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Validation of an *in vitro* exposure system for toxicity assessment of air-delivered nanomaterials

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Abstract

To overcome the limitations of *in vitro* exposure of submerged lung cells to nanoparticles (NPs), we validated an integrated low flow system capable of generating and depositing airborne NPs directly onto cells at an air–liquid interface (ALI). The *in vitro* exposure system was shown to provide uniform and controlled dosing of particles with 70.3% efficiency to epithelial cells grown on transwells. This system delivered a continuous airborne exposure of NPs to lung cells without loss of cell viability in repeated 4 h exposure periods. We sequentially exposed cells to air-delivered copper (Cu) NPs *in vitro* to compare toxicity results to our prior *in vivo* inhalation studies. The evaluation of cellular dosimetry indicated that a large amount of Cu was taken up, dissolved and released into the basolateral medium (62% of total mass). Exposure to Cu NPs decreased cell viability to 73% ($p < 0.01$) and significantly ($p < 0.05$) elevated levels of lactate dehydrogenase, intracellular reactive oxygen species and interleukin-8 that mirrored our findings from subacute *in vivo* inhalation studies in mice. Our results show that this exposure system is useful for screening of NP toxicity in a manner that represents cellular responses of the pulmonary epithelium *in vivo*.

Keywords

Nanoparticles; Lung cells; Air–liquid interface; Deposition efficiency; Spatial uniformity; Cellular dose

1. Introduction

Metal-based nanoparticles (NPs) find many industrial applications and their extensive use has produced concerns that they may pose risks of significant adverse effects (Fahmy and Cormier, 2009; Schrand et al., 2010). Airborne NPs are of particular concern over human exposure, as they can readily move in ambient air and enter the body through inhalation

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Conflict of interest statement

The authors declare that they have no financial or non-financial competing interests.

(Maynard and Kuempel, 2005). Epidemiological studies have also shown an association between exposure to airborne ultrafine particulate matter (PM_{0.1}) and adverse health effects such as cardiovascular and pulmonary disease including bronchial asthma (Penttinen et al., 2001; Weichenthal et al., 2007). Aerosol delivery of NPs can result in deposition in the conducting airways and alveolar region of the lung and subsequent interaction with alveolar epithelial cells if the NPs are not rapidly cleared by the mucociliary escalator or alveolar macrophages.

In vitro and *in vivo* models are both used for testing of lung toxicity of airborne NPs, but *in vitro* assays as predictive screens for toxicity assessment of NPs in commerce are simpler, faster and more cost-effective (Kroll et al., 2009; NRC, 2006, 2007). *In vitro* models allow for extensive investigation of particle–cell interactions in human lung cells, which may be difficult to conduct *in vivo* (Paur et al., 2008). In such models, NPs are conventionally added to the culture medium as a suspension in which lung cells are submerged. In this process, the properties of the NPs can change due to particle–particle interactions and binding to components in the medium. Although the route of entry for inhaled NPs in the body generally occurs across the alveolar epithelium with its very large surface area and thin barrier thickness (Elder et al., 2009; Oberdörster et al., 2005), interaction pathways between NPs and alveolar epithelial cells remain largely unknown mainly due to the lack of an appropriate NP-cell exposure system. Thus, an optimal *in vitro* testing system should have several important features, namely that it uses cell types that represent those targeted by the routes of NP exposure, it allows accurate measurement of cellular dose and the aerosol deposition mechanism mimics real conditions that occur in the human lung.

Specification of the NP dose in a conventional *in vitro* testing system can cause significant misinterpretation of cellular responses and NP uptake (Teeguarden et al., 2007). In an attempt to improve the accuracy and predictive power of *in vitro* system for assessing NP toxicity, Teeguarden and colleagues have identified challenges associated with *in vitro* dosimetry and provided critical considerations on the cellular dose issues in the cytotoxicity of NPs and the need for accuracy in their measurements (Hinderliter et al., 2010; Teeguarden et al., 2007). They demonstrated using a computational model that cellular dose in cell culture media is a function of physical characteristics (e.g., size, shape, and agglomeration state) and surface chemistry of NPs. Thus, cellular dose of NPs in an *in vitro* testing system should be carefully considered before carrying out dose–response studies with NPs.

Since the respiratory system is susceptible to damage resulting from inhalation of particles, it is a prime target for potential adverse effects of NPs including direct lung injury, induction of lung inflammation and impairment of host defense (Card et al., 2008; Kim et al., 2011; Oberdörster et al., 2005; Stern and McNeil, 2007; Tetley, 2007). Epithelial cells lining the airway are the first lines of defense against inhaled inflammatory particles (Donnelly, 2001). The human airway epithelium forms a physical barrier between the lumen and the underlying-cells in the lung and participates in the inflammatory response in the lung (Karp et al., 2002). It produces a number of cytokines and other pro- and anti-inflammatory agents as well as secretes airway surface liquid (ASL) covering the epithelium. The ASL includes immunoglobulin A (IgA) and antimicrobial factors that form part of the defensive surfactant film that protects the airways and lungs from infection at the air–liquid interface (ALI) (Witherden and Tetley, 2001). One of the limitations in conventional submerged *in vitro* culture systems is that the ASL and mucus that cover the epithelial surface is removed or diluted. This does not reflect the physiological condition of lung epithelial cells that are exposed to air and separated by a thin liquid-protein monolayer lining the ALI of the alveoli. In exposed humans, inhaled NPs gain access to the systemic circulation by deposition from the airstream onto airway and alveolar epithelial membranes and their associated ASL.

Thus, an *in vitro* model system for NP toxicity testing should ideally replicate this architecture and deposition mechanism.

There is great interest in developing rapid screening methods that predict *in vivo* toxicity. Epithelial cells grown at the ALI have well-differentiated structures and functions compared to cells grown immersed (Kameyama et al., 2003). Thus, several different NP generation and deposition systems employing ALI have been developed and evaluated for NP toxicity testing (Bitterle et al., 2006; Lenz et al., 2009; Rothen-Rutishauser et al., 2009; Savi et al., 2008; Stringer et al., 1996; Tippe et al., 2002). It is necessary to fully characterize their performance in terms of NP delivery, uptake and impacts on the epithelial cells during NP exposure.

Although *in vitro* screening systems for assessing NP toxicity have beneficial advantages compared to *in vivo* assays, very few systematic attempts have been made to compare the results from *in vitro* studies to *in vivo* toxicity effects on the same materials using the same NP generation system for both types of studies. In this work, we first integrated a NP generation system utilized for our *in vivo* inhalation studies (Grassian et al., 2007a,b; Kim et al., 2011; Pettibone et al., 2008; Stebounova et al., 2011) with a commercially available ALI culture system to assess the pulmonary toxicity of NPs. The integrated *in vitro* exposure system was evaluated by determining physical (efficiency and spatial distribution of particle deposition) and biological performance (effects of the operating conditions on cell viability and cellular dosimetry) of the system prior to studies of NP toxicity. We also evaluated cellular responses after sequential (repeated low-dose) ALI exposure of A549 cells (human alveolar type-II-like cancer cells) to copper (Cu) NPs using the integrated *in vitro* exposure system.

