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Pilot Study of Identifying Hazardous Engineered Nanoparticles Using Human Cell

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Introduction:

Nanoparticle toxicity is amongst the rapidly upcoming area of environmental toxicological research due to its widespread implications. Different types of nanomaterials are now on the market either as raw materials or components of final products, causing great concerns over their impact on the environment and human health. This project aimed to identify hazardous nanoparticles (NPs) through their toxic effect on human cells. A549 carcinoma lung epithelial cells were used to mimic route of exposure via inhalation, as they have many characteristics in common with the human type II lung epithelial cells. Silica and polymer NPs were studied for their toxicity potential as they have wide industrial applications. Their physiochemical properties were analysed by hydrodynamic light scattering assay, transmission electron microscopy and scanning electron microscopy. Cellular toxicity endpoints, including cell viability, morphology and oxidative stress were investigated after NP exposure. All the NPs investigated induced cytotoxicity at a concentration of 25 µg/ml or above in a time dependent manner. Cell viability was assessed by MTT assay. This measures the activity of mitochondrial enzyme and is shown by viable cells having higher absorbance than dead cells. The EC50 was determined by the MTT assay as 80 µg/ml and 45 µg/ml for SiO2 NPs (7 nm) and methyacrylate NPs (80 nm) respectively at 72 h, and 180 µM for H2O2 (positive control) at 48 h. To further investigate the mechanisms of toxicity, the cellular level of reactive oxygen species (ROS) was assessed using the intercellular oxidative dye 2,7-dichlorofluoroscein diacetate as a biomarker of oxidative stress. All NPs except MA NPs increased ROS in A549 cells. In conclusion, this study has identified a toxic effect and the associated dose of several NPs, suggesting that more studies are needed to assess the health risk of human exposure to different engineered NPs.

Research Aim and Objectives

Nanotechnology offers great potential for improving the functionality of a wide range of products. However whether the application of such novel nanoproducts will have negative effect on human health is still uncertain. Due to the unique physical and chemical properties, NPs are potentially capable of interacting with biological system. The concerns over their environmental health and safety (EHS) are urgently needed to be addressed as their use in consumer products is rapidly increasing.

Aim of the project

This project aimed to identify and characterize toxic NPs by studying a number of toxicity endpoints using in vitro human cell models, with human A549 carcinoma lung epithelia cell lines. In association with an ongoing research project within Cranfield Nano research group, this project selected a number of NPs including SiO2 NP and MA NP.

Objectives

1. To characterize NP size and dispersity in a culture medium
2. To find the doubling time of cell growth using the specified cell line in this work.
3. To determine the effective cell seeding density for toxicity study
4. To study the effect of NP on cell viability, determining EC50
5. To determine whether the toxicity is associated with oxidative stress
6. To determine whether NP can initiate inflammatory response, using IL-8 as a biomarker.

Literature Review

Nanomaterials can be best defined as materials having one or more external dimensions in the range of 1-100 nm, including those having nanostructures (Lead and Wilkinson., 2006). A nanoobject is defined as a "discrete piece of material consisting one or more external dimensions on the nanoscale and a nanoparticle as a nanoobject with all the three dimensions on the nanoscale. Nanorods are nanoparticles comprising of two dimensions on the nanoscale whereas the third dimension lies outside the nanoscale that is consistently larger than the two other external dimensions (Lead and Wilkinson., 2006) as shown in (Figure 1-1).

According to the United States National Nanotechnology initiatives, Nanotechnology is defined as a "Research and technology development at the atomic, molecular or macromolecular levels, in the length scale approximately 1-100 nm; the use of the systems structures and devices possessing the novel properties and functions because of the small size; and the ability to be controlled and manipulated on the atomic scale" (NNI., 2004).

