A Comparison of Three Dispersion Media on the Physicochemical and Toxicological Behavior of TiO₂ and NiO Nanoparticles

Erika R. Gutierrez¹, Richard M. Kamens¹, Michael Tolocka², Kenneth Sexton¹, Ilona Jaspers^{3*}

¹Department of Environmental Sciences and Engineering, Gillings School of Global Public Health ²Department of Chemistry and Biochemistry, Rowan University, ³Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, University of North Carolina-Chapel Hill, Chapel Hill, North Carolina, USA

*Correspondence: Center for Environmental Medicine, Asthma, and Lung Biology CB#7310, 104 Mason Farm Road, Chapel Hill, NC, USA 27599; Telephone: (919)-966-8657; ilona_jaspers@med.unc.edu

Keywords: NiO, TiO₂, nanoparticles, A549 cells, dosimetry

Abstract

Nanomaterials represent a burgeoning field of technological innovation. With the onset of environmental release and commercial product exposure associated with nanomaterial manufacture and proliferation, the concomitant effects on human health remain unknown and demand further investigation. Agglomeration of nanomaterials in biologically relevant media used in *in vitro* methods further complicates dosing in toxicological study. Objective: To compare the effects of *in vitro* dispersion techniques on the physicochemical and toxicological dosimetry of TiO₂ (<50 nm) and NiO (<20 nm) nanoparticles and some resulting toxicological endpoints to test for potential effects. Methods: Three media were prepared for A549 and 16hbe14o cells with varying concentrations of TiO₂ and NiO nanoparticles. Physicochemical effects were analyzed with dynamic light scattering, ICP-MS, SEM, and TEM. Toxicological effects were determined after stimulation of cells with nanoparticles for 4 and 24 hours followed by analysis of inflammatory and oxidative stress markers with ELISA and RT-PCR. Our data show that dispersion media differentially affect physicochemical properties and toxicological endpoints. Therefore, we conclude that *in vitro* nanotoxicology models that use re-suspension methods of exposure yield inconsistent and misleading biological results due to physicochemical variation of particle characteristics and transport processes.

Introduction

Nanomaterials represent a growing field of technological innovation. With the onset and continuing increase of environmental release associated with nanomaterial manufacture and proliferation, the concomitant effects on human health need to be determined (Karlsson et al.

2009a, Whatmore August 2006, Warheit et al. 2007). Of particularly expedient concern is the effect on respiratory health, since inhalation presents an important route of exposure. The occupational health effects encumbered by workers during nanoparticle manufacture and remediation remain unquantified, as are the risks to consumers of nanomaterial-based products (Warheit 2010, USEPA 2005).

Currently, the most common and cost-effective method to study the respiratory health effects of nanoparticles involves the use of *in vitro* toxicological models combined with a resuspension technique (Bakand et al. 2005). Nanoparticle resuspension techniques involve the direct addition of nanoparticles to cell culture medium. However, fluctuations between physicochemical characteristics in the colloidal state lead to the agglomeration of nanoparticles in solution, which consequently presents inherent difficulties to the exposure and accurate evaluation of toxicity (Oberdorster et al. 2005). These dynamic interactions of, and between particle size, chemical composition, surface modifications, and particle number that lead to agglomeration will elicit an irregular and non-uniform deposition pattern (Warheit, Sayes & Reed 2009, Hinderliter et al. 2010, Oberdorster et al. 2005, Sayes et al. 2006). In addition, processes of particle deposition onto the cell monolayer change with the size and status of the agglomerated particles (Teeguarden et al. 2007).

At large particle sizes or agglomerates (d_p [particle diameter], or d_{ag} [agglomerate diameter] $>100\text{nm}$), sedimentation processes dominate. In the nanomaterial range ($10\text{-}100\text{nm}$), a combination of both sedimentation and diffusion processes occur. At particle sizes $d_p < 10\text{nm}$, the process of deposition onto the cell monolayer involves properties related to the mobility of the particle in solution through Brownian motion and diffusion (Hinderliter et al. 2010, Bird, Stewart & Lightfoot 1960). To date, the majority of *in vitro* resuspension studies have used doses that

suggest agglomeration rates and physical processes that involve sedimentation onto the cell monolayer, assume uniform deposition, and/or ignore the physical process leading to nanoparticle uptake into the cell monolayer. Some degree of agglomeration takes place during manufacture and in the air in exposure conditions, which have been characterized (Dahm et al. 2012). The goal is to use methods, which achieve and control representative degrees of agglomeration and resulting size and to maintain these conditions during toxicity exposure tests. Therefore, issues of quality control arise when the dose is extrapolated to lower, more environmentally and biologically relevant levels, chronic and sub-chronic exposure levels, and the primary and/or agglomerated particle size is ≤ 100 nm (Teeguarden et al. 2007, Limbach et al. 2005). Thus, the consistency of the exposure is at stake due to the mixture of physical processes occurring in solution: diffusion, Brownian motion, and sedimentation, which control the behavior, their natural agglomeration state, and exposure of the nanoparticles to the cell monolayer (Warheit 2010). These complications present obstacles for risk assessment and exposure regulation since the exposure may no longer be representative of particles in the nano-scale range, but the sub-micron to micro-range, as well as vary between exposure in different dispersants, cell culture media, and media depth used for the exposure (Warheit 2010, Teeguarden et al. 2007, Walker, Bucher 2009).

