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Tracking translocation of industrially relevant engineered nanomaterials (ENMs) across alveolar epithelial monolayers *in vitro*

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Abstract

Relatively little is known about the fate of industrially relevant engineered nanomaterials (ENMs) in the lungs. Inhalation exposure and subsequent translocation of ENMs across the epithelial lining layer of the lung might contribute to clearance, toxic effects or both. To allow precise quantitation of translocation across lung epithelial cells, we developed a method for tracking industrially-relevant metal oxide ENMs *in vitro* using neutron activation. The versatility and sensitivity of the proposed In Vitro Epithelial Translocation (INVET) system was demonstrated using a variety of industry relevant ENMs including CeO₂ of various primary particle diameter, ZnO, and SiO₂-coated-CeO₂ and ZnO particles. ENMs were neutron activated, forming gamma emitting isotopes ¹⁴¹Ce and ⁶⁵Zn respectively. Calu-3 lung epithelial cells cultured to confluency on transwell inserts were exposed to neutron-activated ENM dispersions at sub-lethal doses to investigate the link between ENM properties and translocation potential. The effects of ENM exposure on monolayer integrity was monitored by various methods. ENM translocation across the cellular monolayer was assessed by gamma spectrometry following 2, 4 and 24 hours of exposure.

Our results demonstrate that ENMs translocated in small amounts (e.g. <0.01% of the delivered dose at 24 h), predominantly via transcellular pathways without compromising monolayer integrity or disrupting tight junctions. It was also demonstrated that the delivery of particles in suspension to cells in culture is proportional to translocation, emphasizing the importance of accurate dosimetry when comparing ENM-cellular interactions for large panels of materials. The reported INVET system for tracking industrially relevant ENMs while accounting for dosimetry can be a valuable tool for investigating nano-bio interactions in the future.

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DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health

DECLARATIONS OF INTEREST

The authors declared no personal interests related to this study.

Keywords

nanotoxicology; *in vitro*; translocation; nanotechnology; dosimetry

INTRODUCTION

The increasing manufacture and use of engineered nanomaterials (ENMs) in electronics, cosmetics, toner formulations, and biomedical applications, raises questions regarding their environmental and occupational health and safety(Aitken, 2006, Bello et al., 2012, Pirela S et al., 2013). Human exposure via inhalation in particular is a growing concern. Studies have demonstrated inhaled ENMs can pass from the lungs into the bloodstream and enter extrapulmonary organs(Choi et al., 2010, Geiser et al., 2005, Kreyling et al., 2009), thereby increasing risk of cardiovascular morbidity and mortality(Brain et al., 2009, Mills et al., 2009). However, relatively little is known for industrially relevant ENMs regarding their fate, potential toxicity, and mechanisms of translocation in biological cells, tissues and organs(Kim YH et al., 2010, Yacobi et al., 2010).

Inhaled ENMs likely enter the systemic circulation across the alveolar epithelium, a very thin biological barrier (~0.5µm) that constitutes greater than 95% the surface area (~100m² in humans) in distal airspaces of the lung(West, 2008). ENMs may translocate across the alveolar epithelium transcellularly via transcytosis (Conner and Schmid, 2003) and other nonendocytic mechanisms, or paracellularly via tight junctions. While hydrophilic solutes of hydrodynamic radius <6nm have been reported to passively translocate via paracellular pathways by restricted diffusion(Kim and Crandall, 1983, Matsukawa et al., 1997), nanoparticles greater than 10nm in diameter are expected to be excluded from tight junctions under normal conditions(Yacobi et al., 2010). If, however, ENM exposure disrupts tight junctions and compromises the epithelial monolayer barrier, paracellular transport could be increased. The effect of specific ENM properties on translocation across the alveolar epithelium, and whether transport occurs via a predominantly transcellular or paracellular pathway is not well-understood. The mechanism and extent of ENM translocation across epithelial monolayers hold implications for ENM clearance from the lungs as well as pulmonary and cardiovascular toxicity.

One major challenge in performing such studies is employing tracer particle models for tracking industrially relevant ENMs. Most *in vitro* studies utilize fluorescently labeled polystyrene beads for tracking particle-cell interactions(Rothen-Rutishauser et al., 2005, Yacobi et al., 2010, Xia et al., 2008b). These particles, however, are not representative of industrially relevant ENMs, are generated via wet-synthesis methods(Yang et al., 2013, Rothen-Rutishauser et al., 2005, Yacobi et al., 2010, Xia et al., 2008b), and there is low likelihood of inhalation exposure to these particles in aerosolized form. Furthermore, fluorescently labeled nanoparticles exhibit relatively low sensitivity for detecting small concentrations of particles, label instability, altered physicochemical properties such as surface chemistry and charge, and photobleaching from laser exposure(Tenuta et al., 2011, Sotiriou et al., 2012, Nel et al., 2009). Moreover, ENM transformations and agglomeration in liquid suspension and the subsequent effects on particle transport and delivery to cells in

culture are often overlooked (Cohen et al., 2012, Teeguarden et al., 2007, Hinderliter et al., 2010, Deloid G et al., submitted July 2013)

An optimal tracer particle model for ENM translocation studies should enable tracking of industrially relevant ENMs. One particular class of ENMs with many industrial uses is flame generated metals and metal oxides. Flame-generated synthesis of ENMs is the currently preferred route by nanotechnology industry, and they account for the highest volume of ENMs in production (Wegner, 2003).

