Journal of Advanced Laboratory Research in Biology
E-ISSN: 0976-7614
Volume 5, Issue 1, January 2014
PP 7-11
https://e-journal.sospublication.co.in

Research Article

Relation of Particle size with Toxicity of Calcite Particles

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Abstract: The importance of certain types of nanomaterials and mineral nanoparticles, namely clays and the smallest mineral colloids, has been known for a long time. Mineral nanoparticles also behave differently than larger micro and macroscopic crystals of the same mineral. The variations in chemical properties are most likely due to differences in surface and near surface atomic structure, as well as crystal shape and surface topography as a function of size in this smallest of size regimes. Although most of the nanotoxicological studies were performed using unrealistic exposure conditions. Knowledge about potential human and environmental exposure combined with dose response, toxicity information will be necessary to determine real or perceived risks of nanomaterials following inhalation, oral or dermal routes of exposure. Because the respiratory tract is the major portal of entry for airborne nanoparticles, this exposure route can be used as an example to discuss some key concepts of nanotoxicology, including the significance of dose, dose rate, dose metric and biokinetics.

Keywords: Nanoparticles, Clay particles, Chemical properties, Particle size.

1. Introduction

Every year, there are 50 million cases of occupational respiratory diseases caused by inhalation of toxic dust and chemicals, which are allergenic and carcinogenic agents. A lot of dust and gases are generated in rock crushing and mining industries. Some dusty occupations impair lung function and cause pneumoconiosis (Osim *et al*., 1996; Wang *et al*., 1997; Kampalath *et al*., 1998). Unfortunately, our knowledge about what dusts and chemicals cause disease and how, is imprecise (Ellenhorn and Barceloux, 1998).

The importance of certain types of nanominerals and mineral nanoparticles, namely clays and the smallest mineral colloids, has been known for a long time. What has been generally recognized more recently is that nanominerals and mineral nanoparticles commonly behave differently as a function of their size within the nanoscale size range? Mineral nanoparticles also behave differently than larger micro- and macroscopic crystals of the same mineral. The variations in chemical properties are most likely due, to differences in surface and near surface atomic structure, as well as crystal shape and surface topography as a function of size in this smallest of size regimes.

Here, the atomic and electronic structure of nanoparticles may vary with size even without a phase transformation, and surface-to-volume ratios change dramatically. Such particles are minerals that are as small as roughly 1nm and as large as several tens of nanometers in at least one dimension. Limiting size in one, two, or three dimensions results in a nanofilm (or nanosheet), a nanorod, or a nanoparticle, respectively. Minerals can be found in all of these shapes, although this review will concentrate on nanoparticles. For any particular composition, each mineral expresses a set of specific physical and chemical properties. Nanominerals and mineral nanoparticles satisfy these criteria, except that even with a fixed composition, they express a range of physical and chemical properties depending on their size and shape.

Although, researchers can now engineer nanostructures to direct the intracellular or *in vivo* biodistribution, but the final metabolic fate is still unknown, and strategies for avoiding secondary unintentional behaviours are lacking. With a systematic and thorough quantitative analysis of the pharmacokinetics-absorption, distribution, metabolism, and excretion of nanoparticles are missing.

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Due to lack of cytotoxic studies in this area, we decided to perform cytotoxic studies and explore the potential role of oxidative stress in the adverse effects nanoparticles of calcite. The central hypothesis is that the ability of nanoparticles to cause oxidative stress underlies the association between increased exposure to different size particles and both exacerbations of lung disease and lung cancer. Pulmonary inflammation may increase by nanoparticles, although the mechanisms of the pulmonary inflammation of nanoparticles are not well understood. Nanoparticles are a complex mixture of various particle types and several of the components of nanoparticles are likely to be involved in the induction of oxidative stress. The most likely of these are transition metals, particle surfaces, and organic compounds. In support of this hypothesis, oxidative stress arising from nanoparticles have been shown to activate a number of redox-responsive signaling pathways in lung target cells. These pathways are involved in expression of genes that play a role in responses relevant to inflammation and pathological change, including MAPKs, NF-κB, AP-1, and histone acetylation. Oxidative stress from nanoparticles is also likely to play an important role in the carcinogenic effects associated with nanoparticles and hydroxyl radicals.

