Abstract

Erika Rasche Gutierrez
A Comparison of Three Dispersion Media on the Physicochemical and Toxicological Behavior of TiO₂ and NiO Nanoparticles
(Under the direction of Ken Sexton, Rich Kamens, and Ilona Jaspers)

Nanomaterials represent a burgeoning field of technological innovation. With the onset of environmental release associated with nanomaterial manufacture and proliferation, the concomitant effects on human health remain unknown and polemic. Agglomeration of nanomaterials in biologically relevant media further complicates dosing in toxicological study. Objective: To compare the effects of dispersion techniques on the physicochemical and toxicological dosimetry of TiO₂ (<50 nm) and NiO (<20 nm) nanoparticles. Methods: Three media were prepared for A549 and 16hbe14o cells with varying concentrations of -TiO2 and NiO nanoparticles. Physicochemical and toxicological effects were analyzed with dynamic light scattering, ICP-MS, SEM, TEM, ELISA, and stimulation of A549 cells with nanoparticles for 4 and 24 hours followed by analysis of inflammatory and oxidative stress markers with RT-PCR. Dispersion media physicochemical properties affect toxicological endpoints. In vitro nanotoxicology models that use re-suspension methods of exposure yield inconsistent biological results due to physicochemical transport processes.
Acknowledgments

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

ABSTRACT .................................................................................................................. II

ACKNOWLEDGMENTS ............................................................................................... III

TABLE OF CONTENTS ............................................................................................... IV

LIST OF TABLES .......................................................................................................... V

LIST OF FIGURES ........................................................................................................ VI

LIST OF ABBREVIATIONS .......................................................................................... VII

INTRODUCTION ............................................................................................................ 1

MATERIALS AND METHODS ...................................................................................... 4

NANOPARTICLES .......................................................................................................... 4

DISPERSION MEDIA COMPOSITION ........................................................................... 4

PREPARATION OF DISPERSION MEDIA WITH NANOPARTICLES ....................... 5

PHYSICOCHEMICAL ANALYSIS .................................................................................. 5

  Dynamic Light Scattering (DLS) and ζ-Potential ...................................................... 5
  Scanning Electron Microscopy (SEM) ...................................................................... 5
  Transmission Electron Microscopy (TEM) ................................................................. 6
  Inductively Coupled Plasma Mass Spectrometry (ICP-MS) ..................................... 6

TOXICOLOGICAL ANALYSIS .................................................................................... 7

  In vitro co-incubation with nanoparticles and collection ........................................ 7
  Determination of Dispersant and Nanoparticle Interference on ELISA .................... 7
  RT-qPCR ................................................................................................................... 7

STATISTICAL ANALYSIS .......................................................................................... 8

RESULTS ....................................................................................................................... 8

PHYSICOCHEMICAL ANALYSIS ................................................................................ 8

  Dynamic Light Scattering: NiO ................................................................................ 8
  Dynamic Light Scattering: TiO2 .............................................................................. 10
  ζ-potential ................................................................................................................ 11
  ICP-MS ..................................................................................................................... 12
  SEM ........................................................................................................................... 15
  TEM ........................................................................................................................... 16

TOXICOLOGICAL ANALYSIS .................................................................................... 17

  ELISA ........................................................................................................................ 17
  RT-PCR ..................................................................................................................... 19

DISCUSSION ............................................................................................................... 26

APPENDIX ................................................................................................................... 33
List of Tables

Table 1: 2-potential measurements for TiO$_2$ in three dispersion media......................................................... 11

Table 2: Measured concentration of Ni vs. theoretical concentration of Ni descriptive statistics. As the theoretical concentration increases above 1 mg/mL, the measured concentration begins to deviate significantly from the targeted concentration. ........................................................................................................ 14

Table 3: Effect of media height. Relative expression level of two endpoints in A549 cells in different media........................................................................................................ 21
List of Figures

FIGURE 1: DYNAMIC LIGHT SCATTERING DATA FOR NiO IN A549 DISPERSION MEDIA. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. .......................................................... 9