2. Material and methods

2.1. Source and characterization of NPs

Cu NPs with a manufacturer's stated average particle size of 25 nm were used as received (Nanostructured and Amorphous Materials, Inc, Houston, TX, USA). Since we used these Cu NPs in our prior *in vivo* studies (Kim et al., 2011; Pettibone et al., 2008), they were selected to evaluate the integrated *in vitro* exposure system and to compare responses *in vivo* and *in vitro*. The Cu NPs were characterized using X-ray diffraction (XRD), transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS) and BET techniques as described previously (Pettibone et al., 2008). Evaluation by TEM of Cu NPs revealed an average primary particle size of 12 ± 1 nm, smaller than the manufacturer's stated particle size of 25 nm. The Cu NPs show a core/shell morphology with a metallic Cu core and an oxidized shell consisting of Cu₂O (Cuprite) and CuO (Tenorite), with CuO on the surface of the particles (Pettibone et al., 2008).

2.2. Endotoxin assay for NPs

To test if NPs were contaminated with endotoxin, the endotoxin content was measured using the kinetic chromogenic *Limulus* amoebocyte lysate (LAL) assay (Lonza, Inc., Walkersville, MD, USA) as described previously (Thorne, 2000). Briefly, all glassware was rendered pyrogen free by heating overnight at 200 °C. For each assay, a 12-point standard curve was generated over the concentration range 0.0244–50.0 EU/mL and referenced to control standard endotoxin (*Escherichia coli* E50-643). Endotoxin standards and 5-fold serial dilutions of sample were assayed in pyrogen-free microtiter plates (Costar No. 3596; Corning, Inc., Corning, NY, USA) in a microplate reader (SpectraMax 384 Plus, Molecular Devices, Sunnyvale, CA, USA) for 90 min at 37 °C. Spectrophotometric measurements were taken at 405 nm at 30 s intervals. The endotoxin level of Cu NPs was below the limit

of detection (0.0244 EU/mL). Thus, these materials were not contaminated with endotoxin and cellular responses induced by NP exposure in the study were attributable to the NP themselves.

2.3. Cell culture

A549 cells were obtained from ATCC (American Type Culture Collection, #CCL-185, Manassas, VA, USA). Cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT, USA) and 1% penicillin (100 units/mL) and streptomycin (100 µg/mL, Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO₂. For ALI exposure experiments, cells were harvested with 0.25% trypsin-ethylenediamine tetraacetic acid without phenol red (Trypsin-EDTA, Invitrogen), counted and seeded at a density of 2.5×10^5 cells/mL into 4.7 cm² commercially available transwell membranes without collagen treatment (No. 3450, polyester, 0.4 µm, Transwell, Corning, NY, USA). Cells were allowed to attach to the transwell for 12 h. When cells had grown to confluence (>85% for toxicity testing and 100% for dosimetry studies), they were cultured at an ALI for 12 h prior to exposure of NPs. Cells were then washed twice with PBS and transferred to the *in vitro* exposure system where they were supplied with medium (phenol-free RPMI 1640) from the basal side through the semi-permeable transwell membrane. A constant pH was maintained by using medium buffered with 25 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES, Invitrogen).

2.4. The *in vitro* exposure system

The integrated *in vitro* exposure system consists of a NP generation system, measurement system and an ALI culture chamber (Fig. 1). For these validation studies, a suspension of NPs in water (Optima grade, Fisher Scientific, Pittsburgh, PA, USA) was nebulized to generate a NP aerosol. A 1 mg NP/mL suspension was ultra-sonicated with a high frequency probe set at 30% of the maximum amplitude (20 kHz, model 550, Fisher Scientific, Pittsburgh, PA, USA) for 10 min to minimize the degree of agglomeration. The suspension was placed in a 3-jet Collison nebulizer (BGI Inc., Waltham, MA, USA) supplied with filtered, pressurized air controlled at a precise flow rate (4.75 L/min) and pressure (15 psig). Aerosolized droplets were dried by passing the airstream through a 30-cm, 110 °C brass drying column and vapor condenser consisting of a flow-through 1-L bottle submerged in an ice bath. The dry NP aerosol was passed through a ⁸⁵Kr charge neutralizer (model 3077A, TSI Inc., Shoreview, MN, USA) to remove electrostatic charges on the particles prior to entering the ALI chamber. A scanning mobility particle sizer (SMPS, model 3080 Electrostatic Classifier with model 3081 Differential Mobility Analyzer and model 3785 Condensation Particle Counter, TSI Inc.) was connected to the exposure system for continuous monitoring of the NP aerosol count distribution.

NP aerosols were delivered in a dynamic mode (particles carried by convective transport) to the manifold of a commercial ALI cell culture chamber (6-well module, Vitrocell®, Waldkirch, Germany) through an aerosol distribution unit. Particle-laden air flowed to the aerosol distribution unit at a rate of 4.75 L/min and was supplemented with CO₂ (0.25 L/min) to maintain cells at 5% CO₂ and physiologic pH. The ALI exposure system consisted of the aerosol inlet, the cell culture insert (transwell) and the medium container (well compartment). The aerosol flow delivered to the cells (5 mL/min) was regulated by separate mass flow controllers (GFC17, Aalborg Instruments, Orangeburg, NY, USA) downstream of the ALI cell culture chamber. This flow rate was selected to avoid mechanical stress and dehydration of the cells.

Culture medium (18 mL) was individually supplied to each well compartment without recirculation. The ALI culture system was maintained at 37 ± 0.1 °C by circulating temperature-controlled water through baffles within the stainless steel module housing. After exposure, the transwells were taken out of the module and cells were transferred to the CO₂ incubator. The cells were incubated at 37 °C in 5% CO₂ for specific time points and analysis of selected endpoints.

2.5. Particle deposition efficiency

To determine the efficiency of particle deposition onto the transwells in the exposure system, we generated polydisperse ammonium fluorescein particles with a count median diameter (CMD) of 60 nm by nebulizing a solution of 0.19% (by volume) fluorescein (C₂₀H₁₂O₅) (Acros Organics, Fairlawn, NJ, USA) in 0.01N ammonium hydroxide (NH₄OH) as described previously (Vanderpool and Rubow, 1988) with a Collison nebulizer (BGI). The ammonium fluorescein particles were collected for 2 h at the inlet and outlet of the exposure system using Nuclepore® polycarbonate membranes (0.4 µm pore size, Whatman Inc., Florham Park, NJ, USA). They were also collected on the transwell (deposition). The locations for particle collection are shown in Fig. 1 (① inlet, ② transwell and ③ outlet). The fluorescein particles were then dissolved in 0.01 N NH₄OH to release the fluorescent dye into solution and the fluorescence intensity was measured with a fluorometer (Modulus™, Turner Biosystems, Sunnyvale, CA, USA). Particle mass concentrations were determined from these intensity measurements using a standard curve generated with five known mass concentrations of fluorescein in 0.01 N NH₄OH. Particle deposition efficiency was defined as the ratio of the mass of fluorescein measured on the transwells to the mass at the inlet, expressed as a percent.