We present here a novel *in vitro* method for tracking ENM translocation across Calu-3 human lung epithelial monolayers. The proposed system utilizes well-characterized industrially relevant metal oxide ENMs, using a neutron activation technique. This radiotracer technique is a well-established method that enables tracking of flame-generated metal oxide nanoparticles for biodistribution studies *in vivo*, but has yet to be used extensively for *in vitro* studies (Chen JK, 2010, Yeh et al., 2012, He et al., 2010, Wang et al., 2010). A number of industry relevant ENMs such as CeO₂ and ZnO nanoparticles were utilized in this study to show the versatility of the proposed method. Those ENMs were selected here due to their range in toxicity as well as their extensive presence in consumer products (including cosmetics, fuel additives, polishing slurries, etc. (Gass et al., 2013, Demokritou et al., 2013)). We also included “core shell” composite ENMs, consisting of either a CeO₂ or ZnO core and a hermetic nano-thin coating of amorphous SiO₂ (Gass et al., 2013), in order to assess the effect of surface chemistry, particle size, and chemical composition on translocation across cellular monolayers.

MATERIALS AND METHODS

Nanomaterial synthesis and characterization

ENMs investigated are listed in Table 1. All ENMs were generated using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES), as previously described (Demokritou et al., 2010, Sotiriou et al., 2011, Gass et al., 2013, Demokritou et al., 2013). ENMs were characterized for primary particle size and specific surface area. Specific surface area, *SSA*, defined as the area per mass (m²/g), was determined by the nitrogen adsorption/Brunauer-Emmett-Teller (BET) method (Micrometrics Tristar Norcross, GA, USA). The equivalent primary particle diameter, *d_{BET}*, was calculated, assuming spherical particles, as:

$$d_{BET} = \frac{6}{SSA \times \rho_p} \quad (1)$$

where ρ_p is the particle density, which was obtained for each particle from the densities of component materials, at 20°C, reported in the CRC handbook of Chemistry and Physics (Haynes, 2012). Primary particle diameter was also determined by X-ray diffraction using a Scintag XDS2000 powder diffractometer (Cupertino, CA) (Cu K α (λ = 0.154 nm), –40 kV, 40 mA, step size = 0.02°) following previously described methods (Gass et al., 2013), and reported as *d_{XRD}* (nm). Particle morphology and size were further characterized by

transmission electron microscopy (TEM) (See Supplementary Figure 1) using a Libra 120 TEM (Zeiss, Oberkochen, Germany).

ENM dispersal and characterization in liquid suspensions

ENM dispersion was performed using a protocol previously described by the authors and included calibration of sonication equipment to ensure accurate application and reporting of delivered sonication energy (DSE) in J/ml, as well as determination of the material specific critical delivered sonication energy (Cohen et al., 2012). ENMs were dispersed at 1 mg/ml in 10 ml of solute in 50 ml, conical polyethylene tubes, by sonication with a cup horn Branson Sonifier S-450A (Branson Ultrasonics, Danbury, CT) (maximum power output of 400 W at 60 Hz, continuous mode, output level 3, power delivered to sample: 1.25W). Dispersions were analyzed for hydrodynamic diameter (d_H), polydispersity index (PdI), and zeta potential (ζ) by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Stock solutions prepared in deionized water (DI H₂O) were then diluted to desired concentrations (0, 6.25, 12.5, 25, 50, 100 µg/ml) in cell culture media (MEM/5%FBS) prior to characterization for various particle and media parameters, or application to cells for toxicological evaluation.

Suspensions were also characterized for agglomerate effective density by the Harvard volumetric centrifugation method recently developed by the authors, described elsewhere (Deloid G et al., submitted July 2013, Demokritou P et al., 2012). In brief, one ml samples of 100 µg/ml suspensions of metal oxide ENMs were dispensed into TPP packed cell volume (PCV) tubes (Techno Plastic Products, Trasadingen, Switzerland) and centrifuged at $2,000 \times g$ for one hour. Agglomerate pellet volumes, V_{pellet} , were measured, agglomerate densities were calculated from V_{pellet} values of triplicate samples for each ENM.

In vitro dosimetric considerations

The *in vitro* sedimentation, diffusion and dosimetry (ISDD) model proposed by Hinderliter et. al. and described previously (Hinderliter et al., 2010, Cohen et al., 2012) was used to calculate numerically for all ENMs studied, the fraction of administered particles that would be deposited onto cells as a function of time $f_D(t)$. The agglomerate hydrodynamic diameter, d_H , the measured effective density were used as inputs to the model and are listed in Table 1. Additionally, the following parameters were used as inputs to the ISDD numerical model: Media column height, 1.5 mm; temperature, 310K; media density, 1.00 g/ml; viscosity, 0.00074 Pa s for MEM/5%FBS; and administered (initial suspension) particle concentration, 12.5 µg/ml (Hinderliter et al., 2010).

