Review

Nanotechnology: Safety paradigms

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The versatile utilization of nanotechnology in a variety of products used in different spheres of daily life has made it quite popular. This is attributed due to the enhanced surface related properties of the nanoparticles. But these enhanced properties also make nanoparticles, biologically more active leading to unexpected and unanticipated consequences on interaction with biological systems which may be deleterious to the living organisms. There may be different kinds of pathological conditions arising due to the exposure of nanoparticles, depending on their type, size and route of exposure. Consequently for the risk assessment there is a need for safety evaluation procedure which should be based on various hazardous aspects. The safety evaluation and toxicological assessment of nanoparticles can be further improved by collaboration between the research groups working on similar aspects of nanotechnology, establishment of guidelines along with the standard protocols for developing uniformity in experimental approaches related to nanotoxicology studies, which will help in the development of safer nanoproducts for the benefits of mankind. Thus, safety issues need to be impressed upon the industries and regulators simultaneously, to reduce the lag phase for the development of sustainable nanotechnology.

Keywords: Agglomeration, characterization, inflammation, nanotoxicology, nanoparticles, nanoencapsulation, reactive oxygen species.

INTRODUCTION

Nanotechnology is a highly multidisciplinary field of applied sciences whose unifying theme is to control and fabricate nanoparticles (NP) along with its application for various purposes. Nanoparticles have many advantages over their bigger analogues, which is attributed to their unique physical and chemical properties arising from their small size. The size of nanoparticles lies approximately in the range of 1 to 100 nm. Quantum mechanics play a significant role following reduction of materials to nanoscale that give rise to enhanced electrical, mechanical, and optical properties (Born and Kreyling, 2004), which is due to an increase in surface area to volume ratio. Because of the increased surface and mass ratio of the nanoparticles, owing to their small size, these materials are often much more reactive than their bulk counterparts (Thomas and Sayre, 2005). Consequently, the use of nanotechnology can create more reactive chemical intermediates in industrial processes that have increased catalytic efficiency and therefore can initiate the chemical reactions with less energy input. These properties allow nanoparticles for their improved performance in a wide range of products and various industrial processes including textiles and fabrics, sports equipment, energy generation, drugs and medical devices, drug delivery processes and food processing (Thomas and Sayre, 2005; Das et al., 2009). The most promising uses for nanotechnology are the detection, diagnosis, and treatment of diseases. Some of the complex nanoparticles may probably be used for therapeutic purposes, with the basic aim to enhance the half life in blood, thus enabling its prolonged circulation in the blood so that it can reach specific organs or tissues.

Abbreviations: ROS; Reactive oxygen species, HMM; human monocytes macrophages, SLN; solid lipid nanoparticle, TLR; toll like receptor, CNT; carbon nanotubes, BALF; broncho alveolar lavage fluid, BET; Braunaer Emmett-Teller, TEM; Transmission electron microscopy, DLS; Dynamic light scattering.
with sustained release of drugs (Borm and Kreyling, 2004).

Depending on the source, nanoparticles are divided into two categories:

1) Natural nano-scale particles: There is a broad range of naturally occurring particulates that are of 1 to 100 nm in size, like particulates from forest fire, biological particulates, etc. Further, unintentional anthropogenic particulate by-products from diesel engines, power plants, etc. also have sizes in the range of 1 to 100 nm (Veerman et al., 1998).

2) Engineered nano-scale particles: Engineered nanoparticles in contrast to natural ones are artificially synthesized. Consequently, their properties and other parameters can be manipulated in order to make them better suited for the purpose for which they are synthesized. There are two methods by which nanoparticles can be artificially synthesized and depending on the applications and uses that are desired from the nanoparticle; the protocol for their synthesis is decided. The two techniques used for nanoparticle synthesis are “top down” and “bottom up” techniques.

Top-down technique involves the conversion of the larger single sample of material to the nanoscale material in the desired configuration either by milling or by etching of the large particle (Veerman et al., 1998). Such techniques find applications in lithographic processes used to manufacture computer chips (Jeon et al., 2010).

Bottom up technique assembles the smaller subunits to obtain the larger nanoscale material through processes such as chemical synthesis. Such techniques are new as compared to top-down techniques. Bottom up techniques are used for the production of the carbon nanotubes (Borm and Kreyling, 2004; Riviere et al., 2005).

One of the most important applications of the nanoparticles is their involvement in therapeutic uses including the process of targeted drug delivery (Borm and Kreyling, 2004). During the process of drug delivery the optimal or the desired effects would be achieved by focussing the drug molecule to the target organ. Most of the drug molecules taken orally are metabolized or destroyed by the stomach or liver, and then distributed throughout the entire body. This leads to various side-effects in other organs, and some potentially effective drugs are not being used precisely because of the side-effects resulting from their accumulation in various tissues and organs other than the target organ. Targeted drug-delivery by nanoparticles has the potential to overcome some of these problems, making the treatment more effective with both cost and safety benefits. As they are specific to the target organs only, side-effects resulting from the drug molecules get enormously reduced leading to improvement in the safety aspect of the drug delivery. Because of the increased specificity of the drug molecule towards the target organ, relatively lower doses are required to achieve the desired effect from the drug delivery and therefore reducing the cost involved in the process of treatment.