2.6. Spatial uniformity of particle deposition

To evaluate the spatial uniformity of particle deposition in the exposure system, 40 nm polystyrene latex (PSL) spheres (Duke Scientific Corp., Palo Alto, CA, USA) were aerosolized and deposited onto the cell-free transwell. The membranes were then removed from the transwells with a clean scalpel blade. Samples were allowed to air dry and the membrane of the transwells (24 mm) was mounted on an aluminum stub (25 mm) with double-sided, graphite tape, and the edge of the filter was painted with colloidal silver (Ag) liquid to promote conductivity. The surface of the sample was sputter-coated with gold (K550, Emitech, Ashford, Kent, England) and examined by scanning electron microscopy (SEM, Hitachi S-4800 FE, Tokyo, Japan) by observing particles along the membrane radius (every 2 mm). PSL particles were counted in SEM images (number/5 × 10⁻⁷ cm² of area) using ImageJ software (NIH, USA) along compass point transects to evaluate the distribution of deposited NPs.

2.7. Measuring cell viability

A549 cells were exposed to particle-free air in the system to assess cell viability as determined using Alamar Blue (Sigma, St. Louis, MO, USA), which indicates metabolic mitochondrial function. The viability of cells exposed to particle-free air for 4 h was compared to cells grown under standard cell culture conditions (5% CO₂, 95% humidity, 37 °C) in an incubator. After NP exposure in the system, cells were transferred to an incubator for 4, 8, 12, and 24 h under standard cell culture conditions. After this post-exposure incubation, the cells on the apical side were washed twice with 500 µL of PBS and fresh phenol-free medium containing Alamar Blue (final concentration, 50 µM) was added to the apical side of the cells (1 mL) for 1 h incubation at 37 °C. Next, 200 µL of Alamar Blue solution were withdrawn and placed in the 96-well plate (Corning) and fluorescence (relative fluorescence units, RFU) was quantified at 570 nm (excitation) and 585 nm (emission) using a microplate reader (SpectraMax M5, Molecular Device).

2.8. Characterization of airborne and deposited Cu NPs

The size distribution of the aerosol during Cu NP exposures to A549 cells was measured using a SMPS that measured diameters in the range of 7.4–289 nm. A549 cells from the Cu NP exposure study were processed for TEM-energy dispersive spectroscopy (EDS) analyses. The cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide, dehydrated through graded ethanols and embedded in epoxy resin. Thin sections were cut at ~80–90 nm on a Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and placed on 200 mesh formvar/carbon-coated nickel grids. Elemental analysis of the cells was performed with an EDS system (Thermo Fisher Noran System 7, Waltham, MA, USA) attached to a JOEL JEM-2100F (Tokyo, Japan) field emission TEM. The TEM was operated at an accelerating voltage of 200 kV in scanning mode combined with a high angle annular field detector (HAADF). NSS 2.2 software package was used to acquire and process the data.

2.9. Sequential exposures and cellular dosimetry of NPs

To better model *in vivo* repeated dose protocols we sequentially exposed lung cells to NPs *in vitro*. Exposures beyond 4 h without resting the cells in a CO₂ incubator resulted in loss of cell viability. Thus, we performed sequential NP exposures (4 h-2 h-4 h) in which cells were first exposed to NPs for 4 h in the exposure system, then incubated for 2 h in 5% CO₂ incubator (in 2.0 mL medium from the *in vitro* apparatus) and then exposed for another 4 h in the exposure system (without exchanging the cell culture medium). After the second NP exposure, the transwells were taken out of the module and cells were incubated at 37 °C in 5% CO₂ for specified post-exposure time points in 2 mL of the medium from the *in vitro* apparatus.

The delivery of NPs to the cells and the uptake of NPs by the cells were measured using inductively coupled plasma–mass spectrometry (ICP–MS, X Series, Thermo Scientific, Waltham, MA, USA). For determination of cellular dosimetry, Cu NPs were aerosolized in the nebulizer, drawn through the aerosol distribution system at 5 L/min and deposited on cells in the transwell (4.7 cm²). Confluent A549 cells were sequentially exposed to Cu NPs in the system. After final NP exposure, the membranes were removed from the transwell with a clean scalpel. The membranes including cells and NPs were digested with 4 mL of aqua regia (nitric acid and hydrochloric acid, 1:1 for each) and then heated to 95 °C for 4 h using a hot block digestion system (Environmental Express, Inc., Mt. Pleasant, SC, USA). The dissolved solutions were diluted to 10 mL with Optima water and measured by ICP–MS. The mass concentration of dissolved Cu NPs in basolateral medium was also determined immediately after sequential exposure of NPs (0 h post-exposure). Particle-free basal medium was collected by three rounds of centrifugation at 14,000g. Particle-free cells and medium were prepared similarly and measured to adjust cellular Cu ion content in control cultures. We also measured the mass concentration of Cu in basal medium after 4 h post-exposure of A549 cells in the exposure system to determine the propensity of Cu NPs to dissolve into ions at the ALI after post-exposure.

2.10. Cellular responses to air-delivery of Cu NPs

Cell viability was assessed using the Alamar Blue assay as described above. Of the multiple cytotoxicity assays used for NP toxicity testing, this assay was found to be the most sensitive and reproducible (Davoren et al., 2007). In this assay, cellular mitochondrial enzyme activity was quantified using fluorescence (RFU) measured using a microplate reader (SpectraMax M5) using a commercial assay (Roche Diagnostics, Penzberg, Germany). Lactate dehydrogenase (LDH) release from cells was used to measure membrane integrity and cytotoxicity. For ALI exposure, after 4 h post-exposure, the cells were washed twice on the apical side with 500 µL of PBS and the supernatant was collected by three

rounds of centrifugation at 700g. The supernatant of the basal medium was also collected. Supernatants (100 μ L) were added to the 96-well plate and mixed with 100 μ L of reaction mixture of catalyst and dye solution. After 10 min incubation at room temperature in the dark, the absorbance was measured using a microplate reader (SpectraMax M5). The release of the proinflammatory cytokine, interleukin (IL)-8, was measured by multiplexed fluorescent bead-based immunoassays (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a Luminex xMap system (Bio-Rad) using the cell wash collected for assessment of LDH.

The generation of intracellular reactive oxygen species (ROS) was measured using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA, Invitrogen). Four hours post-exposure, cells were washed twice on the apical side with 500 μ L of PBS after which HBSS with Ca and Mg containing carboxy-H₂DCFDA (25 μ M, final concentration) was added to the apical side of the cells (1 mL) for 30 min incubation at 37 °C. Following incubation, HBSS solution was removed and cells were washed twice with 500 μ L of PBS and were then lysed with 200 μ L of 0.1% Triton X-100 in PBS for 30 min. The suspensions were then centrifuged at 14,000g for 15 min at 4 °C and the supernatant was taken and placed in the 96-well plate for measurement. The fluorescence intensity of each lysate was measured with excitation of 485 nm and emission at 530 nm using a microplate reader (SpectraMax M5).

