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Title: Informing Selection of Nanomaterial Concentrations for ToxCast *In Vitro* Testing based on Occupational Exposure Potential

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DISCLAIMER: *The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.*
ABBREVIATIONS AND DEFINITIONS:
Ag: Silver
CNT: Carbon Nanotube
CPC: Condensation Particle Counter
ENM: Engineered Nanomaterial
FMPS: Fast Mobility Particle Sizer
GI: Gastrointestinal
GSD: Geometric Standard Deviation
HTS: High-throughput Screening
ICRP: International Commission on Radiological Protection
MPPD: Multi-Path Particle Dosimetry
MWCNT: Multi-walled Carbon Nanotube
NCCT: National Center for Computational Toxicology
NIOSH: National Institute for Occupational Safety and Health
NM: Nanomaterial
NP: Nanoparticle
OSHA : Occupational Safety and Health Administration
SMPS: Scanning Mobility Particle Sizer
SWCNT: Single-walled Carbon Nanotube
TiO_2: Titanium Dioxide
TB: Tracheobronchial
V_T : Tidal Volume

ABSTRACT

Background: Little justification is generally provided for selection of *in vitro* assay testing concentrations for engineered nanomaterials (ENMs). Selection of concentration levels for hazard evaluation based on real-world exposure scenarios is desirable.

Objectives: Our goal is to use estimates of lung deposition following occupational exposure to nanomaterials to recommend *in vitro* testing concentrations for the U.S. Environmental Protection Agency's ToxCast^{TM} program. We provide testing concentrations for carbon nanotubes (CNTs), titanium dioxide (TiO_2) and silver (Ag) nanoparticles.

Methods: We reviewed published ENM concentrations measured in air in manufacturing and R&D labs to identify input levels for estimating ENM mass retained in the human lung using the Multiple-Path Particle Dosimetry (MPPD) model. Model input parameters were individually varied to estimate alveolar mass retained for different particle sizes (5-1000 nm), aerosol concentrations (0.1, 1 mg/m^3), aspect ratios (2, 4, 10, 167), and exposure durations (24 hours and a working lifetime). The calculated lung surface concentrations were then converted to *in vitro* solution concentrations.

Results: Modeled alveolar mass retained after 24 hours is most affected by activity level and aerosol concentration. Alveolar retention for Ag and TiO_2 nanoparticles and CNTs for a *working lifetime* (45 years) exposure duration is similar to high-end concentrations (~ 30-400 μg/mL) typical of *in vitro* testing reported in the literature.

Conclusions: Analyses performed are generally applicable to provide ENM testing concentrations for *in vitro* hazard screening studies though further research is needed to improve the approach. Understanding the relationship between potential real-world exposures and *in vitro* test concentrations will facilitate interpretation of toxicological results.

INTRODUCTION

Researchers evaluating toxicity and human exposure potential of engineered nanomaterials (ENMs) are challenged by rapid development of novel materials for new applications as the nanotechnology industry drives forward. These materials can add significant value to industrial or consumer products. ENMs have one or more component with at least one dimension in the range of 1-1000 nm. Components can include nanoparticles (NPs), nanofibers and nanotubes, nanodots, nano-structured surfaces or nanocomposites. Carbon nanotubes (CNTs) and metal oxide nanoparticles (two material types having the highest industrial production volumes) are used in plastics, catalysts, battery and fuel cell electrodes, solar cells, paints, coatings, etc. (Klaine et al. 2008). Nanoparticulate silver (Ag) has the greatest number of consumer product applications. Novel nanomaterial (NMs) types continue to be synthesized based on the value they may add, often without evaluation of implications for human health, toxicity, environmental impact or long-term sustainability. Nanomaterials, especially the ones made of metals, semiconductors and various inorganic compounds, have the potential for post-use risks to humans and the environment (NNI 2008). There is a need to examine and address these concerns before the widespread adoption of nanotechnologies (Oberdorster et al. 2005).

The U.S. Environmental Protection Agency (EPA) is beginning to evaluate exposure and hazard potential of nanomaterials and prioritize them for further animal-based toxicological testing. Prioritization of nanomaterial classes and types for targeted testing is important in the early stages of NM development. Currently, only a small portion of the thousands of commonly used chemicals in the Toxic Substances Control Act (TSCA 1976) inventory (U.S. EPA 2004) have undergone animal testing due to the high cost (millions of dollars) and long timeframe (2-3 years) required per chemical (Judson et al. 2009). Of the unique chemicals (~10,000) that the EPA is most concerned

with, only a fraction have been evaluated for specific classes of toxicity (Judson et al. 2009). The EPA's ToxCast research program was started in 2007 and seeks to predict the potential toxicity of environmental chemicals based on *in vitro* bioactivity profiling at minimal cost as compared to full-scale animal testing (Dix et al. 2007). An initial set of ~300 chemicals (primarily pesticides) was tested in phase I of ToxCast in 467 high-throughput screening (HTS) biochemical and cell-based assays across nine technologies (Judson et al. 2010). A study has been initiated to evaluate the potential of ToxCast methods for screening nanomaterials. A subset of ToxCast *in vitro* HTS cell-based assays will be run on nanomaterials to produce similar bioactivity profiles and toxicity predictions. Most of the cell-based assays have an exposure time of 24 hours. Initial nanomaterial types to be evaluated include single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) along with silver (Ag), titanium dioxide (TiO_2) and gold (Au) nanoparticles.

Design and conduct of ToxCast screening of NMs requires selection of testing concentrations, characterization of materials, and analysis of resulting HTS data. Selection of concentrations used for *in vitro* toxicity studies of nanomaterials often lack scientific justification and are often chosen to be very high to ascertain a toxicological endpoint without consideration of real-world exposure (Oberdorster et al. 2005). Some researchers have utilized particle concentrations causing "overload" (Warheit et al. 2009), a dose where pulmonary clearance becomes severely impaired (Morrow 1988). Although high testing concentrations may be considered to ensure that NMs show bioactivity across the spectrum of assays evaluated, there is also a need for biologically relevant human exposure information to facilitate interpretation of assay results (Cohen Hubal 2009). Authors of the National Academy of Sciences (NAS), "*Toxicity Testing in the 21st Century: A Vision and a Strategy*," noted that human exposure information is required to select doses for toxicity testing facilitating development of environmentally-relevant hazard information (NRC 2007).