The therapeutic potential of currently available drugs is also reduced by the micro-kinetics aspect such as instability issues of the drug molecule in a particular local environment that adversely affects its stability. Also, the available drugs having bulk sizes, often find difficulties in crossing biological barriers such as the blood-brain barrier and placenta (Borm and Kreyling, 2004). Nanoparticles due to their size may help to address these problems, and hence its potential applications in drug delivery are being attempted. Nanoparticle-based drugs have improved solubility, altered pharmacokinetics and biodistribution compared to existing drugs of bigger sizes. Nowadays there are even more medical applications in which nanoparticles are being utilized such as, nanoparticle-based molecular imaging, nanosensors, etc (Kumar et al., 2007). Thus, it is evident that nanoscale materials have the potential for use in the treatment of cancer and neurological and developmental disorders (Nel et al., 2006).

APPLICATIONS

Most nanoparticles, due to their unique properties are able to stimulate the immune system and thus elicit an inflammatory response through the abnormal secretion of various cytokines and other chemicals. In contrast to such nanoparticles, others have been shown to antagonize the inflammation in cells. Cerium oxide nanoparticles were found to be effective in reducing reactive oxygen species (ROS) and inflammatory mediator production and may be used as a novel therapeutic tool for the treatment of inflammation (Hirst et al., 2008). Some nanoparticles are reported to have noninflammogenic or non-immunomodulatory properties, which is a prerequisite for usage in medical therapeutics. Ferumoxtran-10 (a dextran - coated ultra small super paramagnetic iron oxide particle) is one such particle. Its effect on the macrophages, such as viability, cytokine production and oxidative burst, has been studied (Muller et al., 2007). It was found that Ferumoxtran-10 at a concentration of 1 mg/ml showed no toxicity to human monocytes-macrophages (HMM) as it neither activates pro-inflammatory cytokines or superoxide anions nor interferes with Fc-receptor-mediated phagocytosis in vitro. Furthermore, high concentration (>10 mg/ml) of Ferumoxtran-10 had only slight effects on these key activities of macrophages indicating safety of Ferumoxtran-10. The effect of solid lipid nanoparticles (SLN) on interaction with phagocytic cells primarily, macrophages, has been studied and it was observed that SLN at low concentration did not induce cytokine secretion by macrophages. Moreover, change in lipid matrix and size of SLN did not result in macrophages activation. These findings reveal that SLN neither caused
cytotoxic effects on macrophage nor led to secretion of pro-inflammatory cytokines (Muller et al., 2007; Loher et al., 2005). It is for these reasons that SLN is now widely used as carrier in targeted drug delivery applications and in cosmetics. It has been reported that suspension of nano diamond (ND) is quite effective in healing wound and this property of NDs could be exploited as a remedy in cardiology, oncology, dermatology, and vascular diseases (Schrand et al., 2007). However under in vitro condition, ND was found to enhance the production of reactive oxygen species (ROS) in the WBC of human blood (Puzyr et al., 2002). Thus, it is quite likely that the effects induced by ND particles are dependent on the concentration and surface properties of NDs used and hence, more investigations are required to study the hazardous effects under in vivo conditions.

Nutraceuticals and drug delivery

Nanotechnology renders hydrophilic properties to fat soluble and lipophilic features to water soluble nutrients. This property can be utilized with some functional ingredients like carotenoids, phytosterols, and fat soluble antioxidants to be dispersed in water so as to improve their bioavailability in fruit drinks and other products (Sanguansri and Augustin, 2006; Chau et al., 2007; Das et al., 2009). Synthetic nanoparticles are developed and accepted as ‘generally recognized as safe’ (GRAS) for use in food (Wen et al., 2006; Graveland-Bikkera and de Kruija, 2006). Complex coacervates of DNA and chitosan are used as delivery vehicles for treatments of food allergies such as peanut allergy (Li et al., 2004; Zambaux et al., 1998). Furthermore, whey protein nanoparticles (40 nm) have been shown to get internalized in cells, where they are degraded to release the nutraceuticals (Wen et al., 2006).

Food encapsulation

To enhance the bioavailability of active nutrients, minute micelles have been used to encapsulate essential oils, flavour, antioxidant, coenzyme Q10, vitamins, minerals, and phytochemicals. Thus, application of liposomal nanovesicles for the encapsulation and delivery of nutrients and functional ingredients can be used as carriers for nutritional enhancement (Wen et al., 2006). Nanotubes made of milk protein have the potential to be used as novel ingredients for viscosifying the solutions used in food industry. These nanotubes may also be utilized for encapsulation and controlled release purposes (Graveland-Bikkera and de Kruija, 2006). Encapsulating nanoparticles of polyphenols, minerals, and micronutrients helps to protect them from oxidation and to reduce their undesirable off tastes in the finished products (Heller, 2006). Food and beverages are given desired flavours and colours by the addition of nanocapsules, which burst at different microwave frequencies.

