

## Cytotoxicity and cell membrane depolarization induced by aluminum oxide nanoparticles in human lung epithelial cells A549

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The cytotoxicity of 13 and 22 nm aluminum oxide (Al<sub>2</sub>O<sub>3</sub>) nanoparticles was investigated in cultured human bronchoalveolar carcinoma-derived cells (A549) and compared with 20 nm CeO<sub>2</sub> and 40 nm TiO<sub>2</sub> nanoparticles as positive and negative control, respectively. Exposure to both Al<sub>2</sub>O<sub>3</sub> nanoparticles for 24 h at 10 and 25 µg mL<sup>-1</sup> doses significantly decreased cell viability compared with control. However, the cytotoxicity of 13 and 22 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles had no difference at 5–25 µg mL<sup>-1</sup> dose range. The cytotoxicity of both Al<sub>2</sub>O<sub>3</sub> nanoparticles were higher than negative control TiO<sub>2</sub> nanoparticles but lower than positive control CeO<sub>2</sub> nanoparticles (TiO<sub>2</sub> < Al<sub>2</sub>O<sub>3</sub> < CeO<sub>2</sub>). A real-time single cell imaging system was employed to study the cell membrane potential change caused by Al<sub>2</sub>O<sub>3</sub> and CeO<sub>2</sub> nanoparticles using a membrane potential sensitive fluorescent probe DiBAC<sub>4</sub>(3). Exposure to the 13 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles resulted in more significant depolarization than the 30 nm Al<sub>2</sub>O<sub>3</sub> particles. On the other hand, the 20 nm CeO<sub>2</sub> particles, the most toxic, caused less significant depolarization than both the 13 and 22 nm Al<sub>2</sub>O<sub>3</sub>. Factors such as exposure duration, surface chemistry, and other mechanisms may contribute differently between cytotoxicity and membrane depolarization.

**Keywords:** cytotoxicity; aluminum oxide (Al<sub>2</sub>O<sub>3</sub>); nanoparticles; lung cancer cells (A549); depolarization; cell imaging

### Introduction

Nanomaterials are defined by the US National Nanotechnology Initiative as materials that have at least one dimension in the 1–100 nm range. In the past decade, nanotechnology has become one of the leading technologies (Stix 2001). Consequently, public concern about the environmental and health effects of nanomaterials is growing rapidly. Humans may be exposed to nanoparticles via several possible routes, including inhalation, dermal absorption, and gastrointestinal tract absorption. Due to their unique properties such as small size and corresponding large specific surface area, nanomaterials may impose

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different biological effects from their micro-scale material counterparts (Oberdorster, Oberdorster, and Oberdorster 2005; Nel et al. 2006). However, to date, toxicological and environmental effects of nanomaterials remain largely unknown.

As one of the widely studied and used nanomaterials, aluminum oxide ( $\text{Al}_2\text{O}_3$ ) nanoparticles have been applied in catalysis (Jodin et al. 2006), structural ceramics for reinforcements (Bertsch, Jiguët, and Renaud 2004), polymer modification (Cho, Joshi, and Sun 2006), functionalization of textiles (Textor, Schroeter, and Schollmeyer 2006), heat transfer fluids (You, Kim, and Kim 2003), and waste water treatment (Pacheco et al. 2006). In addition,  $\text{Al}_2\text{O}_3$  nanoparticles have shown wide biological applications in biosensors (Li et al. 2001), biofiltration, drug delivery (Popat et al. 2004), and antigen delivery for immunization purposes (Frey et al. 1999). Thus, the environmental and health impact of  $\text{Al}_2\text{O}_3$  nanoparticles is of great interest.

The *in vitro* and *in vivo* toxicity of  $\text{Al}_2\text{O}_3$  particles has been investigated in several studies. For instance, ceramic  $\text{Al}_2\text{O}_3$  particles (1.3  $\mu\text{m}$ , 2.4  $\mu\text{m}$ ) can induce significant cytotoxicity and cytokine release in macrophage cells (Catelas et al. 1998, 1999). Rollin, Theodorou, and Kilroe-Smith (1991) studied the deposition of aluminum in tissues of rabbits exposed to inhalation of low concentration of  $\text{Al}_2\text{O}_3$  dust; they suggested an extensive study to determine a more correct TLV (threshold limit value) and health based permissible concentration for occupational exposure to aluminum. Olivier et al. (2003) investigated the cytotoxicity of 0.43 and 2.81  $\mu\text{m}$   $\text{Al}_2\text{O}_3$  particles toward macrophage and fibroblast cells, and the results demonstrated that incubation with both sizes of  $\text{Al}_2\text{O}_3$  significantly decreased cell numbers in a particle concentration-dependent manner. However, these previous studies only focused on micro-scale  $\text{Al}_2\text{O}_3$  particles. To date, mechanisms of the cytotoxicity induced by  $\text{Al}_2\text{O}_3$  nanoparticles have not been reported. Depolarization is a decrease in the absolute value of a cell's membrane potential; that is, changes in membrane voltage in which the membrane potential becomes less negative indicate depolarization. In cell function, the potential across the membrane plays an important role by controlling ion fluxes across the cell membrane, signal transduction as well as osmotic balance of the cell. Studies have shown that depolarization appears to be an early sign of cell damage because of the cytotoxic activity of toxins (Ordonez et al. 1990; Radosevic et al. 1993).

