NANOPARTICLE DEPOSITION AND DOSIMETRY FOR

IN VITRO TOXICOLOGY

by

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>alternating current</td>
</tr>
<tr>
<td>Ag</td>
<td>silver</td>
</tr>
<tr>
<td>Au</td>
<td>gold</td>
</tr>
<tr>
<td>ALI</td>
<td>air–liquid interface</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>DC</td>
<td>direct current</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>HEPA</td>
<td>high efficiency particle air</td>
</tr>
<tr>
<td>ICP–MS</td>
<td>inductively coupled plasma – mass spectrometry</td>
</tr>
<tr>
<td>IMAGEJ</td>
<td>Imaging Processing and Analysis in Java</td>
</tr>
<tr>
<td>ISDD</td>
<td>In Vitro Sedimentation and Diffusion</td>
</tr>
<tr>
<td>LDE</td>
<td>laser Doppler electrophoresis</td>
</tr>
<tr>
<td>MPPD</td>
<td>Multiple Path Particle Deposition</td>
</tr>
<tr>
<td>NIOSH</td>
<td>National Institute for Occupational Safety and Health</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>OSHA</td>
<td>Occupational Safety and Health Administration</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PdI</td>
<td>polydispersity index</td>
</tr>
<tr>
<td>PM–AEC</td>
<td>Portable Multi–well Aerosol Exposure Chamber</td>
</tr>
<tr>
<td>RH</td>
<td>relative humidity</td>
</tr>
<tr>
<td>SEF</td>
<td>surface enhanced fluorescence</td>
</tr>
<tr>
<td>SMPS</td>
<td>scanning mobility particle sizer</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>UFP</td>
<td>ultrafine particle</td>
</tr>
<tr>
<td>UV–vis</td>
<td>ultraviolet visible spectroscopy</td>
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### LIST OF NOTATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$C_C$</td>
<td>Cunningham correction factor</td>
</tr>
<tr>
<td>$C_{exp}$</td>
<td>exposure concentration in mass per volume</td>
</tr>
<tr>
<td>$C_{SMPS}$</td>
<td>integrated concentration in number per volume measured by the SMPS</td>
</tr>
<tr>
<td>$c_M$</td>
<td>mass concentration</td>
</tr>
<tr>
<td>$c_N$</td>
<td>number concentration</td>
</tr>
<tr>
<td>$c_{SA}$</td>
<td>surface area concentration</td>
</tr>
<tr>
<td>$D$</td>
<td>particle diffusion coefficient</td>
</tr>
<tr>
<td>$d_a$</td>
<td>collision diameter for air</td>
</tr>
<tr>
<td>$d_h$</td>
<td>hydrodynamic diameter</td>
</tr>
<tr>
<td>$d_p$</td>
<td>primary particle diameter</td>
</tr>
<tr>
<td>$E$</td>
<td>electric field strength</td>
</tr>
<tr>
<td>$\sum \vec{F}$</td>
<td>net force</td>
</tr>
<tr>
<td>$\vec{F}_{B,i}$</td>
<td>Brownian force at a single time step</td>
</tr>
<tr>
<td>$\vec{F}_d$</td>
<td>drag force</td>
</tr>
<tr>
<td>$\vec{F}_E$</td>
<td>electric force</td>
</tr>
<tr>
<td>$\vec{F}_g$</td>
<td>gravity force</td>
</tr>
<tr>
<td>$g$</td>
<td>gravity constant</td>
</tr>
<tr>
<td>$G_i$</td>
<td>random number from a Gaussian distribution at a single time step</td>
</tr>
<tr>
<td>$h$</td>
<td>height of the inlet tube discharge from cell layer or ALI</td>
</tr>
<tr>
<td>$h_m$</td>
<td>height of the media layer</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>$L$</td>
<td>characteristic length</td>
</tr>
<tr>
<td>$J$</td>