Food safety/packaging

Nanotechnology ensures safety of food in terms of packaging by adding silver nanoparticles into packaging material that renders preservation of food for longer periods by killing the microorganisms in 6 min (Sanguansri and Augustin, 2006; Graveland-Bikkera and de Kruija, 2006). In addition, silicate nanoparticles films have been developed to reduce the entry of oxygen and retain the moisture, thus preventing food from oxidation. Nylon nanocomposite providing barriers to oxygen and carbon dioxide flow have also been used in food packaging to maintain freshness and block out smells in food, a typical example is multi-layer PET bottles for beer and alcoholic beverages (Sherman, 2005).

Sensors/smart filters

Nano-bioluminescence spray is being used for microbial detection, which binds to the surface of microbes making the food to glow thereby indicating microbial contamination. This new area has offered DNA biochips which are meant to detect bacteria in meat or fish and fruits infested with fungus. Protein-coated nanocantilever, naturally vibrating at a specific frequency, is a new class of ultra-small silicon sensors for the quick detection of viruses, bacteria and other pathogens (El Amin, 2006). When contaminants land on this device, the slight change in mass can cause the nanocantilever to vibrate at a different frequency, thus quickly detecting the contamination. Selective nano filters that can distinguish molecules based on shape as well as size enables the removal of toxins or adjustment of flavour.

The development of synthetic tree-shaped DNA being tagged with colour-coded probes, as a nanobarcode device, enables the identification of food pathogens (Li et al., 2004). A miniature portable micro bio detector has been developed using different nanowires which are tagged with specific pathogens and fluorescent antibodies for the simultaneous detection of toxins, pathogens, and chemicals in foodstuffs. Silver nanoparticles have been incorporated into different products from bandages to refrigerators for suppressing the spread of bacteria and other microbes (Sherman, 2005; El Amin, 2006).

NANOTOXICOLOGY

It deals with various toxicological aspects of the nanoparticles. Nanoparticles may have adverse effects at
their site of entry but some nanoparticles may escape the normal defences and translocate from their site of entry to have diverse effects on the target organs. Thus, the potential threats of nanoparticles need to be addressed.

Due to unique particle size, nanoparticles have higher rate of distribution, modified particle morphology and particle composition, increased surface area, altered surface chemistry and particle reactivity in solution. All these modifications and alterations enable the nanoparticles to enter the human body easily through skin, respiratory and gastrointestinal tract (Dwivedi et al., 2009; Das et al., 2009). This may cause adverse biological reactions and various pathological conditions might arise as a result of nanoparticle toxicity. Environmental contamination and ecosystem disturbance is yet another concern. Furthermore, because of their size and large surface area, nanoparticle binding to protein may result in a series of consequences not expected to occur when proteins bind to large particles (Borm and Kreyling, 2004; Borm et al., 2006). These could include:

a) Complex formation of nanoparticle with protein makes the particle more mobile, thus making it accessible to sites where large particles would not reach.

b) Enhanced protein degradation at the large surface area of NPs may lead to functional changes of those proteins which would not occur at the relatively small surface area of large particles.

Humans are already exposed to large numbers of ambient nanoparticles through environmental air pollution and much attention is now focussed on nanoparticle component, which may be the causative factor for adverse health effects. For some nanoparticles, such as those in sun block cream, dermal exposure is already occurring (Oberdörster et al., 2005a; 2005b). Nanoparticles in food are reported to cross into the gut lymphatics and redistribute to other organs more readily than larger particles. A huge class of nanoparticles are designed to be introduced directly into the body for diagnostic and therapeutic reasons. Thus, the requirement of safety evaluation of these nanoparticles becomes mandatory before these are commercially used.

Carbon nanotubes are long thin structures which can have diameters of a few nanometres, while the length can exceed thousands of nanometres. These nanostructures may have unusual toxicological properties, as they share shape characteristics of both fibres and nanoparticles and consequently they are speculated to be harmful to the lungs (Rasmussen et al., 2009). Easy decontamination of nanoparticles after exposure unlike chemical exposure is not possible due to lesser efficacy of ‘solubilisation’ and ‘dilution’ procedures in case of nanostructures (Shvedova et al., 2003). Therefore the consequences of the interaction of nanoparticles with biological systems are still not completely understood. Investigations are being carried out to study the toxicological aspect of nanoparticles in order to evaluate the sustainability and risk perception of the nanoparticle market product.