In this study, we used DiBAC<sub>4</sub>(3) as the indicator of membrane potential. DiBAC<sub>4</sub>(3) is an anionic probe whose distribution across the cell membrane depends on membrane potential. When depolarization occurs, more dye enters the cells, resulting in an increase of fluorescence inside the cell.

The first objective of this study was to evaluate the cytotoxicity of  $\text{Al}_2\text{O}_3$  nanoparticles (13 nm, 22 nm) in human bronchoalveolar carcinoma-derived cells (A549). This cell line has been widely used in *in vitro* cytotoxicity studies (Huang, Khor, and Lim 2004; Upadhyay 2003). Twenty nanometer  $\text{CeO}_2$  nanoparticles were used as a positive control because our previous study had showed that  $\text{CeO}_2$  nanoparticles induce significant cytotoxicity at 5–25  $\mu\text{g mL}^{-1}$  dose range (Lin et al. 2006b). Forty nanometer  $\text{TiO}_2$  nanoparticles were selected as negative control because of its well-known no or low cytotoxicity (Lee, Trochimowicz, and Reinhardt 1985; Hussain et al. 2005). In the present study, particles were dispersed in the cell culture medium and diluted to 25, 10, and 5  $\mu\text{g mL}^{-1}$  doses and then immediately applied to cells. Cytotoxicity was measured by determining cell viability with the sulforhodamine B (SRB) method (Skehan 1990). The second objective of this study was to investigate a possible depolarization mechanism caused by nanoparticles. To accomplish this, we used a single molecule and single cell

imaging system (SMIS) with freshly-suspended nanoparticles in a free solution to monitor the initial interactions between nanoparticles and cells (Ma et al. 2001; Gai et al. 2005).

## Experimental

### Nanoparticles

Cerium oxide ( $\text{CeO}_2$ , 20 nm) and aluminum oxide ( $\text{Al}_2\text{O}_3$ , 13 nm, 22 nm) were synthesized with the room temperature homogeneous nucleation method (Zhou, Anderson, and Huebner 2002). Titanium oxide ( $\text{TiO}_2$ , 40 nm) was purchased from NanoScale Materials, Inc. (Manhattan, KS, USA). Particle size and distribution were analyzed by a Philips EM430 transmission electron microscopy (TEM) (Philips Electron Optics, Eindhoven, Holland). Crystal structures were characterized by a Scintag XDS 2000 diffractometer (Scintag, Inc., Cupertino, CA, USA). The surface area of the particles was determined by a Quantachrome Autosorb 1-C (Boynton, FL, USA). The characterization results are shown in Table 1.

Due to their nanoscale size and surface properties, nanoparticles tend to aggregate or precipitate in suspensions. In the present experiment, no dispersing agent such as a surfactant was used to improve the dispersion. As a result, the cells were exposed to and interacted with aggregated particles instead of individual particles. To understand the existing form of nanoparticles, the hydrodynamic size (aggregation size) of these four nanoparticles in cell culture medium was determined by dynamic light scattering using a Nanotrak NPA250 (Microtrac, North Largo, FL, USA).

In the cytotoxicity study (SRB assay), each stock suspension of nanoparticles was freshly prepared in the cell culture medium and dispersed using a sonication bath (FS-60H, 130 W, 20 kHz, Fisher Scientific, Pittsburg, PA, USA) for 5 min. Then the suspension was diluted to three doses (5, 10, and  $25 \mu\text{g mL}^{-1}$ ) and immediately applied to the A549 cells. The selection of  $5\text{--}25 \mu\text{g mL}^{-1}$  dose range was based on our preliminary dose response experiments of  $\text{Al}_2\text{O}_3$  nanoparticles (13 nm, 22 nm) at  $1\text{--}200 \mu\text{g mL}^{-1}$  dose range for 24 h exposure time (data not shown). The doses higher than  $25 \mu\text{g mL}^{-1}$  caused severe precipitation during the exposure time, so the doses higher than  $25 \mu\text{g mL}^{-1}$  were not used and doses lower than  $5 \mu\text{g mL}^{-1}$  showed no cytotoxicity in A549 cells.

The nanoparticle suspensions in cell imaging buffer (see Chemicals section) for the cell imaging experiments were freshly prepared and diluted to  $25 \mu\text{g mL}^{-1}$ , which was the highest dose in the cytotoxicity study.

Table 1. Characterization of  $\text{Al}_2\text{O}_3$  (13 nm, 22 nm), 20 nm  $\text{CeO}_2$  and 40 nm  $\text{TiO}_2$ . The particle size, surface area, crystalline structure, and hydrodynamic size were determined by TEM, BET surface area analyzer, X-Ray Diffraction (XRD), and dynamic light scattering spectroscopy, respectively.