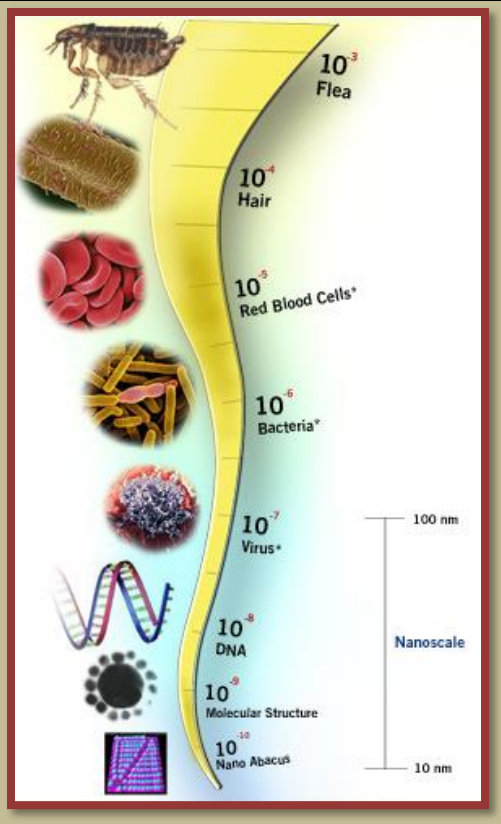

Figure 1 1. Differentiation of particles on a nanoscale based on their size (Cook., 2005).

Nanoparticles and the environment

A wide range of NPs exist in the environment. In urban and areas close to combustion sources particularly, particles exist in nature such as ferritin (i.e NPs with iron in it). Humic substances in soils and viruses are all in nanoscale. NPs that exists in the atmosphere are named as primary NPs, whilst the particles that formed in the atmosphere are named as secondary NPs. Environmental NPs can exist either in solid or liquid state and may even contain partially or completely volatile materials or exist as gas-to particle equilibrium (Van Dingenen et al., 2004).

The potential source of NP in the environment depends upon the various factors such as the industrially derived chemicals or manufactured NPs and their discharge into the environment during the manufacturing, transport, and application by consumers. Among the manufactured NPs, metal NPs such as gold, silver and copper, were the first to be prepared by man. The method of preparation depends on the reduction of the dissolved metal salt in the presence of surfactant or the capping agent. NPs can also occur in the environment following the processes of erosion and degradation of minerals and metals of natural sources on the earth (Nowack and Bucheli., 2007).

Exposure of nanoparticles.

Human beings are always exposed to airborne ultrafine particles from forest fires, volcanic eruptions or indoor fire places. However engineered NPs have a greater impact on many aspects of society and economy. There exists a wide range of scenarios such as work place, ambient air and consumers products application where the human beings can be exposed to the engineered NPs (Colvin., 2003).

The public may be exposed to NPs due to the contamination of air, water or the food chain or by the consumption of product that contains NP, or due to the results of discharge of materials into the environment as a waste or pollutant from the industry. Dermal exposure is a major concern about consumer exposure as a wide range of NPs have been used in cosmetic products. There is a possibility that the NPs can penetrate into the dermis causing local damage or reaching the blood stream causing systemic effects (Schneider et al., 1999, Hodgson et al., 1993).

Work place exposure to nanoparticles

Considering the occupational scenario during the development of a new material there always exists a possibility of workers exposure through inhalation, dermal contact or accidental spillage. Once the material reaches the commercial stage the exposure could occur during the synthesis, packaging, transport and storage, which are the most highlighted area as the quantity of material handling is larger under these circumstances. These particles enters the body react with the cells and cause tissue damage (ICTA., 2010)

To minimize the possible exposure the UK government developed a strategy in March 2010 that aimed to benefit the country and the consumer by the use of technology in a safe, reasonable and sustainable way that could benefit and fulfil the needs of public, industries and academia (UK Nanotechnology strategy March 2010). DEFRA (The department for environment, food and rural affairs) in the UK has worked in collaboration with Organisation for Economic Co-operation and Development (OECD) and the International Standards Organisation (ISO), aiming to develop regulations specific for engineered NPs, which has been recognized as a environmental health and safety issue and currently regulated under the Control of Substances Hazardous to Health (COSHH). As a result, the NPs manufacturers are now required to provide a complete report on;

- Identification of the nanomaterial used

- Estimation of the quantity of nanomaterial intended to be placed on the market per year
- Size of the NP used in the product
- Physical and chemical properties of the nanomaterial
- Toxicological profile and safety data for foreseeable exposure conditions relating to the category of cosmetic product.