Neutron activation of industry relevant ENMs

ENM powders were irradiated with neutrons for up to 24 hours at the Nuclear Reactor Laboratory (Massachusetts Institute of Technology, Cambridge, MA), and the radioactive ¹⁴¹Ce ENMs (CeO₂, SiO₂-coated CeO₂), and ⁶⁵Zn ENMs (ZnO, SiO₂-coated ZnO) were stored in irradiation tubes. ¹⁴¹Ce and ⁶⁵Zn are gamma emitters with a half-life of 32 days and 244 days respectively, and the successful production of radioactive ENMs following irradiation was confirmed by gamma energy spectrometry using a Packard gamma

counter (Cobra Quantum, Packard Instrument, IL). ENMs were quantified as either percentage of total administered mass dose, or percentage of the estimated delivered dose, based on total measured radioactivity (counts per minute, CPM). Concentration calibrations were performed for each ENM by measuring radioactivity for a measured and known total ENM mass in suspension, and measuring radioactivity of dilutions by half down to 5ng.

Cell culture and cytotoxicity experiments

Immortalized human lung epithelial cells [CalU-3 (HTB-55), ATC, Manassas, VA] were cultured in Eagle's Minimum Essential Medium (MEM) culture media supplemented with 5% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10mM HEPES. Culture media components were obtained from Sigma Aldrich (St. Louis, MO). All incubations were performed at 37°C/5% CO₂. Cellular metabolic activity and cytotoxicity were measured via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assay respectively according to the standard protocols previously reported (Demokritou et al., 2013; Gass et al., 2013). All experiments were conducted in triplicate for each ENM.

Absorbance values measured for LDH and MTT *in vitro* assays were normalized with negative control (particle free media) and positive control (Triton X-100; Sigma Aldrich, St. Louis, MO) absorbance values, to a metric of cell viability (%) using the following equation:

$$\text{cell viability (\%)} = \frac{\text{Sample Absorbance} - \text{Negative Control Absorbance}}{\text{Positive Control Absorbance} - \text{Negative Control Absorbance}}$$

Variance of toxicity results for each ENM was tested by post hoc one-way ANOVA with significance set at p 0.05. One-tailed unpaired student T test was used for significance testing, with significance set at p 0.05.

Monitoring of formation and barrier integrity of cellular monolayer

Several methods were used to monitor the formation and integrity of cellular monolayers and tight junctions. In more detail:

Trans Epithelial Electrical Resistance (TEER)—Calu-3 cells were seeded at 5×10^5 cells per well onto 6.5mm Transwell ® tissue culture treated inserts (polyester membrane with 3.0 µm pores and 0.33 cm² growth area) with 100µl of particle free MEM/5%FBS in the apical region and 600µl particle free MEM/5%FBS in the basal region, and cultured at 37°C in humidified air with 5% CO₂. Resistance of the cell monolayer within each well, reported in ohms per cm² of growth area (Ωcm²), was monitored daily prior to exposure using the Epithelial Voltometer and STX2 electrode (World Precision Instruments, Sarasota, FL), and the resistance of filters without cells was subtracted from all sample measurements. Wells reporting resistance greater than 500 Ωcm² were assumed to have achieved monolayers with tight junctions, supported by previous findings in the literature (Geys et al., 2006, Geys et al., 2007, Rothen-Rutishauser et al., 2005),

Electrical Cell-substrate Impedance Sensing (ECIS)—In order to continuously monitor the electrical resistance of cellular monolayers cultured *in vitro* following exposure to industrially relevant ENMs, we utilized electrical cell-substrate impedance sensing (ECIS). Calu-3 cells were seeded onto ECIS 8W10E+ Cultureware slides (Applied BioPhysics, Inc, Troy, NY) with a growth area of 0.8cm² at a density of 8×10^5 cells per well, and cultured in MEM/5%FBS at 37°C in humidified air with carbon dioxide. ECIS slides were connected to electrodes on the ECIS Model 1600R (Applied BioPhysics, Inc.), and resistance measurements were collected every 10 minutes. Once wells reached 2000Ω, culture media was replaced with ENM suspensions at a concentration of 12.5μg/ml, and resistance measurements were collected every 10 minutes for the next 48 hours. All experiments were conducted in triplicate.