<td>particle flux</td>
</tr>
<tr>
<td>$J_R$</td>
<td>regional particle flux (respiratory tract)</td>
</tr>
<tr>
<td>$J_{y=0}$</td>
<td>particle flux at the cell layer (two-dimensional geometry)</td>
</tr>
<tr>
<td>$J_{z=0}$</td>
<td>particle flux at the cell layer (three-dimensional geometry)</td>
</tr>
</tbody>
</table>
\( m_p \) \hspace{1cm} \text{particle mass} \\
\( n_e \) \hspace{1cm} \text{number of electron charges} \\
\( n_{mol} \) \hspace{1cm} \text{number of molecules per volume} \\
\( N_p \) \hspace{1cm} \text{number of particles} \\
\( N_{\text{micrographs}} \) \hspace{1cm} \text{number of micrographs} \\
\( Pe \) \hspace{1cm} \text{Péclet number} \\
\( Q \) \hspace{1cm} \text{air flow rate} \\
\( q \) \hspace{1cm} \text{electron charge} \\
\( r \) \hspace{1cm} \text{deposition radius} \\
\( r_0 \) \hspace{1cm} \text{discharge radius} \\
\( Re \) \hspace{1cm} \text{Reynolds number} \\
\( Re_p \) \hspace{1cm} \text{particle Reynolds number} \\
\( SA_{\text{ALI}} \) \hspace{1cm} \text{surface area of the air–liquid interface} \\
\( SA_{\text{cells}} \) \hspace{1cm} \text{surface area of the cell layer} \\
\( SA_{\text{micrograph}} \) \hspace{1cm} \text{surface area of a micrograph} \\
\( SA_R \) \hspace{1cm} \text{regional surface area (respiratory tract)} \\
\( t \) \hspace{1cm} \text{time} \\
\( T \) \hspace{1cm} \text{temperature} \\
\( \Delta t \) \hspace{1cm} \text{time step} \\
\( V \) \hspace{1cm} \text{applied voltage potential} \\
\( V_{ch} \) \hspace{1cm} \text{chamber volume} \\
\( V_p \) \hspace{1cm} \text{particle volume} \\
\( V_R \) \hspace{1cm} \text{regional volume (respiratory tract)} \\
\( V_m \) \hspace{1cm} \text{media volume} \\
\( v \) \hspace{1cm} \text{media velocity} \\
\( v_a \) \hspace{1cm} \text{air velocity} \\
\( v_E \) \hspace{1cm} \text{electrical drift} \\
\( v_p \) \hspace{1cm} \text{particle velocity} \\
\( v_s \) \hspace{1cm} \text{sedimentation velocity} \\
\( y \) \hspace{1cm} \text{vertical distance travelled by a particle}
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$Z_p$</td>
<td>electrical mobility</td>
</tr>
<tr>
<td>$\dot{\gamma}_{z=0}$</td>
<td>shear rate at the cell layer</td>
</tr>
<tr>
<td>$\epsilon_{exp}$</td>
<td>experimental deposition efficiency</td>
</tr>
<tr>
<td>$\epsilon_{theor}$</td>
<td>theoretical deposition efficiency</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>mean free path of air</td>
</tr>
<tr>
<td>$\mu$</td>
<td>dynamic viscosity</td>
</tr>
<tr>
<td>$\rho$</td>
<td>media density</td>
</tr>
<tr>
<td>$\rho_a$</td>
<td>air density</td>
</tr>
<tr>
<td>$\rho_p$</td>
<td>particle density</td>
</tr>
<tr>
<td>$\sigma_m$</td>
<td>relative permittivity of the media layer</td>
</tr>
<tr>
<td>$\tau$</td>
<td>residence time</td>
</tr>
<tr>
<td>$\tau_{z=0}$</td>
<td>shear stress at the cell layer</td>
</tr>
<tr>
<td>$\chi_R$</td>
<td>regional deposition fraction (respiratory tract)</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>zeta</td>
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Nanoparticle Deposition and Dosimetry for In Vitro Toxicology

