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ORIGINAL ARTICLE

Impact of Particle Size on Toxicity of Calcite Particles

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ABSTRACT

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.

Key words: *Nanoparticles, Clay particles, Chemical properties, Particle size*

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INTRODUCTION

Calcite is a carbonate mineral and the most stable polymorph of calcium carbonate (CaCO3). The Mohs scale of mineral hardness, based on scratch hardness comparison, defines value 3 as "calcite". Other polymorphs of calcium carbonate are the minerals aragonite and vaterite. Aragonite will change to calcite over timescales of days or less at temperatures exceeding 300 °C and vaterite is even less stable. Calcite is derived from the German Calcit, a term coined in the 19th century from the Latin word for lime, calx (genitive calcis) with the suffix -ite used to name minerals. It is thus etymologically related to chalk. When applied by archaeologists and stone trade professionals, the term alabaster is used not just as in geology and mineralogy, where it is reserved for a variety of gypsum; but also for a similar-looking, translucent variety of fine-grained banded deposit of calcite.

Although researchers can now engineer nanostructures to direct the intracellular or in vivo bio-distribution 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 is missing. 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 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, dosemetric and biokinetics. The most likely of these are transition metals, particle surfaces, and organic compounds. In support of this hypothesis, oxidative stress arising from nanoparticles has 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.

MATERIALS & METHODS

Particles:

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

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 A_{549} 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 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 do 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 X 10^3 and 1 X 10^4 viable cells/cm^2. Do not exceed 7 X 10^4 cells/cm^2. 7. Incubate cultures at 37°C. **Interval:** Maintain cultures at a cell concentration between 6 X 10^3 and 6 X 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

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.

Fig.1: Showing Culture Equipmments

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.

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 non permeable 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

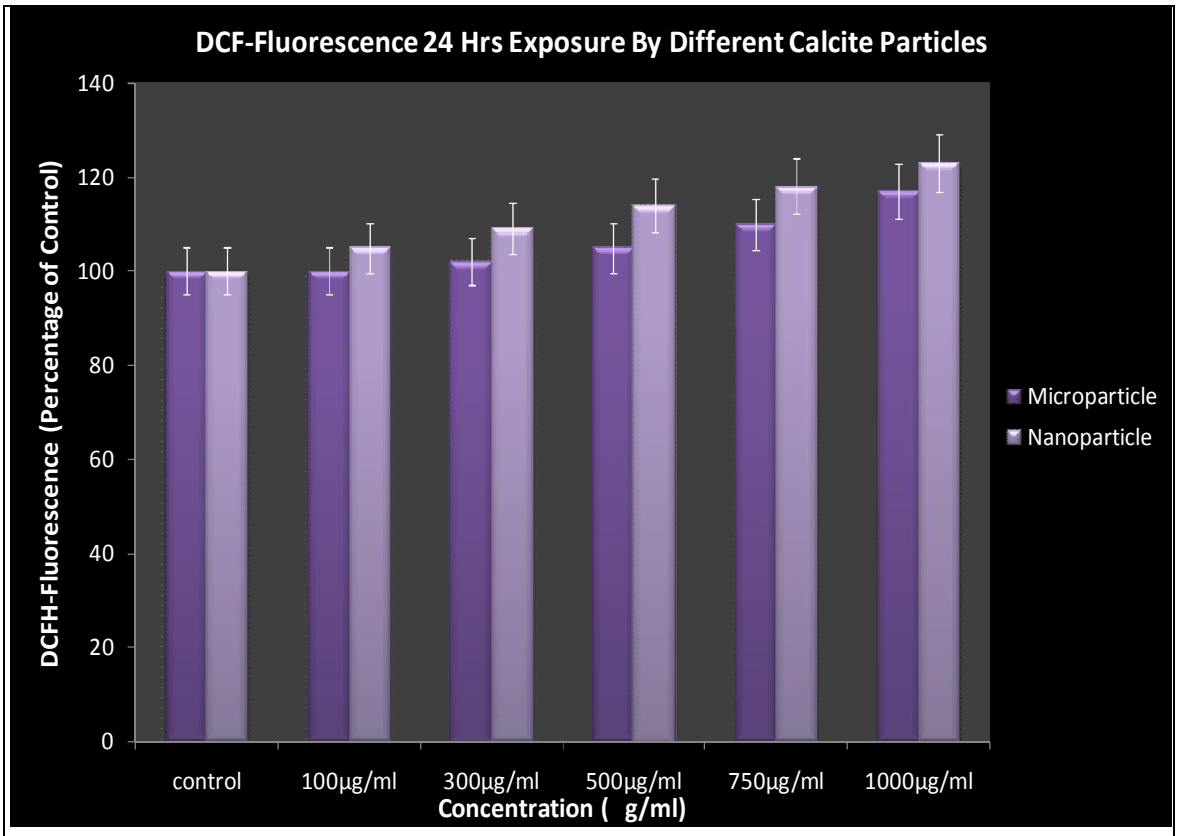
dichlorofluorescin (DCFH) and diacetate by cellular esterases. Now DCFH becomes oxidized with intracellular ROS to form the highly fluorescent compound dichlorofluorescin (DCF) that may be cell permeable.

