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

Jong Sung Kim [a], Thomas M. Peters [a,b], Patrick T. O'Shaughnessy [b], Andrea Adamcakova-Dodd [b], Peter S. Thorne [a,b,*]

[a] Interdisciplinary Graduate Program in Human Toxicology, University of Iowa, Iowa City, IA 52242, USA
[b] Department of Occupational and Environmental Health, University of Iowa, Iowa City, IA 52242, USA

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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*.

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 (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

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, *Limulus* 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.

* Corresponding author at: Department of Occupational and Environmental Health, The University of Iowa, 105 River St., S341A CPHB Iowa City, IA 52242-5000 USA. Tel.: +1 319 335 4216; fax: +1 319 384 4138.

E-mail addresses: jongsung-kim@uiowa.edu (J.S. Kim), thomas-m-peters@uiowa.edu (T.M. Peters), patrick-oshaughnessy@uiowa.edu (P.T. O'Shaughnessy), andrea-a-dodd@uiowa.edu (A. Adamcakova-Dodd), peter-thorne@uiowa.edu (P.S. Thorne).

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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 Cu_2O (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* amebocyte 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% CO_2. 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 cm^2 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