While assessing the exposure it is mandatory to define the route of entry of NP into the human body. In occupational exposure most importance has been kept on inhalation, dermal exposure and ingestion.

Toxicity of nanoparticles

The effect of noxious chemical substances on human health is known as toxicology, which is dose and duration of exposure related. Often the chemical substances show toxicity when they reach the human body at relatively higher doses. Considering asbestos and crystalline SiO2 (alpha-quartz) as respirable particles, they have been found to be noxious in rats when they are inhaled at a dose of 6.2 mg/m3 at 6 h per day for 6 weeks intermittently to 108 mg/m3 for 6 hours/day for 3 days intermittently reported by National Toxicology Program (2009), and have been classified as carcinogens by the International Agency for Research on Cancer (IARC). In recent years there has been emerging alarming evidence on NPs toxicology. Much of the attention has been given to the engineered NPs as these particles are diverse in their chemistry, shape and size and they have found their use widely in the consumer products such as sunscreens, cosmetics, food additives, clothing and medicine. The severity of toxicity depends upon the physico-chemical properties as these are important determinants of their interaction with the biological systems (Hristozov and Malsc., 2009).

Silica nanoparticles

The application of Silica (SiO2) NP in recent years has extensively increased. They have been used as additives in the drugs, cosmetics, biomedical and biotechnological fields, in the development of Biosensors to measure the hypoxanthine levels in rats striatum (Zhang et al., 2005), in cancer therapy (Hirsch et al., 2003), DNA delivery, drug delivery (Lin et al., 2006), and enzyme immobilization (Barik et al., 2008). NPs have also wide applications in the nonmedical field as polishes, varnishes and in the food industry as packaging and additives.

Toxicity of nanomaterial SiO2 depends upon its crystalline state. Respirable SiO2 (alpha-quartz) is of great concern as the inhaled SiO2 found to be associated with the development of chronic respiratory diseases such as Silicosis or diffused fibrosis (Park and park., 2009) and cancer of the lung (Donaldson et al., 2007). Considering the noxious property of crystalline SiO2 and asbestos they are considered as carcinogens by the International Agency for Research on Cancer (IARC., 1997).

Toxicity associated with SiO2 is dose and size dependent. SiO2 at micro-scale size is found to be associated with the development of several autoimmune diseases, such as systemic sclerosis, rheumatoid arthritis, lupus, and chronic renal disease, while the crystalline SiO2 polymorphs may cause silicosis and lung cancer (IARC., 1997; Shi et al., 1998).A study conducted in vitro by Lin et al., (2006) considered the toxicity of SiO2 NP with size of 15 and 46 nm in bronchoalveolar carcinomic cell line. The results showed that the generation of ROS led to oxidative stress. On the other hand the study conducted by Kaewamatawong et al., (2006) reported the acute to moderate inflammatory reactions with the instillation of colloidal SiO2 of size 15 nm and 46 nm with the concentration ranging from 30-100 µg/ml into the lung of mice. The toxicity was associated with apoptosis and oxidative DNA damage. There are variety of SiO2 NPs differing in size, surface chemistry and methods of production. The difference in physiochemical properties will have a different influence on the ability of NP to interact with biological systems.

Results

Physico-Chemical Characterisation

DLS

The dynamic light scattering method (DLS) was employed to measure the particle hydrodynamic size, which demonstrates the extent of aggregation of particles in a suspension. (Fig. 4-1) shows a peak with a hydrodynamic diameter greater than 100 nm when SiO2 NPs of 7 nm was dispersed in water and sonicated for 20 min, suggesting the formation of aggregates. However, when the NPs were dispersed in culture medium and sonicated for 20 min, a peak with smaller hydrodynamic diameter appeared (Fig. 4-2). The peak representing large sized aggregates was still present and the intensity increased with time after sonication, suggesting that SiO2 NPs have a tendency to form aggregates.