Particles	Size and distribution nm (mean $\pm$ SD)	Surface area ( $\text{m}^2/\text{g}$ )	Crystalline structure	Hydrodynamic size in culture medium, nm (mean $\pm$ SD)
13 nm $\text{Al}_2\text{O}_3$	$13 \pm 2$	126.9	Crystal	$143 \pm 57$
22 nm $\text{Al}_2\text{O}_3$	$22 \pm 4$	128.5	Crystal	$157 \pm 60$
20 nm $\text{CeO}_2$	$20 \pm 3$	44.0	Crystal	$185 \pm 61$
40 nm $\text{TiO}_2$	$40 \pm 8$	560.4	Amorphous	$150 \pm 56$

### ***Chemicals***

Fetal bovine serum was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Ham's F-12 medium with l-glutamine, KCl, Na<sub>2</sub>HPO<sub>4</sub>, and NaHCO<sub>3</sub> was purchased from Fisher Scientific (Pittsburg, PA, USA). Penicillin-streptomycin, trichloroacetic acid (TCA), acetic acid, FeSO<sub>4</sub>, MgSO<sub>4</sub>, and ZnSO<sub>4</sub> were purchased from Sigma (Saint Louis, MO, USA). The CaCl<sub>2</sub>, NaCl, and D-glucose were obtained from Aldrich (Milwaukee, WI, USA). Sulforhodamine B (SRB) was bought from ICN Biomedicals (Irvine, CA, USA). The fluorescent marker bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC<sub>4</sub>(3)) was purchased from Molecular Probes (Eugene, OR, USA). Ultrapure DI-water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

The buffer used for cell imaging experiments contained CaCl<sub>2</sub>, NaCl, d-glucose, FeSO<sub>4</sub>, MgSO<sub>4</sub>, ZnSO<sub>4</sub>, KCl, Na<sub>2</sub>HPO<sub>4</sub>, and NaHCO<sub>3</sub> at the same concentrations found in Ham's F-12 culture medium. The buffer was adjusted to pH 7.2 and filtered using a sterile 0.2 μm polyethersulfone filter. The dye DiBAC<sub>4</sub>(3) was added to the imaging buffer to a concentration of 1.5 μM immediately before each experiment.

### ***Cell culture***

The human bronchoalveolar carcinoma-derived cell line, A549, was purchased from ATCC (Manassas, VA, USA). Cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum and 100 units mL<sup>-1</sup> penicillin, 100 μg mL<sup>-1</sup> streptomycin, and grown at 37°C in a 5% CO<sub>2</sub> humidified environment. For the SRB assay, A549 cells were plated into a 24-well plate at a density of 2.0 × 10<sup>4</sup> cells per well in 1.0 mL culture medium and allowed to attach for 48 h. For the single cell imaging experiment with SMIS, the cells were cultured on a sterile No. 1.5, 22 mm square glass cover plate.

### ***Assessment of cytotoxicity***

After the exposure of cells to nanoparticles, the medium was removed and the number of cells in each well was determined using the sulforhodamine B (SRB) assay. Briefly, cells were fixed with 500 μL of cold 10% trichloroacetic acid (TCA) for 1 h at 4°C. The TCA solution was then discarded, and the cells were washed three times with distilled water followed by drying completely in a fume hood. Five hundred microliters of 0.2% sulforhodamine B in 1% acetic acid were added to each well to stain the cells for 30 min at room temperature. The staining solution was discarded, and the cells were washed with 1% acetic acid to eliminate excess dye. After complete drying, the dye in each well was solubilized in 300 μL of cold 10 mM Tris buffer (pH 10.5). One hundred microliters of dye solution were transferred into a 96-well plate and absorbance was measured at 550 nm using a microplate reader (FLOURstar, BMG Labtechnologies, Durham, NC, USA).

### ***Single cell imaging assembly***

A high purity glass cover plate with cells cultured on one side was used in the study of cell-nanoparticle interactions. The plate was placed on the hypotenuse face of a right-angle fused-silica prism (Melles Griot, Irvine, CA; Prism UVGSFS, A = B = C = 2.54 cm) with the cultured cells facing down towards the prism surface. Before each set of experiments, the prism surface was washed with approximately 300 μL of 1.0 M HCl and approximately 500 μL of ultra pure water. The distance between the cell glass plate and prism surface was fixed at 127 μm using two PTFE film strips under opposite edges of the plate. The plate

was strapped firmly in place to prevent it from shifting or floating. Sample suspensions were first introduced into the channel using hydrostatic pressure at one open edge of the resulting 15 mm channel, while excess sample suspension was removed with a wick at the opposite edge. A 3 mL reservoir coupled to polytetrafluoroethylene (PTFE) tubing and a 75  $\mu\text{m}$  inner diameter fused silica capillary delivered a continual flow of sample suspension at a rate of 3.5  $\mu\text{L min}^{-1}$  of the 25  $\mu\text{g mL}^{-1}$  nanoparticle suspension. A continual flow design was used because of the very small volume of sample suspension contained in the 15 mm channel. While cells in the cytotoxicity assessment experiment were exposed to a nanoparticle suspension in the milliliter volume range, the nanoparticle suspension volume in the imaging channel was approximately 40–50  $\mu\text{L}$ . The difference in volumes meant that the cells exposed in the cytotoxicity experiment experienced contact with a “bulk suspension,” and therefore a nanoparticle number limited only by the rate of diffusion of the particles within that suspension. However, it was possible that cells exposed in the 15 mm channel would bind nanoparticles, thereby decreasing the number and concentration of nanoparticles in the extracellular environment. The slow yet continual stream of sample suspension assured that the concentration of nanoparticles in the small experiment chamber was constantly refreshed, without introducing a very large flow that might influence experimental results. The experimental setup is shown in Figure 1.