FIGURE 2: DYNAMIC LIGHT SCATTERING DATA FOR NiO IN 16HBE140- DISPERSION MEDIA. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. ......................................................... 10

FIGURE 3: DYNAMIC LIGHT SCATTERING DATA FOR TiO₂ IN A549 DISPERSANTS. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. .......................................................... 11

FIGURE 4: MEASURED CONCENTRATION OF Ni VS. THEORETICAL CONCENTRATION OF Ni. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. .......................................................... 13

FIGURE 5: MEASURED CONCENTRATION OF Ni VS. THEORETICAL CONCENTRATION OF Ni. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. .......................................................... 14

FIGURE 6: 1 mg/mL NiO IN BSSM. Magnification: 50000X .......................................................... 16

FIGURE 7: BSSM. Magnification: 50000X .......................................................... 16

FIGURE 8: 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. .......................................................... 18

FIGURE 9: EFFECT OF TIME POINT ON THE RELATIVE EXPRESSION LEVEL OF HO-1 mRNA. CO-INOCULATION IN DIFFERENT MEDIA FOR 4 AND 24 HOURS AT 1·mL OF MEDIA HEIGHT. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. .......................................................... 20

FIGURE 10: EFFECT OF CELL LINE ON THE RELATIVE EXPRESSION LEVEL OF HO-1. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. (*) DENOTES STATISTICAL SIGNIFICANCE AT THE ALPHA=0.05 LEVEL. .......................................................... 22

FIGURE 11: EFFECT OF CELL LINE ON THE RELATIVE EXPRESSION LEVEL OF HO-1 AND RESPONSE TO PARTICLE SIZE. ERROR BARS REPRESENT THE MEAN ± ONE STANDARD DEVIATION. (*) DENOTES STATISTICAL SIGNIFICANCE AT THE ALPHA=0.05 LEVEL (#) DENOTES PARTICLE SIZE ≤100 NM .......................................................... 24

FIGURE 12: THE INTRICATE DYNAMICS OF PHYSICOCHEMICAL PROPERTIES, DISPERSION MEDIA, AND CELLS ON THE UPTAKE AND DOSIMETRY OF NiO AND TiO₂ NANOPARTICLES .......................................................... 31
List of Abbreviations

16hbe14o: a bronchial transformed cell line that was derived from normal human bronchial epithelium

A549: adenocarcinomic human alveolar basal epithelial cells

BSSM: F-12K cell culture medium/0.01% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, semisynthetic (DPPC)/0.6 mg/mL BSA/1% P/S

F-12K: A cell culture medium used to culture human cells and a modification of Ham’s F-12K nutrient mixture.

FBS: Fetal Bovine Serum

F-12K + FBS: “F12K + FBS” is composed of F-12K cell culture medium/10% FBS/1% P/S

F-12K + F68: F-12K cell culture medium/0.1% Pluronic F68 nonionic surfactant/1% P/S

HO-1: hemeoxygenase-1

IL-8: interleukin-8

MEM: A cell culture medium used to culture human cells; Modified Eagle’s Medium.

MEM + FBS: MEM cell culture medium/10% FBS/1% L-glutamine/1% P/S

MEM + F68: MEM cell culture medium/0.1% F68 nonionic surfactant/1% L-glutamine/1% P/S

P/S: Penicillin/Streptomycin

RT-qPCR: real-time quantitative polymerase chain reaction
Introduction

Nanomaterials represent a burgeoning field of technological innovation. With the onset of environmental release associated with nanomaterial manufacture and proliferation, the concomitant effects on human health remain unknown and polemic (Karlsson et al. 2009a, Whatmore August 2006, Warheit et al. 2007) . Of particularly expedient concern is the effect on respiratory health, since the health effects on occupational workers during their manufacture and remediation remain unquantified, as well as the risk 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 an in vitro toxicological model combined with a resuspension technique (Bakand et al. 2005) . Nanoparticle resuspension techniques involve the direct addition of nanoparticles to the 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) . 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_{ae} [agglomerate diameter] >100nm), sedimentation processes dominate. In the nanomaterial range (10-100nm), a combination of both sedimentation and diffusion processes occur. At particle sizes d_p<10nm, 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. 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 and exposure of the nanoparticles to the cell monolayer (Warheit 2010). Furthermore, 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 and cell culture media (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 will be 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). NiO and 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).
Materials and Methods