General toxicity

The effects of nanoparticles on immunological functions including antigen presentation by macrophages and dendritic cells and the subsequent effects on immune responses in vitro are important aspects to be studied. The accumulation of particles in the spleen, a major site for immune processing and lymphoid maturation, may have consequences for immune response and immunopathology. The kind of toxicity produced by the nanoparticle depends on its properties and its route of infection into the biological system. It has been reported that some nanoparticles cause cytotoxicity by producing free radicals and by generation of ROS and thus leading to the intracellular damage. Some nanoparticles may also cause tumor formation to occur by causing change in the levels of certain substances which are required for the normal functioning of the cells (Oberdörster et al., 2005a; 2005b).

Immune system modulation

Very small particles and structures may have a range of effects that are not seen with conventional particles. For instance they may not be detected by the normal phagocytic defences, allowing them to gain access to the blood or the nervous system (Oberdörster, 2001). Very small particles are smaller than bulk molecules and could act like haptons to modify protein structures and thereby either altering their function or rendering them antigenic property, raising the potential for autoimmune effects (Oberdörster et al., 1994; Kreyling et al., 2002). However, the effect of nanoparticles on the immune system has hardly been studied, while most existing research data clearly suggests that nanoparticles interact with the immune system and have the potential of inducing immunostimulatory or immunosuppressive reactions by interacting with the cells of immune system (Hussain et al., 2006).

Pro-inflammatory and inflammatory effects of nanoparticles on immune cells

Nanoparticles can penetrate deeply into the body, having larger surface area because of small size and possess charge on their surface; therefore they can cause a greater inflammatory response (Nemmar et al., 2002). The cells of the immune system recognize nanoparticles by their surface properties and core composition and trigger the inflammatory response depending upon their
physiochemical properties (Donaldson et al., 2002; Brigger et al., 2002). When nanoparticles enter the bloodstream, they are exposed to a complex environment of plasma-proteins and immune cells (Dwivedi et al., 2009). Due to their surface-charge nanoparticles uptake by immune cells may occur by monocytes, platelets, leukocytes, dendritic cells and most significantly by macrophages which may lead to inflammatory responses (Dobrovolskaia and McNeil, 2007).

Macrophages are among the first immune cells recruited to a site of invasion, and come into contact with foreign agents through toll-like receptor (TLR), which in turn are involved in initiation of inflammatory response. Macrophages have three major function; phagocytosis, antigen presentation, and immunomodulation through production of various cytokines and growth factors, which may play a critical role in the initiation, maintenance, and resolution of inflammation (Lucarelli et al., 2004). The binding ability of nanoparticles to macrophages is dictated by their charge and the moieties they display. Since, macrophages display negatively charged sialic acid on their surface, therefore positively charged nanoparticles are more likely to affect macrophages and induce inflammation than anionic or neutral particles (Zhu et al., 2008).

Studies have indicated that different nanoparticles can modulate inflammatory response of macrophages in different ways (Zhu et al., 2008; Dick et al., 2003). It has also been reported that nanoparticles significantly affect the expression of TLR and therefore may play a role in affecting cell reactivity to infections by altering the expression of innate receptors. Nano-sized ferric oxide [Fe₂O₃] particles caused inflammatory reactions by overloading of phagocytised nanoparticle in alveolar macrophage (Zhu et al., 2008). The most common inflammatory reactions include increase in inflammatory and immune cells, clinical pathological changes like; follicular hyperplasia, protein effusion, pulmonary capillary vessel haemorrhage and alveolar lipoproteinosis, increase in micro vascular permeability and cell lysis in lung epithelium. Investigators have suggested that low toxicity low solubility (LTLS) nanoparticles elicited greater inflammatory response (Dick et al., 2003; Tran et al., 2006). Thus, surface area and surface reactivity of nanoparticles is important in the induction of cytokine expression in vitro and in vivo, while surface area of nanoparticles was responsible for predicting pro-inflammatory effects (Duffin et al., 2007). It has been suggested that hydroxyapatite crystals have the ability to stimulate tumor necrosis factor-α (TNF-α) secretion from macrophage, which finally leads to NF-kappa β activation (Nadra et al., 2008). The process of TNF-α secretion and NF-kappa β activation was related to particle and pore size, that is, particles of 1-2 µm diameters and pore size of 10-50 A˚ found to be most bioactive.

The role of particle size and surface chemistry of nanoparticle in initiating proinflammatory effects was observed in A549 human lung epithelial cells on treatment with native as well as surface methylated forms of fine (40 to 300 nm) and ultrafine (20 to 80 nm) TiO₂ (Singh et al., 2007). Native as well as methylated form of ultrafine TiO₂ significantly evoked stronger oxidant generation and IL-8 release that led to inflammatory responses (Oberdörster et al., 2005a; Oberdörster 2001). The inflammatory responses occur because of IL-8 release and may be due to the large surface area of ultrafine TiO₂. Further, it has been demonstrated that nano carbon black directly affects the endothelium, causing cytotoxic injury, inflammatory responses by increased production of chemokines and inhibition of cell growth (anti-proliferative activity) in a dose dependent manner (Yamawaki and Iwai, 2006). It shows that nanoparticles may directly affect endothelial cells causing vascular injury that might be responsible for the development of atherosclerosis.