#### *Cell labeling for single cell imaging*

The fluorescent marker DiBAC<sub>4</sub>(3) was used to monitor the cells, as its fluorescence intensity is membrane potential-dependent (Laskey et al. 1992; Cornfield 1994). Each glass plate with adhering cultured cells was individually incubated at 37°C in fresh dye-spiked imaging buffer for 30 min. The glass plate was then removed, the side with cells was rinsed with 0.5 mL cell imaging buffer, and the plain glass side was carefully washed with ultra-pure water, dried, and prepared for the oil immersion objective with a drop of immersion oil. The glass was held at such an angle so that the ultra-pure water did not come into contact with the cells.

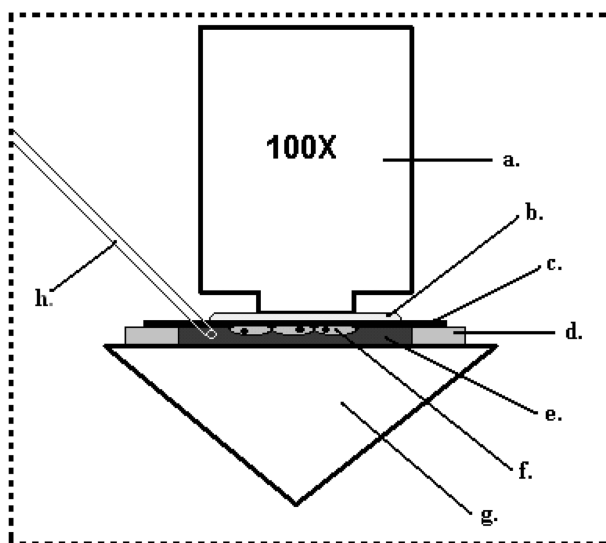


Figure 1. Sample introduction system for single cell imaging: (a) Objective; (b) Immersion oil; (c) Glass cover plate; (d) PTFE support; (e) Buffer; (f) Cells; (g) Prism; (h) Capillary.

**Equipment setup**

The experimental setup for this study represents a minor modification of the data acquisition system used in our previous experiments (Ma et al. 2001; Gai et al. 2005) and is shown in Figure 2. An Intensified Charge-Coupled Device (ICCD) camera (Princeton Instruments, Trenton, NJ, USA) was mounted on top of an Olympus upright microscope. The digitization rate of the camera was 5 MHz (12 bits), the controller gain was set at 70 and the camera was kept at  $-20^{\circ}\text{C}$ . The camera was operated in the internal synchronization mode and in the frame-transfer mode. The excitation source was an argon ion laser at 488 nm (Spectral Physics, Mountain View, CA, USA). Extraneous light and plasma lines from the laser were eliminated prior to its entry into the observation region with the aid of an equilateral dispersing prism, optical pinholes, and a 488 nm bandpass filter. The laser beam was passed through the right angle prism at an angle of  $50^{\circ}$  relative to the normal of the glass plate surface. At this angle, the laser light was totally reflected from the top surface of the glass plate to prevent scattered light from reaching the camera. A 488 nm holographic notch filter (Kaiser Optical, Ann Arbor, MI; HNFP) with optical density of  $>6$  and a 514–533 nm bandpass filter (Omega Optical, Brattleboro, VT; No. XF3003) were placed between the objective and the ICCD. An Olympus 100 $\times$ , 1.3 NA UPlanFl oil microscope objective was used to collect the fluorescence from the labeled cells. Imaging data were collected using Winview/32 software (Princeton Instruments, Trenton, NJ, USA).

The imaging data were analyzed as follows: for each sample, an image data file consisting of 25 video frames was collected every 5 min. Within each imaging file, a single cell was selected. Coordinates for the fluorescence signal at the brightest part of the cell were used to select that region of interest for numerical data analysis. The minimum size of this region of interest was typically  $10 \times 15$  pixels in size. The software calculated the average fluorescence signal in the region of interest. This average was calculated for all 25 frames of each data file, and these 25 averages were averaged in order to arrive at a single value for the fluorescence intensity of the cell's response in that imaging data file.

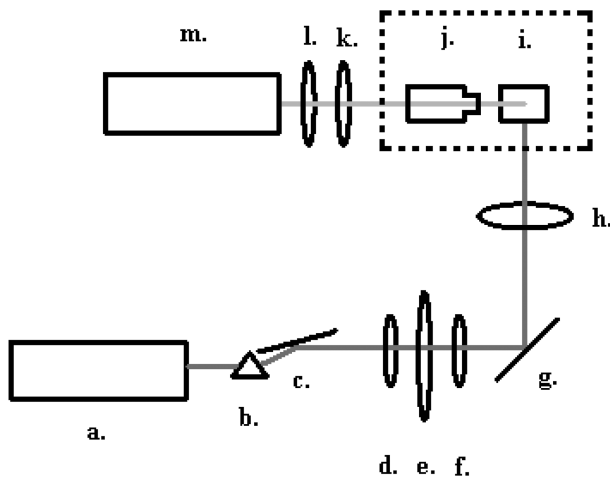


Figure 2. Optical setup for single cell imaging: (a) Laser; (b) Prism; (c) Mirror; (d) Pinhole; (e) Shutter; (f) Pinhole; (g) Mirror; (h) 488 Bandpass filter; (i) Sample and prism; (j) Microscope objective; (k) 488 notch filter; (l) Emission bandpass filter; (m) Camera.