Immunofluorescence staining—The effect of ENM exposure on cellular tight junctions was also assessed by immunofluorescence staining for intracellular tight junction protein zonula occludens-1 (ZO-1). In brief, following 24-hour exposure, transwells were washed in PBS, and cells were fixed in 3.7% formaldehyde (MeOH free, J.T. Baker, Phillipsburg, NJ) for 10 minutes at room temperature. Cells were then washed twice in PBS, and incubated with 50mM of glycine in PBS for 5 minutes at room temperature. Fixed cells were permeabilized and blocked with 0.5% saponin/1%BSA/1.5% goat serum for 30 minutes at room temperature. Rabbit antibody against ZO-1 (Invitrogen, Carlsbad, CA) was diluted 1:100 in 0.5% saponin/1%BSA/1.5% goat serum and incubated with these monolayers at room temperature for 1.5 hours. Cells were then incubated with primary antibody (diluted in 0.5% saponin/1%BSA/1.5% goat serum at room temperature for 1.5 hours, followed by rinsing 3 times with 0.5% saponin in PBS at room temperature for 15 minutes. Monolayers were then incubated with secondary antibody [Alexa Fluor 488 goat-anti-rabbit IgG(H+L); Invitrogen, Carlsbad, CA] diluted 1:100 in 0.5% saponin/1%BSA/1.5% goat serum for 1 hour at room temperature in the dark, followed by rinsing 3 times in 0.5% saponin/PBS for 15 minutes, and mounted on microscope slides with 20μl ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Monolayers not exposed to ENMs were similarly incubated with primary and secondary antibodies and processed, serving as negative controls. Images were acquired with a Zeiss Axioplan 200/Axiovert 200 confocal microscope system (Zeiss, Germany).

In Vitro Epithelial Translocation System (INVET)

The experimental setup for tracking translocation of industrially relevant ENMs across cellular monolayers *in vitro* is depicted in Figure 1. 100μl of ENM suspensions at a concentration of 12.5 μg/ml were applied to both transwells without cells, and transwells with Calu-3 cells cultured to confluence, for 2, 4, and 24 hours. The 12.5 μg/ml dose was pre-determined to be below the lethal dose via LDH and MTT cytotoxicity assays (Supplementary Figure 3). Following exposure, all supernatant and transwell inserts were collected from culture plates and set aside for analysis by gamma spectroscopy. All liquid present in the basolateral compartment of the transwell was then collected, and each basal well was washed 3 times with PBS and collected. Gamma counts were measured for apical compartments (including supernatant and the transwell insert), as well as for the basal compartment (including collected media and PBS wash) by gamma spectroscopy. A mass

balance of ENMs measured in the apical and basal compartments was compared to gamma readings for ENM suspensions of equivalent total particle mass (1.25µg). All experiments were conducted in triplicate.

TEM microscopy

Calu-3 cells were seeded onto 60mm tissue culture plates with a growth area of 28.0 cm² at 2×10^5 cells/plate, and cultured in MEM/5%FBS at 37°C in humidified air with 5% CO₂. Once cells formed a confluent layer, they were exposed to ENM suspensions at concentration of 12.5µg/ml. Following 24 hours exposure, cells were washed twice in PBS, removed from the culture plate with 0.25% Trypsin (Gibco, Carlsbad, CA), and centrifuged at $1000 \times g$ for 10 minutes.

Supernatant was decanted, and samples were fixed in Karnovsky's fixative (2.5% Gluteraldehyde and 2.5% Paraformaldehyde in 0.1 M Soidum Cacodylate Buffer; Electron Microscopy Science, Hatfield, PA), then post-fixed in 2% Osmium Tetroxide (Electron Microscopy Science, Hatfield, PA) for one hour. Samples were then dehydrated and embedded in epon, the blocks were thin sectioned and stained with uranyl acetate and lead citrate (Electron Microscopy Science, Hatfield, PA). Images were photographed on a JEOL 1220 transmission electron microscope (Peabody, MA).

RESULTS

Nanomaterial characterization

Properties of ENM powders and dispersions used in this study are summarized in Table 1. Our nanopanel includes CeO₂ ENMs of $d_{XRD} = 28.4$ (CeO₂ small) and $d_{XRD} = 119\text{nm}$ (CeO₂ large), ZnO ($d_{XRD} = 28.0\text{nm}$), as well as SiO₂-coated CeO₂ of $d_{XRD} = 28.4\text{nm}$ (CeO₂ coated) and SiO₂-coated ZnO of $d_{XRD} = 27.6\text{nm}$ (ZnO coated). The variety of properties of the ENM panel allows for parametric comparison of the effects of primary particle size and surface chemistry on ENM translocation across cellular monolayers using the proposed INVET system. Representative electron microscopy images presented in Figure S1 illustrate the fractal structure of aggregates, indicative of flame generated ENMs. In the case of the SiO₂ coated ENMs, a smooth and relatively homogenous 2-4nm SiO₂ coated layer is visible around the core material (CeO₂ and ZnO respectively). The hermetic encapsulation of the core ENMs by SiO₂ with minimal effects on the core material has been previously described (Gass et al., 2013, Demokritou et al., 2013).

DLS characterization data presented in Table 1 demonstrates that in general, all ENMs formed agglomerates several times larger than their respective primary particle sizes in both DI H₂O as well as cell culture media, with agglomerate diameters ranging from 200-230nm for all suspensions in MEM/5%FBS. ENM agglomerates were stable for up to 48 hours, the duration of our cellular exposures (data not shown), consistent with previous measurements for these materials (Gass et al., 2013, Cohen et al., 2012).

Effect of ENMs on monolayer barrier integrity

As described above and depicted in Figure 1, Calu-3 cell monolayers were cultured on transwell inserts, and monolayer development was monitored by TEER. Monolayer formation was determined when electrical resistance readings measured by TEER exceeded $500\Omega\text{cm}^2$ (Supplementary Figure 3), consistent with previous reports in the literature (Geys et al., 2006, Geys et al., 2007).

