nanomaterials is their size in the transitional zone between individual atoms or

Table 1
Biological effects due to physicochemical properties of nanomaterials.

Physicochemical property		Toxicokinetic findings	Biological effects	References
Size	15 nm gold nanoparticles (NPs)	Most widespread organ distribution including blood, liver, lung, spleen, kidney, brain, heart, stomach in mice	Biodistribution of the nanoparticles	Sonavane et al. (2008)
	15 and 50 nm gold NPs	Pass blood–brain barrier (BBB) in mice	Blood Brain Barrier (BBB) permeability	Sonavane et al. (2008)
	40–50 nm gold NPs	Activation of membrane receptors in SK-BR-3 cells		Jiang et al. (2008)
	50 nm gold NPs	Maximum uptake by Hela cells		Chithrani et al. (2006)
	50 nm quantum dots	Efficient receptor-mediated endocytosis in Hela cells		Osaki et al. (2004)
	1–10 nm silver NPs		Exclusively attach to HIV-1	Elechiguerra et al. (2005)
	1–10 nm silver NPs	Penetrate inside the bacteria		Morones et al. (2005)
Shape	Open-ended Single-walled carbon nanotubes (SWNTs)	Efficient blocking of ion channels in CHO cells	Spherical shaped close-ended SWNTs are comparatively less reactive	Park et al. (2003)
	Spherical gold NPs	Higher uptake by Hela cells	Rrod-shaped gold NPs showed less uptake	Chithrani et al. (2006)
	Carbon particles, except C60CS,	Stimulated human platelet aggregation *in vitro* and accelerated the rate of vascular thrombosis in rat carotid arteries	Biological reactivity: mixed carbon nanoparticles (MCNs) ≥ single-walled carbon nanotubes (SWNTs) > multi-walled carbon nanotubes (MWNTs)	Radomski et al. (2005)
	Filomicelles (Filamentous micelles)		More efficient for drug delivery than their spherical counterparts in rats and mice	Geng et al. (2007)
Surface area/ volume ratio	TiO2 (300 cm^2 Surface area)	Increased lymph-node burdens and Inflammation	More reactive in rats as compared to $BaSO_4$ (200 cm^2 Surface area)	Tran et al. (2000)
	TiO2 and $BaSO_4$ With same surface area	Inflammatory effects were similar	Inflammation	Tran et al. (2000)
	Ultrafine carbon black particles (270 m^2/g surface area)	Cause greater pulmonary toxicity in rats	Increased reactivity in comparison with larger-sized carbon black particles (22 m^2/g surface area)	Nikula et al. (1995), Driscoll et al. (1996)
Chemical composition	Incorporation of 1% (w/w) manganese doping into titania particles	Increase in UVA absorption and reduction in free radical generation *via* surface reactions		Wakefield et al. (2004)
	Carbon nanomaterials	Different geometric structures exhibit quite different cytotoxicity *in vitro*	The cytotoxicity follows a sequence order: SWNTs > MWNTs > quartz > C_{60} on alveolar macrophages isolated from guinea pigs	Guang et al. (2005)
	Metal traces associated with the commercial carbon nanotubes	A dose- and time-dependent increase of intracellular reactive oxygen species and a decrease of the mitochondrial membrane potential in rat macrophages (NR8383) and human A549 lung cells	More reactive as compared to purified carbon nanotubes	Pulskamp et al. (2007)
	Quantum dots core metalloid complexes of Cadmium, Cd	can cross the blood–brain barrier and placenta, and is systemically distributed to all bodily tissues, with liver and kidney being target organs of toxicity	A probable carcinogen	Hardman (2006)
	Quantum dots core metalloid complexes of Selenium, Se		A marked impact on the local ecosystem resulted from elevated environmental concentrations of Se	Hardman (2006)
	Ag, MoO_3, Fe_3O_4, Al, MnO_2 and W (Tungsten)	Ag was highly toxic whereas, MoO_3 moderately toxic and Fe_3O_4, Al, MnO_2 and W (Tungsten) displayed less or no toxicity at the doses tested on *in vitro* rat liver derived cell line (BRL 3A)	Reduced cell proliferation and death	Hussain et al. (2005)
	$La_{0.7}Sr_{0.3}MnO_3$ (LSMO) nanoparticles doped with cerium ($La_{0.7-x}Ce_xSr_{0.3}MnO_3$ where $0 \leq x \leq 0.7$) and $La_{1-y}Sr_yMnO_3$ nanoparticles with different values of y (La/Sr ratio)	Low cytotoxicity in Ce-doped samples as well as in samples with reduced La/Sr ratio as revealed by *in vitro* studies on HT-1080 (human fibrosarcoma) and A431 (human skin/carcinoma) cells	Improved cell proliferation upon Ce doping	Kale et al. (2006)
Surface charge	Neutral NPs and low concentration anionic NPs Cationic NPs		Drug delivery applications to brain in rats	Lockman et al. (2004)

(continued on next page)

Table 1 (*continued*)

Physicochemical property		Toxicokinetic findings	Biological effects	References
			Toxic effect at the blood brain barrier in rats	Lockman et al. (2004)
	Anionic NPs at lower concentrations		Superior uptake rates as compared to neutral or cationic NPs at the same concentrations in rats	Lockman et al. (2004)
	Positive surface charged poly (amidoamine) dendrimers	Deposition into tissues is higher than neutral surface dendrimers in B16 melanoma and DU 145 human prostate cancer mouse tumor model	Higher deposition in tissues	Nigavekar et al. (2004)
	Coating of respirable quartz surface with aluminum lactate or polyvinyl-pyridine-*N*-oxide (PVNO)	Inhibits DNA strand breakage and formation of 8-hydroxy-deoxyguanosine in human lung epithelial cells	Reduction in toxicity	Schins et al. (2002)
Aggregation state	Rope-like agglomerates of carbon nanotubes	Induced more pronounced cytotoxic effects than well dispersed carbon nanotubes in human MSTO-211H cells	Cytotoxicity	Wick et al. (2007)

molecules and the corresponding bulk materials. This can modify the physicochemical properties of the material as well as create the opportunity for increased uptake and interaction with biological tissues (Chithrani et al., 2006; Sonavane et al., 2008). This combination of effects can generate adverse biological responses in living cells otherwise not seen with the same material in larger (bulk) form (Nel et al., 2006). The increase in surface area determines the potential number of reactive groups on the particle surface. Table 1 summarizes the observed biological effects vis-à-vis physicochemical properties and the types of nanomaterials. Shape of the nanoparticles has been shown to have a pronounced effect on the biological activity. It is reported that silver nanoparticles undergo shape-dependent interaction with *E. coli* (Pal et al., 2007); Chithrani et al. (2006) reported better uptake of spherical gold nanoparticles than gold nanorods in HeLa cells. In case of anatase TiO_2 nanomaterial, it was shown that alteration to a fiber structure of greater than 15 μm created a highly toxic particle that initiated an inflammatory response by alveolar macrophages and that length may be an important determinant of nanomaterial biocompatibility (Hamilton et al., 2009). Another study by Journeay et al. (2008) demonstrated that water-soluble rosette nanotube structures display low pulmonary toxicity due to their biologically inspired design and self-assembled architecture. In a review on widely used metal oxide and carbon nanomaterials, Landsiedel et al. (2010) emphasized that physico-chemical characterization of nanomaterials and their interaction with biological media are essential for reliable studies. In a study with 1.5 nm sized gold nanoparticles it was observed that surface charge was a major determinant of their action on cellular processes; the charged NPs inducing cell death through apoptosis and neutral NPs leading to necrosis in HaCaT cells (Schaeublin et al., 2011). Considering the physicochemical properties of various nanomaterials and their interactions with the biological environment, Maynard et al. (2011) state that the challenges presented by simple nanoscale materials such as TiO_2, ZnO, Ag, carbon nanotubes, and CeO_2 are now beginning to be appreciated. But these simple materials are merely the vanguard of a new era of complex materials, where novel and dynamic functionality is engineered into multifaceted substances. Further, according to Maynard et al. (2011), if we are to meet the challenge of ensuring the safe use of this new generation of substances, it is time to move beyond "nano" toxicology and toward a new toxicology of sophisticated materials.

