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Comparative investigation of cellular response of nanoparticles

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ABSTRACT

Nanoparticles are being designed with chemically modifiable surfaces to attach a variety of molecules to improve biosensing, imaging techniques, delivery vehicles, and other useful biological tools. Keeping this in view, the present research work is focused on investigation of cytotoxicity of metal oxide nanoparticles. Different metal oxide nanoparticles (e.g titanium dioxide, zinc oxide, iron oxide, aluminum oxide etc) of different sizes and different concentrations were used to investigate the cellular response. Electron microscopy and colorimetric assays were used to characterize the various process steps. Zirconium oxide nanoparticles were used in suspension form stabilized with stabilizer and the others were used as their aqueous suspension. Results clearly reflect that as the concentration increases, cytotoxicity also increases. As aggregation occurs, cytotoxicity decreases. In suspension with stabilizer, cytotoxicity is more as compared to aqueous suspensions.

Keywords: Zirconium oxide nanoparticles; iron oxide nanoparticles; titanium oxide nanoparticles; aluminum oxide nanoparticles; resazurin sodium.

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Introduction

The field of nanoscience has experienced unprecedented growth during the last decade and as a result has received a great deal of attention from the public, regulatory agencies and the science community. The recent advances in nanotechnology and the corresponding increase in the use of nanomaterials in products in every sector of society have resulted in uncertainties concerning environmental and health impacts. The variety of product available at nanoscale is extensive and all sorts of materials belonging to different classes i.e carbonaceous nanomaterials, metal oxides, semiconductor materials including quantum dots, zero valent metals such as iron, silver and gold, nanopolymers, nanosheets, nanofibers and nanowires are utilized for manufacturing different devices for diverse applications. However, there are many issues that must be properly addressed before we can apply nanomaterials to the field of nanomedicine or conduct science based occupational or environmental exposure risk assessments. The increased production of nanomaterials result in an increase potential for release to the environment, either deliberately in discharge or accidentally in spillages, and a

greater possibility of adverse environmental effects. The demand of insights into the toxicological effects of nanomaterials will continue to grow as new products are produced based on these nanomaterials. The human exposure to airborne nanoparticles has been ongoing since early evolution; the extent of exposure increased dramatically with the advent of the industrial revolution and will continue to raise with the rapid development of nanotechnology as human use and production of these particles increases. Nanoparticles have won enormous popularity in nanotechnology. Metal oxide nanoparticles show promise for many kinds of applications such as catalysis, medical diagnosis and therapy, sensors, cosmetics, solar cells and coatings. They have unique physicochemical properties that are not present in conventional bulk materials. These physicochemical characteristics such as small size and large surface area of nanoparticles are responsible for their toxicity. The metal oxide nanoparticles are similar in size to the major classes of biologically active materials (at nanoscale) used to effect chemical change (proteins), store and process information (DNA and RNA), and provide structure and transport (membranes, actin, microtubules). The similarity in size has prompted concern regarding how synthetic particles might interact with naturally occurring particles within biological systems. Thus, studies related to investigation of adverse effects of nanoparticles on humans have become a pressing issue and many researchers have concluded that nanoparticles can have substantial mobility in a variety of biological tissues. Nanoparticles can potentially cause adverse effects on organs, tissues, cellular, subcellular and protein levels due to their unusual physiochemical properties. Therefore, one hopes that nanoparticles would be one of nanomaterials, whose toxicity has been identified and recognized well before their industrial uses on a large scale. Metal oxide nanoparticles, in particular, have received increasing interest due to their widespread medical, consumer, industrial and military applications. Independent of the very small size of nanoparticles, several parameters play a dominant role in their enhanced magnetic, electrical, optical, mechanical and structural properties. Many of these characteristics have potential implication in nanoparticle toxicity, such as elemental composition, charge, shape, crystalinity, surface area and solubility **[1-5].** The unprecedented freedom to design and modify nanoparticles to accomplish very specific tasks is currently being realized. For example gold nanoparticles have important applications for biological diagnostics, cell labeling, targeted drug delivery, medical imaging and cancer therapy **[6-8]** Furthermore aluminum nanoparticles (Al NPs) have been proposed as drug delivery systems, specifically by encapsulating drugs that are nonionic or non- water soluble, with aluminum–magnesium hybrids to increase solubility, thus avoiding clearance mechanisms and allowing for site-specific targeting of drugs to cells. The use of titanium oxide nanoparticles in cosmetics and silver as an antimicrobial agent are common **[8-10]**. Klabunde and co-workers demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria [**11]**. A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface and structural properties **[12-17]**. Despite their wide application, there is a lack of information concerning their impact on human health and the environment [**18]**. The emitted nanoparticles will ultimately deposit on land and surface water bodies. Nanoparticles reaching land have the potential to contaminate soil, migrate into surface and groundwater, and interact with biota. Particles in solid wastes, wastewater effluents, direct discharges, or accidental spillages can be transported to aquatic systems by wind or rainwater runoff. While nanotechnology looms large with commercial promise and potential benefit, an equally large issue is the evaluation of potential effects on humans and other biological systems. Currently, there are no factual data on concentrations of nanomaterials in the environment, and certainly none on their physicochemical forms or distribution. The present work is focused on cellular response to different nanoparticles in different concentrations.