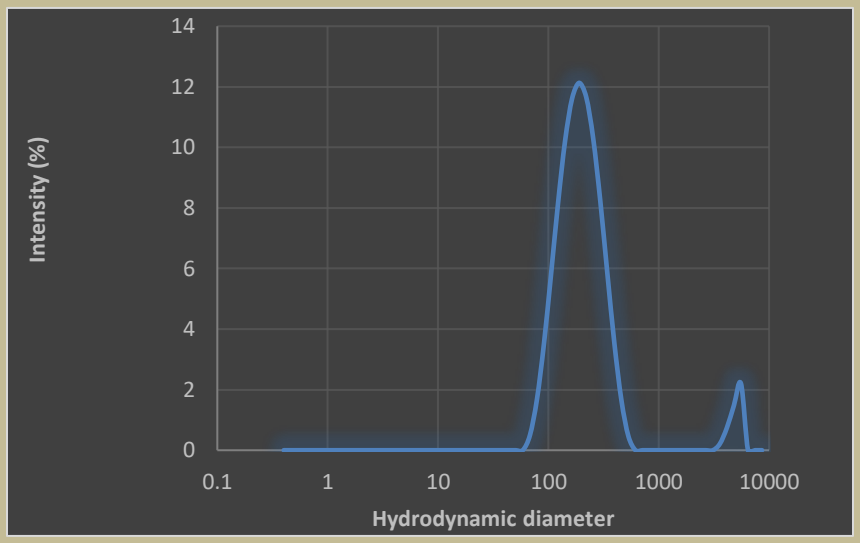

Figure 4 1. DLS measurements of SiO2 NP 7nm diluted with water and sonicated for 20 min.

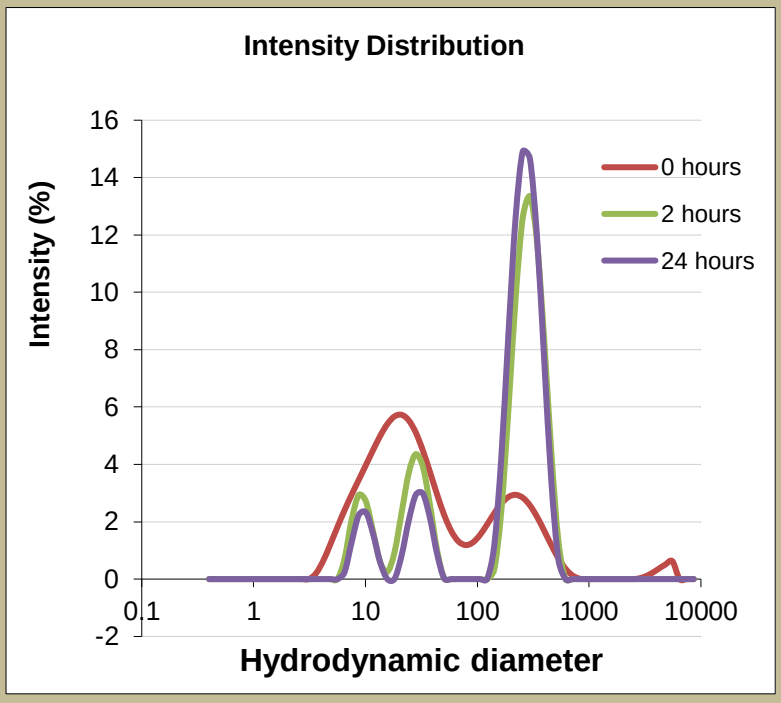

Figure 4 2. DLS measurement of SiO2 NP 7nm in medium.

SiO2 NPs 7 nm were diluted with DMEM medium and sonicated for 20 min. DLS performed at time points 0 h, 2 h and 24 h by using Malvern ZetaSize nano and polystyrene cuvettes. In order to confirm the consistency of the DLS analysis for nanoparticle dispersity and hydrodynamic diameter measurement, the MA NPs that were known as soluble and

monodispersion in water were analyzed. The monodispersion pattern was confirmed and the hydrodynamic diameter was in agreement with the given size of 80 nm (Fig. 4-3.), suggesting that the MA NP do not form aggregates.