Numerous methods and techniques have been developed to resolve the issue of agglomeration. Surfactants are a common additive to control the degree of agglomeration and micelle formation, since there are many biological components of the cell culture medium system that can play a dual role as a de-agglomerating agent and as a cellular nutrient (Tirado-Miranda et al. 2003, Sager et al. 2007). On the other hand, supposedly passive, non-toxic xenobiotic surfactants are used to de-agglomerate nanoparticles in cell culture medium. There

are three types of surfactant-based methods employed in common use to control agglomeration: serum or serum proteins, biologically based pulmonary surfactants such as DPPC and Survanta (Porter et al. 2008, Wang et al. 2010), and non-biologically based surfactants (Ramirez-Garcia et al. 2011). The efficacy of these surfactants has not been inter-compared nor has their subsequent effect on biological endpoints been assessed.

Choosing a particular dispersion method may implicate downstream effects on both the exposure science and the toxicological endpoints. In this study, three different de-agglomerating agents have been chosen for comparison due to their widespread use in research labs and usage in nanotoxicology publications. The following surfactants were used for comparison: 1) fetal bovine serum (FBS), to reflect the traditional cell culture medium surfactants, 2) a synthetic surfactant and non-ionic block copolymer, F68, and 3) a semi-synthetic, plant-derived, but naturally occurring human lung surfactant 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in bovine serum albumin (BSA). Nickel oxide (NiO) and titanium dioxide (TiO₂) metal oxide nanoparticles were chosen due to their unknown and great potential for human health effects, increased environmental and airborne proliferation, occupational safety hazards, and role in atmospheric chemistry (Karlsson et al. 2009b, Lee, Donahue 2011, Leikauf et al. 2001, Warheit 2004). Therefore, our research objective is to compare the effects of dispersion and exposure techniques on the physicochemical and toxicological dosimetry of TiO₂ (<50 nm) and NiO (<20 nm) nanoparticles and resulting toxicological endpoints. The selected toxicological endpoints are meant to serve as an example and proof of principle of the importance of exposure method choices, and not to be suggestive of an all-inclusive set of the many possible toxicological effects.

Materials and Methods

2.1 Reagents and Supplies

Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). F-12K 1x (Ham's F-12K Nutrient Mixture, Kaighn's Modification with L-glutamine) was obtained from Mediatech, Inc. (Manassas, Va). Minimal Essential Medium (MEM) was bought from Gibco (Invitrogen, Carlsbad, CA). Bovine serum albumin and penicillin/streptomycin solution were obtained from Invitrogen (Carlsbad, CA). DPPC and 10% Pluronic F-68 Solution were obtained from Sigma Aldrich (St. Louis, MO). MEM was bought from Invitrogen (Carlsbad, CA). Titanium (IV) dioxide (TiO₂) and nickel (II) oxide (NiO), were obtained from Sigma-Aldrich (St. Louis, MO). The reported mean diameters were TiO₂, <50 nm and surface area 35-65 m²/g, and NiO, <20 nm.

2.2 Cell Culture and Dispersion Media Composition

A549 cells were cultured in F-12K supplemented with 10% FBS and 1% penicillin/streptomycin. 16hbe14o cells were cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were grown to confluency in their respective and traditional medium in 24-well plates. At confluency, cells were treated with the respective dispersion media and nanoparticles described hereafter.

Three media were prepared for A549 cells: "F12K + FBS" is composed of F-12K cell culture medium/10% FBS/1% penicillin/streptomycin, "F12K + F68 Media" is F-12K cell culture medium/0.1% Pluronic F68 nonionic surfactant/ 1% penicillin/streptomycin, and "BSSM" is F-12K cell culture medium/0.01% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, semisynthetic (DPPC)/0.6 mg/mL BSA/1% penicillin/streptomycin. BSSM is modeled after the

biocompatible medium developed by (Porter et al. 2008). The BSSM in this study uses the same surfactant composition as Porter et al. but is adapted to use the appropriate A549 cell culture nutrient mixture.

Two media were prepared for 16hbe14o cells: "MEM + FBS" is MEM cell culture medium/10% FBS/1% L-glutamine/1% penicillin/streptomycin and "MEM +F68" is MEM cell culture medium/0.1% F68 nonionic surfactant/1% L-glutamine/1% penicillin/streptomycin.

Each dispersant contained varying concentrations (0, 0.01, 0.1, 0.5, 10, 100 $\mu\text{g/mL}$) of -
 TiO_2 or NiO nanoparticles.

2.3 Preparation of Dispersion Media with Nanoparticles

1-mg of nanoparticles were weighed and transferred into 100-mL of dispersion medium. The resulting solution was sonicated in a 125-watt Branson Ultrasonic water-bath sonicator for 30 minutes. The solution was immediately diluted to the desired concentration; if serial dilutions were made, vortexing for one minute on high occurred between each dilution. All dispersion medium containing nanoparticles were stored in the dark and experimentations were initiated within 30 minutes of sonication. All solutions were re-vortexed if more than 20 minutes elapsed since the initial sonication and/or vortexing. After 24 hours, solutions were discarded.