Particle-free cells grown under standard cell culture conditions were used as control. The values of cells exposed to the particle-free air or Cu NPs were normalized to control. All experiments were conducted in triplicate and a *p*-value less than 0.05 was considered statistically significant. All data are expressed as mean $\pm$ standard error (SE) of three independent experiments unless otherwise noted.

3. Results

3.1. Particle deposition efficiency

Particle deposition efficiency onto the transwell membranes was determined by mass balance of the exposure system. A deposition efficiency of $70.3 \pm 3.4\%$ was measured (based on $n = 6$) using a test NP with a 60 nm CMD and geometric standard deviation (GSD) of 1.8. There was a 262 ± 9.5 ng deposited mass per unit area of the transwell (cm^2) in 2 h (Table 1). About 26% of the aerosol by mass was exhausted from the exposure system leaving an estimated 4% of the aerosol as wall loss (Table 1). The coefficient of variation in deposited mass between three transwells in the exposure system was approximately 12%. These results demonstrate good uniformity of the exposure system for transwell loading of NP toxicity to lung cells.

We further evaluated particle deposition efficiency in the system as a function of aerosol inlet height above the transwell membrane (1, 2 and 3 mm). The highest deposition efficiency was obtained at a distance of 2 mm between the end of the aerosol inlet and the transwell (Fig. 2; efficiency, 2 mm > 3 mm > 1 mm). The distance between aerosol inlet and the transwell was set at 2 mm in all subsequent experiments.

3.2. Spatial uniformity of particle deposition

The spatial variability of particles deposited onto the transwell was determined by image analysis of 40 nm NP using SEM. In the exposure system, NP aerosol flows are drawn from the distribution unit and particles enter the exposure chamber from the top and exit in annular flow. The particles deposited along the transwell membrane radius (every 2 mm) for 5 radial areas (2, 4, 6, 8 and 10 mm) in eight compass transects are shown in Fig. 3A. Since equal areas were evaluated along the transects, each area would contain 20% of the deposited particles under the assumption of an equal distribution. The test NPs were evenly

deposited along the transwell radius over the entire membrane (Fig. 3). In addition, the spatial uniformity of particle deposition expressed as the standard deviation of differences from perfect uniformity was 4.0% (one-fifth of the particles in each of the five transects).

3.3. Effects of the exposure system operating conditions on cell viability

To evaluate the potential adverse effects of exposure conditions on cell viability during experiments, A549 cells were initially exposed to particle-free air in the exposure system. The viability of cells exposed to particle-free air for 4 h and then incubated for 4, 8, 12 or 24 h was compared to cells grown under standard cell culture conditions (5% CO₂, 95% humidity, 37 °C) in an incubator. Viability for cells exposed to particle-free air in the exposure system did not differ significantly compared to cells grown in the CO₂ incubator (Fig. 4). There was a small but non-significant loss in cell viability 4 or 8 h after exposure to particle-free air. After this time, the viability of air-exposed cells recovered to the level of viability for cells maintained in the incubator. This phenomenon was also observed in other ALI systems (Lenz et al., 2009; Savi et al., 2008). These cell viability data indicate that the exposure system allowed a continuous air-exposure without significant stress to cells.

3.4. Cellular dosimetry and dissolution of Cu NPs

It is necessary to accurately determine the cellular dose of NPs in an *in vitro* testing system to understand cellular responses associated with exposure. We determined the dose of air-delivered Cu NPs on alveolar type II epithelial cells using ICP-MS. Table 2 presents the mass concentration of Cu after sequential exposure (4 h-2 h-4 h) of A549 cells to Cu NPs in the exposure system. The mass concentration of the deposited Cu in/on the cells delivered at a 5 mL/min aerosol flow rate (adjusted for the Cu in particle-free cells) post-exposure was $1.7 \pm 0.1 \mu\text{g Cu}/4.7 \text{ cm}^2$, while the mass of Cu found in the basolateral medium (18 mL) was $2.9 \pm 0.1 \mu\text{g Cu}$. Thus, total air-delivered Cu mass concentration was $4.6 \mu\text{g Cu}$ per transwell with a loading of $1.0 \mu\text{g Cu}/\text{cm}^2$. This demonstrates that a large amount of Cu was dissolved and released to the medium (62% of total mass) during sequential exposure of Cu NPs.

We also examined the degree of Cu NP dissolution by measuring the mass concentration of Cu in basal medium at 0 and 4 h post-incubation of sequential NP exposure. Table 2 presents the mass concentration of dissolved (or released) Cu ion into basal medium and illustrates a significant 2.2-fold time-dependent increase in Cu concentration (4 h vs. 0 h post-exposure, $p < 0.01$).

3.5. Aerosol characterization of NPs

Aerosolization of Cu NPs with primary size of 12 nm in the exposure system produced a geometric mean (GM) particle size of 30.2 nm with a GSD of 1.9 (Fig. 5). Previously, our group evaluated aerosol generation methods to produce a NP aerosol from the bulk powder form for *in vivo* inhalation studies including dry aerosol generators (small-scale powder disperser, acoustical dry aerosol generator/elutriator) and wet aerosol generators (electrospray aerosol generator and Collison nebulizer) (Schmoll et al., 2009). We reported that nebulization was able to produce a consistent aerosol of sufficient concentration. However, the resulting aerosol contained particles attributable to residual salts found in the Optima water used to suspend the nanopowders. Thus, the size distribution of Cu NPs was corrected by subtracting the number of particles in water alone from the number of particles in Cu NPs generated by water.

To further characterize Cu NP delivery in the system, the morphology and elemental composition of the aerosols were analyzed using TEM with EDS. TEM-EDS mapping and EDS spectra of Cu NPs deposited on the cells (Fig. 6) show the particles are Cu (point 1)

and there is no Cu in the background (point 2, particle-free cell; point 3, TEM grid). These results demonstrate that the system successfully delivered Cu NPs to the cells.

3.6. Cellular responses to air-delivery of Cu NPs

The response of A549 cells to Cu NP exposure at the ALI was assessed by determining cell viability, levels of LDH release, intracellular reactive oxygen species (ROS) and interleukin (IL)-8 production. As shown in Fig. 7, there were no significant differences in these markers after sequential exposure to clean air free of NPs compared to cells maintained in a CO₂ incubator (control). However, air-delivered Cu NPs significantly decreased cell viability to 73% ($p < 0.01$) at 4 h post-exposure. Cell membrane damage induced by NPs was also indicated by a significant increase in LDH release into the apical medium at 4 h of Cu NPs ($p < 0.05$). Intracellular ROS generation was examined in response to sequential air-delivery of Cu NPs (0 h post-exposure) at the ALI and resulted in significant increases in ROS production (1.7-fold, $p < 0.05$) compared to control (Fig. 7C). The level of proinflammatory cytokine, IL-8 was measured as a marker of airway inflammation because it plays a critical role in recruiting polymorphonuclear leukocytes (PMN) to the lungs and is a major chemotactic agent (Rahman et al., 2003). As shown in Fig. 7D, a significant increase was observed in the release of IL-8 to the apical medium at the ALI exposure of Cu NPs.