Effect of nanoparticles on cytokine profile

Mononuclear phagocytic cells initiate cytokine mediated immune response. Interleukin-1 (IL-1), TNF-α, interferon gamma (IFN-γ), IL-6, IL-8, IL-12, IL-18 and granulocyte-macrophage colony stimulating factor (GMCSF) are all well characterized as pro-inflammatory cytokines. Activated mononuclear phagocytes play a significant role in the immune reactions by releasing pro-inflammatory cytokines. Secretions of these cytokines may initiate an immune cascade, which ultimately cause severe damage to the host cells. Several studies have been carried out to investigate the effect of nanoparticles on cytokine responses. The potential interference of cobalt nanoparticles on the production of several cytokines including IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α in peripheral blood mononuclear cells (PBMCs) were studied in vitro. It was observed that these nanoparticles induce TNF-α and IFN-γ release and inhibit IL-10 and IL-2 (Petrarca et al., 2006).

Food related toxicity

Various nanoparticles are used in food industry for different applications like in precision farming, maximizing the crop yield by minimum use of fertilizers and pesticides, food packaging, nutritional enhancement, food encapsulation, etc (Das et al., 2009; Sanguansri and Augustin, 2006; Graveland-Blikker and de Kruijf, 2006). Consequently when such food particles are ingested, the nanoparticles in the product gain entry into the body which may lead to different kinds of toxicity. Processes of various food related applications might be accompanied by various unpredictable safety problems and risks (Nemmar et al., 2002; Scholer et al., 2000). Nanostructured ingredients and nutrient delivery systems also involve the risk of carrying other adsorbed foreign substances in the body and thus gaining entry into the
blood circulation system leading to intravenous toxicity.

Nanoencapsulated ingredients and engineered nanoparticulated additives because of their modified and enhanced surface related properties have been found to have increased interaction with the biological systems leading to various damaging effects on gastro-intestinal tract functions, gut micro-flora and other cellular functions in the body mostly accompanied by intracellular damage (Scholer et al., 2002; Shin et al., 2007).

Dermal toxicity

Dermal toxicity is generally caused by the nanoparticles that are used for the cosmetic purposes and therefore when applied on skin cause deleterious effects on human health. The outer layer of the skin is composed of specialized epithelial cells called keratinocytes, which secrete different cytokines that may induce local inflammatory and immunological reactions in the skin on exposure to irritants (Corsini and Galli, 1998; Allen et al., 2000). Nanomaterials like titanium dioxide (TiO$_2$) and zinc oxide (ZnO) are used in sunscreens, cosmetics and personal care products. Topical toxicity data suggest that TiO$_2$ and ZnO nanoparticles exhibit low systemic toxicity and are well tolerated on the skin (Nohynek et al., 2007). There is no evidence of penetration of TiO$_2$ and ZnO nanoparticles into the human epidermis (Dussert et al., 1997). However, recent studies on ZnO nanoparticles have shown oxidative DNA damaging potential in human skin epithelial cell line (A431) (Sharma et al., 2009). TiO$_2$ penetration is associated with hair follicle opening and not due to direct diffusion through the layers of epidermis (Gamer et al., 2006). Titanium dioxide nanoparticles cause cytotoxic effects by forming free radicals and thereby causing intracellular damage.

The effect of dermal exposure to amino acid derivatized fullerenes (substituted phenylalanine derivatives) has been studied by analyzing cell viability and proinflammatory potential in human epidermal keratinocytes (HEK) (Rouse et al., 2006). The decrease in cell viability and elevated levels of the pro-inflammatory cytokines, like IL-8, IL-6 and IL-1β indicate that derivatized fullerenes can initiate a toxic response in HEK at specific concentration. Since nanotization substantially alters the physical and chemical properties (reactivity) of a bulk chemical/drug, the efficacious and toxicological concentrations have to be worked out so that strategies for technological developments can be formulated. However, lack of in vivo studies indicates that more research is needed to investigate the effects of nanoparticles on skin.

Occupational hazards

Continuous nanoparticle exposure to the employees of various industries may lead to the development of pulmonary toxicity. Nanoparticles present as air pollutants can also lead to similar consequences when inhaled by people (Rasmussen et al., 2009). The inhaled nanoparticles gain entry into the body and therefore can cause various deleterious effect to the respiratory system.

Allergic reactions

Studies have indicated that the nanoparticle may be one of the risk factors of the asthma aggravation and allergic manifestation. Diesel exhaust particles (DEP) have been reported to increase allergic asthma and acute lung injury (Last et al., 2004; Veranth et al., 2007). The enhanced expression of different cytokines play an important role in the airway inflammation related to nanoparticles like carbon nanoparticles, in the presence of allergens by inducing the lung expression of thymus and activation-regulated chemokines, granulocyte-macrophage colony stimulating factor, macrophage inflammatory protein-1a, IL-2 and IL-10 as compared to allergen exposure alone (Inoue et al., 2006).