2.4 Physicochemical Analysis

2.4.1 Dynamic Light Scattering (DLS) and ζ -Potential

Measurement of particle size with DLS and solution stability via ζ -potential was determined with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). The size distributions were multimodal for concentrations of 1 and 10 $\mu\text{g/mL}$ and unimodal for 0.01, 0.1, and 0.5 $\mu\text{g/mL}$. The period of time between sonication and exposure was the same for particle

sizing and for exposure to the cells. The gap between sonication and DLS measurement as well as cell exposure was kept to a minimum (<30 minutes) and derived from the fact that the nanoparticle sonication and cell culture equipment were located in different laboratories.

2.4.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Quantitative measurement of nanoparticle concentration and metal contamination was determined with a Varian 820-MS ICP-MS (Agilent Technologies, Santa Clara, CA).

Nanoparticle solutions were first microwave digested according to EPA Method 3052 in hydrogen peroxide and concentrated nitric acid. For ICP-MS analysis, solutions were diluted 1:10, digested nanoparticle dispersion medium: 18-M Ω water.

2.5 Toxicological Analysis

2.5.1 *In vitro* co-incubation with nanoparticles and collection

A549 cells, an immortalized human type-II alveolar epithelial cell line, and 16hbe14o cells, a bronchial epithelial transformed cell line, were used to assess toxicological parameters. For the exposures, cells were plated at density of 200,000 cells/well in a standard 24-well plate. NiO in each dispersion medium was added to the cells for 4 and 24 hours and each cell line was cultured in the appropriate cell line dispersion medium at a volume of either 300 μ L or 1mL. Supernatant and total RNA collected in TRIzol (Carlsbad, CA) were used for analysis.

2.5.2 Determination of Dispersant and Nanoparticle Interference on ELISA

A quality control run was conducted to test potential interference of nanoparticles with the ELISA assay. Four sets of IL-8 standards were made in each of the following solutions: assay diluent (the control and the standard solution for the manufacturer's protocol), F-12K + FBS + 10 μ g/mL NiO, BSSM + 10 μ g/mL NiO, and F-12K + F68 + 10 μ g/mL NiO. For the test,

expression of interleukin (IL)-8 was determined following the manufacturer's protocol with a Thermo Multiskan MCC (Thermo Fisher Scientific Inc., Waltham, MA).

2.5.3 RT-qPCR

The mRNA gene expression levels of IL-8 and HO-1 mRNA were determined using RT-qPCR with β -actin mRNA as the endogenous control as described previously (Bauer et al. 2012, Jaspers et al. 2001). Primer and probe sequences were obtained from Applied Biosystems (Foster City, California) and RT-qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Foster City, California). Data were analyzed according to the $2^{-\Delta\Delta C_t}$ method.

2.6 Statistical Analysis

Each experiment was performed in triplicate, except for the *in vitro* exposures, which were performed in quadruplicate.

The student's t-test was performed to compare the means of each set of results. To use this test, we made three assumptions: data were normally distributed, they have similar variances, and each of the samples produced independent results. Data were presented as mean $\pm$ standard deviation and a p-value < 0.05 was considered to be significant.

Results

3.1 Physicochemical Analysis

3.1.1 Dynamic Light Scattering: NiO

We first measured the size of the particles in dispersion media with dynamic light scattering to compare each solution's ability to maintain particles within the accepted nanoparticle classification range (≤ 100 nm).

Dynamic light scattering measurements were made for each concentration (0, 0.01, 0.1, 0.5, 10 $\mu\text{g/mL}$) of NiO nanoparticles in dispersion media. For NiO, a total of five dispersion media were tested for both the 16hbe14o and A549 cell lines, and for TiO₂, three dispersion media were tested for only A549 cell line media. Samples were tested within 30 minutes of initial preparation. Sedimentation began occurring quickly in 10 $\mu\text{g/mL}$ NiO and 10 $\mu\text{g/mL}$ TiO₂ solutions post-sonication.

Both the 16hbe14o and the A549 cell line dispersion media varied in their ability to maintain particle size ≤ 100 nm and intra- and inter-sample variability was problematic (Figure 1). Amongst the A549 cell line dispersion media, F-12K + FBS maintained particle size below 100 nm the best, with the concentrations up to 0.5 $\mu\text{g/mL}$ meeting the criteria while the highest concentrations of 1- and 10- $\mu\text{g/mL}$ did not (Figure 1A). BSSM did not control the particle size within the optimal range for any concentration. F-12K + F68 showed a dose response relationship with increasing concentration and size of the agglomerated particles; however, the particle size was not maintained below 100nm for any concentration.

For the 16hbe14o cell line, MEM + F68 showed a dose response relationship with respect to particle size and concentration, but as with the related dispersion media for A549 cells, it did not meet the criteria for maintaining particle size under 100 nm for any concentration (Figure

1B). MEM + FBS controlled particle size within the desired range until the highest concentration, 10- μ g/mL.

When both cell line dispersion media are compared, the medium that best controls for the size and agglomeration status below 100 nm is the conventional cell culture medium for that particular cell line. Values meeting the criteria for maintaining particle size are indicated by the asterisks in Figure 1.