2. Materials & Methods

2.1 Particles

Calcite particles in different sizes were measured under phase contrast polarised optical microscope and Dynamic Light Scattering (DLS) was done on raw samples. To prepare MP and NP of uniform size, the powders of these three nanominerals were grinded in a ball mill (PM 100, Retsch, Germany) for 30 and 100 hours, respectively at alternative cycles of grinding (5 min.) and halt (15 min.) at 350 rpm using mixtures of different sizes of balls. The size of the NPs was measured by DLS.

2.2 Cell culture: A549 Cell line

Organism:	***Homo sapiens***
Source:	**Organ:** Lung **Disease:** Carcinoma
Comments:	This line was initiated in 1972 by D.J. Giard *et al*., through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. Further studies by M. Lieber, revealed that A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway. The cells are positive for keratin by immunoperoxidase staining.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** Air 95%; carbon dioxide (CO_2), 5%. **Temperature:** 37.0°C
Subculturing:	**Protocol:** 1. Remove and discard the culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains Trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). (**Note**: To avoid clumping does not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.) 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Cultures can be established between $2*10^3$ and $1*10^4$ viable cells/cm^2. Do not exceed $7*10^4$ cells/cm^2. 7. Incubate cultures at 37°C. **Interval:** Maintain cultures at a cell concentration between $6*10^3$ and $6*10^4$ cell/cm^2. **Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended. **Medium Renewal:** 2 to 3 times per week.
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO. **Storage temperature:** liquid nitrogen vapor phase.
Doubling Time:	About 22 hours

Fig. 1. Showing Culture Equipments.

2.3 Glass/Culture wares

Sterile glass/culture wares were used in the tissue culture studies mainly included, graduated disposable glass pipettes, Fast pipettes, disposable tissue culture flasks (T_{25} and T_{75} Cm^2), screw cap tubes, multiple well plate (96, 6 wells), microfuge tubes, Eppendorf, reagent bottles, micro tips, filter assembly etc.

2.4 Intracellular ROS measurement

The production of intracellular reactive oxygen species (ROS) is measured using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) by the method of Wang and Joseph, 1999.

2.5 Principle

The generation of intracellular ROS was measured using 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) probe, which is a membrane permeable molecule that is enzymatically hydrolyzed by intracellular esterases into DCFH (reduced), a nonpermeable molecule and then oxidized in the presence of ROS to the fluorescent product, DCFH (oxidized). As described by Wang and Joseph (1999), DCFH-DA passively enters the cell where it is broken down into cell impermeable, non-fluorescent reduced dichlorofluorescin (DCFH) and diacetate by cellular esterases. Now DCFH becomes oxidized with intracellular ROS to form the highly fluorescent compound dichlorofluorescin (DCF) that may be cell permeable.

2.6 Reagent Preparation

Stock solution 10mM DCFH-DA made in incomplete medium without phenol red.

2.7 Assay Protocol

a. The cells at 10,000 cells/well were seeded in a 96 well black bottom plate and were treated with Calcite parent, micro and nanoparticles at different concentrations (C, 100, 300, 500, 750, 1000µg/ml) for different time period of 24 and 48 hours.
b. After exposure (for a favourable time period) medium was discarded.
c. Then 100µl of 50µM DCFH-DA in medium (without phenol red) was added and kept in an incubator for 30min at 37°C. Stock solution 10mM DCFH-DA made in incomplete medium without phenol red.
d. The plates were labeled and the fluorescence reading ware took at 480nm excitation and 520nm emissions using a microplate reader.

Fig. 5. Showing 96 well black bottom plates.