Thus it is evident that physicochemical characteristics of the materials are very important with respect to the observed biological effects.

Routes of exposure

The human body has several semi-open interfaces for direct substance exchange with the environment, *i.e.* the skin, respiratory tract and gastrointestinal tract (GIT).

Skin. Skin is the largest primary defense organ in our body and directly comes into contact with many toxic agents. The skin is structured organ comprising three layers: the epidermis, the dermis and the subcutaneous layer. The strongly keratinized *stratum corneum* acts as the primary protecting layer and may be the rate-limiting barrier to defend against the penetration of most micron sized particles and harmful exogenetic toxicants. Skin exposure to nanomaterials can also occur during the intentional application of topical creams and other drug treatments (Curtis et al., 2006; Hagens et al., 2007; Oberdorster et al., 2005b). According to a study by van der Merwe et al. (2009), nanocrystalline magnesium oxide and titanium dioxide applied to dermatomed human skin (as dry powder, water suspension, and water/surfactant suspension) for 8 h did not show dermal absorption through human skin with intact functional stratum corneum. In another study, Gontier et al. (2008) tested penetration of topically applied titanium dioxide (TiO_2) nanoparticles (size range 20–100 nm) in porcine-, healthy human-, and human grafted-skin samples. It was seen that penetration of TiO_2 nanoparticles was restricted to the topmost 3–5 corneocyte layers of the *stratum comeum*. In contradistinction to this finding, there are many reports that show deeper penetration of nanoparticles. Lademann et al. (1999) showed that TiO_2 particles could get through the human *stratum corneum* and reach epidermis and even dermis. Flexing movement of normal skin was shown to facilitate the penetration of micrometer-size fluorescent beads into the dermis (Tinkle et al., 2003). Oberdorster et al. (2005b) demonstrated penetration of a variety of nanoparticles in the dermis and translocation to the systemic vasculature *via* lymphatic system and regional lymph. Further, Ryman-Rasmussen et al. (2006) demonstrated that quantum dots with diverse physicochemical properties could penetrate the intact *stratum corneum* barrier and get localized within the epidermal and dermal layers. In a clinical study, treatment of burns using nanosilver coated dressings (Trop et al., 2006) led to abnormal elevation of blood silver levels and argyria (blue or gray discoloration of the skin due to silver accumulation in the body over time which is a 'cosmetic problem'). Though nanosilver-based dressings and surgical sutures have received approval for clinical application and good control of wound infection is achieved, their dermal toxicity is still a topic of scientific debate and concern. Despite laboratory and clinical studies confirming the dermal biocompatibility of nanosilver-based dressings (Chen et al., 2006a, 2006b; Muangman et al., 2006; Supp et al., 2005; Wright et al., 2002) several other researchers have demonstrated the cytotoxicity of these materials. Paddle-Ledinek et al. (2006) exposed cultured keratinocytes to extracts of several types of silver containing dressings. Of these, extracts of nanocrystalline silver coated dressings were most cytotoxic. Similar observations were also reported by Lam et al. (2004) in another study. Fullerene-based peptides were also shown to be capable of penetrating intact skin and mechanical stressors could facilitate their traversal into the dermis (Rouse et al., 2007). Intradermally administered quantum dots could enter subcutaneous lymphatics (Gopee et al.,

2007) and regional lymph nodes (Kim et al., 2004). Topically applied fine and ultrafine beryllium particles can be phagocytosed by macrophages and Langerhans cells possibly leading to perturbations of the immune system (Tinkle et al., 2003). Epidermal keratinocytes have also been shown to be capable of phagocytosing a variety of engineered nanoparticles and setting off inflammatory responses (Monteiro-Riviere et al., 2005). It is worth noting that some other types of nanoparticles, *i.e.* single-/multi-wall carbon nanotubes, quantum dots with surface coating and nanoscale titania, have been shown to have toxic effects on epidermal keratinocytes and fibroblasts and are capable of altering their gene/protein expression (Christie et al., 2006; Ding et al., 2005; Monteiro-Riviere et al., 2005; Ryman-Rasmussen et al., 2006; Sarkar et al., 2007; Tian et al., 2006; Witzmann and Monteiro-Riviere, 2006; Zhang et al., 2007).

Respiratory tract. The respiratory system serves as a major portal for ambient particulate materials. Pathologies resulting from airborne particle materials, *e.g.* quartz, asbestos and carbon have long been thoroughly researched in occupational and environmental medicine (Alfaro-Moreno et al., 2007; Donaldson et al., 2001; Gillissen et al., 2006; Kanj et al., 2006; Lam et al., 2006; Ovrevik et al., 2005; Parks et al., 1999; Warheit, 2001). Recently, the pathogenic effects and pathology of inhaled manufactured nanoparticles have received attention (Donaldson et al., 2006; Lam et al., 2006; Nel et al., 2006; Oberdorster et al., 2005a). Being different than micron sized particles that are largely trapped and cleared by upper airway mucociliary escalator system, particles less than 2.5 μm can get down to the alveoli. The deposition of inhaled ultrafine particles (aerodynamic-diameter <100 nm) mainly takes place in the alveolar region (Curtis et al., 2006; Hagens et al., 2007). After absorption across the lung epithelium, nanomaterials can enter the blood and lymph to reach cells in the bone marrow, lymph nodes, spleen and heart (Hagens et al., 2007; Oberdorster et al., 2005a). The latter could be of significance since the association between inhaled ambient ultrafine particles and cardiovascular events such as coagulation and cardiac rhythm disturbances has been proven (Nurkiewicz et al., 2006; Yeates and Mauderly, 2001). Other targets after translocation include the sensory nerve endings embedded in the airway epithelia, followed by ganglia and the central nervous system *via* axons (Oberdorster et al., 2005b; Oldfors and Fardeau, 1983). Takenaka et al. (2001) have demonstrated that in both inhalation and instillation experiments, ultrafine silver particles were taken up by alveolar macrophages and aggregated silver particles persisted there for up to 7 days. Aggregated silver nanoparticles and some other nanomaterials have been shown to be cytotoxic to alveolar macrophage cells as well as epithelial lung cells (Soto et al., 2007).