3.1.2 Dynamic Light Scattering: TiO₂

All concentrations of TiO₂ in the conventional A549 cell culture medium, F-12K + FBS, with the exception of 10 μ g/mL, which rapidly sediments post-sonication, maintained particle size below 100 nm. Neither BSSM nor F-12K + F68 sufficiently dispersed TiO₂ to meet the particle size criteria (Figure 2).

3.1.3 ζ -potential

The ζ -potential of TiO₂ in A549 cell dispersion media was measured (Table 1). ζ -potential is an analytical measurement that determines the electrostatic interactions between particles in colloidal solutions. The measurement provides information about the degree of stability in colloidal or dispersed solutions. Knowing the level of stability in each dispersion medium will provide insight on the interactions between the nanoparticles, de-agglomerating agents, and cell culture medium. A value ± 40 or greater is an indication of good stability. None of the dispersion media were in the stable range and the behavior between nanoparticles, de-agglomerating agent, and cell culture medium can be assumed to be unstable and rapidly flocculating. Since NiO is magnetic, ζ -potential was not attempted due to measurement interference.

3.1.4 ICP-MS

Once the dispersion media were evaluated for particle size and stability, the question remained as to whether or not the delivered dose equaled the targeted dose. Due to its ability to keep the average particle size smaller than the other dispersion media, F-12K + FBS was chosen to measure potential contamination by other toxic metals and for concentration validation.

Quantitative measurement of the concentration of Ni in the F-12K + FBS medium at concentrations of 0.01, 0.1, 0.5, 1.0, and 10 µg/mL NiO confirmed that the serial dilutions reflected the targeted concentrations (Figure 3A). A linear regression was fit to the data with an R^2 of 0.9961.

A panel of metals (Al, Ti, V, Cr, Cu, As, Ag, Cd, Pb) was measured to determine the presence of undesirable metal contamination. No significant contamination was measured.

Due to presence of sedimentation in the highest concentration, 10 µg/mL, we added a higher concentration, 100 µg/mL, to determine if the delivered dose deviated even further from the targeted dose with increasing concentration (Figure 3B).

The highest measured concentrations' means differed significantly from the targeted, or theoretical dose of Ni (Table 2). This trend indicates that at concentrations of 10 µg/mL and above, there is a rapid loss of material in solution due to sedimentation.

We also attempted to measure the concentration of TiO₂ in the same range (0.01, 0.1, 0.5, 1.0, 10 µg/mL) with ICP-MS. Despite multiple attempts utilizing various digestion techniques, technical limitations prohibited us from analyzing TiO₂ with ICP-MS.

3.2 Toxicological Analysis

Although the aforementioned physicochemical properties are important in consideration of the exact exposure conditions, the environment within the human respiratory tract may

produce equally, if not greater, potential for variation. Therefore, we tested whether and how changes in physicochemical properties affect biological and toxicological responses in relevant cell culture models.

3.2.1 ELISA

An ELISA quality control run was designed to test for the potential interference of nanoparticles with the ELISA assay. All three dispersion media supplemented with 10 µg/mL NiO nanoparticles produced a significantly lower concentration of IL-8 when compared to the assay diluent for the highest concentrations of IL-8 (Figure 4, see Supplementary Table A.1 for p-values). The implications that arise from these data suggest that a significant under prediction of IL-8 protein levels will likely occur in solutions that contain NiO nanoparticles.

3.2.2 RT-PCR

Since the NiO nanoparticles interfered with protein detection in the ELISA assay, mRNA levels were measured to detect biomarkers of exposure to nanoparticles. To see how physicochemical properties of dispersion media affect biological endpoints, we conducted a dose-response and time course study of A549 cells stimulated with NiO and TiO₂. Initially we focused our analyses on the expression of hemoxygenase 1 (HO-1), a marker of oxidative stress. Briefly, A549 cells were co-incubated with NiO nanoparticles for 4 and 24 hours.. In Figure 5, we see that the relative expression level of HO-1 varied at different time points and between dispersion media. There was high variability in HO-1 expression among the different NiO concentrations and different dispersion media. No significant HO-1 induction was seen in either of the three dispersion media for either 1 or 10 µg/ml NiO at 4 hours. At 24 hours, an increase was observed for 10g/ml NiO using the F12K+F68 dispersion media, which however did not reach the level of statistical significance at $p < 0.05$.

To determine whether physicochemical forces affected intra-sample variability of the relative expression level of HO-1, we reduced the media height from 1-mL to 300- μ L, since at lower volumes, more particles would be in contact with the cells, delivered more rapidly, and a greater fraction would potentially interact with the monolayer rather than stay suspended in dispersion media (Hinderliter et al. 2010). Table 3 shows that less variability occurs in the measurement of HO-1 at 300- μ L media height, indicating that media height is a physicochemical property that affects biological endpoints.

Figure 6 illustrates that induction of HO-1 expression in response to stimulation with NiO particles is both dispersion medium and cell line dependent. A549 cells show a dose response to F-12K + F68, with the highest concentration 10- μ g/mL, showing statistical significance over the control. There was no response in A549 cells to the F-12K + FBS dispersion medium. Conversely, 16hbe14o- cells responded to MEM + FBS with the highest concentration showing statistical significance over the control. There was no statistically significant response in 16hbe14o- cells stimulated with NiO particles in MEM + F68 dispersion medium.