CHRISTIN MARIE GRABINSKI

ABSTRACT

This thesis addresses several issues in the field of in vitro toxicology involving particle exposure. First, controlling and assessing particle dosimetry in in vitro models is addressed. Traditional in vitro models expose cells grown at the bottom of a dish to nanoparticles under static conditions, regardless of the exposure route of interest (e.g. inhalation, ingestion, systemic translocation). We demonstrated the use of flowing media to reduce sedimentation and simplify dosimetry. The shear stress introduced at the cell layer was found to be much lower than physiological conditions, avoiding potential damage to the cells, and allowing this model to serve as a straightforward approach for dosimetry that can be easily adopted using standard cell culture tools for a broad range of cell types. For the second and third studies, aerosol methods were used to represent inhalation exposure. In the second study, the deposition of ultrafine (<100 nm) and fine (100–2500 nm) aerosol particles onto cells at an air–liquid interface (ALI) was investigated. We studied the effect of chamber design and electrostatics on aerosol deposition using both theoretical and empirical techniques to provide guidelines for controlling the dose of submicron aerosols, which has been a significant challenge to date. Third, we designed and validated a scaled-up portable multi-well aerosol exposure chamber (PM–AEC). The device contains eight wells for cell culture inserts arranged
radially, a heated water bath which maintains internal temperature, and electrodes above and below the ALI to allow for electrostatic deposition of particles flowing past the cell culture inserts. The key unique feature of the chamber is the ability to access cell culture media outside of a sterile environment by penetrating resealable silicone walls. To demonstrate portability, the chamber was loaded with live cell cultures and transported to a field environment where the cells were exposed to emissions from energetic material impaction testing. Overall, the PM-AEC was demonstrated as a useful tool for relevant exposure of aerosolized particles to cells cultured at the ALI, showing promise for assessing particle toxicity in realistic environments.
CHAPTER I
INTRODUCTION AND REVIEW OF LITERATURE

History has shown that it is imperative to be proactive towards understanding adverse health effects from exposure to chemicals and materials. For example, the correlation between inhalation of coal dust and coal miner’s pneumoconiosis was officially recognized in the United States in the late 1960s (Centers for Disease Control and Prevention, 2014). Once standards were established, mortality associated with this type of exposure was drastically reduced from a peak of 2900 deaths in 1972 to an annual average of 400 from 2008–present. Conversely, exposure standards have been in place for asbestos since 1972, but mortality related to asbestos exposure did not begin to decline until the early 2000s due to a 10–20 year latency period between exposure and onset of disease (American Lung Association, 2006). This indicates the importance of bridging the gap between assessing exposure levels and the toxicological consequences on human health.

In general, inhalation of particulate matter can cause acute and chronic pulmonary irritation, chronic obstructive pulmonary disease, lung cancer, and exacerbations of existing pulmonary–related conditions (Driscoll et al., 2004; World Health Organization, 2010). Occupational particle exposure can also lead to pneumoconiosis, a restrictive lung disease marked by shortness of breath, which can advance to progressive massive fibrosis or scarring of the lungs. Comorbidities of lung disease due to particle exposure include cardiovascular disease and cancer (Brook et al., 2010). Airborne particles are ubiquitous,
so it is not straightforward to regulate exposure. The source of particles and their properties play a major role in the ability to produce hazardous outcomes upon exposure.

In the United States, environmental particle exposure is regulated by the Environmental Protection Agency (EPA), and occupational particle exposure is regulated by the Occupational Safety and Health Association (OSHA). The EPA establishes 24 hr and annual outdoor air standards for both coarse (2.5–10 microns) and fine (≤ 2.5 microns) particle size fractions, where exposure limits are lower for fine particles (EPA, 2014). The OSHA establishes Permissive Exposure Limits, which are 8 hr time weighted average standards for specific particle compositions and particles otherwise not regulated. They can be found in the National Institute for Occupational Safety and Health (NIOSH) Pocket Guide to Chemical Hazards (NIOSH, 2010). Exposure limits are often lower for particles in respirable (50% cut–point of 4 microns) versus inhalable (50% cut–point of 100 microns) size ranges.