Gastrointestinal tract (GIT). Nanomaterials can reach the GIT after mucociliary clearance from the respiratory tract through the nasal region, or can be ingested directly in food, water, cosmetics, drugs, and drug delivery devices (Hagens et al., 2007; Oberdorster et al., 2005b). The utility of biodegradable nanoparticles in the delivery of oral vaccines has been proposed for antigens known to be susceptible to proteolysis (Russell-Jones, 2000). Apparently studies on toxicity of nanomaterials post oral ingestion are limited. Chen et al. (2006a, 2006b) determined the acute toxicity of copper particles (bulk) and nanocopper in mice and found that nanocopper was several folds toxic than bulk copper (LD_{50} for nanocopper 413 mg/kg; bulk copper >5000 mg/kg). Nanocopper was also reported to cause pathological damage to liver, kidney and spleen. Chung et al. (2010) recently reported occurrence of systemic argyria after ingestion of colloidal nanosilver proves its translocation from the intestinal tract. Earlier Smith et al. (1995) reported the uptake of fluorescently labeled polystyrene nanoparticles by intestinal lymphatic tissue (Peyer's patches).

Biodistribution

"Do nanoparticles show a different biodistribution profile than large sized particles? How long do they accumulate in tissues/organs? Do they exhibit organ specificity? Can clearance of nanoparticles be accurately assessed? Does chemical composition of nanomaterial play an important role in biodistribution?" are some of the questions with reference to studies on *in vivo* interactions of nanoparticles. Studies carried out so far point at involvement of physical clearance processes (viz., mucociliary movement, epithelial endocytosis, interstitial translocation, lymphatic drainage, blood circulation translocation and sensory neuron translocation) and chemical clearance processes such as dissolution, leaching and protein binding (Oberdorster et al., 2005b). Certain kinds of nanoparticles can pass through the GIT and are rapidly eliminated in feces and in urine indicating that absorption across the GIT barrier and entry into the systemic circulation (Curtis et al., 2006; Oberdorster et al., 2005b). However, some nanoparticulates can accumulate in the liver during first-pass metabolism (Oberdorster et al., 2005b). After intravenous administration, nanoparticles get distributed to the colon, lungs, bone marrow, liver, spleen, and the lymphatics (Fabian et al., 2008; Hagens et al., 2007; Huang et al., 2008). Such distribution is followed by rapid clearance from the systemic circulation, predominantly by action of the liver and spleenic macrophages (Moghimi et al., 2005). Clearance and opsonization of nanoparticles depends on size and surface characteristics (Curtis et al., 2006; Moghimi et al., 2005). Differential opsonization translates into variations in clearance rates and macrophage sequestration of nanoparticles (Moghimi et al., 2005). To increase the passive retention of nanomaterials in systemic circulation, the suppression of opsonization events is necessary at desired sites or anatomical compartments. For example in case of hydrophobic particles, a coating with poly(ethylene) glycol (PEG), would increase their hydrophilicity, hence increasing the systemic circulation time (Garnett and Kallinteri, 2006). In another study with PEGylated (Polyethylene glycol coated) gold nanoparticles Myllynen et al. (2008) observed that 10–30 nm sized particles did not cross the perfused human placenta and were not detected in fetal circulation.

A study by Takenaka et al. (2001) carried out in rats revealed that inhaled ultrafine silver nanoparticles were distributed in liver, lungs and brain. The authors have shown considerable amount of silver could be detected in rat brain following inhalation of silver nanoparticles. Few other studies with Inhaled nanoparticles demonstrate distribution of particles to the lungs, liver, heart, kidney, spleen and brain (BeruBe et al., 2007; Hagens et al., 2007; Medina et al., 2007; Oberdorster et al., 2002) and clearance *via* phagocytosis in the alveolar region by macrophages (Curtis et al., 2006; Garnett and Kallinteri, 2006; Oberdorster et al., 2005b). In addition, at least one clinical report has associated impaired liver function to silver nanoparticles released from a wound dressing (Trop et al., 2006).

Jong et al. (2008) demonstrated size dependent tissue distribution of gold nanoparticles with the smallest (10 nm) nanoparticles showing the most widespread distribution (blood, liver, spleen, kidney, testis, thymus, heart, lung and brain) whereas the larger particles (50, 100 and 250 nm) were detected only in blood, liver and spleen. In another study on biodistribution of gold nanoparticles, Niidome et al. (2006), detected most of gold stabilized with hexadecyltrimethylammonium bromide (CTAB) in the liver whereas 54% of PEG-modified gold nanoparticles were found in blood at 0.5 h after intravenous injection.

Owing to characteristic internalization and systemic distribution of inorganic and polymeric nanoparticles, there is a growing interest in exploring their uses for imaging, systemic delivery of drugs, target specific killing of cancerous cells etc. Understanding the relationship between the physico-chemical properties (size, surface charge, hydrophilicity etc.) of nanoparticles and their ADME (absorption, distribution, metabolism and elimination) characteristics is critical to

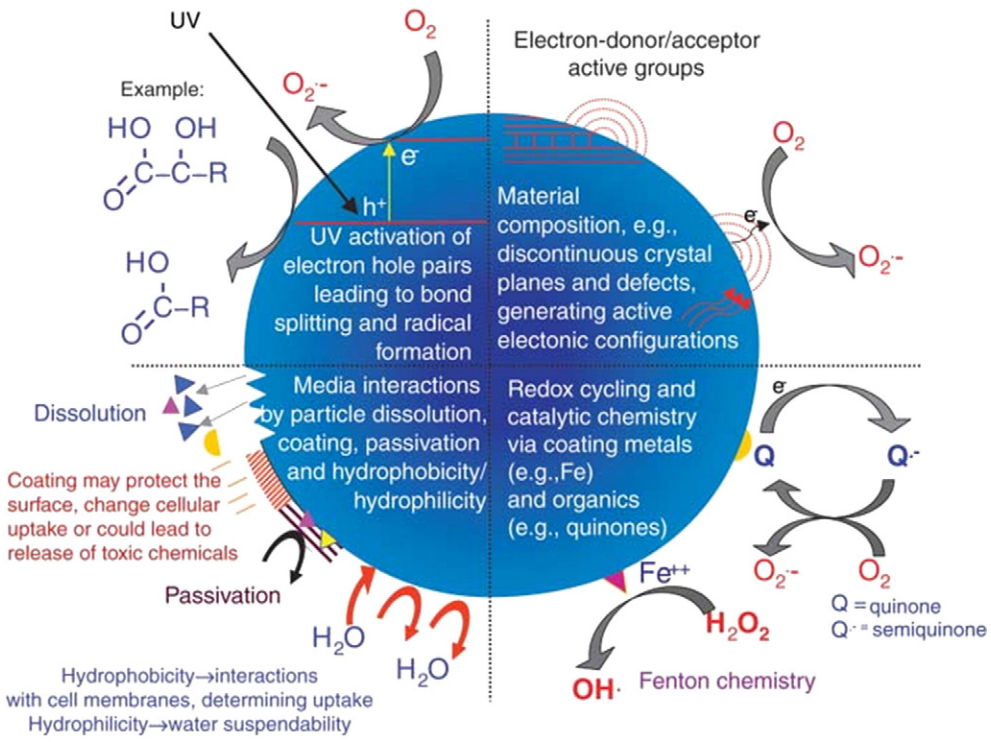

Fig. 2. Possible mechanisms by which nanomaterials interact with biological tissue. Examples illustrate the importance of material composition, electronic structure, bonded surface species (*e.g.*, metal-containing), surface coatings (active or passive), and solubility, including the contribution of surface species and coatings and interactions with other environmental factors (*e.g.*, UV activation) From Nel et al. (2006) Science 311, 622–627. Reprinted with permission from AAAS.