4. Discussion

The ALI exposure system to assess NP toxicity has received considerable attention in recent years (Lenz et al., 2009; Rothen-Rutishauser et al., 2009; Volckens et al., 2009). One of the challenges of establishing an *in vitro* model employing an ALI for nanotoxicity testing is the need to maximize the particle deposition efficiency of the system for NPs which deposit by diffusion rather than impaction or sedimentation. Various groups have reported particle deposition efficiencies for *in vitro* ALI systems, however, comparing these values is complicated by the lack of a clear definition of *efficiency* and full specification of how it was assessed. We defined the deposition efficiency in our exposure system as the mass of fluorescein particles (CMD = 60 nm, GSD = 1.8) measured spectrophotometrically deposited on the transwells divided by the mass of the same fluorescein particles collected at the inlet measured spectrophotometrically in the same assay (refer to Fig. 1 for the locations of particle collection).

An air-liquid interface cell exposure system for NPs was validated by Lenz et al. (2009). They defined the deposition efficiency as the ratio of dried salts (sodium chloride, ammonium sulphate) or NP (ZnO, Au, Carbon black) mass (measured gravimetrically) deposited on aluminum foil placed in the *in vitro* system to the salts or NPs mass loaded into the nebulizer and reported deposition efficiency of 57%. An electrostatic deposition system was tested by Savi and colleagues (2008) to increase particle deposition efficiency in an ALI exposure system. They used monodisperse PSL particles in the size range of 50–600 nm and measured the number concentration of particles by SMPS at the outlet of the deposition chamber. In this manner they reported a deposition efficiency in the range of 15–30% (Savi et al., 2008). The electrostatic aerosol *in vitro* exposure system evaluated by Volckens et al. was reported to have a collection efficiency of 88%. However, this was for non-nanosized sodium chloride particles (0.5 μm) assessed with an aerodynamic particle sizer (Volckens et al., 2009). The relatively high deposition efficiency and low variance of the estimate in our exposure system for nanosized materials may be ascribed to the low particle velocity (~ 1 mm/s) and our use of the same measurement technique for assessing particle mass at the inlet and the transwell. Ideally, particle deposition efficiency studies should be conducted using comprehensive size-resolved characterization. In our validation we used polydisperse fluorescein particles. Despite this limitation, the fluorescein technique had the advantage of being extremely sensitive and detecting a range of particle masses with a limit of detection

of 0.08 ng/mL. Thus, it is more sensitive than measuring the particles deposited in the system gravimetrically or by counting particles by microscopy. The primary mechanism of NP deposition is diffusion under stagnation flow conditions at low flow velocities (~32 mm/s) (Bitterle et al., 2006; Tippe et al., 2002). An uneven distribution of NPs could cause artifactual localized cellular responses at overexposed areas on the transwell due to excessive localized NP loading. Previous studies reported that a rotational symmetric stagnation point flow showed a homogeneous particle deposition over the membrane (Bitterle et al., 2006; Tippe et al., 2002) with at a Reynolds number of 6.3. At our estimated Reynolds number of 0.5 we also used radially symmetric stagnation point flow to produce reasonably uniform particle deposition onto the membrane. However, somewhat fewer particles were deposited at the outer edge of the membrane (at 10 mm from the center, Fig. 3) compared to 6 mm from the center. This decrease is likely due to the outer diameter of the aerosol delivery tube (22 mm) being smaller than that of the filter membrane (24 mm) and the higher velocity of the outer particles (Desantes et al., 2006; Tippe et al., 2002). This observation is in agreement with findings reported by Savi et al. (2008).

Since alveolar epithelial cells are among the first lung cells to be exposed to inhaled toxicants, selection of the cells that represent the lung epithelium in an *in vitro* model for NP toxicity testing to lung cells at the ALI is very important. While primary human lung epithelial cells are optimal, A549 cells represent a reasonable surrogate transformed cell line for developmental work. These cells retain several features of type II alveolar epithelial cells, including the production of alveolar surfactant (Stringer et al., 1996). Type II cells are stimulated to proliferate and differentiate into type I cells when type I cells are damaged. However, the type II epithelium itself represents a small fraction of the alveolar surface area as compared to the type I epithelium which accounts for 95% of the alveolar surface and furthermore, type II cells do not grow in contact with each other in the normal lung (Fujino et al., 2011). Therefore, future experiments will examine the viability of primary human epithelial cells over time.

Our *in vitro* dosimetry data are consistent with the findings of Midander et al. (2009) showing significant Cu release from nanosized Cu particles within the first 4 h of exposure *in vitro* (Midander et al., 2009). These findings are also in agreement with our previous studies of Cu NP-exposed mice (Kim et al., 2011). In particle-free bronchoalveolar lavage (BAL) fluid, the concentration of dissolved Cu ions increased in a dose-dependent manner over a short time.

In submerged cell culture systems, the dose of NPs is generally expressed as a mass concentration of NPs added to a specified volume of media. However, this mass concentration reflects poorly the actual cellular dose, because cells respond to NPs that are in contact with the cells, not those that remain suspended in media (Teeguarden et al., 2007). Despite this limitation, we compared the delivered dose of NPs to cells in our study (1 $\mu\text{g Cu/cm}^2$) to the dose per unit area in submerged cell culture conditions calculated from the mass concentration in the medium ($\mu\text{g/mL}$) assuming 100% of dosed NPs reach the cells in submerged culture (12-well plate, 3.8 cm^2). Based on this calculation, the dose of air-delivered Cu NPs of 1.0 $\mu\text{g/cm}^2$ in our study corresponds to a concentration delivered in an *in vitro* submerged culture system of 3.8 $\mu\text{g/mL}$. This estimated equivalent mass concentration is below the level required to induce cytotoxicity in submerged cells.

Air-delivery of NPs at the low flow rate in our *in vitro* exposure system sets a lower limit on the dose that can be delivered. However, it has been suggested that the development of cell culture methods are needed for continuous and repeated low-dose testing and it should be carried out in a similar manner to exposure *in vivo* (Andersen and Krewski, 2009). Thus, a successful *in vitro* testing system for NP toxicity assessment should have the capability of

predicting *in vivo* repeated dose toxicity. Toxicity testing for NPs has often employed extraordinarily high doses that far exceed human exposures (Oberdörster et al., 2007). The use of high-dose toxicity tests for predicting human health risks of NPs has remained controversial. It is recognized that biological effects induced by high-dose exposure frequently will not occur at lower doses of NPs in animals or cells. Thus, the induction of irrelevant responses in exposed cell cultures at high doses may lead to the misinterpretation of toxic effects of NPs.