Cellular monolayers with TEER $500\Omega\text{cm}^2$ were then exposed to ENMs at an administered dose of $12.5\mu\text{g}/\text{ml}$, pre-determined to be below the lethal dose via LDH and MTT cytotoxicity assays (Supplementary Figure 4), which is consistent with toxicity results from the literature (Gass et al., 2013, Demokritou et al., 2013, Kroll et al., 2011, Xia et al., 2008a). Figure 2a presents TEER readings pre- and post-exposure at each time point, which suggest that ENM exposure did not result in a compromised monolayer and disturbance of tight junctions. Figure 2b presents the ECIS resistance measurements taken every 10 minutes following exposure, providing further support that ENM exposure at $12.5\mu\text{g}/\text{ml}$ does not compromise tight junctions or monolayer integrity. This data is consistent with previous studies investigating the effect of ZnO particles on cellular monolayers at similar administered mass concentrations (Kim YH et al., 2010).

Immunofluorescence staining for tight junction protein zonula occludens-1 (ZO-1) demonstrated negligible differences between Calu-3 monolayers exposed apically to $25\mu\text{g}/\text{ml}$ of each ENM suspension for 24 hours, compared with cells exposed to particle-free media (Supplementary Figure 5), suggesting ENM exposure leads to negligible disruption of continuity of tight junctions. These images are consistent with previously published confocal slices of ZO-1 stains, exhibiting high density of ZO-1 proteins principally at cell borders (Ye et al., 2009). This data further support our TEER and ECIS results, suggesting that ENM exposures at the low concentrations investigated do not compromise Calu-3 monolayer barrier function, nor do they disrupt tight junctions.

Tracking ENM translocation across cellular monolayers

ENM translocation across transwell filter membrane without cells – particle loss assessment—The ability of our ENM agglomerates to successfully pass through the $3\mu\text{m}$ pores of the transwell insert membrane in the absence of cells following 24-hour incubation was investigated in order to account for potential particle losses within the system. All five ENMs investigated easily permeated through the naked porous filter ($<4\%$ of the administered ENMs were collected from the transwell membrane, Supplementary Figure 6). A complete mass balance of administered particles was achieved for all ENMs, where the sum of ENMs measured in each compartment (supernatant, transwell membrane, and basolateral) was equal to the administered dose value, suggesting all ENMs are accounted for in our proposed *in vitro* translocation system (Supplementary Figure 6).

In vitro dosimetric considerations—The recently developed by the authors methodology to determine the delivered to cell dose was employed for all ENMs investigated (Deloid G et al., submitted July 2013), whereby ENM deposition *in vitro* via sedimentation and diffusion was estimated based on careful characterization of agglomerate

parameters including hydrodynamic diameter and agglomerate effective density. Supplemental Figure 6 reports the estimated delivered to cell dose after 24 hours exposure for each material investigated, which in general strongly agreed with the translocated through the membrane dose for each material after 24 hours exposure (defined as the sum of ENMs measured on the transwell membrane and in the basolateral compartment), a clear indication of the validity of the proposed *in vitro* dosimetry methodology.

Figure 3 presents the estimated particle deposition curves over time for the three ENMs investigated for translocation across cellular monolayers at various time exposures. Large differences were estimated in the rate of particle delivery to cells across the ENMs studied. For example, after 24 hours exposure time, 99% of CeO₂ large is delivered to cells, while only 58% of CeO₂ small is delivered to cells. This discrepancy is largely due to differences in effective density of the two ENMs (2.368 and 1.365 g/cm³ respectively), which is directly related to sedimentation speed.

ENM translocation across cellular monolayers—Figure 4a summarizes the amount of each ENMs detected in the basolateral compartment by gamma spectroscopy after 24 hour exposure duration using the INVET systems. These values are reported as both a percentage of the administered dose (Figure 4a), and normalized to the percentage of the delivered to cell dose based on the estimated delivered dose fractions (Figure 4b). Following 24 hours of exposure, very low amounts of each ENM were measured in the basolateral compartment (<0.01% administered dose) (Figure 4a). The results from ANOVA indicate the mean values for each ENM are statistically different from each other, though the low values of translocated particles make ranking the translocation potential of all ENMs investigated difficult. When dosimetry is taken into consideration, the difference between the two sized CeO₂ particles (d_{XRD} =28.4nm and 119nm respectively) is decreased, indicative of the importance of dosimetry *in vitro*. ZnO still exhibits the greatest translocation ability per delivered to cell dose (Figure 4b).