The same regions of interest were used for all imaging data files of a given sample, to ensure that the same specific region of the same specific cell was measured every 5 min. The process was repeated to collect cell responses from multiple cells within each sample. Imaging data file examination showed that the cell response in the dimmer regions of the cell mirrored the trend in the bright regions, so the bright fluorescence regions were chosen arbitrarily for analysis. In addition, it was determined that within the 25 frames of an imaging data file, no great trend was typically visible in cell response from frame 1 to frame 25. For this reason, the 25 data frames were averaged together. Multiple cells were investigated for all samples, though cell response within each sample was very similar from cell to cell. Since cells in the same sample under the same conditions may yield slightly different levels of fluorescence intensity (Brauner, Hulser, and Strasser 1984), the percent change in fluorescence was compared over the course of the experiment. Finally, in order to account for environmental changes such as laser power fluctuations, a sample region containing no cells was analyzed and averaged, and then subtracted from the cellular fluorescence signal prior to the calculation of percent intensity change.

### *Statistical analysis*

In SRB assay, data were expressed as the mean  $\pm$  SD from three independent experiments. One-tailed unpaired Student's *t*-test was used for significance testing, using a *p*-value of 0.05. In cell imaging experiment, the same *t*-test was performed to determine if the relative fluorescence signal of the cell imaging data were significantly different from control samples.

## **Results**

### *Particle characterization*

The results from the characterization of Al<sub>2</sub>O<sub>3</sub>, CeO<sub>2</sub>, and TiO<sub>2</sub> nanoparticles were summarized in Table 1. The mean size and distribution of two sizes of Al<sub>2</sub>O<sub>3</sub> nanoparticles were 13  $\pm$  2 and 22  $\pm$  4 nm. The surface areas of these two sizes of Al<sub>2</sub>O<sub>3</sub> by BET measurement were 126.9 and 128.5 m<sup>2</sup> g<sup>-1</sup>, respectively. Thirteen and 22 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles demonstrated almost the same specific surface area, probably due to their aggregation. The XRD analysis clearly showed the crystalline structure of both Al<sub>2</sub>O<sub>3</sub> nanoparticles.

The hydrodynamic size of nanoparticles measured by a dynamic light scattering method indicates the extent of particle aggregation in culture medium (Table 1). The hydrodynamic sizes of 13 and 22 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles in medium were 143  $\pm$  57 and 157  $\pm$  60 nm (*p* > 0.05), respectively, indicating that aggregation occurred. The hydrodynamic sizes of CeO<sub>2</sub> and TiO<sub>2</sub> nanoparticles were 185  $\pm$  61 and 150  $\pm$  56 nm, respectively.

### *The cytotoxicity of Al<sub>2</sub>O<sub>3</sub> (13 nm, 22 nm) nanoparticles*

After A549 cells were exposed to Al<sub>2</sub>O<sub>3</sub> nanoparticles (13 nm, 22 nm) at 5, 10, and 25  $\mu$ g mL<sup>-1</sup> for 24 h, cell viability decreased as a function of dose levels (Figure 3). A dose of 5  $\mu$ g mL<sup>-1</sup> of 13 nm Al<sub>2</sub>O<sub>3</sub> did not affect cell viability, while cell viability decreased significantly to 86.0 and 82.8% at 10 and 25  $\mu$ g mL<sup>-1</sup> doses, respectively (*p*'s < 0.05), in comparison with the control group. The dose response curve of the 22 nm Al<sub>2</sub>O<sub>3</sub> particles showed a similar trend; 10 and 25  $\mu$ g mL<sup>-1</sup> doses decreased the cell viability

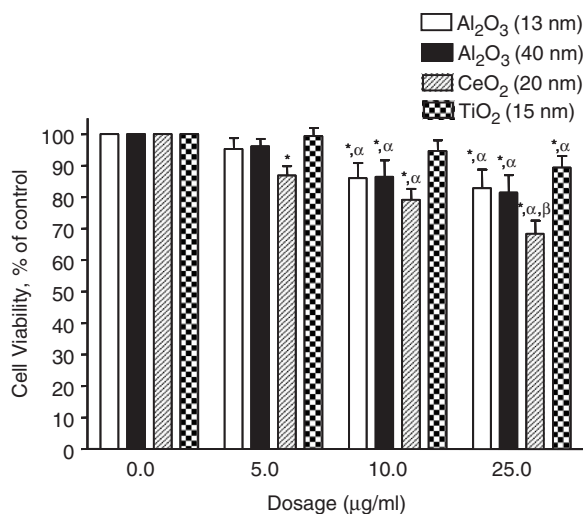


Figure 3. Viability of A549 cells after 24 h exposure to 5, 10, or 25  $\mu\text{g mL}^{-1}$  of  $\text{Al}_2\text{O}_3$  (13 nm, 22 nm),  $\text{CeO}_2$  (20 nm), and  $\text{TiO}_2$  (40 nm).  $\text{CeO}_2$  and  $\text{TiO}_2$  were used as positive and negative control, respectively. Values are mean  $\pm$  SD from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Significance indicated by: \* $p < 0.05$  vs. control cells,  $^{\alpha}p < 0.05$  vs. cells exposed to 5  $\mu\text{g mL}^{-1}$  dose,  $^{\beta}p < 0.05$  vs. cells exposed to 10  $\mu\text{g mL}^{-1}$  dose.

significantly to 86.4 and 81.4%, respectively ( $p$ 's  $< 0.05$ ). Interestingly, the cytotoxicity of both  $\text{Al}_2\text{O}_3$  particles had no significant difference from one another at 5–25  $\mu\text{g mL}^{-1}$ .