Assuming that the concentrations delivered were equivalent based on mass concentration, one would conclude from Figure 6 that each cell line responds to different dispersion media and that the "responsive" dispersion media for the respective cell lines was the best exposure delivery method with which to evaluate nanotoxicity. Additionally, one would conclude that both cell lines are not responsive to concentrations of nanoparticles below 10 μ g/mL. However, when the physicochemical property of particle size is stacked with the toxicity response, one sees that the two cell lines that have statistically significant HO-1 induction (as indicated by the *) are actually responding to different sized agglomerated particles, even though the concentration delivered is equivalent (Figure 7). More specifically, although neither cell line responds to a

particle size in the nanoparticle range ≤ 100 nm (as indicated by the #), the 16hbe14o- cells have a statistically significant response at an average particle size of 163.3 nm (concentration: 10 μ g/mL, dispersion medium: MEM + FBS) compared to the A549 cells, which elicit a statistically significant response at an average particle size of 636.2 nm (concentration: 10 μ g/mL, dispersion medium: F-12K + F68).

Discussion

Three dispersion media were compared for their ability to control agglomeration in NiO and TiO₂ nanoparticles and to investigate how and if they produced different effects on physicochemical and toxicological endpoints.

Dynamic light scattering experiments confirm that each dispersion medium varied in its ability to control particle size, irrespective of nanoparticle type (NiO or TiO₂). Dispersion media containing FBS outperformed those with surfactants F68 and DPPC based on maximum average particle size criteria (≤ 100 nm). In general, all dispersion media observed the trend that particle size increases with increasing concentration. Furthermore, there was high intra-sample variability and polydispersity within the particle size measurements. These findings suggest that dosimetry is a unique characteristic of both the interaction between nanoparticles and dispersion media, rather than an inherent property of discrete components to be evaluated separately.

Originally employed purely as a quality control tool, ICP-MS was used to validate whether or not the measured concentration of nanoparticles approximated the theoretical dose, based on mass concentration. For concentrations of nanoparticles in the range < 10 μ g/mL, the measured concentration of NiO nanoparticles accurately represented the theoretical dose. However, we noticed that at the 10 μ g/mL dose, the concentration dropped. Due to the concern about polydispersity, large particle size, and high intra-sample variability observed in the highest

concentrations measured in dynamic light scattering, we wondered whether or not this was an indication of settling. Repeating the same experiment with the addition of a higher dose, 100 $\mu\text{g/mL}$, resulted in a continuation of the trend that at high concentrations, $>10 \mu\text{g/mL}$, the measured concentration deviated from the theoretical mass concentration. These high concentrations underpredicted the theoretical dose, which was an indication of gravitational sedimentation transport processes. The lower concentrations, $<10 \mu\text{g/mL}$, accurately predicted the dose, and as such, reflect different solution transport processes. Overall, ICP-MS proved to be an invaluable tool to understand the relationship between actual vs. theoretical dosimetry of mass-based exposure metrics. Additionally, ICP-MS measurements confirmed the dynamic light scattering measurements in that they showed a mixing of transport processes that become more pronounced with increasing concentration. At this juncture, we had exhausted the analytical techniques that could characterize the dispersion media's physicochemical properties. The results of the physicochemical measurements indicated that each dispersion medium would provide a different exposure since the dispersion medium and nanoparticle transport processes are mixed in this concentration range, thereby providing a non-uniform exposure, particle size polydispersity, and high intra-sample variability.

If we are to assume that the physicochemical properties and solution dynamics are an inherent property of the dose itself, we assume that a constant exposure metric based on mass concentration occurs between each media and their respective nanoparticle transport processes during co-incubation with cells. Whether or not this is the case, was the subject of the subsequent toxicological analysis that sought to determine how and if physicochemical properties affected biological endpoints.

Since the physicochemical analysis revealed that the exposure conditions were dynamic and that we had no absolute indication of exposure conditions, we employed ELISA and RT-PCR to study consequent biological effects. The effects of dispersion media and nanoparticles on the ELISA assay found that all three dispersion media produced significantly lower concentrations of IL-8 compared to the control (assay diluent). The implications of these data are that a significant underprediction in IL-8 expression will be observed in samples that contain nanoparticles. Even more significant than this interference is that it occurs in concentrations lower than 10 $\mu\text{g/mL}$ (due to the serial dilutions) and it should be noted that most nanotoxicological research is carried out at higher concentrations. Thus, the data presented in Figure 4 indicate potential obstacles to assess accurate mediator production in cell culture supernatants and further reinforce the effects of nanoparticles on protein-binding (Tirado-Miranda et al. 2003).

Since nanoparticles significantly affected the ELISA assay, we next studied the effect of dispersion media on mRNA expression using RT-PCR. Since the isolation of mRNA involves a series of washes, chemical reactions, and centrifugations, we decided that these processes would sufficiently remove most of the nanoparticle contamination in the cell lysate.