Figure 5 presents time resolved translocation data for CeO₂ (for both primary particle sizes) and SiO₂ coated CeO₂ after 2, 4 and 24 hour exposure. Overall, very small amounts of all three ENMs successfully translocated into the basolateral compartment (<0.01%, Figure 5a). CeO₂ large exhibited slightly greater translocation than CeO₂ small at each time period. Additionally, for all three particles used here, increased translocation associated with increasing exposure duration from 4 to 24 hours was observed (e.g. CeO₂ d_{XRD} =28.4nm: 0.00038% vs 0.0020%). However, when normalized to delivered to cell dose, the amount of CeO₂ small and CeO₂ large measured in the basolateral compartment were found to be are roughly equal, and differences in translocation associated with increased exposure time also disappeared (Figure 5b). For example, after 24 hours exposure, the percent of delivered to cell particles (by mass) that translocated across the cellular monolayer for CeO₂ small was 0.0035%, and for CeO₂ large was 0.0030%. It is worth noting that SiO₂ coated CeO₂ exhibited greater translocation than both bare CeO₂ particles even when normalized to the delivered dose (Figure 5b), a clear indication that surface chemistry plays an important role in translocation.

Cellular internalization of ENMs

Electron micrographs of cells exposed to ENMs for 24 hours were collected to determine the intracellular localization of ENM particles. Figure 6 presents representative images for Calu-3 cells exposed to CeO₂ large (6a), and CeO₂ small (6b). These images provide qualitative confirmation of cellular internalization, evidence that ENMs translocate across cellular monolayers via a predominantly transcellular pathway. In general, CeO₂ ENMs appear encapsulated within membrane bound vacuoles. There appears to be greater quantities of CeO₂ large particles internalized after 24 hour exposure, consistent with the estimated fast deposition rate and high delivered to cell dose estimated for this particle (Figure 3). It is worth noting that ENM aggregates appear concentrated within large endosomal vacuoles (Figure 6a). The CeO₂ small particles appear internalized within later stage endosomal vacuoles, already fused with lysosomes and consolidated with other cellular debris. Similarly, representative images for the other particles provide evidence for cellular internalization and are included in Supplementary Figure 7.

DISCUSSION

Neutron activated tracer particle system

The data presented here illustrate the high sensitivity of the described radiolabel method which enables rapid and accurate detection of extremely small quantities of ENM in various *in vitro* systems. Furthermore, neutron activation has been shown to induce minimal effects on particle properties (He et al., 2010, Yeh et al., 2012). One limitation of the proposed particle system is the short half-life of some radioisotopes (¹⁴¹Ce, $t_{1/2}$ = 32 days), making necessary careful planning and time management of all cellular studies once ENMs have been irradiated.

Calu-3 In vitro model of alveolar epithelium

It must be noted that the Calu-3 human epithelial monolayer system used here is a simplified model of the alveolar region, consisting of type II alveolar epithelial cells which only account for <5% of the total surface area of the alveolar wall due to their distinct morphology, and are less likely to come into contact with inhaled ENMs than type I cells (West, 2008). Additionally, the distinct morphology of type II pneumocytes may have implications for particle-cell interactions in terms of the time required for particle endocytosis and exocytosis (West, 2008).

Furthermore, our monoculture model lacks phagocytic macrophages largely responsible for clearance and elimination of insoluble deposited particles through the tracheobronchial tree towards the larynx (Semmler et al., 2004, Kreyling et al., 2009, Geiser and Kreyling, 2010). It is worth noting that recent studies report non-specific and sporadic phagocytosis of TiO₂ ENMs by alveolar macrophages *in vivo*, suggesting ENMs are able to evade a major clearance mechanism and that there is an increased likelihood for direct interactions with the alveolar epithelium (Geiser et al., 2008). Few nanotoxicology studies have developed 3 dimensional triple co-culture systems with primary epithelial cells, along with phagocytic macrophages and dendritic cells to simulate more realistic physiological conditions (Lehmann et al., 2011). Such sophisticated co-culture systems are necessary to improve the

physiological relevance of *in vitro* translocation studies (Rothen-Rutishauser et al., 2005, Lehmann et al., 2011, Muller, 2011), and should be adopted in future studies to bring more physiological relevance to *in vitro* studies.

Translocation of industrially relevant ENMs using the proposed INVET system

It is clear from the aforementioned data that only small quantities of the ENMs used in this study are able to translocate transcellularly across Calu-3 cells without compromising the monolayer barrier or disrupting tight junctions. Yacobi et al recently reported similar findings after exposing rat alveolar epithelial cell monolayers to fluorescently labeled non-agglomerating polystyrene nanoparticles (PNPs) (Yacobi et al., 2010). However, PNP's are not industrially relevant particles with high risk of inhalation exposure. The translocation potential of each ENM material used in this study are discussed in more detail below.

CeO₂ ENMs—Both the size- and time-dependent differences in translocation are likely attributable to the differences in the delivered to cell dose of particles at each exposure time point. Limbach et al recently reported cellular uptake of CeO₂ ENMs at low exposure concentrations may be limited to particle delivery to cells (Limbach, 2005). Indeed, when normalized to delivered dose, the amount of CeO₂ small and CeO₂ large we measured in the basolateral compartment are roughly equal, and differences in translocation associated with increased exposure time also disappear (Figure 5b). This data supports the theory that ENM-cell interactions *in vitro* largely depend on particle delivery to cells, emphasizing the importance of accurate dosimetry for *in vitro* nanotoxicology.