Pulmonary toxicity

Some nanoparticles are reported to enhance the harmful effects of some immunomodulating agents like ozone. Ozone (O$_3$) is a gaseous air pollutant and well known to produce acute and chronic toxicity in the respiratory system (Inoue et al., 2005). Combined exposure of carbon nanotubes (CNT) and ozone in C57BL mice has been studied (Han et al., 2008). Low concentrations of ozone gas by itself were found to produce minimal effects, but in CNT-exposed animals, there was a significant increase in total broncho-alveolar lavage (BAL) cells and polymorpho nuclear leukocytes. There is no size cut-off below which particles suddenly become harmful in the lungs. This is because harmful particles have their effects as a consequence of two factors that act together to determine their potential to cause harm: their large surface area, and the reactivity or intrinsic toxicity of the surface. The smaller the particles, more is their surface area and therefore their intrinsic toxicity gets magnified.

Nanoparticles of different types are relatively more toxic than the same material of larger size because of their ability to redistribute from their site of deposition. Following inhalation exposure, nanoparticles have been reported to travel via the nasal nerves to the brain, and may gain access to the blood and other organs (Inoue et al., 2006). It has been found that inhaled ultratrine or nanoparticles produce more potent adverse effects in the form of inflammation and subsequent tumors compared with larger sized particles of identical chemical composition at equivalent mass concentrations or intratracheally-instilled doses. Surface properties may play a significant role in nanoparticle particle toxicity. The very high size-specific deposition of nanoparticles when
inhaled as singlet ultrafine rather than aggregated ones also contributes to their effect. Some evidence suggests that inhaled nanoparticles like carbon nanotubes and ozone following deposition in the alveolar regions of the lung, largely escape lung defence (alveolar macrophage) surveillance and transmigrate from airspace to the interstitial regions of the lung, considered to be a vulnerable anatomical compartment in the respiratory tract (Inoue et al., 2006; Han et al., 2008).

Pulmonary toxicity is generally accompanied by the enhanced expression of different cytokines which might play an important role in airway inflammation related to various nanoparticles. Pulmonary inflammation and pulmonary carcinogenicity are the two major deleterious effects caused by inhalation of nanoparticles which might be accompanied by fibrosis and neoplastic lesions as well (Last et al., 2004).

SAFETY EVALUATION OF NANOPARTICLES

One of the major reasons of toxicology studies on nanoparticles to be lagging behind is that experimental designs recommended by theory is not always practically possible because of the extensive instrumentation and skilled manpower required. At times it is the availability of facilities that decides the type of characterization to be done rather than the study design or experimental need. Due to the previously discussed potential threats and adverse effects of the nanoparticles, the safety evaluation of the nanoscale materials has to be performed in order to completely understand the toxic effects of their exposure. There are different aspects involved in the safety evaluation studies of nanoparticles, which have to be dealt simultaneously. These are as follows:

Problems of agglomeration and surface coating

The phenomenon of agglomeration involves adhesion of nanoparticles to each other mainly due to enhanced Van der Waal's forces, caused by increased surface area to volume ratio (Powers et al., 2007). The tendency of nanoparticles to agglomerate begins during synthesis step, however, the rate of agglomeration is substantially enhanced when dispersed into a liquid medium (Murdock et al., 2008). Consequently during agglomeration, the number of nanoparticles decreases while the mass remains conserved. Therefore agglomerates have their size distribution shifted to higher side and possess a lower density, high mass and volume, slow rates of diffusion compared to primary particles constituting them (Murdock et al., 2008; Teeguarden et al., 2007). In this regard, carbon nanotubes dispersed in biocompatible surfactant PS80 showed more cytotoxicity on mesothelioma cell line compared to their agglomerated counterpart (Balbus et al., 2007). Hence size, specific surface area, number concentration and size distribution which are some of the many key parameters for toxicological assessment, get altered as the particles agglomerate. It is speculated that nanomaterials may cross biological barriers due to their small size; however, agglomeration will increase the size thereby affecting the transport. High surface area is, thus, considered the main factor for studying the adverse biological effects of nanoparticles (Nel et al., 2006; Sager et al., 2007).

There are different procedures to deagglomerate and disperse nanoparticles, for example, sonication, detergents, lung surfactants, polyethylene glycol (PEG), serum etc. ‘Surface coating’ is yet another method to prevent agglomeration of nanoparticles in solutions. This is performed by modifying the nanoparticle surface with polymers followed by dispersing them in ionic or non-ionic surfactants thereby decreasing the surface reactivity (Sager et al., 2007; Skebo et al., 2007). It was observed from various studies that broncho-alveolar lavage fluid (BALF) is an excellent vehicle to suspend nanoparticles without altering their inflammatory or toxic potential (Sager et al., 2007; Long et al., 2007; Warheit et al., 2007). However, surface modifications may also shield or influence the effects of nanomaterials on biological systems (Warheit et al., 2007; Derfus et al., 2004). Thus the durability and stability of such surface coatings inside biological environment have to be considered.