We first investigated the effect of dispersion media on different time points. Most significantly, we found that expression levels varied between different dispersion media. High variability in HO-1 relative expression levels plagued the experiment; however, expression increased at 24 hours compared to 4 hours. In an effort to decrease the intra- and inter-sample variability, we decreased the dispersion media height in the well from 1-mL to 300- μL to increase contact and delivery to the monolayer. A decrement in the intra-sample variability was observed, as well as the continuation of the trend that different dispersion media give rise to

differences in biological endpoints. We can infer from the data that the NiO-induced differences in HO-1 expression were due to transport phenomena, which altered the exposure conditions. Transport processes change with increased proximity to the cell monolayer since a larger fraction of particles is taken up by both physical and cellular processes. The experiment provides insight into how physicochemical properties and transport processes will affect biological responses in cell culture models. We describe media height as a physiochemical property, because of the impact media height likely has on any resulting effects on particle size. At lower media height the greater fraction of mass is in closer proximity of the cells, thus reducing the distance particles need to settle onto the cell per unit time, which in turn is a function of the media type and nanoparticle type. All of these factors likely contribute to biological endpoints.

Since we are investigating whether or not transport processes are an inherent property of the dose itself, overlaying particle size data will help us understand how a physicochemical property affects the deposition (Figure 7). Neither cell line responds to a particle size in the nanoparticle range ≤ 100 nm, however, the 16hbe14o- cells have a statistically significant response at an average particle size of 163.3 nm (concentration: 10 μ g/mL, dispersion medium: MEM + FBS) compared to the A549 cells, which elicit a statistically significant response at an average particle size of 636.2 nm (concentration: 10 μ g/mL, dispersion medium: F-12K + F68). Both cell lines are responding to concentrations that have been implicated in polydisperse particle size and large agglomerates that deposit via sedimentation. Viewed in this manner, we can see that physicochemical transport phenomena in the medium and size are affecting the dosimetry, even though one cell line is more sensitive than the other.

When we consider just the 16hbe14o- cells' response across two different dispersion media (Figure 7b), the data presented seem to violate the relationship that normally exists

between increasing concentration and dose-response. A statistically significant dose response trend is observed with increasing concentration and increasing particle size for MEM + FBS. However, changing the composition of the dispersion medium to MEM + F68 shows no significant dose response. Since time, concentration, media height, and cell line were held constant, the difference in biological response at equivalent concentrations proves that physicochemical properties and transport process are interfering with nanoparticle deposition in the medium. This observation also holds for A549 cells (Figure 7a).

Looking back at the results from the ICP-MS measurements (Figure 3), the concentrations $<10 \mu\text{g/mL}$ are those that do not have sedimentation as a major transport phenomena that dominates dosimetry onto the cell monolayer. So it is very interesting that the cells do not respond to these concentrations. This suggests that the nanoparticles are so well-suspended that they are not leaving the dispersion medium to deposit onto the cells.

In our experimental protocol we attempted to minimize time between the preparation of the solutions and addition to the cell culture conditions. We believe that the time chosen to conduct the tests for characterization and exposure are critical when conditions are implemented which result in continued agglomeration and settling. The DLS measurements were taken at the same time the suspensions were added to the cell culture system, thus representing what was indeed added to the *in vitro* system. Our interpretations of the results shown in Figure 7 are based on data of the particle suspensions at the time of addition. Our data also indicate that some experimental choices will result in dynamic conditions, which make any DLS sizing measurements unreliable and not representative of the exposure conditions during prolonged exposure period. In addition, we cannot rule out that additional agglomeration occurred during

the exposure period, affecting biological responses, further adding to the complexity of the dynamic nature of nanoparticle toxicity..

Conclusions

This paper seeks to demonstrate how nanoparticle dosimetry applied during *in vitro* resuspension studies is a function of both the dispersion medium and the exposure conditions (Figure 8). Highly complex transport phenomena of nanoparticles in dispersion media affect the expression of biological endpoints.

Comprehensive understanding of the transport phenomena and cellular uptake are intricate and a function of dose. At low concentrations ($\leq 10 \mu\text{g/mL}$), it is recommended that *in vitro* resuspension studies should be avoided. Careful selection of exposure conditions at higher concentrations ($> 10 \mu\text{g/mL}$) during resuspension studies should include consideration of how dispersion medium, cell line, particle concentration, size, and deposition patterns will affect biological and physicochemical endpoints. Furthermore, comparison between nanotoxicology resuspension studies for risk assessment and generalization should take heed of how different exposure conditions manipulate biological and physicochemical endpoints. Since uniform deposition in terms of size, concentration, and time are integral to the reproducibility of experiments and assurance of more relevance to human exposures, air-liquid interface methods using aerosolized nanoparticle suspension may present a viable alternative and should be employed to avoid the complications of the transport phenomena that occur between the cell culture medium and the cell monolayer.

Acknowledgments

Thank you to Jose Zavala, Wenli Zhang, Victoria Madden, and Ya-Ru Li for technical support and help with experiments.