Another important characteristic of the toxicity of metal-based NPs is their dissolution ability (release of ions) in a suspending medium or biological environment (Midander et al., 2009; Xia et al., 2008). The solubility of the NPs most likely plays a vital role in the particle toxicity, but it has remained controversial. Thus, determining dissolution of metal-containing NPs is extremely important to investigate whether the observed responses are due to the intact NP or metal ion released from NPs. However, the dissolution phenomenon is a complex, dynamic process and a recent study demonstrated the complexity of dissolution on the nanoscale of Cu particles by measuring particle size distribution in real time with an electrospray atomizer coupled to a SMPS (Elzey and Grassian, 2010). Elzey and Grassian (2010) showed that the particle size was smaller with time as the NPs dissolved and the particle size distribution became more polydisperse. Most dissolution studies have been performed in biological fluids or cell culture medium (without cells). Thus, effects of NP dissolution on their toxicity in an *in vitro* model including target cells should be further investigated. In this respect, air-delivery of NPs on lung epithelial cells grown at the ALI for NP toxicity testing enables us to determine actual cellular dose at conditions that closely mimic the exposure conditions of lung cells *in vivo* to inhaled NPs and are similar to repeated low dose *in vivo* exposures. Consequently, we are able to provide cellular responses associated with actual cellular dose while accounting for both dissolution and Cu NPs retained in the cells.

To accurately assess cellular NP toxicity it is vital that cells are dosed with nano-sized aerosols. Dosing cells with agglomerated NPs in an *in vitro* study can lead to an inaccurate assessment of cellular responses. It has frequently been reported that the NP agglomeration and dispersion problems in submerged exposures make it difficult to accurately determine cellular dose and deliver NPs to cells (Kroll et al., 2009; Sager et al., 2007; Teeguarden et al., 2007; Wittmaack, 2011). These problems in turn are expected to significantly alter cellular responses. Therefore, the demonstration of limited agglomeration of NPs in the exposure system helps ensure the representativeness of cellular responses.

Although TEM analysis in NP research is frequently used to demonstrate cellular uptake of NPs, it has been reported that NPs are often indistinguishable from cellular structures in the same size range by TEM. Electron dense particles such as TiO₂ can be impossible to differentiate from cellular organelles due to the similarity with glycogen granules, mitochondrial matrix granules or ribosomes whereas electron lucent particles (e.g., polystyrene) can be confused with spherical vesicular structures like caveolae (Muhlfeld et al., 2007; Panyam et al., 2003). However, TEM coupled with EDS allows the analysis of the elemental composition and visualization of the spatial distribution of NPs deposited on the cells. By employing several complimentary techniques, such as SMPS, ICP-MS and TEM-EDS, we were able to fully characterize NP aerosols and provide data on the number, size, mass, morphology and elemental composition of NP aerosols delivered to the cells in the exposure system.

To assess the integrated *in vitro* exposure system in the present study as a means to screen NP toxicity and prioritize NPs for *in vivo* testing, we compared *in vitro* measurements to our *in vivo* results using the same nanomaterials and the same NP generation system. Despite the

challenges of comparing in *in vivo* (mouse) and *in vitro* (human cell) studies, our observations in the study are in agreement with our previous findings that Cu NP exposure (sub-acute inhalation and acute instillation) induced severe inflammatory responses with increased recruitment of total cells and neutrophils to the lungs as well as increased total protein, LDH activity and cytokine concentration in BAL fluid (Kim et al., 2011; Pettibone et al., 2008). Taken together, the results of the present study suggest that cell viability, LDH release, intracellular ROS and IL-8 may be useful screening biomarkers to assess NP toxicity using the integrated *in vitro* exposure system. Inflammation and cytotoxicity indices as comparative end points for both *in vitro* and *in vivo* studies have been used for assessing pulmonary toxicity profiles (Sayes et al., 2007). The results of these comparisons suggest that the experiments described in this study show potential for screening NP toxicity with a higher throughput than *in vivo* studies. However, *in vivo* approaches are primary and fundamental tools for assessment of NP pulmonary toxicity and can account for higher level responses that require an intact immune system and whole animal physiology.

In our prior work inflammatory responses were observed in mice exposed sub-acutely (4 h/day, 5 d/week, 2 week) to Cu NPs immediately following NP exposure. Inflammatory markers decreased and resolved to baseline values 3 wk post-exposure. *In vitro* time course studies (multiple time points of post-exposure) combined with repeated low dose exposures are warranted to determine the level of inflammation and oxidative stress induced by NPs which in turn will give us insight into the mechanism of lung injury and repair associated with NP exposure and will allow for the prioritization of more toxic nanomaterials to second tier testing.

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References

- Andersen ME, Krewski D. Toxicity testing in the 21st century: bringing the vision to life. *Toxicological Sciences*. 2009; 107:324–330. [PubMed: 19074763]
- Bitterle E, Karg E, Schroepel A, Kreyling WG, Tippe A, Ferron GA, Schmid O, Heyder J, Maier KL, Hofer T. Dose-controlled exposure of A549 epithelial cells at the air–liquid interface to airborne ultrafine carbonaceous particles. *Chemosphere*. 2006; 65:1784–1790. [PubMed: 16762398]
- Card JW, Zeldin DC, Bonner JC, Nestmann ER. Pulmonary applications and toxicity of engineered nanoparticles. *American Journal of Physiology – Lung Cellular and Molecular Physiology*. 2008; 295:L400–L411. [PubMed: 18641236]
- Davoren M, Herzog E, Casey A, Cottineau B, Chambers G, Byrne HJ, Lyng FM. In vitro toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. *Toxicology In Vitro*. 2007; 21:438–448. [PubMed: 17125965]
- Desantes JM, Margot X, Gil A, Fuentes E. Computational study on the deposition of ultrafine particles from Diesel exhaust aerosol. *Journal of Aerosol Science*. 2006; 37:1750–1769.
- Donnelly, LE. Airway epithelial cells (primaries vs. cell lines). In: Rogers, DF.; Donnelly, LE., editors. *Human airway inflammation*. Humana Press; 2001. p. 127–136.
- Elder A, Vidyasagar S, DeLouise L. Physicochemical factors that affect metal and metal oxide nanoparticle passage across epithelial barriers. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. 2009; 1:434–450. [PubMed: 20049809]
- Elzey S, Grassian VH. Nanoparticle dissolution from the particle perspective: insights from particle sizing measurements. *Langmuir*. 2010; 26:12505–12508. [PubMed: 20590108]