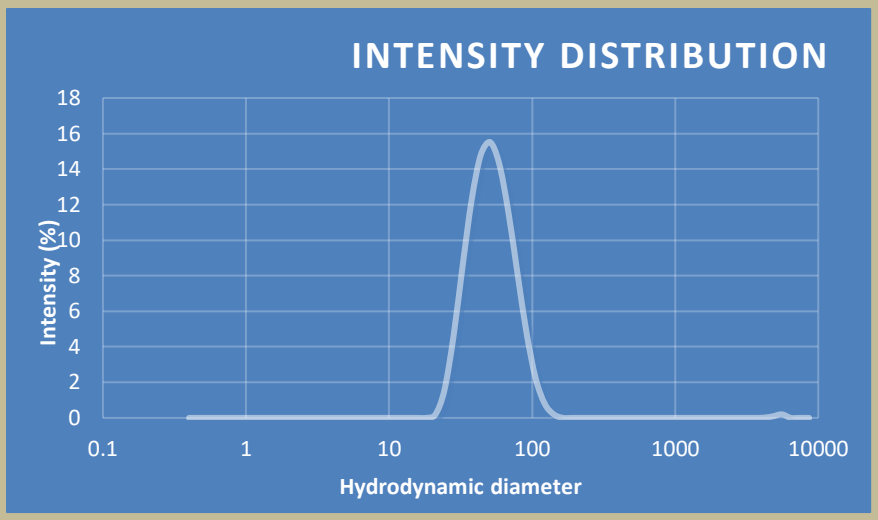

Figure 4 3. DLS measurements of MA NP of 80nm in water.

The formation of aggregates of SiO2 (7 nm) was also confirmed by TEM (Fig. 4 4.) and SEM (Fig. 4-5). The shape of the SiO2 NPs seems to be spherical. NPs appear to be in clusters, suggesting that the SiO2 NPs have the tendency to aggregate/ agglomerate when air-dried.

A. TEM image

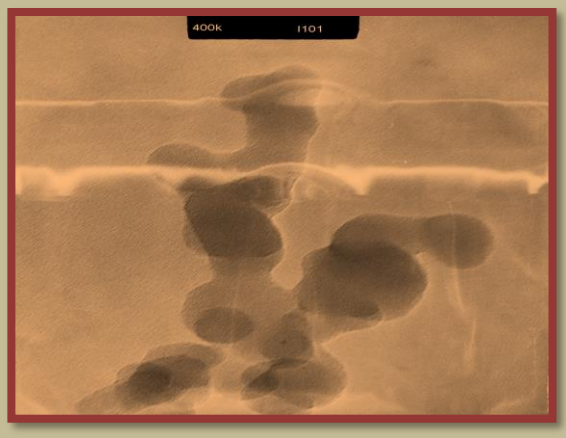

Figure Error! No text of specified style in document.**-1. TEM image of SiO_2 7 nm.**

B. SEM

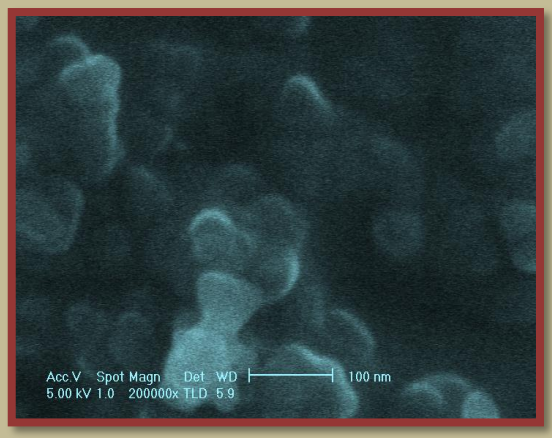

Figure Error! No text of specified style in document.**-2. SEM image of SiO_2 7 nm.**

NPs were diluted in water and sonicated for 20 min. A drop of sample was deposited on a stub and analysed (Images were displayed with permission by Dr. Zhu).