The EPA and OSHA have recognized through regulatory standards that fine and respirable particles are more hazardous than larger size fractions, which is supported by decades of toxicology and epidemiological research (Pope et al., 1995; Brook et al., 2010). Although not officially recognized, recent evidence suggests that there is an additional size fraction, ultrafine (≤ 1 micron), which is an exposure concern. Ultrafine particles (UFPs) are emitted during processes where materials are combusted (e.g. transportation or energy production), heated (e.g. welding) or manipulated with mechanical force (e.g. construction). Studies comparing UFPs to fine particles have shown that toxicity correlates better with particle dose when expressed by surface area rather than mass for particles with the same composition (Donaldson et al., 1998;
Concerns related to UFPs include the ability to deposit efficiently throughout the respiratory tract (Asgharian et al., 2001) and translocate to other organs. UFPs deposited on the nasal membrane have been shown to translocate through the olfactory nerves to the brain (Elder et al., 2006; Oberdörster et al., 2004). The role of macrophages (phagocytic cells) is to internalize foreign particles deposited in the airways and clear them via the mucociliary escalator (Thibodeau and Patton, 2010). However, UFPs deposited in the airways have been shown to diffuse into the interstitial space or blood circulation, indicating they can escape the body’s natural clearance mechanisms (Oberdörster et al., 1992; Nemmar et al., 2002). UFPs have also been shown to be internalized by non–phagocytic cells and trapped in intracellular structures (Grabinski et al., 2014; Untener et al., 2013).

The terms UFPs and nanoparticles (NPs) apply to the same size range (≤ 1 micron), where the latter refers specifically to engineered particles. NPs are an emerging class of materials synthetically produced for a broad range of applications and products, generally referred to as nanotechnology. There were 1638 commercial products voluntarily recorded in 2013 incorporating some form of nanotechnology, a number that has grown exponentially since 2005 (The Project on Emerging Nanotechnologies, 2014). It is important to note that companies are not required to report the use of NPs in their products, so the actual number of commercial products is much higher than what has been recorded. Cumulative government investment in nanotechnology since 2001 now
totals almost $21 billion (National Nanotechnology Initiative, 2015). The widespread application of NPs will undoubtedly lead to exposure during their lifecycle (Maynard and Aitken, 2007). With growing urban populations and increased worldwide growth in nanotechnology, the risk for exposure to UFPs and NPs is continuously growing, and there is potential for a large population to be exposed (Borm and Donaldson, 2007). Proactive toxicology investigations are essential for controlling exposure and mitigating the potential for negative health impacts.

Common methods for studying toxicity include in vivo, or within a living organism and in vitro, or in artificial environments outside living organisms. In general, in vitro experiments carried out on a bench top are overly simplified and do not realistically represent a living organism, requiring in vivo experiments with animal models. However, animal use is costly and requires extended time lengths for data production, and, even then, does not always accurately predict human toxicity (Hartung, 2013). A key advantage of in vitro studies is the possibility to improve the rate of throughput to screen a broad range of new materials being developed (Damoiseaux et al., 2011).

The overarching challenge for establishing in vitro models as a viable tool to assess toxicity is accuracy. In vitro studies are often limited to acute exposures and basic endpoints for cell death, morphology, oxidative stress, and release of inflammatory markers. More advanced in vitro models are under development, with the most promising being the microfluidic platform, where multiple cell types can be incorporated into a well–controlled microenvironment with dimensions and shear rates which correspond to the target organ (Sivagnanam et al., 2013; Huh et al., 2011). Several devices have
demonstrated the ability to mimic whole organ responses in response to disease and insult (Huh et al., 2010; Huh et al., 2012). Although microfluidic technology is promising, it is still under development, and commercial availability of advanced lung models is limited. Further, there are more basic challenges related to in vitro studies, which have yet to be addressed, including dosimetry and relevant exposure.

The fundamental rule in toxicology is that the magnitude of any biological response correlates with dose and time, where nontoxic materials become toxic when delivered in a great enough quantity (Editorial, 2011). Therefore, understanding dosimetry in NP toxicity studies is critical for accurately interpreting results and applying data for hazard assessment. Dosimetry parameters that must be carefully considered include terminology, dose metric, target dose and dose characterization.