Dose response relationship

Assessment of the safety/toxicity of any test material requires the correct assessment of dose-response relationship. The dose is one of the important factors that govern the shape of dose response curve leading to the anticipated mode of action of the particle. In conventional toxicology, dose metric is generally based on exposure concentration of the substance while in the case of nanomaterials; there has always been a debate not only on the issue of dose metrics but also the shape and size, which are important physicochemical factors (Dhawan et al., 2009). Most of the studies are still carried out on the basis of conventional toxicology; however, some studies have considered surface area to be the best parameter for assessing cellular response on exposure to nanoparticles (Stoege et al., 2006; Oberdörster et al., 2007). It has been observed that surface area is the most appropriate parameter to evaluate the inflammatory potential and other toxicological effects on the biological systems (Teeguarden et al., 2007). Thus, size and surface area formulate the two important properties in relation to toxicity. Consequently with decreasing size, proportion of surface atoms increases making the particle surface more reactive (Wittmaack, 2007). It is this enhancement of surface reactivity at the nanoscale which may be accounted for the nanoparticles elicited biological responses. Due to this predominance of surface
dependent properties in case of nanoparticles, many investigators have considered surface area as the dose metric choice in their studies. Apart from mass and surface area, the number of nanoparticles in exposure medium is considered to be important for dose metric studies. It was suggested that particle number tends to work best as dose metric parameter (Teeguarden et al., 2007; Jiang et al., 2008). Selection of a suitable dose metric for in vitro nanotoxicity assessment is not enough for deciding cellular dose without taking into consideration many other factors associated with nanoparticles. In the case of soluble chemicals, the dose reaching the target site in an in vitro system is taken as equivalent to the delivered dose as mass and concentration are considered central attributes having direct impact on cellular responses. However, it is suggested that unlike soluble chemicals, particles can settle, diffuse and agglomerate depending on their size, shape, density and media characteristics. These factors acting together may affect the concentration of nanoparticles reaching the cells largely from the administered dose and subsequently affect the cellular responses. Due to different dose metric parameters being involved there is a considerable amount of variability in the similar studies on the nanotoxicology. Therefore the results obtained, must be analysed with respect to different types of dose metrics for nanotoxicology studies.

Relation of shape and size to toxicity

It has been emphasized that shape and size of nanoparticles can drastically modify its toxicity when compared to the respective bulk molecule. However, documented literature is relatively less on this aspect. Spherical gold nanoparticles of different sizes were not found to be toxic, but CTAB coated- gold nanorods were shown to be highly toxic to human keratinocytes (Wang et al., 2008). The cationic or positively charged gold nanoparticles (2 nm) showed greater toxic effects in Cos-1, RBCs and E. Coli compared to anionic or negatively charged gold nanoparticles of the same size (Yen et al., 2009). A more than 10 fold increase in ROS levels were observed in cells exposed to 15 nm silver nanoparticles when compared to 55 nm silver particles, which suggest that cytotoxicity of silver is mediated through oxidative stress and size is one of the most critical stage of toxic response (Carlson et al., 2008). In another study the effects of nanoparticles of metal oxides (TiO$_2$ and Al$_2$O$_3$) and multiwalled-carbon nanotubes (MWCNT) on two strains of bacteria viz Cupriavidus metallidurans CH34 and Escherichia coli MG1655 was investigated (Simon-Deckers et al., 2009). The results of the study suggest that nanoparticles toxicity depends on their chemical composition, size and shape but not on their crystalline phase. Further, E. coli MG1655 was sensitive to nanoparticles in terms of toxicity whereas C. metallidurans CH34 was not, suggesting that nanoparticles may pose a threat to environment by creating an imbalance in ecosystem (Simon-Deckers et al., 2009).

Characterization

Nanoparticles of different shapes and sizes having different surface related properties such as crystallinity, porosity, agglomeration, charge, solubility etc., may contribute to the responses generated by the nanoparticles in biological systems (Dhawan et al., 2009). Therefore, characterization of nanoparticle is extremely important prior to toxicological studies. There are five general rules that are involved in particle characterization (Powers et al., 2006):

1) Because of the large number of particles in a given mass of a nanoparticle system, it is imperative that the sample of particles measured be representative of the material. This is the foremost principle of particle characterization. The broader the size distribution, the more significant the errors will be if the sample is not representative.

2) Primary particle size and shape characteristics should be measured in the most dispersed state achievable.

3) The most appropriate physical principle of measurement and instrumentation should be selected for the intended application.

4) Enough particles must be measured to ensure that the desired limits of precision and accuracy are achieved. For broad particle size distributions, this may amount to tens of thousands of particles.

5) The particle characteristics should be measured under conditions as close to the point application as possible. For toxicology studies, this should include, if possible, the biological environment.