Nanoparticles

Two types of nanoparticles were used in this study, titanium (IV) dioxide (TiO$_2$) and nickel (II) oxide (NiO), were obtained from Sigma-Aldrich (St. Louis, MO). The reported mean diameters were TiO$_2$, <50 nm and surface area 35-65 m$^2$/g, and NiO, <20 nm.

Dispersion Media Composition

Three media were prepared for A549 cells: “F12K + FBS” is composed of F-12K cell culture medium/10% FBS/1% P/S, “F12K + F68 Media” is F-12K cell culture medium/0.1% Pluronic F68 nonionic surfactant/ 1% P/S, 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% P/S. 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 16hbe140 cells: "MEM + FBS" is MEM cell culture medium/10% FBS/1% L-glutamine/1% P/S and "MEM +F68" is MEM cell culture medium/0.1% F68 nonionic surfactant/1% L-glutamine/1% P/S.

Fetal Bovine Serum (FBS) was bought from Atlanta Biologicals (Norcross, GA). F-12K 1x (Ham's F-12K Nutrient Mixture, Kaighn's Modification with L-glutamine) was bought from Mediatech, Inc. (Manassas, Va). Bovine serum albumin and penicillin/streptomycin solution were bought from Invitrogen (Carlsbad, CA). DPPC and
10% Pluronic F-68 Solution were bought from Sigma Aldrich (St. Louis, MO). MEM was bought from Invitrogen (Carlsbad, CA).

Each dispersant contained varying concentrations (0, 0.01, 0.1, 0.5, 10, 100 μg/mL) of TiO₂ or NiO nanoparticles.

**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 experimentation took place within 24 hours. All solutions were re-vortexed if more than 20 minutes elapsed since the initial sonication and/or vortexing. After 24 hours, solutions were discarded.

**Physicochemical Analysis**

**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 μg/mL and unimodal for 0.01, 0.1, and 0.5 μg/mL.

**Scanning Electron Microscopy (SEM)**

Two techniques were used to view nanoparticles in the dispersion media. The first technique involved dropping 25μL of dispersion medium with nanoparticles onto a slide
and leaving it to dry in a vacuum. The second technique involved pipetting 50\(\mu\)L of dispersion medium with nanoparticles onto a poly-l-lysine coated 12mm coverslip. The solution sat for 60 minutes in a humid chamber. Next, 40\(\mu\)L of 2.5% glutaraldehyde in 0.15M sodium phosphate buffer at pH 7.4 was added. The slide and solutions were left to incubate for 60 minutes. After the elapsed time, the remaining unevaporated solution was drained onto filter paper. A large drop of 0.2 \(\mu\)m filtered, deionized water was dropped on the slide and then drained onto filter paper, and then repeated once more. The slide was then left to air dry. The images were photographed on a Zeiss Supra 25 FESEM (Carl Zeiss Microscopy GmbH, Jena, Germany).

**Transmission Electron Microscopy (TEM)**

Three techniques were used to view nanoparticles in the dispersion media. The first technique used samples of nanoparticles in dispersion media. The samples were viewed with TEM by first pipetting 2\(\mu\)L of sample with a subsequent 2\(\mu\)L aliquot of uranyl acetate to a charged grid. The grid was dried by wicking with filter paper. Next, 2\(\mu\)L of sodium phosphotungstate (pH 7) were added and then dried by wicking with filter paper. The second technique involved viewing the nanoparticles dispersed in deionized water. 2\(\mu\)L of this sample was dropped onto a grid and allowed to air dry. The third technique involved viewing the nanoparticles dispersed in 0.1M HNO3. All images were photographed on a Zeiss EM 910 TEM (Carl Zeiss Microscopy GmbH, Jena, Germany).