achieve desired biological effect (Li and Huang, 2008, Liang et al., 2008). Kunzmann et al. (2011) have extensively reviewed the commonly studied nanomaterials viz., iron oxide nanoparticles, dendrimers, mesoporous silica particles, gold nanoparticles, and carbon nanotubes with reference to their toxicity, biocompatibility, biodistribution and biodegradation. The authors re-emphasize the importance of physico-chemical characteristics of nanoparticles as well as ensuing immunological reactions vis-a-vis the target biological application. Zhi Yong et al. (2009) recommend the use of radiotracer techniques for determining ADME characteristics.

Molecular determinants

When exposed to light or transition metals, nanoparticles may promote the formation of pro-oxidants which, in turn, destabilizes the delicate balance between the biological system's ability to produce and detoxify the reactive oxygen species (ROS) (Curtis et al., 2006; Kabanov, 2006). Size, shape and aggregation are nanomaterial characteristics that can culminate in ROS generation (Shvedova et al., 2005a, 2005b). Properties such as surface coating and solubility may possibly decrease or amplify the size effect as illustrated in Fig. 2.

ROS include free radicals such as the superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($^{\cdot}OH$) and the non-radical hydrogen peroxide (H_2O_2), which are constantly generated in cells under normal conditions as a consequence of aerobic metabolism. When cells are exposed to any insult (chemical/physical), it results in the production of ROS (Luo et al., 2002). But cells are also endowed with an extensive antioxidant defense system to combat ROS, either directly by interception or indirectly through reversal of oxidative damage. Cellular antioxidants can be divided into primary (superoxide dismutase, glutathione peroxidase, catalase and thioredoxin reductase) or secondary defense (reduced glutathione) mechanisms (Stahl et al., 1998). Superoxide dismutase (SOD) converts the highly reactive radical superoxide into the less reactive peroxide (H_2O_2) which further can be destroyed by catalase or glutathione peroxidase (GPx) (Fridovich, 1995). Catalase is a highly reactive enzyme, which converts H_2O_2 to form water and molecular oxygen (Mates and Sanchez-Jimenez, 1999).

Table 2
Possible pathophysiological outcomes due to various nanomaterials.

Experimental NM effects	Possible pathophysiological outcomes
ROS generation*	Protein, DNA and membrane injury,* oxidative stress†
Oxidative stress*	Phase II enzyme induction, inflammation†, mitochondrial perturbation*
Mitochondrial perturbation*	Inner membrane damage*, permeability transition (PT) pore opening*, energy failure*, apoptosis*, apo-necrosis, cytotoxicity
Inflammation*	Tissue infiltration with inflammatory cells†, fibrosis†, granulomas†, atherogenesis†, acute phase protein expression (*e.g.*, C-reactive protein)
Uptake by reticulo-endothelial system*	Asymptomatic sequestration and storage in liver*, spleen, lymph nodes†, possible organ enlargement and dysfunction
Protein denaturation, degradation*	Loss of enzyme activity*, auto-antigenicity
Nuclear uptake*	DNA damage, nucleoprotein clumping*, autoantigens
Uptake in neuronal tissue*	Brain and peripheral nervous system injury
Perturbation of phagocytic function,* "particle overload," mediator release*	Chronic inflammation†, fibrosis†, granulomas†, interference in clearance of infectious agents†
Endothelial dysfunction, effects on blood clotting*	Atherogenesis*, thrombosis*, stroke, myocardial infarction
Generation of neoantigens, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle regulation	Proliferation, cell cycle arrest, senescence
DNA damage	Mutagenesis, metaplasia, carcinogenesis

Effects supported by limited experimental evidence are marked with asterisks (*); effects supported by limited clinical evidence are marked with daggers (†).
From Nel et al. (2006) Science 311, 622–627. Reprinted with permission from AAAS.

Glutathione peroxidase catalyzes the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage and also reducing cellular lipid hydroperoxides (Jornot et al., 1998). Under normal conditions, more than 95% of the glutathione (GSH) in a cell is reduced and so the intracellular environment is usually highly reducing. However, depletion of GSH will lower the reducing capacity of the cell and can therefore induce oxidative stress without the intervention of ROS. Free radicals also attack free fatty acids in cell membranes forming lipid hydroperoxides. Consequently, lipid peroxidation causes damage to cell membrane. Oxidative stress induced by nanoparticles is reported to enhance inflammation through upregulation of redox-sensitive transcription factors including nuclear factor kappa β (NFκβ), activating protein 1 (AP-1), extracellular signal regulated kinases (ERK) c-Jun, N-terminal kinases, JNK, and p38 mitogen-activated protein kinases pathways (Curtis et al., 2006; Kabanov, 2006). The possible pathophysiological outcomes of effects due to nanomaterials have been concisely complied and presented in Table 2. Generally speaking, biological systems are able to integrate multiple pathways of injury into a limited number of pathological outcomes, such as inflammation, apoptosis, necrosis, fibrosis, hypertrophy, metaplasia, and carcinogenesis (Table 2). However, even if nanomaterials do not introduce new pathology, there could be novel mechanisms of injury that require special tools, assays, and approaches to assess their toxicity. Specific biological and mechanistic pathways can be elucidated under controlled conditions *in vitro*; these, in conjunction with *in vivo* studies would reveal a link of the mechanism of injury to the pathophysiological outcome in the target organ (Nel et al., 2006).