Optimization of cell seeding density: To ensure exponential cell growth during NP treatment, optimization of seeding density was carried out. A549 cells were seeded in 24 well plates at 6, 7

and 8 x 104 cells/well (3, 3.5 and 4 x 104 cells/cm2) in triplicate for each density. Cell growth was determined by MTT analysis (Fig. 4-6) at 4 time points. Exponential growth was confirmed during the 96 hour culture period at the chosen seeding densities. It was decided that these seeding densities can be used for toxicity studies when cells will be incubated with testing NPs or positive control for up to 96 h. The doubling time was estimated to be 22-24 h, the values are the mean of triplicate measurements.

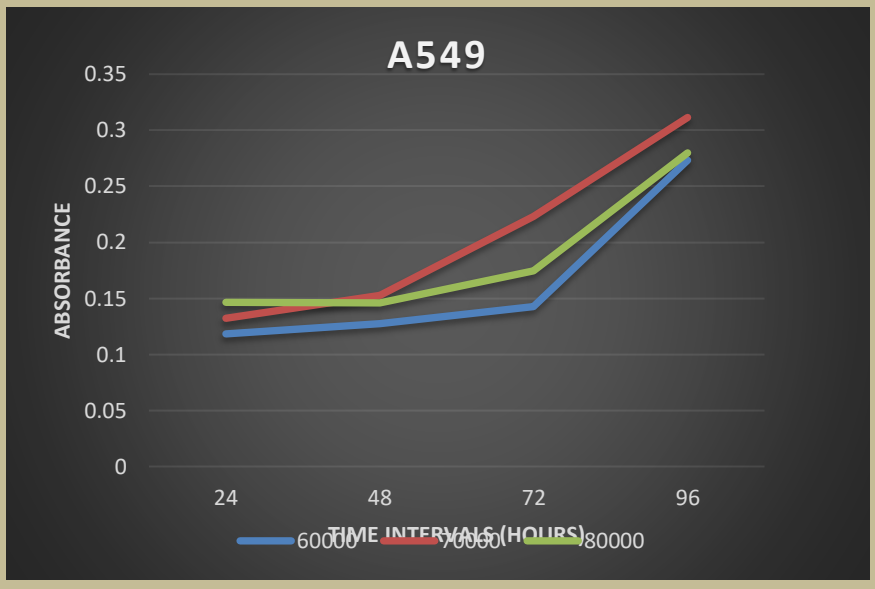

Figure 4 6. Cell growth curve of A549 cells in 24-well plates determined by MTT analysis.

Main findings of the study

- A number of methods have been employed to test the cytotoxicity of NP in vitro, and each has its own testing principle. Their combination will give a broad understanding of the mechanisms of NP toxicity.

- Considering the inhalation toxicity of NP, A549 cells were selected in this study. The doubling time of A549 cells was found to be 22-24 h at seeding density of 1-3 x 104cells/cm2. To study their toxicity potential, NP were first tested for their size and dispersity by means of DLS, TEM and SEM that demonstrated the tendency of NP to form aggregates over time, and the dispersity depends on the solvent employed for dilution. The toxicity doses of H2O2 (as positive control) and NP was studied by performing the cell viability assay by means of MTT. The EC50 for H2O2 was found to be at 180 µM at 48 h of exposure and the EC50 for SiO2 NP 7nm and MA NP were found to be 80 µg/ml and

45 µg/ml, respectively, at 72 h. All the SiO2 NP tested, but not MA NP, induced oxidative stress indicated by the increase in cellular ROS level. ELISA revealed the rate of release of inflammatory cytokine IL-8 was correlated with the cellular level of ROS in response to NP treatment.

- This study has identified toxic effect and dose of a number of NP in human A549 cells. The toxicity mechanisms appear to be distinct between SiO2 NP and MA NP; oxidative stress is associated with SiO2 NP induced toxicity.

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