Size is one of the most important parameter which determines the functionality of a nanoparticle. There are a variety of methods available for determining size distribution and mean size of nanoparticles. However, there is variation in the data obtained from different methods due to the difference in principles involved in various techniques. Therefore one has to be well versed with the principles and technical details of the measurement methods (Dhawan et al., 2009; Tiede et al., 2008). Different techniques require the test sample to be present in different physical states. For example, Brunauer-Emmett-Teller (BET) technique is used for calculating surface area of solids following adsorption of gas molecules on the solid surface. In this procedure, a layer of liquid nitrogen is adsorbed on to the surface of particles, which is subsequently released on vaporization of that layer and measured (Tiede et al., 2008). Thus, BET surface is indicative of the surface area that is accessible to gases and the particle diameter is
If routine protocols may not apply, new methods may be adopted or formulated.

Nanoparticles

Specific size characteristics in vehicle for toxicity assessment

Route of entry

In vitro studies (Primary cells & cell lines)

In vivo animal studies

Risk calculation based on toxicity dose

Extrapolation of toxic doses to human

Risk assessment

- Zeta Potential
- TEM
- SEM

- Inhalation
- Dermal
- Oral

- Most challenging step (computational modeling etc.)

- Bio-absorption & pharmacokinetics
- Acute, sub-chronic, chronic toxicity
- Effects in organs

- Regulatory standards
- Strategies for prevention & intervention

Figure 1. Interdisciplinary research in nanotechnology safety.

calculated from already available specific surface area and density of particles. The advantage of BET is that it provides data in size and surface area simultaneously. The drawback of this procedure is its assumption of a monodispersed spherical system which reports only an average size and does not provide the size distribution of whole particles which is one of the parameter required for size-dependent toxicity assessment (Powers et al., 2007; Weibel et al., 2005).

Transmission electron microscopy (TEM) is the most widely used technique to measure nanoparticle size, distribution and morphology by image analysis. However, it is time-consuming and requires enough number of particles containing fields to be analyzed before any critical assessment can be made. Moreover, it measures the sample in dry form and not in suspensions and involves drying of samples under vacuum which may alter its properties. Another constraint of this technique is that it cannot measure the sample in the dispersed form, which has to be prepared prior to exposure of living systems (Powers et al., 2007). Dynamic light scattering (DLS) provides the information on size and size distribution in specific solvents or medium, which are used for exposure risk assessment. The size of the particles in Brownian movement is measured by applying Stokes-Einstein relationship.

Advantage of using this technique is that it can measure size, under conditions that resemble more to the exposure conditions. DLS also provides the zeta potential of nanoparticles in different solutions at different pH. Compared to TEM, DLS methodology is less time consuming and more cost effective giving an ensemble measurement of particles. The drawback of DLS method is that the size of nanoparticle analyzed is usually more than the size measured by TEM or BET, due to the presence of few solvent layers which enhances the hydrodynamic diameter of the particle (Hradil et al, 2007; Murdock et al., 2008). It is therefore suggested that characterization of nanoparticles at different experimental steps is extremely important so as to understand the specificity of the size and shape related exposure risk, for which data from different techniques must be gathered and compared for reliable, reproducible and useful results.

CONCLUSION

The safety evaluation of nanomaterials will be best conducted through multidisciplinary teams as shown in Figure 1, following physicochemical characterization of nanomaterial; either being assessed in vitro or in vivo.
assays. These investigations may be carried out by actual experiments or with the aid of computation. Thus, establishment of biodistribution on intended or accidental exposure, as well as study of environmental dispersal of different nanomaterials is a priority area. Methods are required to investigate whether nanomaterial retains its size and surface properties in the environment or in the biophase, and the duration for which it does so. There is a need for improvements in environmental risk assessment and nano- and micro-technologies may offer solution for this problem. Protocols should be developed for testing of all materials in the nanoscale, where there is the potential for substantial human exposure referring to the transport and burden (accumulation) of the particles at sites distant to the site of entry. Focussed research should be directed towards the understanding of the relation of size and surface area on the deposition, translocation, and toxicity of small particles. The difference in nanoparticle properties may give rise to different results. Moreover some specific properties of the nanoparticles may influence the results of toxicity tests (Kroll et al., 2008). Therefore, standardized protocols and guidelines for nanomaterials toxicity assessment should be framed after considering the nanoscale properties and reaching an appropriate consensus. Further, more in vivo studies are required to be carried out for the immunotoxicity assessment of the nanoparticles in order to explain the exact mechanism of nanoparticle uptake which is still not well understood. As the applications of nanoparticles in various fields specially in biomedical products is relatively new and no formal guidelines have been approved till yet, it is therefore important to formulate a proper guideline for the purpose of safety evaluation of nanoparticles. Hence, safety issues need to be impressed upon the industries and regulators simultaneously, to reduce the lag phase for the development of sustainable nanotechnology.

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