- Fahmy B, Cormier SA. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. *Toxicology In Vitro*. 2009; 23:1365–1371. [PubMed: 19699289]
- Fujino N, Kubo H, Suzuki T, Ota C, Hegab AE, He M, Suzuki S, Suzuki T, Yamada M, Kondo T, Kato H, Yamaya M. Isolation of alveolar epithelial type II progenitor cells from adult human lungs. *Lab Invest*. 2011; 91:363–378. [PubMed: 21079581]
- Grassian VH, Adamcakova-Dodd A, Pettibone JM, O'Shaughnessy P, Thorne PS. Inflammatory response of mice to manufactured titanium dioxide nanoparticles: comparison of size effects through different exposure routes. *Nanotoxicology*. 2007a; 1:211–226.
- Grassian VH, O'Shaughnessy P, Adamcakova-Dodd A, Pettibone JM, Thorne PS. Inhalation exposure study of titanium dioxide nanoparticles with a primary particle size of 2–5 nm. *Environmental Health Perspectives*. 2007b; 115:397–402. [PubMed: 17431489]
- Hinderliter P, Minard K, Orr G, Chrisler W, Thrall B, Pounds J, Teeguarden J. ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. *Particle and Fibre Toxicology*. 2010; 7:36. [PubMed: 21118529]
- Kameyama S, Kondo M, Takeyama K, Nagai A. Air exposure causes oxidative stress in cultured bovine tracheal epithelial cells and produces a change in cellular glutathione systems. *Experimental Lung Research*. 2003; 29:567–583. [PubMed: 14594656]
- Karp, PH.; Moninger, TO.; Pary Weber, S.; Nesselhauf, TS.; Launsbach, JL.; Zabner, J.; Welsh, MJ. An in vitro model of differentiated human airway epithelia. In: Wise, C., editor. *Epithelial cell culture protocols*. Humana Press; 2002. p. 115–137.
- Kim JS, Adamcakova-Dodd A, O'Shaughnessy P, Grassian V, Thorne P. Effects of copper nanoparticle exposure on host defense in a murine pulmonary infection model. *Particle and Fibre Toxicology*. 2011; 8:29. [PubMed: 21943386]
- Kroll A, Pillukat MH, Hahn D, Schnekenburger J. Current in vitro methods in nanoparticle risk assessment: limitations and challenges. *European Journal of Pharmaceutics and Biopharmaceutics*. 2009; 72:370–377. [PubMed: 18775492]
- Lenz A, Karg E, Lentner B, Dittrich V, Brandenberger C, Rothen-Rutishauser B, Schulz H, Ferron G, Schmid O. A dose-controlled system for air–liquid interface cell exposure and application to zinc oxide nanoparticles. *Particle and Fibre Toxicology*. 2009; 6:32. [PubMed: 20015351]
- Maynard A, Kuempel E. Airborne nanostructured particles and occupational health. *Journal of Nanoparticle Research*. 2005; 7:587–614.
- Midander K, Cronholm P, Karlsson HL, Elihn K, Möller L, Leygraf C, Wallinder IO. Surface characteristics, copper release, and toxicity of nano- and micrometer-sized copper and copper(ii) oxide particles: a cross-disciplinary study. *Small*. 2009; 5:389–399. [PubMed: 19148889]
- Muhlfeld C, Rothen-Rutishauser B, Vanhecke D, Blank F, Gehr P, Ochs M. Visualization and quantitative analysis of nanoparticles in the respiratory tract by transmission electron microscopy. *Particle and Fibre Toxicology*. 2007; 4:11. [PubMed: 17996124]
- NRC. *Toxicity Testing for Assessment of Environmental Agents: Interim Report*. 2006
- NRC. *Toxicity Testing in the 21st Century: A Vision and a Strategy*. 2007
- Oberdörster G, Oberdörster E, Oberdörster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives*. 2005; 113:823–839. [PubMed: 16002369]
- Oberdörster G, Stone V, Donaldson K. Toxicology of nanoparticles: a historical perspective. *Nanotoxicology*. 2007; 1:2–25.
- Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V. Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(d, l-lactide-co-glycolide) nanoparticles. *International Journal of Pharmaceutics*. 2003; 262:1–11. [PubMed: 12927382]
- Paur HR, Mülhopt S, Weiss C, Diabaté S. In vitro exposure systems and bioassays for the assessment of toxicity of nanoparticles to the human lung. *Journal für Verbraucherschutz und Lebensmittelsicherheit*. 2008; 3:319–329.
- Penttinen P, Timonen KL, Tiittanen P, Mirme A, Ruuskanen J, Pekkanen J. Ultrafine particles in urban air and respiratory health among adult asthmatics. *European Respiratory Journal*. 2001; 17:428–435. [PubMed: 11405521]

- Pettibone JM, Adamcakova-Dodd A, Thorne PS, O'Shaughnessy PT, Weydert JA, Grassian VH. Inflammatory response of mice following inhalation exposure to iron and copper nanoparticles. *Nanotoxicology*. 2008; 2:189–204.
- Rahman I, Gilmour PS, Jimenez LA, Biswas SK, Antonicelli F, Aruoma OI. Ergothioneine inhibits oxidative stress- and TNF- α -induced NF- κ B activation and interleukin-8 release in alveolar epithelial cells. *Biochemical and Biophysical Research Communications*. 2003; 302:860–864. [PubMed: 12646250]
- Rothen-Rutishauser B, Grass RN, Blank F, Limbach LK, Mühlfeld C, Brandenberger C, Raemy DO, Gehr P, Stark WJ. Direct Combination of nanoparticle fabrication and exposure to lung cell cultures in a closed setup as a method to simulate accidental nanoparticle exposure of humans. *Environmental Science and Technology*. 2009; 43:2634–2640. [PubMed: 19452928]
- Sager TM, Porter DW, Robinson VA, Lindsley WG, Schwegler-Berry DE, Castranova V. Improved method to disperse nanoparticles for *in vitro* and *in vivo* investigation of toxicity. *Nanotoxicology*. 2007; 1:118–129.
- Savi M, Kalberer M, Lang D, Ryser M, Fierz M, Gaschen A, Ri ka J, Geiser M. A novel exposure system for the efficient and controlled deposition of aerosol particles onto cell cultures. *Environmental Science and Technology*. 2008; 42:5667–5674. [PubMed: 18754491]
- Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles. *Toxicological Sciences*. 2007; 97:163–180. [PubMed: 17301066]
- Schmoll LH, Elzey S, Grassian VH, O'Shaughnessy PT. Nanoparticle aerosol generation methods from bulk powders for inhalation exposure studies. *Nanotoxicology*. 2009; 3:265–275.
- Schrand AM, Rahman MF, Hussain SM, Schlager JJ, Smith DA, Syed AF. Metal-based nanoparticles and their toxicity assessment. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*. 2010; 2:544–568. [PubMed: 20681021]
- Stebounova L, Adamcakova-Dodd A, Kim J, Park H, O'Shaughnessy P, Grassian V, Thorne P. Nanosilver induces minimal lung toxicity or inflammation in a subacute murine inhalation model. *Particle and Fibre Toxicology*. 2011; 8:5. [PubMed: 21266073]
- Stern ST, McNeil SE. Nanotechnology safety concerns revisited. *Toxicol Sci*. 2007 kfm 169.
- Stringer B, Imrich A, Kobzik L. Lung epithelial cell (A549) interaction with unopsonized environmental particulates: quantitation of particle-specific binding and IL-8 production. *Experimental Lung Research*. 1996; 22:495–508. [PubMed: 8886755]
- Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. Particokinetics *in vitro*: dosimetry considerations for *in vitro* nanoparticle toxicity assessments. *Toxicological Science*. 2007; 95:300–312.
- Tetley TD. Health effects of nanomaterials. *Biochemical Society Transactions*. 2007; 035:527–531. [PubMed: 17511644]
- Thorne PS. Inhalation toxicology models of endotoxin- and bioaerosol-induced inflammation. *Toxicology*. 2000; 152:13–23. [PubMed: 11090935]
- Tippe A, Heinzmann U, Roth C. Deposition of fine and ultrafine aerosol particles during exposure at the air/cell interface. *Journal of Aerosol Science*. 2002; 33:207–218.
- Vanderpool RW, Rubow KL. Generation of large, solid, monodisperse calibration aerosols. *Aerosol Science and Technology*. 1988; 9:65–69.
- Volckens J, Dailey L, Walters G, Devlin RB. Direct particle-to-cell deposition of coarse ambient particulate matter increases the production of inflammatory mediators from cultured human airway epithelial cells. *Environmental Science and Technology*. 2009; 43:4595–4599. [PubMed: 19603682]
- Weichenthal S, Dufresne A, Infante-Rivard C. Indoor ultrafine particles and childhood asthma: exploring a potential public health concern. *Indoor Air*. 2007; 17:81–91. [PubMed: 17391231]
- Witherden, IR.; Tetley, TD. Isolation and culture of human alveolar type II pneumocytes. In: Rogers, DF.; Donnelly, LE., editors. *Human airway inflammation*. Humana Press; 2001. p. 137–146.
- Wittmaack K. Excessive delivery of nanostructured matter to submersed cells caused by rapid gravitational settling. *ACS Nano*. 2011; 5:3766–3778. [PubMed: 21446668]

Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, Yeh JI, Zink JI, Nel AE. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano. 2008; 2:2121–2134. [PubMed: 19206459]

Abbreviations

Ag	silver
ALI	air–liquid interface
ASL	airway surface liquid
BAL	bronchoalveolar lavage
CMD	count median diameter
Cu	copper
EDTA	ethylenediamine tetraacetic acid
EDS	energy dispersive spectroscopy
FBS	fetal bovine serum
GM	geometric mean
GSD	geometric standard deviation
HAADF	high angle annular field detector
HBSS	Hanks balanced salt solution
HEPES	hydroxyethyl piperazine ethanesulfonic acid
ICP–MS	inductively coupled plasma–mass spectrometry
IL	interleukin
LAL	<i>Limulus</i> amebocyte lysate
LDH	lactate dehydrogenase
NHBE	normal human bronchial epithelial
NP	nanoparticle
PM	particulate matter
PMN	polymorphonuclear leukocytes
PSL	polystyrene latex
RFU	relative fluorescence units
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SE	standard error
SEM	scanning electron microscopy
SMPS	scanning mobility particle sizer
TEM	transmission electron microscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

Fig. 1.

Schematic of our *in vitro* exposure system. Nanoparticle (NP) aerosol generation system supplies the air–liquid interface (ALI) cell culture chamber to expose epithelial lung cells *in vitro*. The exposure chamber consists of the aerosol inlet, the cell culture insert (transwell) and the medium container. The direct air-delivery of NP aerosols to lung cells using the integrated exposure system mimics the *in vivo* conditions of the lung epithelium for NP toxicity testing.

Fig. 2. Particle deposition efficiency in the exposure system as a function of aerosol inlet distance (1, 2, and 3 mm) to the transwell membrane. Data are expressed as mean $\pm$ SE of two (1 and 3 mm) or three independent experiments (2 mm).

Fig. 3.

The spatial uniformity of particle deposition in the exposure system was evaluated by counting polystyrene particles (40 nm) with scanning electron microscopy (SEM). (A) The number of deposited particles on the transwell showing positions of delivered particles. The red circle indicates entire transwell (diameter, 24 mm). (B) Deposition fraction (mean % $\pm$ SE) of particles in 4 h on the transwell along the membrane radius, every 2 mm. Spatial uniformity is expressed as standard deviation (SD) of the differences between the mean deposition fraction of particles on each area shown by the 5 bars and the expected deposition fraction of particles assuming perfect uniformity (20%). The lines at 16% and 24% demarcate 1 SD below and above 20%.

Fig. 4.

Effects of the *in vitro* exposure system operating conditions on cell viability. Cell viability for A549 cells exposed to particle-free air (air exposure) for 4 h in the exposure system did not differ significantly compared to cells grown in the CO₂ incubator (control). After exposure of A549 cells to particle-free air, the cells were incubated at 37 °C in 5% CO₂ for specific time points (4, 8, 12, and 24 h). Data are expressed as mean $\pm$ SE of three independent experiments.

Fig. 5. Particle size distribution of Cu nanoparticle (NP) aerosols produced by a 3-jet Collison nebulizer in the exposure system. The size distribution of Cu NP aerosols was corrected to the background water used as a carrier.

Fig. 6. Particles found in the cells were identified using transmission electron microscopy (TEM)-energy dispersive spectroscopy (EDS) after direct exposure of A549 cells to Cu nanoparticles (NPs). Cu NP aerosols were delivered to A549 cells for 4 h using the exposure system. (A) TEM-EDS mapping of Cu NPs deposited on the cells and (B) the corresponding energy-dispersive X-ray spectrum of the particles deposited on the cells (point 1) and backgrounds (particle-free cells, point 2 and TEM grid, point 3). The Ni signal in the spectrum is from the nickel TEM grids used for sample preparation.

Fig. 7.

Effects of particle-free air and Cu nanoparticle (NP) exposure on (A) the viability, (B) LDH release, (C) intracellular ROS generation and (D) IL-8 induction of A549 cells at the air–liquid interface (ALI) in the exposure system. A549 cells were sequentially exposed to particle-free air (air alone exposure) and NPs at the ALI in the exposure system and responses were evaluated at 4 h post-exposure. The value was normalized to the cells grown in the CO₂ incubator (control). Data are expressed as mean $\pm$ SE of three independent experiments; *Statistically significant difference as compared to control (* p < 0.05, ** p < 0.01).

Table 1

Particle deposition efficiency of ammonium fluorescein particles aerosolized in the integrated *in vitro* exposure system.

Particle mass concentration (ng/cm ²) ^a		Particle deposition efficiency (%) ^b	
Inlet	Transwell (Deposition)	Outlet	
375 ± 28.5	262 ± 9.5	96.5 ± 3.2	70.3 ± 3.4

^aThe mass concentration of ammonium fluorescein particles was determined by measuring fluorescence intensity using a fluorometer at the aerosol inlet, outlet and deposited amount on the transwells (refer to Fig. 1 for the location of particles collected). Shown are the mean ± SE for 6 trials.

^bDeposition efficiency (%) = (mass of fluorescein deposited on the transwell/mass of fluorescein at the inlet) × 100; Mass balance: Inlet mass = deposition mass (70.3%) + Outlet mass (25.7%) + Loss (4.0%).

Table 2

The mass concentration of Cu after sequential exposure of A549 cells to Cu NPs at the air–liquid interface (ALI) in the integrated *in vitro* exposure system.

Exposure time	Adjusted Cu mass in and on the cells (4.7cm ²)	Adjusted Cu mass in medium (18 mL)	Total delivered Cu mass (cell and medium)
<i>h</i>	μg ± SE		
Sequential exposure (4-2-4)	1.7 ± 0.1	2.9 ± 0.1	4.6 ± 0.1
Post-exposure time		Adjusted Cu mass in medium (18 mL)	
<i>h</i>		μg/L	
0		169 ± 10.2	
4		377 ± 22.7 ^{**}	

Data are expressed as mean ± SE of three independent experiments.

^{**} Statistically significant difference as compared to 0 h post-exposure (^{**}*p* < 0.01).