Genotoxicity and immunogenic potential

Reactive oxygen species (ROS), due to their high chemical reactivity can react with DNA, proteins, carbohydrates and lipids in a destructive manner causing cell death either by apoptosis or necrosis. The most frequently affected macromolecules are those genes or proteins, which have roles in oxidative stress, DNA damage, inflammation or injury to the immune system. For example, sub-micronic to nanometer-sized preparations of SiO_2 were found to increase arachidonic acid metabolism eventually leading to lung inflammation and pulmonary disease as well as expression in genes directly related to inflammation (Driscoll et al., 1996; Englen et al., 1990). Similar results were obtained by Ishihara et al. (1999) for nanometer sized TiO_2 particles and TiO_2 whiskers (width of 140 nm). Based on detailed analyses of studies which investigated the mechanisms of these adverse effects, several researchers have put forth the concept of primary versus secondary genotoxicity (Knaapen et al., 2004; MacNee and Donaldson, 2003; Vallyathan and Shi, 1997). Genotoxicity directly related to the exposure of the 'substance' is referred to as primary genotoxicity. Secondary genotoxicity is the result of the 'substance' interacting with cells or tissues and releasing factors, which, in turn, cause adverse effects such as inflammation and oxidative stress. Most investigations on genotoxicity and cellular interactions of engineered nanomaterials are limited to screening for cytotoxicity. A few studies have focused on immunological responses of nanoparticles. Moghimi et al. (2005) showed that PEG-grafted liposome infusion triggered non-IgE-mediated signs of hypersensitivity whereas peptide-functionalized carbon nanotubes were shown to form immunogenic complexes, enhancing the antibody response (Curtis et al., 2006). These studies highlight the need for undertaking further investigations on the antigenicity (capacity to evoke immune response) of nanoparticles *per se* and their complexes (with cellular biomolecules) as well as the resulting specific immune responses (Curtis et al., 2006; Lanone and Boczkowski, 2006). Interactions of nanomaterials with eukaryotic cells have been recently reviewed by Shvedova et al. (2010) with reference to recognition of engineered nanomaterials by the immune system, and the operating primary cellular defense mechanisms.

Regulatory issues

As far as the safety aspects of nanomaterials are concerned; academia, industry and regulatory governmental agencies should consider the unique biological properties of nanomaterials, and the related potential risks (Curtis et al., 2006; Lanone and Boczkowski, 2006; Nel et al., 2006). Multidisciplinary studies are encouraged to establish nanomaterials classification and testing procedures which would include toxicology, material science, medicine, molecular biology, and bioinformatics (Curtis et al., 2006; Lanone and Boczkowski, 2006). Regulatory aspects on the synthesis, use and disposal of nanoparticles are beyond the scope of this review.

Methods for assessing toxicity of nanomaterials

As with any other man-made materials, both *in vitro* and *in vivo* studies on biological effects of nanoparticles need to be performed. *In vitro* model systems provide a rapid and effective means to assess nanoparticles for a number of toxicological endpoints. They also allow development of mechanism-driven evaluations and provide refined information on how nanoparticles interact with human cells in many ways. Such studies can be used to establish concentration–effect relationships and the effect-specific thresholds in cells. These assays are suited for high-throughput screening of an ever increasing number of new engineered nanomaterials obviating the need for *in vivo* testing of individual materials. They also serve as well defined systems for studying the structure–activity relationships involving nanomaterials.

Some of the distinct advantages of *in vitro* systems using various cell lines include; (1) revelation of primary effects of target cells in the absence of secondary effects caused by inflammation; (2) identification of primary mechanisms of toxicity in the absence of the physiological and compensatory factors that confound the interpretation of whole animal studies; (3) efficiency, rapidity and cost-effectiveness; and (4) scope for improvements in design of subsequent expensive whole animal studies (Huang et al., 2010). Other advantages such as reduction in variability between experiments; reduced requirement of test materials thereby leading to generation of limited amounts of toxic wastes; possibility of using transgenic cell lines carrying human genes etc. have been discussed in a review by Takhar and Mahant (2011). Utility of such assays has also been demonstrated in assessment of pulmonary hazards due to fine and nanoscale materials (Sayes et al., 2009; Warheit et al., 2009).

The potential dangers of exclusive use of *in vitro* testing have been documented by Donaldson et al. (2009) and the authors state that cells in culture do not experience the range of pathogenic effects that are likely to be observed *in vivo*; which are partly related to issues of translocation, toxicokinetics and coordinated tissue responses. The latter is the most under-researched area in toxicology. In another study, Monteiro-Riviere et al. (2009) have observed that classical dye-based assays such as MTT and neutral red (NR) that determine cell viability produce invalid results with some nanomaterials due to interaction and/or adsorption of the dye/dye products. Further, carbon nanomaterials interact with assay markers to cause variable results with classical toxicology assays and may not be suitable for assessing nanoparticles cytotoxicity. Thus the authors indicate the lower utility of *in vitro* assays using human cell lines. The interaction of fluorimetric dyes with dextran coated SPIONS has been reported by Griffiths et al. (2011); such interactions need serious consideration in cytotoxicity assays. In a recent article by Dhawan and Sharma (2010) the methods for both *in vitro* and *in vivo* toxicity of nanomaterials have been reviewed. The authors discussed interferences in *in*

vitro assays (due to the unique physico-chemical properties of nanomaterials), as well as major challenges for *in vivo* assays such as dosimetry, optimization of dispersion, evaluation of interactions and biodistribution etc. Hence it is essential that multiple assays be employed depending on the type of nanomaterial in addition to imaging techniques such as transmission electron microscopy to validate chemical marker-based viability assays.

Currently used in vitro methods in nanotoxicology

Presently, in absence of any clear guideline(s) by the regulatory agencies on the testing/evaluation of nanoparticulate materials, *in vitro* studies (using established cell lines and primary cells derived from target tissues) become extremely relevant and important. In general, all the current experimental techniques of cellular biology and toxicology can be employed for nanotoxicological studies (Monteiro-Riviere and Tran, 2007). The techniques that can be used to assess toxicity of nanomaterials include (1) *in vitro* assays for cell viability/proliferation, mechanistic assays [ROS generation, apoptosis, necrosis, DNA damaging potential] (2) microscopic evaluation of intracellular localization [include SEM-EDS, TEM, AFM, Fluorescence spectroscopy, MRI, VEDIC microscopy] (3) gene expression analysis, high-throughput systems (4) *in vitro* hemolysis and (5) genotoxicity etc.

The first step towards understanding how an agent will react in the body often involves cell-culture studies. Compared to animal studies, *in vitro* studies are less ethically ambiguous, are easier to control and reproduce and are less expensive. In the case of cytotoxicity, it is important to recognize that in addition to the concentration of the potentially toxic agent being tested, cells in culture are sensitive to changes in their environment such as fluctuations in temperature, pH, nutrient and waste concentrations. Therefore, controlling the experimental conditions is crucial to ensure that the measured cell death corresponds to the toxicity of the added nanoparticles versus the unstable culturing conditions. In addition, as nanomaterials can adsorb dyes and can be redox active, it is important that the choice of the cytotoxicity assay is appropriate. Conducting multiple tests is advantageous to ensure valid conclusions are drawn (Lewinski et al., 2008).

In vitro cytotoxicity studies of nanoparticles using different cell lines, incubation times and colorimetric assays with different nanomaterials are increasingly being published. It should also be borne in mind that while the number of nanomaterials types and applications continues to increase, studies to characterize their effects after exposure and to address their potential toxicity are comparatively few (Lewinski et al., 2008). It can be said that relatively fewer number of assays have been used to assess the cytotoxic potential of a whole range of nanomaterials from carbon nanotubes to metallic nanoparticles to semiconductor nanoparticles with completely diverse applications. As is clear from the literature, for nanomaterials, the major biological effects involve interactions with cellular components such as the plasma membrane, organelles or genetic material. It is important to perform cytotoxicity studies for each nanomaterial type because of their unique biological response (Lewinski et al., 2008). Similar observations were reported by Kroll et al. (2011) for 23 engineered nanomaterials which were tested using ten different cell lines in three different assays. According to the authors, *in vitro* toxicity of the analyzed engineered nanomaterials was not attributed to a defined physicochemical property and the accurate identification of nanomaterial cytotoxicity would require a matrix based on a set of sensitive cell lines and *in vitro* assays measuring different cytotoxicity endpoints. Table 3 summarizes the toxicity assays being currently used for several classes of nanomaterials. There is not a single method that is satisfactory for obtaining all the information on the toxicity. Since different nanoparticles elicit different biological responses; to study mechanisms underlying toxicity a combination of assays is often required.