Our results for CeO₂ are also consistent with previously reported findings indicating that extremely small quantities of CeO₂ are able to translocate across a cellular monolayer quickly *in vivo*, with only small increases in total flux at longer exposure durations. A recent study investigating the biodistribution of CeO₂ instilled in the lungs of Wistar rats reported small quantities of CeO₂ nanoparticles are capable of quickly accessing the bloodstream and entering transpulmonary organs within 10 minutes post exposure, though the majority of particles are persistent in the lungs weeks after instillation (64% of instilled particles remained in the lung at 28 days post exposure) (He et al., 2010). It is clear that CeO₂ ENMs are able to translocate across cellular monolayers both *in vitro* and *in vivo* at extremely low levels, and translocation likely occurs via transcellular pathways.

SiO₂ Coated ENMs—Both SiO₂ coated CeO₂ and SiO₂ coated ZnO ENMs exhibited greater translocation than both bare CeO₂ ENMs at 24 hours exposure (Figure 4a). SiO₂ coated CeO₂ exhibited greater translocation than bare CeO₂ even when normalized to the delivered dose (Figure 4b), suggesting surface chemistry plays a significant role in ENM translocation across cellular monolayers. Increases in translocation associated with increasing exposure time (2, 4 and 24 hours) disappear when normalized to delivered dose (Figure 5), supporting the theory that ENM-cellular interactions are greatly influenced by particle delivery to cells and emphasizing the importance of dosimetry considerations for *in vitro* nanotoxicology.

Our findings for both SiO₂ coated ENMs are consistent with recently reported *in vivo* investigations reporting translocation of pure SiO₂ particles. Amorphous SiO₂ ENMs of

various primary particle size were instilled in the lungs of Wister rats at relatively high doses (2, 5 and 10mg/Kg by weight), and exhibited the ability to pass through the alveolar epithelium into systemic circulation and induce cardiovascular toxicity(Du et al., 2013). However, the exceedingly high doses used in this study may have resulted in lung particle overload and impaired alveolar macrophage mediated particle clearance, leading to increased particle accumulation in the lungs and increased potential for lung injury, compromise of the air-blood barrier and increased translocation events to occur(Oberdorster, 1995). In spite of this limitation, our results support the growing body-of-evidence that amorphous SiO₂ particles and SiO₂ coated ENMs are capable of translocation across the alveolar epithelium into the systemic circulation via transcellular pathways.

ZnO ENMs—ZnO exhibited the greatest amount of translocation following 24-hour exposure (Figure 4a). It is important to note that the measured translocation for ZnO ENMs does not distinguish between ZnO particles and Zn²⁺ ions associated with partial solubility of the ZnO. While Zhang et al recently reported 35% dissolution of ZnO ENMs following 24-hour incubation in cell culture media(Zhang et al., 2012), time-resolved solubility studies for this material will be required to determine the dissolution rate over time in cell culture media to further elucidate this issue. Significantly, ZnO particles of agglomerate diameter 230nm were measured by DLS in the basolateral media, providing evidence of particle translocation following 24-hour exposure. Future studies are required to further distinguish the translocation kinetics of particulate ZnO vs. translocation of zinc ions.

Cellular Internalization of ENMs

While our study provides some insight into understanding ENM translocation across epithelial barriers, more work is necessary to determine the mechanisms of cellular internalization. Several groups report a relationship between ENM properties (diameter, surface charge, etc) and endocytic mechanisms, though often with conflicting results (Nel 2009). For example, Chithrani et al. reported an optimal diameter of 200nm for clathrin mediated uptake, and 500nm for caveolin mediated uptake (Chithrani et al. 2006). In contrast, Gratton et al. report the optimal diameter ranges from 50-80nm for clathrin mediated uptake, and is 120nm for caveolin mediated uptake (Gratton et al. 2008). With regards to particle translocation, Yacobi et al. reported higher translocation rates for amidine-modified polystyrene nanoparticles compared with carboxylate-modified PNPs, suggesting that direct interactions between PNPs and cell membranes may be the predominant mechanism for PNP internalization and translocation across primary rat alveolar epithelial cell monolayers (Yacobi et al. 2010).

It is clear that additional *in vitro* and *in vivo* studies are necessary to further elucidate the specific endocytic and exocytic mechanisms underlying translocation of industrially relevant ENMs, and our group is currently working to investigate these issues utilizing the developed INVET platform.