Twenty nanometer  $\text{CeO}_2$  nanoparticles and 40 nm  $\text{TiO}_2$  nanoparticles were used as the positive control and negative control, respectively, to evaluate the relative cytotoxicity of  $\text{Al}_2\text{O}_3$  nanoparticles (Figure 3). The cell viability of the groups exposed to 5, 10, and 25  $\mu\text{g mL}^{-1}$   $\text{CeO}_2$  particles decreased significantly to 86.8, 79.1, and 68.3%, respectively, compared to the control group ( $p$ 's  $< 0.05$ ). However, only 25  $\mu\text{g mL}^{-1}$  dose of 40 nm  $\text{TiO}_2$  nanoparticles significantly decreased cell viability to 89.3% ( $p < 0.05$ ). The cytotoxicity order of these particles is: 20 nm  $\text{CeO}_2 > 13$  nm  $\text{Al}_2\text{O}_3 = 22$  nm  $\text{Al}_2\text{O}_3 > 40$  nm  $\text{TiO}_2$  nanoparticles.

#### ***Time course and dose-dependent cytotoxicity of 13 nm $\text{Al}_2\text{O}_3$ nanoparticles***

Due to the similar cytotoxicity, only 13 nm  $\text{Al}_2\text{O}_3$  nanoparticles was used in the subsequent time course cytotoxicity study. A549 cells were exposed to  $\text{Al}_2\text{O}_3$  at 5, 15, and 25  $\mu\text{g mL}^{-1}$  dose levels for 6, 12, and 24 h. No doses after 6 h exposure showed significant different of cell viability compared with control group (Figure 4). However, the 25  $\mu\text{g mL}^{-1}$  dose showed significant cytotoxicity after 12 h exposure. After 24 h exposure, cell viability decreased further to 95.3, 86.0, and 82.8%, compared to the control, indicating the time-dependent cytotoxicity of  $\text{Al}_2\text{O}_3$  nanoparticles.

#### ***Single cell imaging to study the interaction of nanoparticles with cells***

To investigate the initial interactions of nanoparticles with the cells, we conducted a single cell imaging experiment using a SMIS. The nanoparticles chosen for investigation were 20 nm  $\text{CeO}_2$ , 13 nm  $\text{Al}_2\text{O}_3$ , and 22 nm  $\text{Al}_2\text{O}_3$ , because they showed greater cytotoxicity in



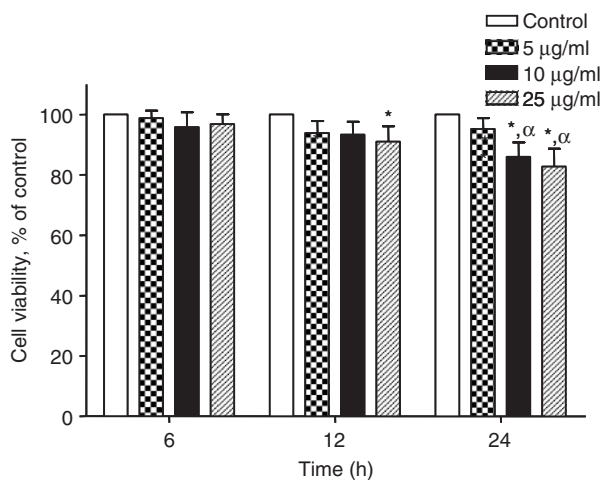


Figure 4. Viability of A549 cells after 6, 12, and 24 h exposure to 5, 10, or 25  $\mu\text{g mL}^{-1}$  of 13 nm  $\text{Al}_2\text{O}_3$  nanoparticles. Values are mean  $\pm$  SD from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Significance indicated by: \* $p < 0.05$  vs. control cells,  $^{\alpha}p < 0.05$  vs. cells exposed to 5  $\mu\text{g mL}^{-1}$  dose.