Hemolysis . *In vitro* hemolysis is a test to evaluate the biocompatibility of nanoparticles. In this assay the impact of physico-chemical characteristics of nanoparticles viz., size, porosity and surface functionality on human red blood cells (RBCs) is evaluated by quantifying the release of hemoglobin. Mesoporous SiO_2 and amine-modified SiO_2 were observed to exhibit reduced hemolysis in comparison with bare SiO_2 (Yu et al., 2011).

Genotoxicity assays. The cytotoxic effects for almost all kinds of metallic, metal oxide, semiconductor nanoparticles, polymeric nanoparticles and carbon based nanomaterials etc. have been reported. For establishing 'safe' nanotechnology it would be necessary to prove non-genotoxic nature of the nanomaterial in question. Several genotoxicity assays can be carried out *in vitro*. For example, in a recent article by Gonzalez et al. (2011) the applicability of *in vitro* micronucleus (MN) assay as described in OECD guideline for testing nanomaterials is reviewed. Several types of nanomaterials were shown to induce a significant increase of MN frequencies. Based on the micronucleus test (MNinv) data on 21 nanomaterials, it was proposed that the in *vitro* MN test is quite appropriate to screen nanoparticles for potential genotoxicity. However it was recommended that protocols should be formulated to as to achieve maximum sensitivity and avoid false negatives. Determination of the cellular dose, cytochalasin-B treatment, time of exposure, serum levels and choice of cytotoxicity assay was advised for a better interpretation of MN frequency results.

The comet assay is a widely used *in vitro* assay in fundamental research for DNA damage and repair, in genotoxicity testing of novel chemicals and pharmaceuticals, environmental biomonitoring and human population monitoring. It has been employed for toxicity assessment of nanoparticles. In the article by Karlsson (2010) at least 46 cellular *in vitro* studies and several *in vivo* studies using the comet assay have been reviewed. These studies had used the comet assay to investigate the toxicity of manufactured nanoparticles. Findings indicate that majority of the nanoparticles exhibited high reactivity and cause DNA strand breaks or oxidative DNA lesions. Considering the sensitivity of the assay it can enable the assessment of their relative potency. However, the author also states that, additional methods to measure DNA damage/genotoxicity should be employed and more studies investigating mutagenicity would prove valuable.

Ames Test (or Bacterial Reversion Mutation Test) is yet another *in vitro* assay used to assess the genotoxic potential of nanomaterials. The test employs histidine dependent (auxotrophic) mutant strains of *Salmonella typhimurium*. This test is usually employed as an adjunct technique because it is difficult to interpret the data generated in a prokaryotic system to a eukaryotic genotoxicity testing. Furthermore results could be ambiguous in some instances when certain nanomaterials are not able to cross the bacterial wall or in situations where the nanomaterials are bactericidal.

Singh et al. (2009) have reviewed the abilities of metal nanoparticles, metal-oxide nanoparticles, quantum dots, fullerenes, and fibrous nanomaterials, with reference to their potential to damage or interact with DNA. In these studies chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles have largely been assessed based on *in vitro* assays for the diverse group of materials studied. Studies on neurological effects of nanoparticles have been reviewed by Yang et al. (2010); most studies focus on the interaction between CNS neuronal lines (PC-12, CA1 and CA3) and nanoparticles (including Cu, CuO, Zn and Ag). According to the authors, more studies should be focused on biological cells of hippocampal membrane. In a recent review Becker et al. (2011) have stated that with the available tests/assays,

Table 3
Summary of toxicity assays for different nanoparticles.

Assay	Purpose	Used for nanoparticles	References
Light microscopy	Morphological observations	Single-walled carbon nanotubes (SWNTs)	Fiorito et al. (2006)
		Silver nanoparticles	Arora et al. (2008); Arora et al. (2009)
Neutral red assay	Cell viability (lysosomal activity)	Carbon nanotubes,	Flahaut et al. (2006); Monteiro-Riviere and Inman (2006)
		Silver, molybdenum, aluminum, iron oxide and titanium dioxide nanoparticles	Hussain et al. (2005)
		Titanium dioxide nanoparticles	Shukla et al. (2011)
Colony formation assay	Proliferative capacity	Carbon based nanomaterials	Herzog et al. (2007)
Trypan blue	Cell viability/cell growth (membrane integrity)	Gold nanoparticles	Goodman et al. (2004)
		SWNTs	Bottini et al. (2006)
Calcein acetoxymethyl (calcein AM)/ ethidium homodimer	Cell viability (cell metabolic activity/ membrane integrity)	Fullerenes,	Sayes et al. (2004)
		Gold nanoshells	Hirsch et al. (2003) Loo et al. (2004)
Lactate dehydrogenase (LDH)	Cell viability (membrane integrity)	Carbon nanoparticles	Sayes et al. (2004); Muller et al. (2005), Uo et al. (2005)
Tetrazolium salts (MTT, MTS, XTT, WST)	Cell viability/cell growth (cell metabolic activity)	Fullerenes	Sayes et al. (2004)
		Carbon nanoparticles	Flahaut et al. (2006) Monteiro-Riviere and Inman (2006)
		Silver nanoparticles	Arora et al. (2008), Arora et al. (2009)
		TiO_2, SiC nanoparticles or multi-walled carbon nanotubes (MWCNT).	Barillet et al. (2010)
		23 engineered nanomaterials including TiO_2, CeO_2, carbon black AlOOH, Ti–Zr, Al–Ti–Zr, ZrO_2, $BaSO_4$, $SrCO_3$	Kroll et al. (2011)
Resazurin or Alamar blue	Cell viability/cell growth (cell metabolic activity)	Quantom dots,	Selverstov et al. (2006), Selvan et al. (2005)
Propidium iodide	Cell viability/cell growth/apoptosis (membrane permeability)	Carbon nanoparticles	Pantarotto et al. (2004), Kam et al. (2004), Kostarelos et al. (2007)
		SiO_2 nanoparticles	Yu et al. (10.1021/nn2013904
LDH assay	Cell death	23 engineered nanomaterials including TiO_2, CeO_2, carbon black AlOOH, Ti–Zr, Al–Ti–Zr, ZrO_2, $BaSO_4$, $SrCO_3$	Kroll et al. (2011)
DNA laddering	Biochemical hallmark of apoptosis	Silver nanoparticles	Gopinath et al. (2008), Arora et al. (2008)
Acridine orange/ethidium bromide	Apoptosis/necrosis	Silver nanoparticles	Gopinath et al. (2008), Arora et al. (2008), Arora et al. (2009), Jain et al. (2009)
Caspase-3 activity	Apoptosis	Silver nanoparticles	Arora et al. (2008), Arora et al. (2009), Jain et al. (2009)
Levels of reduced (GSH) and oxidized (GSSG) glutathione, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CT), ROS production	Oxidative stress	Polymeric nanoparticles	Fernandez-Urrusuno et al. (1997)
ROS production and levels of GSH	Oxidative stress	Silver, molybdenum, aluminum, iron oxide and titanium dioxide nanoparticles,	Hussain et al. (2005)
Vitamin E, levels of GSH and lipid peroxidation	Oxidative stress	SWNTs	Shvedova et al. (2003)
Levels of GSH and lipid peroxidation	Oxidative stress	C_{60} fullerenes	Sayes et al. (2005)
Levels of GSH, GPx, SOD, catalase (CT) and lipid peroxidation	Oxidative stress	Silver nanoparticles	Arora et al. (2008), Arora et al. (2009), Jain et al. (2009)
ROS generation	Oxidative stress	Titanium dioxide nanoparticles	Shukla et al. (2011)
DCF assay	Oxidative stress	23 engineered nanomaterials including TiO_2, CeO_2, carbon black AlOOH, Ti–Zr, Al–Ti–Zr, ZrO_2, $BaSO_4$, $SrCO_3$	Kroll et al. (2011)
		TiO_2, SiC nanoparticles or multi-walled carbon nanotubes (MWCNT).	Barillet et al. (2010)
Transmission electron microscopy	Visualization of intracellular localization	Fullerene derivatives	Foley et al. (2002)
		Ultrafine particulates	Li et al. (2003)
		Silver nanoparticles	Arora et al. (2009), Jain et al. (2009)
		Titanium dioxide nanoparticles	Shukla et al. (2011)