**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-MΩ water.

Toxicological Analysis

*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 cultured in our
laboratory. For the co-incubation exposures, plating density was 200,000 cells/well in a
standard 24-well plate. NiO in each dispersion medium was co-incubated with 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 300μL or 1mL. Supernatant and the lysed cell monolayer in
TRizol (Carlsbad, CA) were collected for analysis.

**Determination of Dispersant and Nanoparticle Interference on ELISA**

A quality control run was conducted to test the effect of nanoparticles on the
response of 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 + 10μg/mL NiO, BSSM + 10μg/mL NiO, and F-
12K + F68 + 10μg/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).

**RT-qPCR**

The mRNA gene expression levels of IL-8, HO-1, and COX-2 mRNA were
determined using RT-qPCR with β-actin RNA as the endogenous control. Total RNA
was isolated from frozen cell lysate in TRizol according to the manufacturer's protocol.
First strand cDNA synthesis and real-time RT-PCR was performed according to work described previously (Bauer et al. 2012, Jaspers et al. 2001). Primer and probe sequences were obtained from Applied Biosystems (Foster City, California). RT-qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Foster City, California). Data were analyzed according to the 2^\text{-}\Delta\text{ΔCt} method.

**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 ± standard deviation and a p-value < 0.05 was considered to be significant.

**Results**

**Physicochemical Analysis**

**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 µg/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 μg/mL NiO and 10 μg/mL TiO₂ solutions post-sonication.

Both the 16hbe14o and the A549 cell line dispersion media varied widely in their ability to maintain particle size ≤100 nm and intra- and inter-sample variability was problematic (Figure 1).

![Dynamic Light Scattering: NiO in A549 Cell Culture Media](image)

**Figure 1**: Dynamic light scattering data for NiO in A549 dispersion media. Error bars represent the mean ± one standard deviation.

Amongst the A549 cell line dispersion media, F-12K + FBS maintained particle size below 100 nm the best, with the lower concentrations meeting the criteria until the highest concentrations of 1- and 10-μg/mL. 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 2). MEM + FBS controlled particle size within the desired range until the highest concentration, 10-μg/mL.

![Dynamic Light Scattering: NiO in 16hbe14o Cell Culture Media](image)

**Figure 2:** Dynamic light scattering data for NiO in 16hbe14o-dispersion media. Error bars represent the mean ± one standard deviation.

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. All dispersion media produced significantly different particle sizes at each concentration.

**Dynamic Light Scattering: TiO₂**

All concentrations of TiO₂ in the conventional A549 cell culture medium, F-12K + FBS, with the exception of 10 μg/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 3).
Dynamic Light Scattering:
TiO\textsubscript{2} in A549 Cell Culture Media

![Graph showing Z-Average (d/\text{nm}) vs. Concentration NiO (\mu g/mL) for BSSM, F-12K + F68, and F-12K + FBS.]

\textbf{Figure 3:} Dynamic light scattering data for TiO\textsubscript{2} in A549 dispersants. Error bars represent the mean ± one standard deviation.

\textbf{ζ-potential}

The ζ-potential of TiO\textsubscript{2} in A549 cell dispersion media was measured. ζ-potential is an analytical measurement that measures 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.

\textbf{Table 1:} ζ-potential measurements for TiO\textsubscript{2} in three dispersion media.

<table>
<thead>
<tr>
<th>TiO\textsubscript{2} (0, 0.01, 0.1, 0.5, 1.0, 10 \mu g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSSM</td>
</tr>
<tr>
<td>Media</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>F-12K + FBS</td>
</tr>
<tr>
<td>BSSM</td>
</tr>
<tr>
<td>F-12K + F68</td>
</tr>
</tbody>
</table>

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 in the ≤100 nm range, 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 4). A linear regression was fit to the data with an R² of 0.9961.
Figure 4: Measured Concentration of Ni vs. Theoretical Concentration of Ni. Error bars represent the mean ± one standard deviation.