Recognizing the critical need for exposure information to inform chemical design, evaluation and health risk management, the EPA's ExpoCast™ program was initiated in 2010 to meet challenges posed by new toxicity testing approaches (Cohen Hubal et al. 2010). The goal of ExpoCast is to advance characterization of exposure required to translate findings in computational toxicology to information that can be directly used to support exposure and risk assessment. Combining information from ToxCast with information from ExpoCast will help the EPA prioritize nanomaterials and chemicals for further evaluation based on potential risk to human health.

Human exposures to ENMs are likely to be higher for workers in occupational settings than for the general population including consumers (Bergamaschi 2009) and may thus provide upper bounding estimates of exposure potentials. For consumers the greatest exposure to ENMs likely comes from products that are ingested or that come into intimate contact with the body (Kessler 2011). Though ingestion and dermal exposures must also be considered during the product life cycle (manufacturing, usage, and disposal of EMNs) (Oberdorster et al. 2005; U.S. EPA 2010), inhalation may be the key route of human exposure in nanotechnology manufacturing and R&D facilities (Bergamaschi 2009; Hoet et al. 2004). As such, many studies have focused on the inhalation exposure route for ENMs and consider potential airborne releases of nanomaterials from facilities. Following intake of nanoparticle-containing aerosols, high deposition fractions in the alveolar region (for particles < 100 nm in size) and the head region (diameter < 5 nm) are predicted by the Multiple-Path Particle Dosimetry (MPPD) and International Commission on Radiological Protection (ICRP) models (U.S. EPA 2009). Nanomaterial exposure of the lung parenchyma is of concern due to long-term retention in this lower region and potential for particles to cause cytotoxicity and translocate.

GENERAL APPROACH

The aim of this study is to use information on potential ENM exposure in the occupational setting to recommend *in vitro* testing levels for bioprofiling in EPA's ToxCast program. The general approach taken (Figure 1) was to assume the inhalation exposure route for NMs to be of primary concern for humans in occupational settings. Occupational aerosol levels of nanomaterials reported in the literature were reviewed and used as inputs for lung dosimetry modeling. These reported NM concentrations from manufacturing and R&D laboratory facilities were assumed to provide a high end potential for real-world nanomaterial exposure to the general population, higher than exposures that may result from consumer products (Bergamaschi 2009).

The maximum reported NM aerosol concentrations (mass per m^3 of air) were taken as an input for the MPPD model to estimate deposition, clearance and mass retained in the alveolar region of the human lung. A sensitivity analysis was performed to evaluate MPPD input parameters that most affect NM alveolar retention after 24 hours of exposure. Two exposure scenarios were considered for further modeling: exposure over the course of 24-hours (based on the standard assay exposure duration) and 45 years (a full occupational lifetime). For each scenario, we varied the significant parameters to estimate the mass of particles retained in the alveolar region per surface area. Model results of lung surface mass concentrations were then converted (using the reported well bottom surface area and volume delivered) to suggest testing solution mass concentrations for *in vitro* screening. All of the applied material is assumed to deposit on the bottom of the well. The results suggest upper and lower bounding HTS assay testing concentrations based on potential for real world NM exposures at short and long durations via the inhalation route in an occupational setting. The concentrations were subsequently compared to *in vitro* concentrations found in recent literature. Although we have chosen here to consider aerosol mass concentration, we recognize that

other lung deposition metrics (based on particle number or particle surface area) are also potentially important for understanding health risk.

A small fraction of nanoparticles deposited in the alveolar region may be cleared into the blood stream by absorption. Particles that deposit in the respiratory tract can also be cleared to the gastrointestinal (GI) tract via the pharynx or to the regional lymph nodes (LN) via lymphatic channels. Only lung surface cells would receive the same concentration of NPs as estimated here for inhalation. Modeling exposure to other cell types is beyond the scope of this paper but these exposures would likely be significantly lower concentrations than that calculated for lung cells.

DATA AND METHODS

Nanomaterial air concentrations: We reviewed occupational exposure studies that measured airborne levels of ENMs. The instruments used to obtain particle number concentrations were typically the condensation particle counter (CPC), scanning mobility particle sizer (SMPS), and the fast mobility particle sizer (FMPS). In some cases, personal air samples collected nanoparticles on filters from the breathing zone of workers during the work day. The SMPS and FMPS instruments provide real-time temporal changes in particle size. The data give particle number concentrations (particles/cm^3 of air) versus particle diameter across the size distribution. The CPC provides particle number concentration (particles/cm^3 of air) for particles in the range from 2.5 to >1000 nm. The instruments can also report the change in particle number concentration versus time. We searched for the highest aerosol particle number concentrations for TiO_2 and Ag nanoparticles, and CNTs (including MWCNTs) in manufacturing and R&D settings (Table 1). Background particle number concentrations were subtracted from the maximum particle number concentrations if they were reported. Typically, particle counts per volume (cm^3) of air are reported while exposure limits are set as mass concentrations (mg/m^3). To convert from reported particle

count concentration to mass concentration, TiO_2 and Ag NPs were assumed to be spherical and the reported size (taken to be geometric particle diameter) was used to calculate a particle volume. The CNTs were assumed to be cylindrical and reported diameter and length were used to obtain particle volume. A CNT length of 0.5 μm was assumed if it was not reported. A density of 4, 10, and 2 g/cm^3 was assumed for TiO_2 NPs, Ag NPs and CNT, respectively, based on specifications of similar materials from supplier websites (http://www.sigmaaldrich.com and http://www.nanoamor.com/). The high-end reported particle counts were approximated to mass concentrations by multiplying particle volume by the assumed density (Table 1).