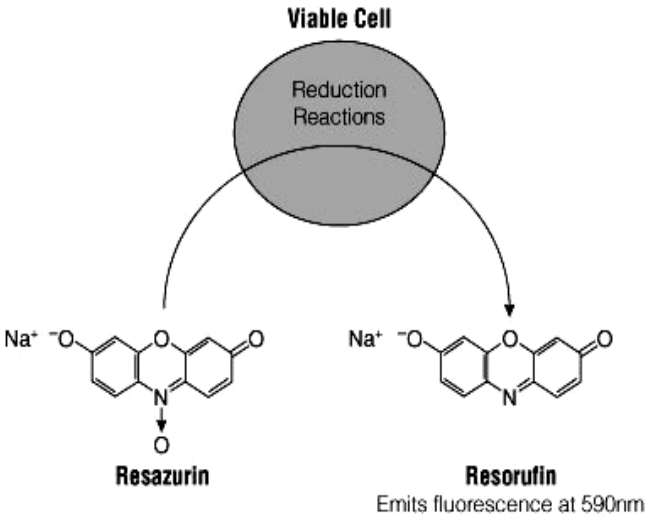

Fig. 1. Schematic representation of resazurin dye reduction.

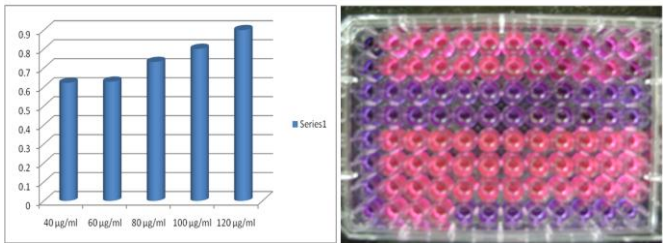

Fig. 2. 96 well plates after the resazurin assay.

Experimental

Cytotoxicity studies

The cytotoxic activity of nanoparticles was assessed by colorimetric assay using resazurin dye (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide, HiMedia) which is blue in color and non-fluorescent until it is reduced (**Fig. 1**) to the highly fluorescent pink colored resorufin (**Fig. 2**). Zinc

oxide, aluminum oxide, titanium oxide (90% pure, Nanostructured and Amorphous materials, USA) and zirconium oxide (Sigma-Aldrich) nanoparticles were used in the present study. Briefly, vero cell lines at a density of 1×10^4 per well were cultured in a 100-µL volume of cell culture medium (EMEM supplemented with 10% fetal bovine serum and antibiotics) in a 96-well cell culture plate. After 24 hours, cultured cells were treated with different concentrations of nanoparticles (40 µg/ml to 120 µg/ml of zinc, aluminium and titanium oxide nanoparticles in powder form and zirconium oxide nanoparticles in liquid form from 2 µl/ml to 10 µl/ml) well dispersed in 100µl of deionized water with sonication and incubated at various time points. After incubation, the samples were treated with 20 µl of the resazurin solution prepared in DMEM media and incubated for 4 hrs. After 4 hrs the pink colored resorufin is formed and absorbance was observed by spectrophotometer (ELISA plate reader) at 590 nm. With the help of absorbance cytotoxicity was calculated as absorbance is directly proportional to cytotoxicity because dead cells are responsible for high absorbance or we can say that viable or live cells increase the fluorescence. Nanoparticles used were in the size range of 10-40 nm and size was confirmed by SEM (scanning electron microscope) and TEM (transmission electron microscope).