References

- Bakand, S., Winder, C., Khalil, C. & Hayes, A. 2005, "Toxicity Assessment of Industrial Chemicals and Airborne Contaminants: Transition from In Vivo to In Vitro Test Methods: A Review", *Inhalation toxicology*, vol. 17, no. 13, pp. 775-787.
- Bauer, R.N., Brighton, L.E., Mueller, L., Xiang, Z., Rager, J.E., Fry, R.C., Peden, D.B. & Jaspers, I. 2012, "Influenza enhances caspase-1 in bronchial epithelial cells from asthmatic volunteers and is associated with pathogenesis", *Journal of Allergy and Clinical Immunology*, vol. 130, no. 4, pp. 958-967.e14.
- Bird, R.B., Stewart, W.E. & Lightfoot, E.N. 1960, *Transport Phenomena*, .
- Cozens, A.L., Yezzi, M.J., Kunzelmann, K., Ohnishi, T., Chin, L., Eng, K., Finkbeiner, W.E., Widdicombe, J.H. & Gruenert, D.C. 1994, "CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells.", *American Journal of Respiratory Cell and Molecular Biology*, vol. 10, no. 1, pp. 38-47.
- Dahm, M. M.; Evans, D. E.; Schubauer-Berigan, M. K.; Birch, M. E.; Fernback, J. E. 2012, "Occupational exposure assessment in carbon nanotube and nanofiber primary and secondary manufacturers." *Ann Occup Hyg*, vol. 56, no. 5, pp. 542-56.

- Hinderliter, P., Minard, K., Orr, G., Chrisler, W., Thrall, B., Pounds, J. & Teeguarden, J. 2010, "ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies", *Particle and Fibre Toxicology*, vol. 7, no. 1, pp. 36.
- Jaspers, I., Zhang, W., Fraser, A., Samet, J.M. & Reed, W. 2001, "Hydrogen peroxide has opposing effects on IKK activity and IkappaBalpha breakdown in airway epithelial cells", *American journal of respiratory cell and molecular biology*, vol. 24, no. 6, pp. 769-777.
- Karlsson, H.L., Gustafsson, J., Cronholm, P. & Möller, L. 2009a, "Size-dependent toxicity of metal oxide particles—A comparison between nano- and micrometer size", *Toxicology letters*, vol. 188, no. 2, pp. 112-118.
- Karlsson, H.L., Gustafsson, J., Cronholm, P. & Möller, L. 2009b, "Size-dependent toxicity of metal oxide particles—A comparison between nano- and micrometer size", *Toxicology letters*, vol. 188, no. 2, pp. 112-118.
- Lee, J. & Donahue, N.M. 2011, "Secondary Organic Aerosol Coating of Synthetic Metalâ€“Oxide Nanoparticles", *Environmental science & technology*, vol. 45, no. 11, pp. 4689-4695.
- Leikauf, G.D., McDowell, S.A., Wesselkamper, S.C., Miller, C.R., Hardie, W.D., Gammon, K., Biswas, P.P., Korfhagen, T.R., Bachurski, C.J., Wiest, J.S., Willeke, K., Bingham, E., Leikauf, J.E., Aronow, B.J. & Prows, D.R. 2001, "Pathogenic mechanisms for particulate matter induction of acute lung injury and inflammation in mice.", *Research Report-Health Effects Institute*, , no. 105, pp. 5.
- Limbach, L.K., Li, Y., Grass, R.N., Brunner, T.J., Hintermann, M.A., Muller, M., Gunther, D. & Stark, W.J. 2005, "Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations", *Environ Sci Technol*, vol. 39, pp. 9370-9376.
- Oberdorster, G., Maynard, A., Donaldson, K., Castranova, V., Fitzpatrick, J., Ausman, K., Carter, J., Karn, B., Kreyling, W., Lai, D., Olin, S., Monteiro-Riviere, N., Warheit, D.,

- Yang, H. & A report from the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening, Working Group 2005, "Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy", *Particle and Fibre Toxicology*, vol. 2, no. 1, pp. 8.
- Porter, D., Sriram, K., Wolfarth, M., Jefferson, A., Schwegler-Berry, D., Andrew, M. & Castranova, V. 2008, "A biocompatible medium for nanoparticle dispersion", *Nanotoxicology*, vol. 2, no. 3, pp. 144-154.
- Ramirez-Garcia, S., Chen, L., Morris, M.A. & Dawson, K.A. 2011, "A new methodology for studying nanoparticle interactions in biological systems: Dispersing titania in biocompatible media using chemical stabilisers", *Nanoscale*, vol. 3, no. 11, pp. 4617-4624.
- Sager, T.M., Porter, D.W., Robinson, V.A., Lindsley, W.G., Schwegler-Berry, D. & Castranova, V. 2007, "Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity", *Nanotoxicology*, vol. 1, no. 2, pp. 118-129.
- Sayes, C.M., Wahi, R., Kurian, P.A., Liu, Y., West, J.L., Ausman, K.D., Warheit, D.B. & Colvin, V.L. 2006, "Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells", *Toxicol Sci*, vol. 92, pp. 174-185.
- Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D. & Pounds, J.G. 2007, "Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments", *Toxicological Sciences*, vol. 95, pp. 300-312.
- Tirado-Miranda, M., Schmitt, A., Callejas-Fernandez, J. & Fernandez-Barbero, A. 2003, "The aggregation behavior of protein-coated particles: a light scattering study", *European Biophysics Journal With Biophysics Letters*, vol. 32, no. 2, pp. 128.
- USEPA 2005, "External Review Draft Nanotechnology White Paper", , pp. 1-134.
- Walker, N.J. & Bucher, J.R. 2009, "A 21st century paradigm for evaluating the health hazards of nanoscale materials?", *Toxicol Sci*, vol. 110, pp. 251-254.