the dose response experiment. Cells were exposed to the same concentration (25  $\mu\text{g mL}^{-1}$ ) of nanoparticles, while the environment for control cells was imaging buffer. The concentration of nanoparticles chosen was stable for at least 1.5 h in this particular SMIS system. The cells were first labeled with the fluorescent membrane potential marker DiBAC<sub>4</sub>(3). An increase in fluorescence signal is an indication that the potential across the cell membrane is less negative than the membrane potential in healthy control cells. The fluorescence intensity of the control cells changed little over the course of 1 h. In contrast, cells exposed to the various nanoparticles showed a distinct increase in fluorescence intensity over the same period of time (Figure 5). Specifically, at the end of one hour, 13 nm  $\text{Al}_2\text{O}_3$ -treated cells showed an average signal increase of 86%, which was the greatest signal change seen during the experiment. Cells dosed with 22 nm  $\text{Al}_2\text{O}_3$  showed an average signal increase of 45%, while the 20 nm  $\text{CeO}_2$ -treated cells showed the least degree of signal increase, 37%. The control exhibited a relatively stable fluorescence signal, with the maximum average signal change being observed at 14%. Nanoparticle-exposed samples were statistically different from the average control signal at different times. The 22 nm  $\text{Al}_2\text{O}_3$ -treated cells showed a significant difference from the control by 10 min ( $p < 0.05$ ,  $N = 19$ ), yet the percent change in signal leveled out by approximately 30 min. The 13 nm  $\text{Al}_2\text{O}_3$ -treated cells showed a significant increase in signal by 15 min ( $p < 0.05$ ,  $N = 12$ ), and 20 nm  $\text{CeO}_2$ -treated cells by 55 min ( $p < 0.05$ ,  $N = 7$ ). The signal change in the  $\text{Al}_2\text{O}_3$ -treated cells was visibly evident. Cell morphology remained generally unchanged throughout the experiment.

## Discussion

### Cytotoxicity of $\text{Al}_2\text{O}_3$ nanoparticle

The purpose of this study was to compare the potential cytotoxicity of two sizes of  $\text{Al}_2\text{O}_3$  nanoparticles (13 nm, 22 nm) in cultured human bronchoalveolar carcinoma-derived cells. To date there are very few studies investigating the toxic effects and mechanisms of

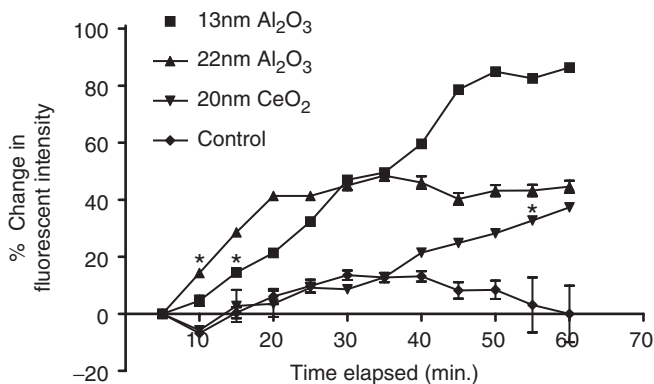


Figure 5. Averaged percent change in fluorescence intensity for Al<sub>2</sub>O<sub>3</sub> and CeO<sub>2</sub> nanoparticles, and control. Data were expressed as mean  $\pm$  coefficient of variation. The coefficient of variation is calculated by multiplying the standard deviation by 100 and dividing by the average. Nanoparticle data with an asterisk (\*) above them designate the first point in each respective nanoparticle data series to be significantly different from the controls. This significance for 13 nm Al<sub>2</sub>O<sub>3</sub> ( $N=12$ ), 22 nm Al<sub>2</sub>O<sub>3</sub> ( $N=19$ ) and 20 nm CeO<sub>2</sub> ( $N=7$ ) was calculated using Student's *t*-test ( $p < 0.05$ ).

Al<sub>2</sub>O<sub>3</sub> nanoparticles. Our results showed that the both 13 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles and 22 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles induced significant cytotoxicity at the levels of 5–25  $\mu\text{g mL}^{-1}$ . In addition, the cytotoxicity of 13 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles at the dose levels of 5–25  $\mu\text{g mL}^{-1}$  for 6, 12, and 24 h exposure showed that the cytotoxicity is dose- and time-dependent.

To determine whether the cytotoxicity of Al<sub>2</sub>O<sub>3</sub> particles (13 nm *versus* 22 nm) is size-dependent was one of our objectives. It is generally perceived that smaller particles cause greater toxicity because of their relatively larger specific surface area. For instance, in a previous study using titanium of diameters 3, 10, 50, and 150  $\mu\text{m}$  and titanium oxide of diameters 3  $\mu\text{m}$ , 50, and 500 nm, the survival rate of neutrophils decreased as the particle size decreased (Tamura et al. 2004). Brown et al. (2001) have shown the size-dependent proinflammatory effects of ultrafine polystyrene particles. However, our data showed similar cytotoxicity between 13 and 22 nm Al<sub>2</sub>O<sub>3</sub> particles in the 5–25  $\mu\text{g mL}^{-1}$  dose range. This result may mainly be due to aggregation of Al<sub>2</sub>O<sub>3</sub> nanoparticles in the cultured medium. Based upon the hydrodynamic size data, the 13 and 22 nm Al<sub>2</sub>O<sub>3</sub> particles aggregated to form the similar hydrodynamic sizes of  $143 \pm 57$  and  $157 \pm 60$  nm, respectively. The similar hydrodynamic sizes might provide an explanation as to why they showed similar cytotoxicity. Our previous study of cytotoxicity of 15 and 64 nm SiO<sub>2</sub> nanoparticles also showed the similar hydrodynamic size and cytotoxicity (Lin et al. 2006a). Because metal oxide nanoparticles, such as Al<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>, and CeO<sub>2</sub> tend to aggregate in cell culture medium and the aggregation size (hydrodynamic size) is quite different from its original particle size, the aggregation size of nanoparticles should also be considered in an *in vitro* study. Because of the complexity and diverse properties of particles, such as composition, size, shape, aggregation, surface activities, surface treatment, and crystallinity, a complete evaluation of the effects of various particle properties is warranted (Oberdorster, Oberdorster, and Oberdorster 2005).