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 5).
Figure 5: Measured Concentration of Ni vs. Theoretical Concentration of Ni. Error bars represent the mean ± one standard deviation.

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.

Table 2: Measured Concentration of Ni vs. Theoretical Concentration of Ni descriptive statistics. As the theoretical concentration increases above 1 μg/mL, the measured concentration begins to deviate significantly from the targeted concentration.

<table>
<thead>
<tr>
<th>Theoretical Concentration, Ni (ppm)</th>
<th>Measured Concentration Ni (ppm)</th>
<th>Mean</th>
<th>Confidence Interval</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1.017885667</td>
<td>(0.975, 1.061)</td>
<td>no</td>
</tr>
</tbody>
</table>
We also attempted to measure the concentration of TiO$_2$ in the same range (0.01, 0.1, 0.5, 1.0, 10 μg/mL) with ICP-MS. After multiple attempts utilizing various digestion techniques, we found that TiO$_2$ was too difficult to study within the confines of our study design and detection limit. TiO$_2$ continually clogged sample lines in the concentration range, even with the addition of digestion. Dilution was attempted, however, this compromised the sensitivity of the instrument for detecting our concentration range.

**SEM**

Since dynamic light scattering and ICP-MS measurements indicated that complex transport processes were occurring due to the physicochemical properties of the nanoparticles in dispersion media, a qualitative approach was sought out to confirm the particle sizes, agglomeration status, and uniformity of the dispersion media. Several methods were used to verify shape, particle size, and degree of agglomeration of nanoparticles in media with SEM. The first method, which dried the nanoparticle-dispersant solution in a vacuum, yielded poor results on SEM due to large particle size and the inability to distinguish nanoparticles from artifacts in the dispersion medium. The second method used fixatives and a series of washes to maintain the particles as true to original size as possible. This attempt also yielded no practical result because there were still a significant amount of artifacts present in addition to the inability to distinguish NiO nanoparticles from the components of the dispersion medium.
TEM

After SEM proved itself unusable, several methods were used to verify shape, particle size, and degree of agglomeration with TEM. The first method, which involved fixatives and wick-drying, was attempted in hopes of producing a minimally invasive technique in which to view nanoparticles in dispersion medium. However, the nanoparticles were indistinguishable from the medium (Figure 6 and Figure 7).

Figure 6: 1 μg/mL NiO in BSSM. Magnification: 50000X.

Figure 7: BSSM. Magnification: 50000X.

Since the desire was to view the nanoparticles in dispersion media in as close to the original state as possible, further attempts to distinguish the nanoparticles from the dispersion medium were not continued after these less invasive techniques failed.
Toxicological Analysis

At this point, we decided that we had exhausted all analytical techniques that could characterize the nanoparticles in dispersion media. We know from physicochemical measurements that the average diameter of the agglomerates is highly variable, produces a non-uniform size distribution, and the delivered dose deviates from the targeted dose at high concentrations. Furthermore, we cannot qualitatively verify the status of our dispersion with EM without first adulterating the samples. Since we know we are seeing a mixture of transport processes and solution dynamics, we cannot fully understand the exposure conditions to which our cells will be exposed.

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.

Currently, many in the fields of toxicology and exposure science justify their exposure conditions by using the argument that environmental and biological conditions are highly variable in- and of-themselves and that those conditions freely force agglomeration anyway. 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.

ELISA

An ELISA quality control run was designed to test for the potential interference of nanoparticles with the ELISA assay or the proteins in supernatant. All three dispersion media 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 8, see Appendix Table A.1 for p-values).

Figure 8: 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.

The implications that arise from this data suggest that a significant under prediction of IL-8 protein levels will occur in solutions that contain nanoparticles. During and after experimentation, the dispersion media, which becomes the supernatant once co-incubated with the cells, will still contain nanoparticles after it is collected for protein analysis. The concentration and size of the particles in this solution is usually not measured. Even more significant than this interference is that it occurs in concentrations lower than 10 μg/mL (due to the serial dilutions) and it should be noted that most nanotoxicological research is carried out at higher concentrations. The data presented in Figure 8 present obstacles to accurate protein quantitation and further reinforce the effects of nanoparticles on protein-binding (Tirado-Miranda et al. 2003) .
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. Although nanoparticles that were absorbed into or onto the cell monolayer were collected in the cell lysate, we assumed that the series of washes, chemical reactions, and centrifugation that occur during mRNA isolation purged the samples of nanoparticle contamination.