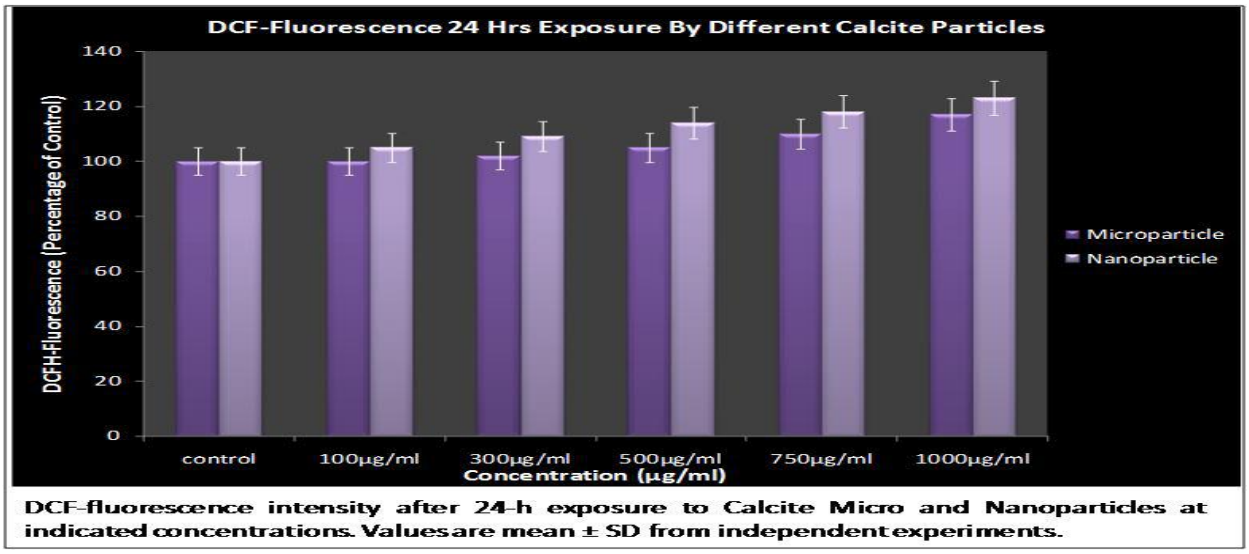

Fig. 1. The ability of calcite micro- and nanoparticles to induce intracellular oxidant production in A549 cells was assessed by measuring DCF fluorescence of ROS generation. The DCF fluorescence intensity increased after 24 h exposure to 2%, 5%, 10%, 17% for concentrations 300, 500, 750, 1000µg/ml, respectively for microcalcite particles. Nanocalcite at concentrations of 100, 300, 500, 750, 1000µg/ml, evaluated and found to increase ROS production by 5%, 9%, 14%, 18%, 23% respectively. The highest fluorescence obtained was that for indigenous nanocalcite at 1000µg/ml.