(continued on next page)

Table 3 (*continued*)

Assay	Purpose	Used for nanoparticles	References
Synchrotron radiation based techniques	Biodistribution of nanoparticles *in vitro* and *in vivo*, interactions with biological systems including ROS generation, chemical speciation etc.	Nanoscale Zerovalent iron, Titanium dioxide, ZnO, CeO_2 etc.	Wang et al. (2010)
Cellular uptake using radiolabelled nanoparticles	Cellular uptake	Cobalt nanoparticles	Ponti et al. (2009)
In vitro micronucleus test	Genotoxicity	Several classes of nanoparticles	Gonzalez et al. (2011)
Allium cepa chromosome damage test	Genotoxicity	Chitosan/Poly methyl methacrylate nanoparticles	de Lima et al. (2010)
Comet assay	DNA damage	metal nanoparticles, carbon based nanomaterials, magnetic nanomaterials, metal oxide nanoparticles etc.	Karlsson (2010)
		TiO_2, SiC nanoparticles or multi-walled carbon nanotubes (MWCNT).	Barillet et al. (2010)
Colony forming efficiency test	Cytotoxicity,	Cobalt nanoparticles	Ponti et al. (2009)
Hemoglobin estimation	Hemolysis	SiO_2 nanoparticles	Yu et al. (2011)

carcinogenicity of nanomaterials can only be assessed on a case-by-case basis.

Newer methods to assess nanomaterial toxicity

Based on measurements of certain physical parameters such as size, zeta potential and biological property such as lactate dehydrogenase release, Sayes and Ivanov (2010) have developed a mathematical model to provide insights on how engineered nanomaterial features influence cellular responses. The study proves that predictive computational models for biological responses caused by exposure to nanomaterials can be developed and applied to assess nanomaterial toxicity.

With the advent of nanotechnology, increasingly large numbers of compounds have been introduced in the environment and data on toxicity of these materials is required. In such cases, traditional toxicity testing using animal models is often not possible because it is often time-intensive, low capacity, expensive and assesses only a limited number of endpoints. North and Vulpe (2010) propose mechanism-centered high-throughput testing as an alternative approach to meet this pressing need for analysis of responses due to the large number and types of nanomaterials. According to the authors this approach along with functional toxicogenomics (which is the global study of the biological function of genes on the modulation of the toxic effect of a compound), can play an important role in identifying the essential cellular components and pathways involved in toxicity response.

Genome arrays have been used to assess the effects of nanoparticles. According to Lee et al. (2010) the inhaled silver nanoparticles caused modulation of the expression of several genes associated with motor neuron disorders, neurodegenerative disease and immune cell function, indicating potential neuro- and immune-toxicity. According to the authors these genes may assist in the development of surrogate markers for silver nanoparticles exposure and/or toxicity.

Jin et al. (2010) have reported the utility of high-throughput screening (HTS) methods for screening the effect of silver nanoparticles on bacterial cells. This helps for monitoring the ecological effects of nanoparticles. Similar studies were performed with ZnO and iron doped ZnO particles (Li et al., 2011). Sadik et al. (2009) describe portable, dissolved oxygen electrochemical sensor arrays capable of detecting the engineered nanomaterials (quantum dots and fullerenes) as well as provide rapid nanotoxicological information. Such sensors will be of utility because of their portable nature. Feliu and Fadeel (2010) have extensively reviewed HTS methods developed in miniaturized devices for screening of nanomaterials toxicity. The authors clearly state the goal of HTS: to utilize rapid, automated screening approaches to provide detailed and comparable toxicity data ('signatures') for thousands of different nanomaterials in order to promote the safe development of such materials. The authors also point out that, HTS will not replace conventional toxicology but could aid in the prioritization of nanomaterials for further testing; including animal testing. HTS may also allow for the development of models that predict behavior of nanoparticles in biological systems. Similar to the above report, George et al. (2011) describe use of multi-parametric, automated screening assay that incorporates sublethal and lethal cellular injury responses to perform high-throughput analysis of a batch of commercial metal/metal oxide nanoparticles (nano-ZnO, Pt, Ag, SiO_2, Al_2O_3) with the inclusion of a quantum dot (QD1). The data on *in vitro* assays was co-related with *in vivo* data using zebra-fish embryos. The approach was used to predict toxicity and prioritize nanomaterials for *in vivo* testing.

Eco-toxicology

To ensure a 'safe' nanotechnology industry the need for proactive research in the area ecotoxicology of nanomaterials has been emphasized Nel et al. (2006). Several assays for eco-toxicological testing of nanomaterials have been developed. Literature on the toxicity of metallic nanoparticles to bacteria has been reviewed by Niazi and Gu (2009). Various mechanisms that govern toxicity as well as usefulness of bacterial systems to study toxicity of manufactured nanoparticles have been explained. In another study, C_{60} suspensions have been shown to be toxic to bacteria (Lyon et al., 2005, 2006), fathead minnows (*Pimephales promelas*) (Zhu et al., 2006), and zebrafish embryos (Usenko et al., 2007; Zhu et al., 2007). Toxicity of single-walled carbon nanotube (SWNT)-based nanomaterials to an estuarine copepod (*Amphiascus tenuiremis*), Daphnia, and rainbow trout have been reported (Roberts et al., 2007; Smith et al., 2007; Templeton et al., 2006). Adams et al. (2006) compared the ecotoxicities of TiO_2, ZnO, and SiO_2 nanoparticles suspended in water using *Escherichia coli* and *Bacillus subtilis* as two model bacterial species and it was reported that ZnO was toxic to *Bacillus subtilis*. Experiments on embryonic zebrafish demonstrated similar results; ZnO nanoparticles were more toxic than TiO_2 or Al_2O_3 nanoparticles (Zhu et al., 2008). Moreover, Hund-Rinke and Simon (2006) reported the first results on the toxicity of TiO_2 nanoparticles to Daphnia (a common freshwater zooplankton) and green algae (*Desmodesmus subspicatus*). In a comprehensive study on the 48-h acute toxicity of water suspensions of six manufactured nanomaterials (*i.e.*, ZnO, TiO_2, Al_2O_3, C_{60}, SWCNTs, and MWCNTs) to *Daphnia magna*, using immobilization and mortality as toxicological endpoints, a dose dependence in acute toxicity was demonstrated (Zhu et al., 2009). With fish hepatocyte cultures as model system Scown et al. (2010) have noted their suitability for studies investigating the cellular uptake of