To see how physicochemical properties of dispersion media affect biological endpoints, we stimulated A549 cells at different time points because we did not know when the biomarkers would appear, the rate of response, or the route of toxicity, if there was any. We also did not know how the physicochemical properties of dispersion media, such as size, agglomeration rates and status, dispersant behavior (does it stick to the agglomerates as it did during the ELISA assay?), non-uniform deposition pattern, and delivered concentration would cross-react with the biological characteristics.

A549 cells were co-incubated with NiO nanoparticles for 4 and 24 hours. In Figure 9, 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 within individual dispersion media concentrations and between different dispersion media. All three dispersion media did not show a response at four hours. At 24 hours, a response was seen, but due to the high degree of variability and standard deviation within the measurements, there was no statistically significant difference between the concentrations within any individual dispersion media. However, dispersion media produced differences in HO-1 expression level.
Since 24 hours produced a response in F-12K + FBS and F-12K + F68 media, we decided to drop BSSM from further consideration. Additionally, BSSM produced large and inconsistent particles during the physicochemical analysis, which further warranted its discontinuation in the study (see Figure 1).

To see how the physicochemical forces affected intra-sample variability of the relative expression level of HO-1, I reduced the media height from 1-mL to 300-μL. (Hinderliter et al. 2010) state that at lower volumes, more particles would be in contact with the cells, delivered more rapidly, and a greater fraction would interact with the monolayer rather than stay suspended in dispersion media. According to these findings, lowering the media height will allow for increased contact between the nanoparticles and cells and increase the possibility of a more targeted exposure.
Table 3 shows that less variability occurs in the measurement of both IL-8 and HO-1 at 300-µL media height.

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

<table>
<thead>
<tr>
<th></th>
<th>1-mL</th>
<th></th>
<th>300-µL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>σ</td>
<td></td>
<td>σ</td>
</tr>
<tr>
<td>0</td>
<td>8.32</td>
<td>11.96</td>
<td></td>
<td>1.37</td>
</tr>
<tr>
<td>F-12K+</td>
<td>0.1</td>
<td>n=3</td>
<td>9.22</td>
<td>9.97</td>
</tr>
<tr>
<td>FBS, IL-8</td>
<td>1</td>
<td>18.13</td>
<td>15.17</td>
<td>n=3</td>
</tr>
<tr>
<td></td>
<td>3.95</td>
<td>2.61</td>
<td>1.57</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>2.57</td>
<td>1.37</td>
<td>1.72</td>
<td>0.66</td>
</tr>
<tr>
<td>F-12K+</td>
<td>0.1</td>
<td>n=4</td>
<td>6.18</td>
<td>1.72</td>
</tr>
<tr>
<td>F68, HO-1</td>
<td>1</td>
<td>6.58</td>
<td>4.72</td>
<td>n=4</td>
</tr>
<tr>
<td></td>
<td>9.42</td>
<td>2.21</td>
<td>2.83</td>
<td>0.34</td>
</tr>
</tbody>
</table>

From the data presented in Table 3, we can see how the media height affects the contact between nanoparticles and the cell monolayer. As the height of the media decreases, the standard deviation decreases, and thus, a more repeatable exposure deposition occurs. Since the impact of media height is a physicochemical property, we can conclude that physicochemical properties affect both the exposure and the measurement of biological endpoints.

As demonstrated by adjusting the media height, the assumption based on mass concentration equivalency between dispersion media and their transport processes does not produce a constant exposure metric. Clearly, transport processes change with
increased proximity to the cell monolayer since a larger fraction of particles are uptaken by both physical and cellular processes.