Proper terminology is essential to avoid misconceptions in NP toxicity investigations. Particularly for in vitro studies, the term dose is often misused. Dose describes the amount of NPs (i.e. mass, number, surface area) that reach the biological target of interest, while the terms treatment or exposure are more appropriate to describe the amount of NPs delivered to the system.

The selection and standardization of a dose metric has been widely debated, with possible dosing approaches including mass, particle number and surface area (Wittmaack et al., 2007). Currently, the majority of NP toxicity studies report exposure concentration based on a mass. However, many have presented data to argue that the most appropriate metric for comparing biological effects is based on surface area (Duffin et al., 2007; Oberdörster et al., 2005). While there is no standard metric agreed upon by the community as a whole, it is safe to conclude that each metric must be characterized or
calculated, so this information is available as the nanotoxicology field continues to develop. Further, it is essential to report the exposure in the amount (mass, number, surface area) of NPs per surface area of cells or tissue. In many studies, concentrations are expressed per volume, which has little meaning when comparing results across studies where cell culture conditions and treatment volumes are not consistent.

The target dose and exposure range must also be considered for *in vitro* studies. Many published studies expose NPs to biological systems at concentrations that are much higher than any real world situation. Dosimetry models and literature relevant to the biological target should be referenced when establishing a target dose. For example, in case of an inhalation model, theoretical deposition of NPs per surface area in the airway generation of interest can be predicted using a published computational approach, such as the Multiple Path Particle Dosimetry (MPPD) model.

When choosing an exposure concentration, it is important to consider that 100% of the exposed particles will not reach the biological target of interest. When estimating the fraction of an exposure concentration that will become the dose, it is essential to recognize that NP dosimetry is unique from chemical dosimetry in that NP transport is strongly dictated by physicochemical properties (Teeguarden *et al.*, 2007). *In vitro* studies are typically carried out by dispersing particles in cell culture media (Chairuangkitti *et al.*, 2008; Carlson *et al.*, 2008; Grabinski *et al.*, 2007; Hussain *et al.*, 2005). During the dispersion step, complex agglomerates are formed consisting of particles and adsorbed media components, such as proteins (Nel *et al.*, 2009; Lundqvist *et al.*, 2008). Additionally, cell culture media does not have the same properties as bodily
fluids, so agglomeration trends will inevitably be different from in vivo (Han et al., 2012).

Traditional in vitro cell models for toxicity analyses expose cells grown at the bottom of a dish to NPs under static conditions and assume 100% deposition (Teeguarden et al., 2007). Further, in vitro studies investigating the effect of NP size assume equivalent dose (Coradegnhini et al., 2013; Yu et al., 2009; Chithrani et al., 2006). These assumptions are misleading and not useful for hazard assessment in the absence of dosimetry measurements. In a static upright system, NPs are deposited onto cells through a combination of sedimentation (i.e. deposition due to the force of gravity on an object with mass) and Brownian forces (i.e. deposition due to random Brownian motion). The sedimentation force is a function of primary particle size, agglomerate size and density, while the Brownian force only depends on agglomerate size (Hinderliter et al., 2010; Mason and Weaver, 1924).

Progress has been made in understanding NP dosimetry through the theoretical In Vitro Sedimentation and Diffusion Deposition (ISDD) model (Hinderliter et al., 2010). Assumptions in the ISDD model include a static upright system, spherical NP morphology, and uniform agglomerate size and density. Until recently, agglomerate density was calculated based on assumptions related to the packing and fractal structure. More recently, validated methods for measuring the density of NP agglomerates using volumetric centrifugation and corresponding updates to the ISDD model have been developed (Cohen et al., 2014; DeLoid et al., 2014).

In addition to characterizing dosimetry, the exposure route should represent realistic conditions. For example, when particles are exposed to the lung via inhalation,
they are deposited in the gas phase onto cell layers covered in a thin layer of mucus. Therefore, studies at the ALI are appropriate for investigating the inhalation toxicity of particles. Exposure to aerosolized particles has been found to be a more sensitive model than traditional exposure to aqueous particle dispersions, particularly in the assessment of toxicity resulting from inhalation (Lichtveld et al., 2012; Lenz et al., 2009; Shvedova et al., 2008). Further, lung cells prefer to grow at the ALI, and certain cells display differentiated features when grown using this approach (Huh et al., 2010; Wengst and Reichl, 2010). Therefore, as more advanced in vitro cell models are implemented, bringing aerosols in from the gas phase will become a requirement.