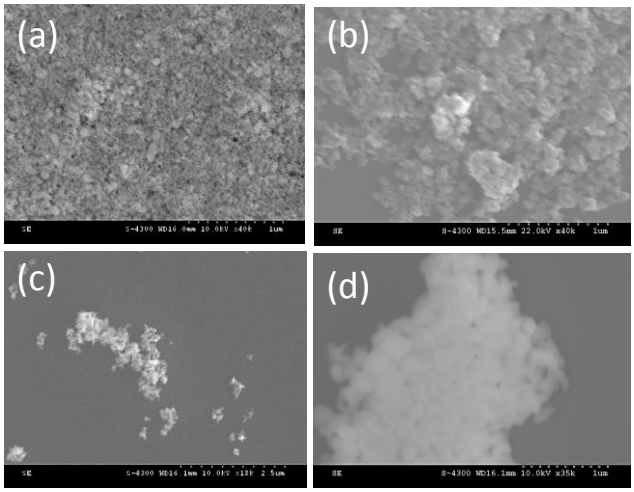

Fig. 3. SEM images of (a) ZrO, (b) Fe_3O_4, (c) TiO_2 and (d) Al_2O_3 particles.

Results and discussion

Fig. 3 shows the SEM micrographs of nanoparticles. The micrographs were obtained by placing the powdered sample of nanoparticles on the silver tape. **Fig. 4** reflects the TEM image of liquid nanoparticles. The image was taken by placing the suspension of nanoparticles on carbon coated copper grid. **Fig. 3 a-b** show that the nanoparticles are in cluster form and the size of nanoparticles varies between 30-40 nm. In **Fig. 3c**, the nanoparticles appear in aggregated form and individual particle size appears to be in the range of 50-70 nm. **Fig. 3d** reflects the SEM micrograph of aluminium oxide nanoparticles in aggregated form. **Fig. 4** i.e., TEM micrograph clearly represents that the individual particles are of size range 20-50 nm.

The fabrication of novel devices based on nanoparticles or hybrid materials incorporating nanomaterials demands proper evaluation especially when these devices are meant to be used inside human body. The toxicity mechanisms for most of nanoparticles have not yet been completely elucidated. The possible mechanisms include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, and interruption of energy transduction, formation of reactive oxygen species and release of toxic constituents. **Fig. 5a** shows that there is a regular increase in cytotoxicity with increase in concentration of iron oxide nanoparticles and there is a small difference in first two concentrations used.

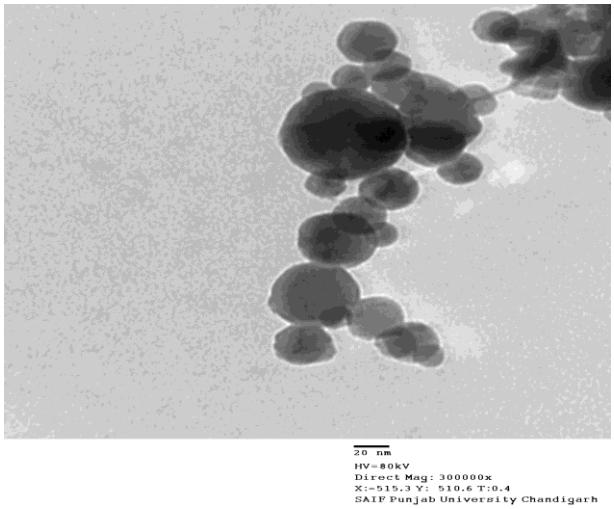

Fig. 4. TEM image of titanium oxide nanoparticles.

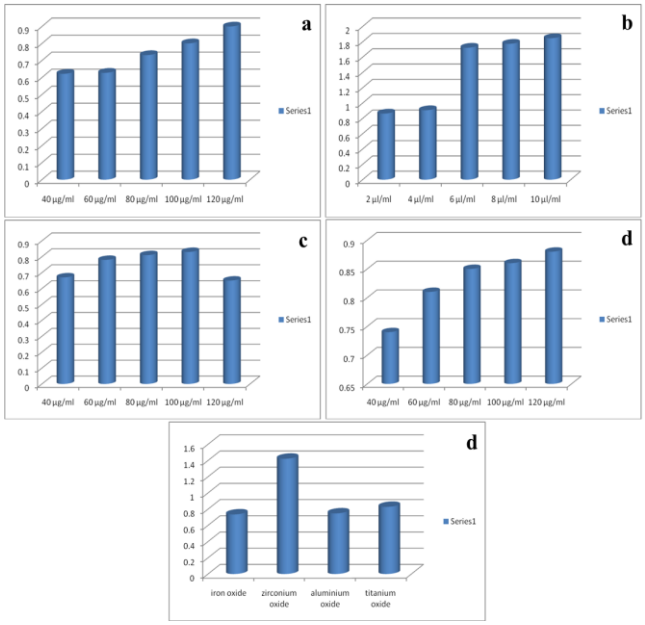

Fig. 5. Cytotoxicity graphes of Fe_2O_3 (a), ZrO (b), Al_2O_3 (c), TiO_2 (d), and comparative toxicity of different particles depends on various concentrations.