The calculated mass concentrations were typically less than ~ 0.1 mg nanomaterial per volume (m^3) of air (Table 1). One study on MWCNTs reported a higher mass concentration of 0.3208 mg/m^3 (Lee et al. 2010). However, this value was from personal sampler filters with typical sampling durations of 183-409 min. The mass concentration would be lower if calculated over the time duration. Data normalized over exposure characterization duration from a liquid-phase production facility of Ag NPs yielded a mass concentration of 0.46 mg/m^3 for one minute (Park et al. 2009). In this study, both change in particle number concentration versus time and total number of particles (with diameters between 10-250 nm) counted over a range of time are reported. A conservative aerosol concentration of 1 mg/m^3 was taken to be an upper exposure limit. Though the U.S. National Institute for Occupational Safety and Health (NIOSH) does not have a recommended exposure limit (REL) for TiO_2 NPs, a draft NIOSH bulletin recommends "0.1 mg/m^3 for ultrafine TiO_2, as time-weighted average concentrations (TWA) for up to 10 hr/day during a 40-hour work week" where ultrafine is defined as the fraction of respirable particles with primary particle diameter < 100 nm (NIOSH 2005). A recent draft NIOSH bulletin has proposed a REL of 0.007 mg/m^3 for CNTs and carbon nanofibers (NIOSH 2010). Using a different approach, an occupational exposure limit (OEL) of 0.05 mg/m^3 was derived for Baytubes, a more flexible MWCNT type

(Pauluhn 2010). There is no limit set of silver nanoparticles in the US. However, the Occupational Safety and Health Administration (OSHA) established a permissible exposure limit (PEL) of 0.01 mg/m^3 (which is the same REL set by NIOSH) for all forms of airborne silver (Miller et al. 2010). The American Conference of Governmental Industrial Hygienists (ACGIH) set a threshold limit value (TLV) of 0.1 mg/m^3 for metallic silver and 0.01 mg/m^3 for soluble silver compounds (Miller et al. 2010). The mass concentrations derived based on measured aerosol levels were taken as a basis and used as inputs to model the mass of nanoparticles that could deposit and be retained deep in human lungs.

Lung Dosimetry Modeling:

MPPD model application**:** Particle deposition and clearance in human lungs was estimated using the recently developed, publicly-available MPPD model (version 2.1 for nanoparticles, presently supported by Applied Research Associates, Inc.). The model can be used to estimate particle dosimetry in both human and rat airways (Anjilvel and Asgharian 1995; Asgharian et al. 2001). It calculates deposition and clearance of particles (with size ranging from ultrafine (0.001 μm) to coarse (100 μm) in the respiratory tract based on user-provided input on airway morphometry, clearance rates, particle properties (density, diameter, and size distribution), and exposure scenario (aerosol concentration and activity breathing pattern, and exposure duration). There are three main particle deposition mechanisms (impaction, sedimentation and diffusion) incorporated in the model and deposition in different regions of the lung are calculated using published analytic formulas (Anjilvel and Asgharian 1995). Clearance from each lung region is treated competitively between absorption into the blood and particle transport processes (from the respiratory tract to the GI tract and to lymph nodes, and from one region to another) (ICRP 1994). Retention in the human alveolar-interstitial region is represented by three compartments which clear at fast, medium and slow rates to the lymph nodes and the bronchiolar region (ICRP 1994). Though

the clearance kinetics in the MPPD model were based on studies of micro-sized particles, evidence suggests efficient surface macrophage uptake and clearance of both micro- and nanoparticles as well as penetration of both sized particles through the human lung epithelium into the interstitial region, from which they are slowly cleared (Geiser and Kreyling 2010). In addition, the MPPD model (version 2.1) incorporates improved estimates of particle losses from the airway by diffusion and includes particle-specific axial diffusion and dispersion effects in the transport equation (Asgharian and Price 2007). This updated model provides for more realistic assessment of regional deposition of diffusion-dominated (nano-sized) particles in the lung (Asgharian and Price 2007). A MPPD model version (obtained directly from Applied Research Associates, Inc.) that incorporates length to diameter aspect ratio to predict inhaled nanofiber/nanotube deposition in the human lung was used for nonspherical CNTs/MWCNTs (NIOSH 2008). This model incorporates altered analytical expressions for deposition efficiency of nanofibers of a given aspect ratio by adjusting for the viscous drag and nanofiber orientation in the deposition efficiency equation for spherical particles. The clearance calculations are valid only for spherical particles.

An initial baseline set of MPPD inputs (Table 2) were selected based on data from the ICRP report (ICRP 1994), which provides morphological characteristics and physiological parameters for the human respiratory tract. We organized the MPPD model input parameters into three categories: individual characteristics, exposure scenario, and material properties. For the individual characteristics input, the airway morphometry selected was the human Yeh/Schum symmetric lung model (Yeh and Schum 1980). Default values were selected for the clearance rates and other parameters. For the exposure scenario input, 0.1 mg/m^3 aerosol concentration was selected and light exercise activity breathing pattern for an adult male were assumed with 20 breaths/min frequency at 1250 mL tidal volume, V_T (ICRP 1994). Oronasal-mouth breather was selected for breathing scenario as humans typically switch to breathing partly through the mouth and through the nose at

ventilation rates between light and heavy exercise (ICRP 1994). For the particle properties input, a particle count median (CMD) diameter of 40 nm was selected, assuming a single mode of log-normal size distribution with size geometric standard deviation (GSD) of 1.25 based on the ICRP report. Inhalability was not considered since it approaches 100% for small (< 5 μm) particles (ICRP 1994). The length to diameter aspect ratio was set to 1.

Sensitivity analysis**:** Key determinants of MPPD model predictions of mass (mg) retained in the alveolar region were determined by systematically altering each input baseline parameter one at a time, while holding the others constant, and re-running the model based on a 24 hour exposure duration with 1 week of total time (Table 3) to allow for clearance. For the individual characteristic inputs, two different size (based on total number of airways) human stochastic lung models were evaluated because these provide more realistic lung geometry than the symmetric lung model. Calculations were also performed using an age-specific symmetric lung model for a three year old child. Though, this group is unlikely to be exposed occupationally, we wanted to check model results for a vulnerable population group. The alveolar interstitial rate constants for fast, medium, slow, and lymph node human clearance were doubled, halved, and increased by and decreased by an order of magnitude. Tracheal mucosal velocity was not considered since it only affects TB clearance rates and residence times and will not affect long term alveolar burden. For the exposure scenario inputs, the aerosol concentration was decreased by one order of magnitude from 0.1 mg/m^3. As a conservative estimate in case the mass per air volume concentration is much higher than what is reported, the aerosol mass concentration was also increased by one and two orders of magnitude. Both heavy exercise and resting breathing patterns were evaluated as well as purely nasal and oral breathing. For the particle properties inputs, we considered a low size diameter of 5 nm, a high diameter of 100 nm, a low GSD of 1 (monodisperse diameter distribution), and a high

GSD of 4 (polydisperse diameter distribution). Additionally, aspect ratios from 4-1000 were evaluated with a length GSD of 1.0 (as a conservative estimate) and density of 2.