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

Assay Protocol:

1. 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.
2. After exposure (for favourable time period) medium was discarded.
3. Then 100 µl of 50µM DCFH-DA in medium (without phenol red) was added and kept in incubator for 30min at 37°C. Stock solution 10mM DCFH-DA made in incomplete medium without phenol red.
4. The plates were labeled and the fluorescence reading ware taken at 480nm excitation and 520nm emissions using a microplate reader.

RESULTS AND DISCUSSION

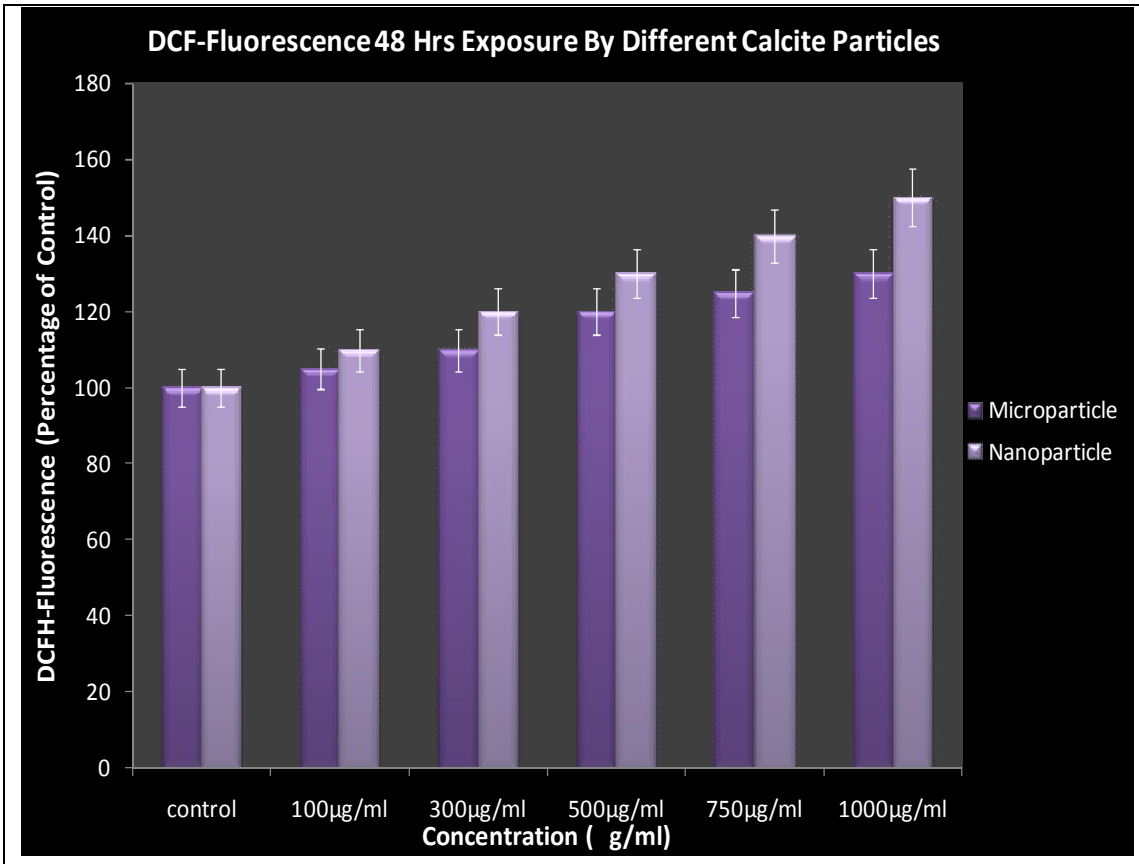


DCF-fluorescence intensity after 24-h exposure to Calcite Micro and Nanoparticles at indicated concentrations. Values are mean ± SD from independent experiments.

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

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.



DCF-fluorescence intensity after 48-h exposure to Calcite Micro and Nanoparticles at indicated concentrations. Values are mean ± SD from independent experiments.

Fig. 3: The ability of calcite micro- and nanoparticles to induce intracellular oxidant production in A549 cells was assessed by measuring DCF fluorescence of ROS generation

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.

Here, 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 1 nm 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 sharp.

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, nano calcite resulted higher ROS generation than microcalcite.

In recent researches, two different sets of Miller indices are used to describe directions in calcite crystals - the hexagonal system with three indices h, k, l and the rhombohedral system with four indices h, k, l, i. To add to the complications, there are also two definitions of unit cell for calcite. One, an older "morphological" unit cell, was inferred by measuring angles between faces of crystals and looking for the smallest numbers that fit. Later, a "structural" unit cell was determined using X-ray crystallography. The morphological unit cell has approximate dimensions a = 10 Å and c = 8.5 Å, while for the structural unit cell they are a = 5 Å and c = 17 Å. For the same orientation, c must be multiplied by 4 to convert from morphological to structural units. As an example, the cleavage is given as "perfect on {1 0 1 1}" in morphological coordinates and "perfect on {1 0 1 4}" in structural units. (In hexagonal indices, these are {1 0 1} and {1 0 4}.) Twinning, cleavage and crystal forms are always given in morphological units.

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 clue about the consumption rate of GSH in enzymatic detoxification of ROS. The activity of antioxidant enzymes can therefore provide further insight in 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 there by 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, proinflammatory 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 been resulted differential toxicity.

In conclusion, we have demonstrated the toxicity response elicited by the Calcite natural powder, Micro particles and nanoparticles depending on the size which showed that nano particles are more toxic then parent calcite powder and microparticles on A549 cells. Nano particles 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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