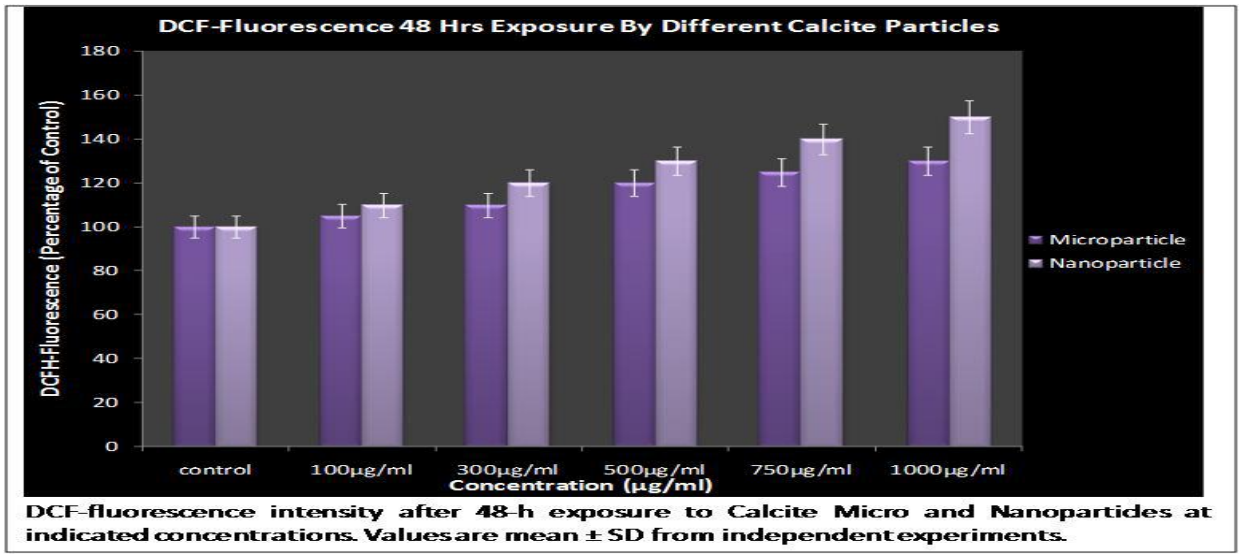

Fig. 2. The ability of calcite micro- and nanoparticles to induce intracellular oxidant production in A549 cells was assessed by measuring DCF fluorescence of ROS generation. The DCF fluorescence intensity increased after 48 h exposure by 5%, 10%, 20%, 25%, 30% for concentrations 100, 300, 500, 750, 1000µg/ml, respectively for microcalcite particles. Nanocalcite at concentrations of 100, 300, 500, 750, 1000µg/ml, evaluated and found to increase ROS production by 10%, 20%, 30%, 40%, 50% respectively. The highest fluorescence obtained was that for indigenous nanocalcite at 1000µg/ml.

3. Results and Discussion

In the present study, calcite micro and nanoparticles induced significantly higher ROS generation compared with untreated A549 cells when using the fluorescent dichlorofluorescin probe. Moreover, nanocalcite resulted higher ROS generation than microcalcite.

Since, calcite particles may also generate ROS through activation of NADPH oxidase by frustrated phagocytosis, leading to the initiation of pulmonary diseases particularly in occupationally exposed workers. Oxidative stress is known to elicit varying effects on the activity of antioxidant enzymes. The three primary scavenger enzymes involved in detoxifying ROS in mammalian systems are catalase, superoxide dismutase and glutathione peroxidase (Mates *et al*., 1999). For example, the activity of GPx can provide important clues about the consumption rate of GSH in enzymatic detoxification of ROS. The activity of antioxidant enzymes can, therefore, provide further insight into understanding the mechanism of

toxicity caused by talc particles and is currently under investigation.

For a given mass compared with larger particles, the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size. Particle size is thereby an essential determinant of the fraction of reactive groups on particle surface (Oberdorster *et al*., 2005; Nel *et al*., 2006). For example, several studies found that ultrafine particles are more toxic than its larger counterparts having the same chemical composition (Donaldson *et al*., 1998; Gilmour *et al*., 1997; Oberdorster *et al*., 1992, 1995; Oberdorster, 1996, 2000). Similarly, surface area-dependent induction of oxidative stress and consequently, pro-inflammatory effects have been found to correlate in case of polystyrene particles by Brown *et al*., (2001) and Lin *et al*., (2006). Where smaller nanoparticles of titania had effects comparable to larger nanoparticles of titania but showed a phase-dependent differential toxicity where anatase titania (photoactive phase), able to generate ROS more strongly, was 100 times more toxic than an equivalent sample of rutile titania. In the present study, different size particles would have resulted in differential toxicity.

In conclusion, we have demonstrated the toxicity, response elicited by the Calcite natural powder, Microparticles and nanoparticles depending on the size which showed that nanoparticles are more toxic than parent calcite powder and microparticles on A549 cells. Nanoparticles of calcite produced more ROS in A549 cells. Parent calcite had no such effect and partially decreased the ROS production. Results suggest that exposure of calcite, particularly nanopowder, should be protected in humans at risk of occupational as well as domestic exposure.

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