For this sensitivity analysis, if the alveolar mass retained using the new setting resulted in a percentage change greater than or equal to 10 % of the baseline amount, the parameter was considered to be significant and was evaluated further. If the alveolar mass retained using the new setting yielded a negative percentage change compared to the baseline setting, then the input was not considered since we are interested in a conservative exposure approach that may overestimate particle deposition and retention deep in the lungs. If alveolar retention output did not change linearly with change in input, additional input changes were considered to better characterize model behavior over the relevant range. The MPPD input parameters determined to be significant were evaluated further to calculate mass retained in the alveolar region per alveolar surface area based on two exposure durations: a short-term exposure duration of 24-hours and a long-term occupational lifetime exposure. The long-term scenario assumed a 45 year *full working lifetime* (Schulte et al. 2010) with 8 hours inhalation per day, 5 days per week, 52 weeks per year. The alveolar surface area (~ 106,350 cm^2) was obtained from the MPPD model results report by summing the pulmonary surface area for lung generations 17 to 24. This alveolar surface area only accounts for surface area of the airways (alveolar ducts) and not the alveolar sacs and thus is a low estimate of the actual alveolar surface area. The MPPD calculations were performed for different particle sizes (5, 10, 20, 30, 40, 50, 60, 70, and 100 nm), aerosol concentrations (0.1 and 1 mg/m^3), and exposure durations. Larger particle sizes (200, 500 and 1000 nm) were also run as particle aggregation of nano-sized particles may occur in air (Maynard et al. 2004; Methner et al. 2010a) or inside the human respiratory tract. For CNTs, an aspect ratio of 167 was selected based on material dimensions (5 μm length, 30 nm diameter) of one sample to be tested in ToxCast. Aspect ratios of 2, 4 and 10 were also run for the different particle sizes. These aspect ratios were chosen based on

electron images of SWCNT aggregates from the literature (Baron et al. 2008). Searching for realistic airborne CNT aspect ratios was challenging since many exposure studies found no evidence of carbon-based nanotubes or nanotube bundles in air samples (Bello et al. 2008; Bello et al. 2009). In one study of seven CNT handling workplaces, TEM micrographs reveal clumped structures with aspect ratio of ~ 8-10 and diameter of ~ 100 nm (Lee et al. 2010). However, these particle aggregates are mostly metal components rather than CNTs.

Determining *in vitro* concentrations: Based on MPPD model predictions, associated *in vitro* concentrations were determined by calculating mass retained in the alveolar region of the lung per alveolar surface area for each particle size, at two aerosol concentrations (0.1 and 1 mg/m^3) and for each exposure scenario (24 hours and 45 years). We assumed that the NM mass retained at the lung surface can be directly correlated to NM mass sedimented on the bottom surface of a well. To convert to mass of nanomaterial per volume of solution, the resulting mass per alveolar surface area concentration ($\mu g/cm^2$) was multiplied by the bottom surface area of a single well in a 12-, 96- or 384-well plate and divided by the culture medium volume added to each well (as obtained from the assay contractors). The 12-, 96- and 384-well plates had a single well bottom surface area of 3.8, 0.32, and 0.056 cm^2 and volume presented of 1000, 200, and 50 μL, respectively. The converted concentrations were compared to *in vitro* concentrations tested using human, mouse and rat cell lines in the literature (see Supplemental Material Tables S1, S2, and S3). Though final selection of concentration will be include consideration of the MPPD model output and conversion, there will still be a need to test at levels based on where bioactivity has been demonstrated in the literature, bounded by concentration levels that can be dispersed with long-term stability in cell culture media.

Another method to determine high range *in vitro* concentrations to test could be to evaluate a NM steady state mass in the alveolar region of the lung. Steady state occurs when the clearance rate

equals the rate of deposition and the NM mass retained reaches a constant value. It has been reported to take more than 10 years to reach a steady state lung burden for insoluble 1 μm-sized particles for a 0.01 mg/m^3 aerosol concentration based on resting human breathing pattern (Brown et al. 2005).

RESULTS AND DISCUSSION

Key MPPD model input parameters: For the MPPD baseline settings used here (Table 2), a steady state retention dose will take more than 80 years to achieve for 40 nm particles based on inhaling an aerosol concentration of 0.1 mg/ m^3 for 8 hours a day, 5 days a week. Due to the long time to achieve steady state, this method was not utilized. Instead, we focused on modeling potential exposure scenarios and understanding implications of associated model inputs. Model results for the baseline input parameters (Table 2) resulted in 1.22 mg alveolar mass retained.

Results of the sensitivity analysis (alveolar mass retained, percentage change in model output and input, and sensitivity percentage [output % change by input % change]) are presented in Table 3. Though interactions between input parameters may occur, we assumed that key parameters could be uncovered by varying one parameter per run. Based on this analysis, aerosol concentration and heavy exercise breathing pattern were the most important MPPD input parameters as these increased alveolar retention by more than 10 percent. For variations to the individual characteristic inputs (Table 3), the choice of airway morphometry with the human stochastic lung model resulted in a lower retention when compared to the symmetric lung model. The age-specific symmetric lung model for a three year old resulted in lower mass retained at 0.16 mg because of lower intake

(functional residual capacity, upper respiratory tract volume, and V_T) compared to the adult male default baseline condition. Thus, the Yeh/Schum symmetric model provided a conservative estimate of nanomaterial particle dosimetry. Nasal and oral breathing scenario did not significantly affect the results and was set to the baseline of oronasal breathing. Increasing and decreasing the default alveolar-interstitial rate constants did not significantly affect the result as indicated by the sensitivity percent in Table 3. The alveolar-interstitial rate constants were set to the default values and the lung model to symmetric to further calculate alveolar mass retained per alveolar surface area.