We assumed dose equivalency with media height and found that this assumption was false due to physicochemical interactions. Can we rule out that the physicochemical interactions are not a property of the A549 cell line? Studies have show that this cell line is not as sensitive and investigators have increasingly chosen to move away from using tumor-derived cell lines. 16hbe14o- is a bronchial transformed cell line that was derived from normal human bronchial epithelium. The cell line is prized for its ability to retain differentiated epithelial morphology and function (Westmoreland et al. 1999, Cozens et al. 1994).

Over the same range of concentrations and with two different surfactants, FBS and F68, we find that the two cell lines are responsive to different dispersion media (Figure 10).

![Co-incubation of NiO in Varied Media with Different Cell Lines for 24h: Relative Expression Level of HO-1](image)

**Figure 10: Effect of Cell Line on the Relative Expression Level of HO-1.** Error bars represent the mean ± one standard deviation. (*) denotes statistical significance at the alpha=0.05 level.
Figure 10 illustrates that the response to HO-1 is both dispersion medium and cell line dependent. Additionally, each cell line is responsive to different dispersion media, rather than a singular surfactant producing the same dispersion effect across cell lines. A549 cells show a dose response to F-12K + F68, with the highest concentration 10-μg/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 to 16hbe14o- cells in MEM + F68 dispersion medium.

Assuming that the concentrations delivered were equivalent based on mass concentration, one would conclude from Figure 10 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 μg/mL. However, when the physicochemical property of particle size is overlaid, one sees that the two cell lines that have statistically significant responses are actually responding to different sized particles, even though the concentration delivered is equivalent (Figure 11). Although neither cell line responds to a particle size in the nanoparticle range ≤100 nm, the 16hbe14o- cells have a statistically significant response at an average particle size of 163.3 nm (concentration: 10μg/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: 10μg/mL,
dispersion medium: F-12K + F68).

**Figure 11:** Effect of Cell Line on the Relative Expression Level of HO-1 and Response to Particle Size. Error bars represent the mean ± one standard deviation. (*) denotes statistical significance at the alpha=0.05 level. (#) denotes particle size ≤100 nm.

One would now conclude that not only are the different dispersion media producing different effects on biological endpoints (Figure 9 and Figure 10), but also that the A549 and 16hbe14o- cell lines are responsive to different sized particles and different exposure methods (Figure 11).

The larger implication of the data presented in Figure 11 comes from evaluating each cell line separately amongst its responses to different concentrations in varied dispersion media. When we consider just the 16hbe14o- cells' response across two different dispersion media (Figure 11b), 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, 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. This observation also holds for A549 cells (Figure 11a).
**Discussion**

Three dispersion media were intercompared for their ability to control agglomeration in NiO and TiO₂ nanoparticles. The effect of each dispersion medium on the dosimetry of the nanoparticles in solution was related to physicochemical and biological 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 μg/mL, the measured concentration of NiO nanoparticles accurately represented the theoretical dose. However, we noticed that at the 10 μg/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 μg/mL, resulted in a continuation of the trend that high concentrations, >10 μg/mL, deviated from the
theoretical mass concentration. These high concentrations underpredicted the theoretical
dose, which was an indication of the transport process of gravitational sedimentation. The
lower concentrations, <10 μg/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.

The results of the dynamic light scattering and ICP-MS experiments confirmed
that we were studying polydisperse and highly dynamic solutions. Since the variability in
these measurements indicated the presence of complex transport processes, we decided to
use EM to qualitatively confirm the solution status and expected dosimetry medium.
SEM and TEM were both attempted with several methodologic variations to view
particles in dispersion media. These attempts yielded no practical results due to the
presence of artifacts created by the media in the micrographs as well as the inability to
distinguish NiO nanoparticles from the components of the dispersion medium. Since the
desire was to view the nanoparticles in media in as close to their natural state as possible,
further attempts and variations were dismissed due to further alteration of the original
material.

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 and that 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.