The **Fig. 5b** represents variation of cytotoxicity for zirconium oxide nanoparticles and shows large increase of cytotoxicity at concentration of 6 µl/ml. As the

concentration increases, cytotoxicity also increases. A dip at higher concentration (120 µg/ml) in **Fig. 5c** may be due to some experimental error or due to less penetration of nanoparticles in cells because of aggregation of nanoparticles at high concentration or may be a result of interference with the chemical probes, differences in the innate response of particular cell types, or other factors. Thus, problems may occur at high dose levels of nanoparticles where stability of the nanoparticle suspension may be compromised (due to aggregation). **Fig. 5d** reflects there is a gradual increase in cytotoxicity with increase in concentration because as the concentration of nanoparticles increases, number of particles in contact with cells and penetrating the cells also increases.

A comparative study in **Fig. 5e** shows that zirconium oxide nanoparticles are highly toxic than the other nanoparticles. Zirconium nanoparticles were taken in liquid form with stabilizer incorporated for retaining better dispersion. The aggregation does not occur and particles remain in segregated form and penetrate more in the cell. Aluminum oxide and iron oxide nanoparticles are almost same cytotoxic. However titanium oxide nanoparticles are somewhat more toxic. There can be many reasons for cytotoxic behavior of nanoparticles.

We need to have proper information on facts like: do nanoparticles retain their nominal nanoscale size and original structure and reactivity, what kind of association of nanoparticles exist with other colloidal and particulate constituents present around them, and what are the effects of solution and physical (temperature, flow, viscosity etc) conditions on nanoparticles long term stability. It is not yet clear that how human physiology responds to nanoparticles and one should be very careful with application of these materials inside human body. Nanoparticles may interact with proteins and enzymes within mammalian cells and they can obstruct the antioxidant defense mechanism leading to reactive oxygen species generation, the beginning of an inflammatory response and perturbation and damage of the mitochondria causing apoptosis or necrosis.

After systemic administration, nanoparticles may be able to penetrate very small capillaries throughout the body and efficiently distribute to certain tissues. In this case, nanoparticles passing through epithelia and biological membranes can potentially affect the physiology of any cell in the body. NPs may provoke oxidative stress and generate free radicals that could disorder the endothelial cell membrane. This interruption may cause blood–brain barrier dysfunction follow-on in the entry of nanoparticles into the central nervous system. After breathing of nanoparticles, cells in the respiratory system such as macrophages and epithelial cells that line the lungs may come into straight contact with NPs. Further translocation to the lymphatic system could induce secretary immune responses. In contrast, when nanoparticles enter the circulation, they may control endothelial cell membrane toxicity and/or disrupt the tight junctions of the blood–brain barrier and increase access into the cerebral environment.

Conclusion

Nanoparticles (NPs) can potentially cause adverse effects on organ, tissue, cellular, subcellular, and protein levels due to their extraordinary physicochemical properties (e.g., small size, high surface area to volume ratio, chemical composition, crystallinity, electronic properties, functional groups and surface structure reactivity, inorganic or organic coatings, shape, solubility, and aggregation behavior). The health and environmental risks posed by nanomaterials cannot be assessed easily because of their above mentioned unusual properties. It is observed that as particle size decreases, some metal-based NPs are showing increased toxicity, even if the same material is relatively inert in its bulk form. Another important factor which affects the cytotoxicity is the aggregation of nanoparticles. As aggregation occurs, particle size increases and cytotoxicity decreases. Size can be maintained with the help of sonication but it is not a permanent solution because after some time particles again aggregate. The cytotoxic investigation of zirconium oxide nanoparticles represent that aggregation can be avoided with the help of stabilizer which prevents the segregated particle to come closer. It is suggested that each new nanomaterial must be subjected to new health and safety assessment prior to its commercial use as it is very difficult to predict the toxic risks associated with any nanomaterial.

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