For the exposure scenario inputs evaluated (Table 3), the correlation between alveolar mass retained and aerosol concentration was linear—the amount retained in the alveolar region changed linearly by one order of magnitude as the exposure aerosol concentration (and thereby the intake) was increased or decreased by one order of magnitude, yielding a sensitivity of 100%. Using resting or heavy exercise breathing pattern resulted in a sensitivity of approximately 100 percent, indicating alveolar mass retained changed almost linearly with minute ventilation (breathing frequency by V_T). To further calculate alveolar mass retained per alveolar surface area, the breathing scenario was set to light exercise based on the assumption that this was the most realistic for a full working lifetime. The aerosol concentration of 1 mg/m^3 was taken as a conservative estimate of potential worker exposure.

Particle property input changes to particle diameter, size GSD, and aspect ratio (length:diameter) did not result in linear changes to output alveolar mass retention as observed in the sensitivity % column in Table 3. A particle size of 20 nm resulted in maximum alveolar mass retained (1.51 mg) for diameters between 5-100 nm and was a ~24% change increase in output compared to baseline 40 nm size. A GSD value of 1 (monodisperse size distribution) yielded a higher mass amount retained, but it was only 9.08 % more than the baseline size GSD value of 1.25

and did not meet the sensitivity analysis requirements. All other input changes for diameter and GSD lowered the alveolar mass retained as compared to the baseline settings. One report on TiO_2 particles listed a GSD of 1.66 (Hameri et al. 2009) and another report on Ag NPs listed GSD values of 4.63-6.3 (Park et al. 2009). Although a higher size GSD value is expected for realistic size distribution of nanomaterials, this parameter was set to 1.25 as a conservative estimate increasing alveolar retention. Changes to the aspect ratio input at constant aerosol concentration and minute ventilation lowered the alveolar mass retained compared to the baseline (aspect ratio 1 in Table 3). Only an aspect ratio of 20 slightly increased the alveolar mass retained. The sensitively % to the aspect ratio parameter was low.

Concentrations recommended for *in vitro* testing: Results of the deposition modeling are presented as a function of material characteristics for the two exposure scenarios of interest in Figure 2. Since the MPPD alveolar mass retention is linearly proportional to the inputted aerosol concentration, mass per lung surface area *per inputted aerosol concentration* versus particle diameter were plotted. The alveolar retention per surface area for silver and TiO_2 spherical nanoparticles for a *full working lifetime* was highest for 20 nm diameter particles at 48.9 $\mu g/cm^2$ based on an exposure aerosol concentration of 1 mg/m^3 (Figure 2A). Relative to this peak lung surface concentration, the amount decreased to 20.3 $\mu g/cm^2$ as size was increased to 100 nm and decreased to 25.1 $\mu g/cm^2$ for 5 nm particles. For the 12-, 96- and 384-well plates used by the different assay contractors, the peak lung surface concentration equates to 186 [i.e., 48.9 $\mu g/cm^2$ x (3.8 cm^2/1 mL)], 78.2, and 54.8 μg/mL, respectively. These amounts for a *full working lifetime* lie within the range of the highest *in vitro* assay concentrations tested in the literature for Ag nanoparticles and TiO_2 nanoparticles on human, rat, and mouse cell lines. The highest amount tested for Ag NPs ranges from 1.6 – 500 μg/mL, whereas for TiO_2 nanoparticles the high-side range

is 100-1000 μg/mL or 20-520 μg/cm^2 (see Supplemental Material, Tables S1, S2). Most of the Ag NPs concentrations tested fall within 50-400 μg/mL, whereas the TiO_2 NPs fall within 100-250 μg/mL. Note that since the MPPD model uses a low estimate of alveolar surface area, a more realistic estimate would result in lower alveolar mass retained per surface area (by approximately one order of magnitude), which would correspond to a lower *in vitro* concentration for a given exposure duration. For a full working lifetime exposure duration at 0.1 mg/m^3, the peak lung surface concentration was 4.9 μg/cm^2 (for particle diameter of 20 nm) and the range was 2.0-4.9 μg/cm^2 for particle diameters 5-100 nm (Figure 2A). Since alveolar retention is directly proportional to aerosol concentration, reducing the input aerosol concentration by a factor of 10, results in a linear reduction of the calculated well plate concentration (μg/mL) by a factor of 10. The calculated well plate concentration for a *full working lifetime* is similar to the low range (1.6 to 10.8 μg/mL) of the highest concentrations tested using *in vitro* assays for Ag nanoparticles, but is below the range tested for TiO_2 NPs (see Supplemental Material, Tables S1, S2).

The lung surface concentration for a 24-hour exposure duration at 1 mg/m^3 aerosol concentration to TiO_2 or Ag nanoparticles ranges from 0.061-0.15 μg/cm^2 for particles sizes 5-100 nm (Figure 2A). This range is lower by more than two orders of magnitude than the range found for a full working lifetime (Figure 2A). The peak lung surface concentration equates to 0.570, 0.240, and 0.168 μg/mL for the 12-, 96- and 384-well plates, respectively. From the literature, the lowest amount tested for Ag nanoparticles ranges from 0.108-25 μg/mL, whereas for TiO_2 nanoparticles the range was 0.002-10 μg/mL or 0.0052-5 μg/cm^2 (see Supplemental Material, Tables S1, S2). For 24 hours exposure duration, the alveolar surface concentrations range calculated using the MPPD model fell within the range (closer to the lower end) of lowest *in vitro* concentrations tested. The literature concentrations thus are similar to the lower bound assay test concentrations derived using

the estimated lung retention after 24 hours of exposure. Each of the studies had a set exposure duration ranging from 1-144 hours for Ag nanoparticles and from minutes to 24 hours to days for TiO_2 nanoparticles (see Supplemental Material, Tables S1, S2). Re-running all the baseline settings for 20 nm particles for an exposure duration of 24 hours would require *a very high aerosol concentration* of ~ 330 mg/m^3 to result in a similar peak alveolar surface concentration (of ~ 48.9 μg/cm^2) for TiO_2 and Ag nanoparticles.