engineered nanoparticles. Another model system for judging nanomaterials toxicity is zebrafish embryos; the model also being useful for comparative biology because of the similarities between the zebrafish and human genomes, early life development and disease processes. In a study on ZnO toxicity in rodent lung and zebra fish embryo's, data indicated reduced toxicity in the latter system upon doping of Fe in ZnO (Xia et al., 2011).

Release of nanomaterials to the environment during recycling and disposal is of particular concern for nanoparticles incorporated into limited use and/or disposable products. Once released these nanomaterials would readily undergo transformations *via* biotic and abiotic processes. Understanding environmental transformations and fate of engineered nanomaterials will enable design and development of environmentally benign nanomaterials, as well as their use as environmental tracers, in environmental sensing and in contaminant remediation. This was demonstrated in a biomimetic hydroquinone-based Fenton reaction which provides a new method to characterize transformations of nanoscale materials expected to occur under oxidative environmental conditions (Metz et al., 2009). Current computational techniques are being used to study interactions of nanoparticles with biological systems and these have been reviewed by Makarucha et al. (2011). Such studies could also be used to complement the experimental data on toxicity.

Data gaps and research needs

Taking into consideration the routes of exposure to nanoparticles, to better understand dermal absorption of nanomaterials more research on regular skin, dry skin and damaged skin is necessary as pointed out by Zwart et al. (2004); Hagens et al. (2007). More studies on gastrointestinal lymphatic uptake and transport and direct toxicological effects on the GIT are required (Lanone and Boczkowski, 2006). Similarly questions such as penetration of placental barrier by nanomaterials would require attention. For such studies suitable *in vitro* models need to be developed with subsequent *in vivo* studies. Cellular interactions with certain nanomaterials may not introduce any new pathological conditions, but one cannot ignore novel mechanisms of injury that require special tools, assays and approaches to assess their toxicity. The number of engineered nanomaterials is increasing day-by-day, and it is expected that materials will be more complex and will have unique chemistries; therefore in order to ensure 'safe' nanotechnology, 'Nanotoxicology' studies would require a standard set of protocols for *in vitro*, *in vivo* toxicity (including genotoxicity, teratogenecity), ecotoxicity. Aspects such as biomagnification would require serious consideration. Ecotoxicity studies with anaerobic bacteria are specifically relevant with the manufactured materials. Quantitative data on toxicological effects of nanoparticles are still scarce even at the single organism level. Ecotoxicological information on nanoparticles is required at several levels (single organisms, simplified communities and whole ecosystems) for risk assessment and regulatory purposes. Currently, neither the fate of nanosize materials nor their impact on animals, plants and soil communities have been investigated *in situ* although it would be necessary for the validation of models proposed for the environmental risk assessment of nanoparticles (Kahru and Dubourguier, 2010). Physico-chemical characteristics of particles after they react with cultured cells *in vitro* needs to be evaluated, and there is also a need for more research on effects of long term exposure to nanomaterials. A five tier system for toxicity evaluation has been proposed by Savolainen et al. (2010). This is a comprehensive study including physicochemical characterization as the first step. Despite this kind of a proposed system, there are challenges particularly the validation of *in vitro* tests with appropriate predictive power for *in vivo* effects in whole organisms.

Concluding remarks

Nanotechnology is growing at an exponential rate and will undoubtedly have both beneficial and toxicological impact and consequences on health and the environment. According to some estimates, nanotechnology promises to far exceed the impact of the Industrial Revolution and is projected to become a US$ 1 trillion market by 2015 (Drobne, 2007). The importance of nanotechnologies to our well being is beyond debate, but its potential adverse impacts need to be studied all the more. Nanotoxicology as a new discipline should make an important contribution to the development of a sustainable and safe nanotechnology. An improved understanding of the risk factors related to nanomaterials in the human body and the ecosystem will aid future development and exploitation of a variety of nanomaterials. Issues related to new nanoparticles are in the headlines due to the fear of their escaping into the environment. In fact, we have lived with sub-micron sized particles around us forever. The introduction of man-made versions has just brought to light the fact how little we know about their toxic effects. Awareness is growing about the need to develop an infrastructure for characterizing and measuring nanomaterials in complex matrices and for developing reference materials, both for calibration of instruments used for assessing exposure and dosimetry and for benchmarking toxicity tests. Public expects that new or emerging technologies meet higher safety requirements than tried and tested technologies. Failure to meet these requirements may result in public fear or even rejection of nanotechnology-based products, which often essentially improve the quality of life of individuals, groups of people, or even nations.

Nanotechnology has the potential to revolutionize everything from medicine to clothing and electronics. Indeed many nanomaterials are already on the market. Whilst this technology has enormous potential benefits, there are concerns that the unique properties of nanoparticles will also lead to human health problems. Many reviews have recently considered approaches to investigate the toxicology of nanoparticles and have recognized that preliminary toxicity data can be usefully obtained from *in vitro* studies. *In vitro* studies of the possible toxicological effects of nanoparticles should be undertaken before *in vivo* studies. We have listed a large number of *in vitro* studies that could usefully be applied to nanoparticles. Those appropriate in a given instance will need to be considered on a case by case basis. We note that current concerns about the use of animals in research are making *in vivo* work more difficult, but recognize that in only a few areas have *in vitro* studies been validated for regulatory purposes. *In vitro* studies are likely to provide initial data on comparative toxicity of different sized materials, with the findings having to be followed up by *in vivo* studies in animals.

From the above discussion and the research presented in this review, the need for more toxicology research on manufactured nanomaterials is clear. In addition to standard tests, there is a need to develop better and rapid screening methods and to move into more predictive toxicology. The former will help prevent risk by knowing where to control exposure; the latter will help prevent risk by helping with design parameters to remove toxicity by design. There are some significant gaps in knowledge that need to be addressed. In the meantime it should be assumed that the safety evaluation of nanoparticles and nanostructures cannot rely solely on the toxicological profile of the equivalent bulk material. Toxicology studies are the basis for protection of human health and the environment relating to nanotechnology. It is only through addressing the issues raised by toxicological studies that nanotechnology will be able to realize its full potential.

Conflict of interest

There is not any conflict of interest.

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