- Wang, L., Castranova, V., Mishra, A., Chen, B., Mercer, R., Schwegler-Berry, D. & Rojanasakul, Y. 2010, "Dispersion of single-walled carbon nanotubes by a natural lung surfactant for pulmonary in vitro and in vivo toxicity studies", *Particle and Fibre Toxicology*, vol. 7, no. 1, pp. 31.
- Warheit, D.B. 2004, "Nanoparticles: Health impacts?", *Materials Today*, vol. 7, pp. 32-35.
- Warheit, D.B., Sayes, C.M. & Reed, K.L. 2009, "Nanoscale and fine zinc oxide particles: can in vitro assays accurately forecast lung hazards following inhalation exposures?", *Environ Sci Technol*, vol. 43, pp. 7939-7945.
- Warheit, D.B. 2010, "Debunking Some Misconceptions about Nanotoxicology", *Nano Letters*, vol. 10, no. 12, pp. 4777-4782.
- Warheit, D.B., Borm, P.J.A., Hennes, C. & Lademann, J. 2007, "Testing Strategies to Establish the Safety of Nanomaterials: Conclusions of an ECETOC Workshop", *Inhalation toxicology*, vol. 19, no. 8, pp. 631-643.
- Westmoreland, C., Walker, T., Matthews, J. & Murdock, J. 1999, "Preliminary Investigations into the use of a Human Bronchial Cell Line (16HBE14o-) to Screen for Respiratory Toxins In Vitro", *Toxicology in Vitro*, vol. 13, no. 4-5, pp. 761-764.
- Whatmore, R.W. August 2006, "Nanotechnology—what is it? Should we be worried?", *Occupational Medicine*, vol. 56, no. 5, pp. 295-299.

Tables with Captions

Table 1: ζ -potential measurements for TiO_2 in three dispersion media.

TiO_2 (0, 0.01, 0.1, 0.5, 1.0, 10 $\mu\text{g/mL}$)	
Media	ζ -potential (mV)
F-12K + FBS	-6.60, -11.97, -11.73, -12.07, -11.39, -11.73
BSSM	-0.32, -0.22, -0.47, -0.56, -0.85, -1.13
F-12K + F68	-1.18, -3.43, -3.28, -6.72, -7.16, -10.07

Table 2: Measured Concentration of Ni vs. Theoretical Concentration of Ni descriptive statistics. As the theoretical concentration increases above 1 $\mu\text{g/mL}$, the measured concentration begins to deviate significantly from the targeted concentration.

	Measured Concentration Ni (ppm)		
Theoretical Concentration, Ni (ppm)	Mean	Confidence Interval	p-value
1	1.0179	(0.975, 1.061)	0.458888
10	8.3765	(7.800, 8.957)	0.005398
100	68.5711	(61.486, 75.657)	0.001107

Table 3: Effect of Media Height. Relative Expression Level of Two Endpoints in A549 cells in Different Media.

		1-mL		300- μ L	
		$\bar{x}$	σ	$\bar{x}$	σ
F-12K + F68, HO-1	0	2.57	1.37	1.72	0.66
	0.1	6.18	1.72	2.14	0.41
	1.0	6.58	4.72	2.37	0.46
	10	9.42	2.21	2.83	0.34

Figure Captions

Figure 1: Dynamic light scattering data for NiO in A) A549 and B) 16hbe14o- dispersion media. Error bars represent the mean $\pm$ one standard deviation. (*) denotes average particle size that meets criteria of $\leq 100\text{nm}$.

Figure 2: Dynamic light scattering data for TiO₂ in A549 dispersants. Error bars represent the mean $\pm$ one standard deviation.

Figure 3: Measured Concentration of Ni vs. Theoretical Concentration of Ni. A) 0.001 – 1 $\mu\text{g/mL}$; B) 1.0 – 100 $\mu\text{g/mL}$. Error bars represent the mean $\pm$ one standard deviation. (*) denotes concentrations that deviate significantly from the theoretical concentration.

Figure 4: ELISA quality control run. IL-8 standards were prepared in assay diluent (control) and three dispersion media with nanoparticles. The (*) above the points denotes a significant difference between the measured concentration of IL-8 in assay diluent from the measured concentration in all three dispersion media.

Figure 5: Effect of Time Point and Concentration on the Relative Expression Level of HO-1 mRNA. Stimulation of A549 cells with NiO particles suspended in different Media for 4 and 24 hours at 1-mL of media height. Error bars represent the mean $\pm$ one standard deviation.

Figure 6: Effect of Cell Line and dispersion media on the Relative Expression Level of HO-1 at 24 hours. Error bars represent the mean $\pm$ one standard deviation. (*) denotes statistical significance at the $\alpha=0.05$ level.

Figure 7: Effect of Cell Line on the Relative Expression Level of HO-1 (solid bars), overlaid with Particle Size in the respective dispersion medium (hashed bars). Error bars represent the mean $\pm$ one standard deviation. (*) denotes statistical significance at the $\alpha=0.05$ level. (#) denotes particle size $\leq 100\text{ nm}$.

Figure 8: Proposed schematic of the intricate dynamics of physicochemical properties, dispersion media, and cells on the uptake and dosimetry of NiO and TiO₂ nanoparticles.

Figure 1a revised

Figure 1b revised

Figure 2 revised

Figure3a

Figure3b

Figure4

Figure 5 revised

Figure 6 revised

Figure7

Figure8