The alveolar mass retention per surface area for CNTs (with length to diameter aspect ratio of 167) for a full working lifetime exposure to 1 mg/m^3 aerosol concentration ranges from 12.4-46.5 μg/cm^2 (Figure 2B), similar to the range for spherical particles. As CNT diameter decreases from 100 nm, the mass retained per surface area increases to a maximum of 46.5 μg/cm^2 for 5 nm diameter nanotubes. The highest amount tested *in vitro* for CNTs ranges from 50 – 1000 μg/mL (see Supplemental Material, Table S3). Most of the CNT concentrations tested fall within 50-400 μg/mL. For the more realistic aspect ratios of 4 and 10, there is a peak mass per surface area concentration at 40 and ~25 nm, respectively (Figure 2B). This peak concentration decreases with increasing diameter (Figure 2B). Based on the literature, it is possible that the CNTs will form aggregates of larger diameter and lower aspect ratios (Baron et al. 2008). Particles having aspect ratios ~20 were found to have maximum deposition fraction in the alveolar region using the model. For the 2, 4, and 10 aspect ratios, the mass per surface area retained for diameters greater than 40 nm follows a similar trend. The lung surface concentration of aspect ratio 2 is similar to the trend for spherical particles (Figure 2A) at the same aerosol concentration and exposure duration.

Applications of approach: The approach taken here was a simple screening-level assessment that attempts to utilize the latest nanomaterial quantitative aerosol level data in occupational settings to determine concentrations that may deposit and be retained deep in the

human respiratory tract. The methodology used and alveolar retention results obtained here can be generally applied to inform *in vitro* study designs, which include other NM types. Wherever possible, conservative MPPD input parameters were selected so that results would indicate a higher alveolar retention, though we have attempted to choose realistic inputs as well. Our results indicate that a full lifetime occupational exposure to a concentration of 1 mg/m^3 (one order of magnitude higher than what has typically been reported, Table 1) is required to reach the highest concentrations currently being tested *in vitro* in most studies (see Supplemental Material, Tables S1, S2, and S3). Since i*n vitro* studies use different cell culture containers, in order to convert the lung surface concentrations provided here, the specific well bottom surface area and medium volume presented to each well are required.

Note that we are comparing lung surface concentrations to concentrations being tested in a range of cell types and would expect only a small percentage of particles to reach cells in other organs of the body following absorption into the bloodstream (Figure 1). Nevertheless, retention of nanoparticles in the deep lung alveolar region is of importance as these particles can potentially quickly be absorbed into the bloodstream. Such a phase of rapid absorption is observed immediately after inhalation, even with relatively insoluble materials (ICRP 1994). Recently, researchers have found that NPs having hydrodynamic diameters less than 6 nm with zwitterionic surface charge can rapidly enter the bloodstream from the lung in rats, and then be subsequently cleared by the kidneys and that NPs smaller than 34 nm with a noncationic surface charge translocate rapidly from the lung to the mediastinal lymph nodes (Choi et al. 2010). However, for the case of Technetium–radiolabeled 100 nm, 35 nm and 4-20 nm diameter carbon particles, no significant systemic translocation of particles has been observed in humans (Mills et al. 2006; Moller et al. 2008; Wiebert et al. 2006). Gold NPs (5-8 nm) have been found at low fraction (0.03 to 0.06% of lung concentration) in the blood of rats from 1 to 7 days post-inhalation (Takenaka et al. 2006). The type

and amount of surface charge or coating may be a key factor for translocation of particles and needs to be evaluated. There is also a potential for larger NM mass amounts per lung surface area to be deposited in the tracheobronchial (TB) region. All airway surfaces may not receive the same amount of deposited particles and localized "hot spots" for deposition in the vicinity of airway bifurcations have been predicted (up to 100-1000 times higher than the average mass per surface area for particles > 10 nm) using mathematical modeling techniques (Farkas et al. 2006; U.S. EPA. 2009). However, mass retained in this region was not considered herein since a large portion of the particles deposited are assumed to be cleared within 24-48 hours by action of the mucociliary escalator (U.S. EPA. 2009). Potential future work will be to consider GI tract exposure to nanoparticles cleared from the TB region as it may be significant for aggregated NPs at heavy exercise breathing conditions.

Limitations of approach: There remain a number of limitations in estimating concentrations to test *in vitro* from the latest available nanoparticle aerosol level data in occupational settings. The instrumentation technology to measure spherical NPs typically provide non-specific particle counts over a broad size range. For example, the SMPS provides particles counts in a size range of 2.5-1000 nm, whereas the CPC used across a number of studies (Table 1) measures particles in the size range of 10-1000 nm. The particle counts become increasingly insensitive to particles sizes approaching the lower limit of detection (Maynard and Aitken 2007). Measurements for non-spherical particles such as CNTs may not be reliable and may need to be corrected since these instruments are designed to count spherical particles. Additionally, in order to compare particle number concentration for the same type of materials across different occupational settings or manufacturing processes, the FMPS and SMPS data reported needs to be divided by the number of channels to normalize it. The instrument data is not always normalized and is thus may be reported as a higher count over a particle size distribution than what actually occurs. It is not

currently possible to distinguish between nanoparticles, aggregates of the same compounds, aggregates of a mixture of particles, dust, and other airborne particle types. Nanoparticles often can agglomerate in air, which is why results for potentially more realistic sizes larger than 100 nm (Figure 2A, 2B) are provided. Instruments such as the Universal NanoParticle Analyzer (UNPA), which utilize a CPC, a Differential Mobility Analyzer (DMA), and a Nanoparticle Surface Area Monitor (NSAM), are being developed to determine the primary particle size and measure the number, surface area, and volume distributions of gas-borne NP agglomerates (Wang et al. 2010). In order to distinguish nanoparticles from background particles, real-time instrumentation measurements as well as qualitative analysis by electron microscopy are required (Ono-Ogasawara et al. 2009). Also, chemical analysis is necessary for quantitatively assessing exposure to nanomaterials at facilities with high levels of background NPs. Models are being developed to predict the change in nanoparticle number concentration for a defined source and a defined environment based on a given background aerosol concentration (Seipenbusch et al. 2008). The NPs do not reach the receptor in their original size as an aerosol, but change their size and number concentration by coagulation either within the same type of materials or by interaction with a background aerosol (Seipenbusch et al. 2008).