CONCLUSIONS

In this study we present a novel *in vitro* model for tracking the translocation of industrially relevant metal oxide ENMs across cellular monolayers *in vitro*. While neutron activated

industrially relevant ENMs have been used for biodistribution studies *in vivo*, this is the first time such an approach has been used *in vitro* to assess particle-cell interactions. With the ever-increasing number and variety of engineered nanomaterials (ENMs) entering the consumer market, efficient and inexpensive *in vitro* models such as the one presented here are needed to correlate ENM properties with biological activity. The versatility and high sensitivity of this approach was demonstrated using a panel of industrially relevant ENMs of variable size and chemical composition and surface chemistry. We report that CeO₂ of various primary particle sizes, ZnO, SiO₂-coated CeO₂ and SiO₂-coated ZnO ENMs are able to translocate transcellularly across Calu-3 cellular monolayers at low doses, without compromising the alveolar epithelial barrier integrity, nor disrupting tight junctions at cell-cell borders. Electron micrographs of exposed cells exhibiting ENMs internalized by cells localized near large endosomal vacuoles provide supporting evidence that the translocation pathway is transcellular rather paracellular. Currently, there is indirect validation of our *in vitro* results published in the literature for each material based on instillation experiments *in vivo*. Additionally, our group is currently preparing manuscripts summarizing the results of *in vivo* pharmacokinetic studies investigating translocation of the particular materials used in our INVET system and reported on here. Though it was observed that very few particles were able to translocate across healthy cellular monolayers, the kinetics and mechanisms of ENM translocation may be different for unhealthy monolayers with disrupted tight junctions. For example, ozone exposure is known to increase the permeability of the air blood barrier, with implications for increased translocation of ENM particles. Furthermore, the results of this study highlight the importance of dosimetry for accurate *in vitro* nanotoxicity studies. Although additional *in vitro* and *in vivo* studies are required to further elucidate the specific endocytic and exocytic mechanisms underlying ENM translocation at physiologically relevant exposure doses, it is clear that industrially relevant metal oxide ENMs are capable of crossing healthy alveolar monolayers without compromising the epithelium barrier integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Experimental setup of In Vitro Epithelial Translocation system (INVET).

Figure 2.

Effect of ENM exposure on monolayer electrical resistance. a) TEER of Calu-3 monolayers pre and post ENM exposure, error bars represent standard deviation; b) continuous resistance measurements via Electrical Cell-substrate Impedance Sensing (ECIS) during 48-hour exposure to ENM suspensions, error bars represent standard deviation.

Figure 3.
Estimation of particle delivery to cells over 24-hour exposure period, $f(24)$: fraction of administered dose delivered to cells following 24 hours exposure

Figure 4.

Translocation of ENMs across cellular monolayer *in vitro*. a) Percent of administered dose measured in basolateral compartment of *in vitro* system following 24 hours exposure. b) Percent of delivered dose for ENMs measured in basolateral compartment following 24 hours exposure. All experiments were done in triplicate, error bars represent standard deviation. # indicates mean values for each material are not all equal (ANOVA) for $p < 0.05$.

Figure 5.

a) Percent of administered dose for insoluble ENMs measured in basolateral compartment of *in vitro* system following 2, 4 and 24 hours exposure. b) Percent of delivered dose for insoluble ENMs measured in basolateral compartment following 2, 4 and 24 hours exposure. All experiments were performed in triplicate. Error bars represent standard deviation.

Figure 6.
TEM images of ENMs internalized by Calu-3 cells following 24 hours exposure. a) CeO₂ large; b) CeO₂ small

Table 1

Characterization of ENM powders and liquid suspensions. SSA: (specific surface area) by nitrogen adsorption/ Brunauer-Emmett-Teller (BET) method. d_{BET} : particle diameter determined from SSA and particle density, ρ , as described in methods. d_{XRD} : particle diameter by X-ray diffraction; ρ : material density; d_{H} : hydrodynamic diameter, PDI: polydispersity index, ζ : zeta potential; π_{E} : agglomerate effective density

Material	SSA (m ² /g)	d_{BET} (nm)	d_{XRD} (nm)	ρ (g/cm ³)	Media	d_{H} (nm)	ζ (mV)	ρ_{E} (g/cm ³)
CeO ₂ - small $d_{\text{XRD}}=28.4\text{nm}$	28.0	28.0*	28.4	7.65	DI H ₂ O	136±1.06	34.5 ±3.15	NA
					MEM/5%FBS	184±2.40	-12.5±0.289	1.625
CeO ₂ - large $d_{\text{XRD}}=119\text{nm}$	11.0 31.3	71.9* 29.2	119 28.4	7.65 6.29	DI H ₂ O	231±2.93	34.5 ±3.15	NA
					MEM/5%FBS	215±3.42	-9.77±0.497	2.368
SiO ₂ -coated CeO ₂ $d_{\text{XRD}}=28.4\text{nm}$	40.14 66.0	26.7 18.5	28.0 27.6	5.6 4.13	DI H ₂ O	208±2.87	-26.8±0.277	NA
					MEM/5%FBS	227±3.17	-11.6±0.451	1.600
ZnO $d_{\text{XRD}}=28.0\text{nm}$	11.0 31.3	71.9* 29.2	119 28.4	7.65 6.29	DI H ₂ O	218±3.9	23.0±0.462	NA
					MEM/5%FBS	235±2.55	-15.5±3.29	1.485
SiO ₂ -coated ZnO $d_{\text{XRD}}=27.6\text{nm}$	40.14	26.7	28.0	5.6	DI H ₂ O	165±1.71	-15.9±1.27	NA
					MEM/5%FBS	206±4.52	-12.6±5.77	1.655

* d_{BET} assumes spherical particles, CeO₂ particles are hexagonal so d_{XRD} is a more accurate description of primary particle size