Frequent counters to the target-tissue paradigm argue that the lung’s natural processes that freely force agglomeration will trump these physicochemical measurements and adjustments. If we are to assume that this is indeed the case, we assume that the physicochemical properties and transport phenomena remain constant with each mass-concentration based dose. Whether or not this is the case, was the subject of the subsequent toxicological analysis that sought to determine how or if physicochemical properties affected biological endpoints.

Since the physicochemical analysis revealed that the exposure conditions were dynamic and we had no absolute indication of exposure conditions, we employed ELISA and RT-PCR to study consequent effects.

The effect 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 implication of this data is that a significant underprediction in IL-8 expression will be observed in samples that contain nanoparticles. This would be routine during the ELISA process since cellular supernatant is used. Even more alarming is that the interference occurs at such low concentrations - the original dose is diluted serially - and that most nanotoxicological research evaluates higher concentrations of nanoparticles.

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 both IL-8 and 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 differences in the media height experiment were due to transport phenomena, which altered the exposure conditions. The larger implication of this particular experiment comes from the expansion from the model system to the actual human lung. The human respiratory tract is lined with a thin layer of fluid. Since the thickness of the fluid lining in the respiratory tract is variable upon location and internal conditions, the ability for nanoparticles to penetrate this layer and deposit in- or onto the epithelia is entirely dependent upon transport phenomena. The experiment just presented provides insight into how physicochemical properties and transport processes will affect exposure through this fluid lining.

Since the delivered dose changed with media height, we wondered whether or not this was a property of the A549 cell line. Although A549 cells, which are a type-II adenocarcinomic alveolar epithelial cell line, have been well studied and characterized for decades, many investigators and research labs are moving away from their use. We
decided to compare the response of the A549 cell line to the 16hbe14o- cell line, which is known for its ability to retain differentiated epithelial morphology and function.

We found that the two cell lines produce statistically significant responses to nanoparticles in different dispersion media. A549 cells were more sensitive to nanoparticles in F-12K + F68 and 16hbe14o- were more sensitive to nanoparticles in MEM + FBS. Most importantly, if only one dispersion media had been employed across the board, it would only have a response in one cell line. This finding implicates transport processes and cell line differences in the ability to detect a biological response to nanoparticles. Additionally, neither cell line produced statistically significant results at concentrations below 10 µg/mL vs. control. If we remember the results from the physicochemical measurements, the concentrations <10 µg/mL are those that do not have sedimentation as a major transport phenomena that dominates cellular dosimetry, 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.

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. 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: 10µg/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: 10µg/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 are affecting the dosimetry, even though one cell line is more sensitive than the other. Additionally, this particular experiment begs the question of whether one should first design an experiment for biological response or around a physicochemical property.

![Diagram](image)

**Figure 12:** The intricate dynamics of physicochemical properties, dispersion media, and cells on the uptake and dosimetry of NiO and TiO₂ nanoparticles.

This paper seeks to outline how nanoparticle dosimetry in *in vitro* resuspension studies is a function of the dispersion medium and the exposure conditions (Figure 12). 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 (≤10µg/mL), it is recommended
that \textit{in vitro} resuspension studies should be avoided. Since uniform deposition in terms of size, concentration, and time are integral to the reproducibility of experiments air-liquid interface methods should be employed to avoid the pitfalls of complex transport phenomena that occur between the cell culture medium and the cell monolayer.
Appendix

Table A.1: ELISA quality control run. IL-8 standards were prepared in assay diluent (control) and three dispersion media with 10 μg/mL NiO nanoparticles. F-values of each

<table>
<thead>
<tr>
<th>Theoretical Concentration of IL-8 in Assay Diluent (Control)</th>
<th>p-value</th>
<th>vs. F-12K</th>
<th>vs. BSSM</th>
<th>vs. F68</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>0.014</td>
<td>0.013</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.044</td>
<td>0.006</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.003</td>
<td>0.023</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.003</td>
<td>0.005</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0.045</td>
<td>0.148</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>6.25</td>
<td>0.076</td>
<td>0.047</td>
<td>0.052</td>
<td></td>
</tr>
<tr>
<td>3.125</td>
<td>0.001</td>
<td>0.002</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>
References