We have provided MPPD results assuming no changes to the original aerosol concentration and have taken the reported size of the particles as the size to simulate. If particles have a tendency to aggregate and agglomerate above an aggregate size of 100 nm, the amount deposited and retained in different regions of the lung will be less (Figure 2A, 2B). In the case of SWCNTs, large aggregates more than 100 micrometers in diameter can form by diffusion and van der Waals interactions between nanotubes in air or in aqueous solutions (Mutlu et al. 2010). Other drawbacks present based on the method used are limitations with the MPPD model including a low estimate of alveolar surface area. Distinctions between nanomaterial types cannot be made currently based on

NM physicochemical characteristics. The only input that can be made in the latest version of the model is length to diameter aspect ratio for cylindrical particles. The clearance calculations in the model are based on experimental data for spherical particles and fibers with elongated structures may have different clearance kinetics. Nanomaterials have unique physicochemical characteristics that may affect their deposition, retention and toxicity. These include particle shape and shape distribution, large surface area to volume ratio, chemical composition and crystalline form, surface composition/coating, and surface charge. There is a need to understand which physicochemical characteristics most affect the deposition and alveolar retention of nanoparticles and to further incorporate these key parameters into the model.

Another limitation in the approach may be the conversion of lung surface concentrations to *in vitro* test concentrations, assuming that the nanomaterials will quickly (relative to the duration of the assay) settle onto the cells at the bottom of the well plate. If the particle transport (diffusion, sedimentation) time is slower than the *in vitro* assay testing time (which could possibly be the case for particle agglomerates depending on their mass, size and density) (Hinderliter et al. 2010), then the localized NM concentrations near the cells at the bottom of the well may be lower than what we estimate. A recently developed computational model of particokinetics (sedimentation, diffusion) and target cell dosimetry for *in vitro* systems addresses this issue (Hinderliter et al. 2010) and could be used to calculate dose rates and target cell doses to compare to the total assay exposure time. Further, bioactivity profiles attained for nanomaterials would need to take the localized concentration into account.

CONCLUSIONS

Consideration of potential exposures during design of *in vitro* toxicity tests would improve interpretation of hazard screening results for use in risk assessment. The methodology described here is a first step toward improving selection of nanomaterial concentrations to test *in vitro* based on real-world inhalation exposure potential. The results obtained can be generally applied to other *in vitro* study designs and for other nanomaterial types. The approach here reveals that current high range *in vitro* testing concentrations being utilized are similar to predicted lung surface area concentrations based on occupational setting inhalation exposure to nanomaterials of a high aerosol concentration over the course of a full working lifetime. This methodology can be improved by better measurements of nanomaterials in occupational settings, addition of particle property input parameters to the MPPD model, and considerations of delivered dose to cells.

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Table 1. Nanomaterial exposure concentrations in lab and manufacturing sites. Abbreviations: CPC, Condensation Particle Counter; SMPS, Scanning Mobility Particle Sizer; FMPS, Fast Mobility Particle Sizer; REL, Recommended exposure limit; PEL, Permissible exposure limit; OEL, Occupational exposure limit.

NM	Highest particle count (#/cm^3)	Mnfg. / Lab	Particle size (nm)	Calculated Mass concentration (mg/m^3)	Instrument used for Detection	Reference
Ag NPs	72900	Mnfg.	35	0.02	CPC	(Methner et al. 2010a; Methner et al. 2010b)
	1.102e7 (over 55 min)	Mnfg.	76	0.46 (over 1 min)	SMPS	(Park et al. 2009)
	7000	Lab	150	0.12	FMPS	(Tsai et al. 2009)
	995000 (over 15-30 min)	Mnfg.	10	0.005 0.094 (over full work shift) [0.01 (OSHA PEL—airborne silver)]	FMPS Personal filter sample	(Miller et al. 2010)
MWCNTs	35800	R&D Lab	20 nm diam., 0.5 μm length	0.01	CPC	(Methner et al. 2010a; Methner et al. 2010b)
	-	-	-	[0.05 (researcher suggested OEL)]	-	(Pauluhn 2010)
	75000 – during metal catalyst preparation	Mnfg. - 7 sites	25 nm diam., No reported length	0.037 0.32	SMPS Personal air	(Lee et al. 2010)
CNTs	7000 – no detectable CNTs or bundles	Lab	10 and 100 diam.	0	FMPS and CPC	(Bello et al. 2008)
	5000000 (CNT carbon composites – no detectable CNTs or bundles)	R&D Lab	-	0	FMPS	(Bello et al. 2009)
	-	-	-	0.007 (draft REL for elemental carbon)	-	(NIOSH 2010)
TiO_2 NPs	111300	Mnfg.	40	0.015	CPC	(Methner et al. 2010a; Methner et al. 2010b)
	-	-	-	0.1 (draft REL)	-	(NIOSH 2005)
	140000	Mnfg.	16	0.001	SMPS	(Hameri et al. 2009)

Table 2. MPPD baseline settings.

MPPD baseline input categories	**Baseline input settings**
A) Individual characteristics (Airway morphometry & Deposition/clearance)	Human Species; Yeh-Schum Symmetric Single Path lung model; FRC=3300 ml; URT Volume=50 ml; Tracheal Mucous Velocity=5.5 mm/min; Fast Human Clearance Rate=0.02 $days^{-1}$; Medium Human Clearance Rate=0.001 $days^{-1}$; Slow Human Clearance Rate=0.0001 $days^{-1}$; Lymph Node Human Clearance Rate=0.00002 $days^{-1}$;
B) Exposure scenario: Constant exposure	Acceleration of Gravity=981.0 cm/s^2; Body Orientation=Upright; Aerosol Concentration=0.1 mg/m^3; Breathing Frequency=20 per minute; Tidal Volume=1250 ml; Inspiratory Fraction=0.5; Pause Fraction=0; Breathing Scenario=Oronasal-Mouth Breather; Number of Hours Per Day=24; Number of Days Per Week=1; Number of Weeks=1; Max. Post-Exposure Days=0
C) Particle properties	Density=4 g/cm^3; Diameter=0.04 μm; CMD checked; Nanoparticle Model checked; Inhalability Adjusment not checked; GSD(diam.)=1.25