### *Single cell imaging and the interaction of nanoparticles with cells*

To investigate possible initial mechanisms of toxicity, we studied changes in cell membrane potential by monitoring the fluorescence intensity of the anionic cell membrane marker DiBAC<sub>4</sub>(3). This marker has been widely used for investigating cell membrane behavior while cells are under environmental stress or other conditions (Xie and Zhuang 2001; Wolff, Fuks, and Chatelain 2003; Allen and Kirk 2004). When depolarization of the cell membrane occurs, the transmembrane dye equilibrium shifts, causing the dye to migrate inside the cell from the extracellular environment. The reverse is also true; hyperpolarization causes the observed fluorescence signal to decrease. In this study, the increase in fluorescence signal indicated depolarization of the cell membrane after the cells were exposed to Al<sub>2</sub>O<sub>3</sub> and CeO<sub>2</sub> nanoparticles. Such a condition could be caused by a number of pathways, including a protein-mediated influx of a specific cation(s), expulsion of a specific anion(s), or by physical breaches of the membrane. Under normal conditions, the potential across the cell membrane is maintained by the active transport of cations through the lipid bilayer (Widmaier, Raff and Strang 2004). Since the concentrations of ions such as Cl<sup>-</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, and Na<sup>+</sup> within the cell help regulate physiological pathways, a change in the concentration of each can activate a variety of mechanisms within the cell (Becker, Kleinsmith, and Hardin 2000). This includes even the triggering of apoptosis, as is the case with Ca<sup>2+</sup> (Orrenius, Zhivotovsky, and Nicotera 2003). Further research is necessary to further understand the exact pathway(s) by which the membrane potential is changed.

Exposure to the nanoparticles tested in the cell imaging experiment caused a distinct increase in the fluorescence intensity of the DBAC<sub>4</sub> dye in the cells (Figure 5). Of the three kinds of particles studied, the 13 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles appeared to have the greatest influence upon the cells. Moreover, the effect was time-dependent. Oscillation of the signal was observed in control cells, but the signal did not increase sharply. An ANOVA of the control data revealed that the control groups were not significantly different from one another ( $p > 0.05$ ). The 13 nm Al<sub>2</sub>O<sub>3</sub> caused the most membrane depolarization during the measured exposure time, followed by 22 nm Al<sub>2</sub>O<sub>3</sub>. Because the Al<sub>2</sub>O<sub>3</sub> particles disrupted the membrane potential more than the CeO<sub>2</sub> particles in the imaging experiment, the data from imaging the cell membrane potential change did not agree with the Al<sub>2</sub>O<sub>3</sub> nanoparticle cytotoxicity data.

In the cytotoxicity study, the 20 nm CeO<sub>2</sub> showed the greatest cytotoxicity. Different factors may contribute to this apparent discrepancy. First, the exposure time during the imaging experiment (60 min) was very short compared to the 24 h exposure in the cytotoxicity experiment. Thus, any changes observed during the imaging experiment represent only the *initial* effects of nanoparticles upon the cells. Cellular environmental conditions limited the length of the imaging study, as neither temperature nor atmospheric gas concentrations could be regulated. Second, it is reasonable to propose that the Al<sub>2</sub>O<sub>3</sub> nanoparticles may exhibit faster kinetics than CeO<sub>2</sub> nanoparticles when moving through the solution and interacting with the cells, due to the lower density of Al<sub>2</sub>O<sub>3</sub> nanoparticles. Third, CeO<sub>2</sub> nanoparticles may produce toxicity via mechanisms that do not involve disrupting the membrane potential. For example, our previous study has shown the cytotoxicity of CeO<sub>2</sub> nanoparticles was related to an oxidative stress mechanism and lipid peroxidation (Lin et al. 2006b). Fourth, since the chemical surfaces of the CeO<sub>2</sub> and Al<sub>2</sub>O<sub>3</sub> nanoparticles are different, they may interact with the membrane differently. The metal atoms on the surface of the two kinds of metal oxide nanoparticles are different in their size, charge (and charge density), valence electrons and valence electron orbital arrangements. These several factors may cause the difference in reaction time with the

cell membrane, as well as affect the number of nanoparticles interacting with the membrane. The presence of such differentiating factors would eventually cause a difference in the levels of cytotoxicity. These are areas meriting further research.

## Conclusion

We have demonstrated that 13 and 22 nm Al<sub>2</sub>O<sub>3</sub> nanoparticles significantly reduce cell viability in a dose- and time-dependent manner in bronchoalveolar carcinoma-derived cells at 5–25 µg mL<sup>-1</sup> doses. Both Al<sub>2</sub>O<sub>3</sub> particles showed similar cytotoxicity at a 5–25 µg mL<sup>-1</sup> doses range after 24 h exposure; this may be due to their similar aggregation size in cell culture medium. The real-time imaging study clearly indicated that cell membrane depolarization induced by two sizes of Al<sub>2</sub>O<sub>3</sub> and CeO<sub>2</sub> nanoparticles may be one of the initial mechanisms of action for nanoparticle cytotoxicity.

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