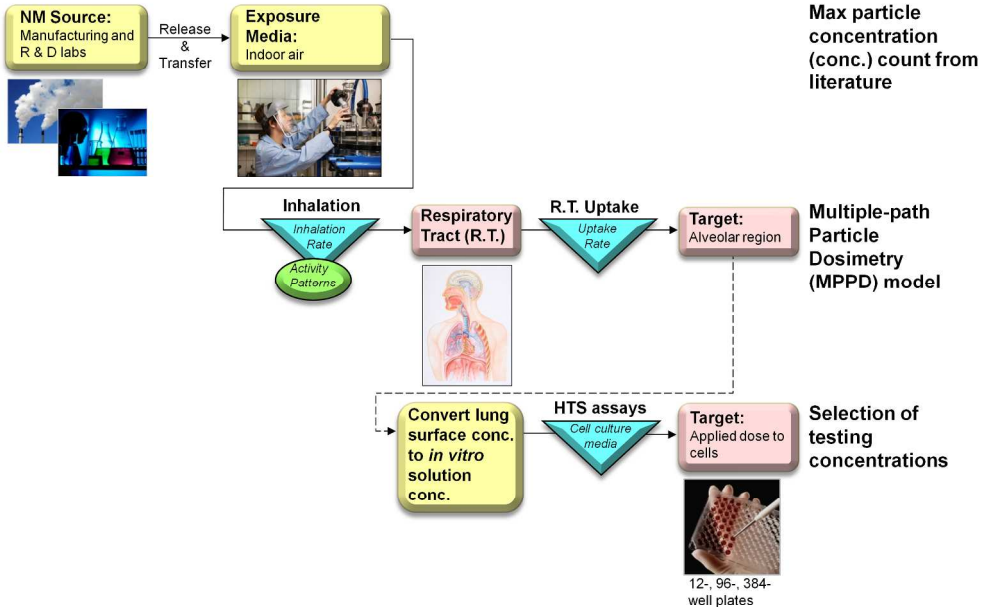
Table 3. MPPD sensitivity analysis

MPPD input category	Altered input setting	Output Alveolar mass retained (mg)	% Change in output	% Change in input	Sensitivity %
Individual characteristics	Human, Stochastic Lung model:				
	1st size percentile	0.81	-34.1	-	-
	60th size percentile	1.01	-17.2	-	-
	Age-specific Symmetric model:				
	3 year old child	0.16	-87.1	-	-
	Clearance rates:				
	0.1 * default alveolar-interstitial rate constants	1.27	3.8	-90	-4.18
	½ * default rate constants	1.25	2.0	-50	-4.09
	1.5 * default rate constants	1.20	-2.0	50	-3.92
	2 * default rate constants	1.18	-3.8	100	-3.76
	10 * default rate constants	0.95	-23.0	900	-2.50
Exposure Scenario	Aerosol Concentration:				
	0.01 mg/m^3	0.12	-90.0	-90	100
	1 mg/m^3	12.2	898	900	99.7
	10 mg/m^3	122.3	9900	9900	100
	Breathing pattern:				
	Resting, 12 breaths/min, 625 mL V_T	0.32	-74.1	-70	106
	Heavy exercise, 26 breaths/min, 1923 mL V_T	2.30	88.0	100	88.0
	Breathing scenario:				
	Nasal	1.20	-2.1	-	-
	Oral	1.25	2.0	-	-
Particle Properties	Diameter:				
	5 nm	0.81	-33.9	-87.5	38.7
	20 nm	1.51	23.5	-50	-46.9
	50 nm	1.07	-12.7	25	-50.7
	70 nm	0.85	-30.7	75	-41.0
	100 nm	0.65	-46.6	150	-31.1
	GSD:				
	1	1.33	9.08	-20	-45.4
	1.6	0.90	-26.7	28.0	-95.5
	2	0.60	-51.2	60	-85.3
	2.8	0.68	-44.3	124	-35.7
	4	0.12	-90.1	220	-41.0
	Aspect Ratio (Length:Diam.):				
	[Baseline] 1 (20 nm : 20 nm)	1.54	0	0	-
	4 (80 nm : 20 nm)	1.26	-18.3	300	-6.09
	20 (400 nm : 20 nm)	1.56	1.69	1900	0.09
	100 (2 μm : 20 nm)	1.12	-27.3	9900	-0.28
	500 (10 μm : 20 nm)	0.61	-60.1	49900	-0.12
	1000 (20 μm : 20 nm)	0.54	-65.0	99900	-0.07

Figure Legends

Figure 1. General approach for recommending *in vitro* testing levels. Exposure to NMs from occupational setting indoor air via the inhalation route resulting in respiratory tract uptake is considered. Estimated exposure potential is converted to levels for nanomaterial testing in HTS cellular assays.

Figure 2. MPPD model results of alveolar mass retained per alveolar surface area per inputted aerosol concentration versus particle diameter in human lungs for TiO_2 or Ag nanoparticles for an exposure duration of (A) 45 years (full working lifetime) and 24 hours. (B) Alveolar mass retained per alveolar surface area per aerosol concentration in human lungs after 45 years of exposure duration to CNTs with aspect ratio of 167, 10, 4, and 2. Both (A) and (B) are based on light exercise breathing pattern. The curves are to guide the eye.



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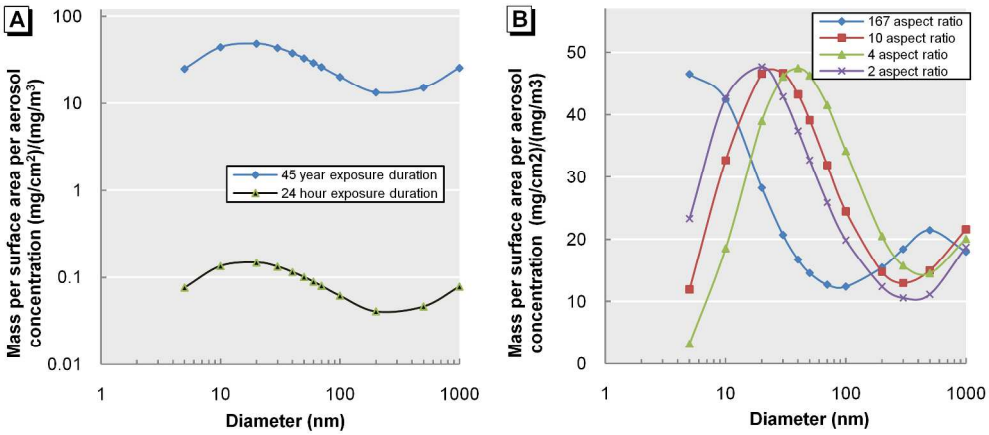

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