A well–designed in vitro aerosol exposure system requires accurate control of aerosol size and concentration with limited stress induced to the cells. Ideally, cells are grown on microporous membranes at an ALI, where cells are fed from the basal (lower) side of the membrane while the apical (upper) part containing cultivated cells is in direct contact with the test atmosphere. Experimental systems for exposing cells directly to aerosol particles have been developed for cell studies over the last decade (Aufderheide, 2005). A less controlled, but straightforward approach is to place cell culture plates with the lid removed into a box where particles are generated or fed (Müller et al., 2010; Rothen–Rutishauser et al., 2009). Another straightforward method, which has been reported, is nebulization of particles to produce a dense cloud which settles as one mass onto cells (Brandenberger et al., 2010; Lenz et al., 2009). This method requires that particles are dispersed in an aqueous media, which is not ideal for many particle materials. It also exposes cells to a large quantity of water, which remains on the apical compartment until removed by pipet.
The most common design is to introduce aerosols to individual wells from central inlet tubes. There are three commercially available models configured in this manner, including VitroCell, Cultex and Minucell, which have been described by Kim et al. (2013), Aufderheide (2008) and Tippe et al. (2002), respectively. All of these systems rely on sedimentation and diffusion to deposit particles and chemicals onto cells, with the exception of later models by Cultex (Aufderheide et al., 2011). Engine exhaust, cigarette smoke, and volatile chemicals are examples of aerosols which may be appropriate for systems using sedimentation or diffusion as mechanisms for dose, as these include a range of particle sizes and chemicals (Knebel et al., 2002; Müller et al., 2010; Aufderheide et al., 2003; Aufderheide, 2005; Pariselli et al., 2009). However, exposures focused specifically on delivering nano–sized aerosols are not appropriate, as particles in this size range do not deposit efficiently out of a gas stream onto a substrate by diffusion or gravity.

Aerosol dosimetry has been investigated for sedimentation and diffusion forces (Fujitani et al., 2014; Comouth et al., 2013; Desantes et al., 2006; Tippe et al., 2002). Results showed that particles in the submicron size range deposit with efficiency of less than 10%, with a dip in efficiency for particles in the range of 0.1 to 1 micron (Fujitani et al., 2014; Comouth et al., 2013; Desantes et al., 2006). The deposition of larger particles occurs by sedimentation, so deposition efficiency depends on the density of the particles. Interestingly, aerosols generally exhibit fractal structure with reduced density than the parent material, further reducing deposition by sedimentation (Comouth et al., 2013). Chamber configuration, such as distance between the nozzle discharge and cell layer
played a minor role, where reducing the distance from 4 to 0.5 mm improved diffusional deposition by about 2% (Fujitani et al., 2014).

Several recent studies have recognized the use of electric fields to deposit charged particles more efficiently using electrostatics (Hawley et al., 2014; Jeannet et al., 2014; Holder and Marr, 2013; Aufderheide et al., 2011; Grigg et al., 2009; Volckens et al., 2009; Savi et al., 2008; Stevens et al., 2008). One unique approach was to charge particles in a corona chamber, then repel the charged particles into a grounded cell culture dish (Hawley et al., 2014; Volckens et al., 2009). In another approach, charged particles were distributed horizontally past inverted cell culture inserts suspended within an electric field, forcing particles to deposit (Holder and Marr, 2013). The more common approach has been to modify the typical exposure method used for diffusional and sedimentation deposition with individual tubes delivering aerosol to individual cell cultures (Jeannet et al., 2014; Aufderheide et al., 2011; Savi et al., 2008). In this case, the tube and a counter electrode placed below the cell layer are conductive, with one at high voltage, and the other electrically grounded, to create an electric field encompassing the cell culture. This method is attractive because it accommodates typical cell culture inserts and is adaptable for different types of test environments. A limitation is that the ideal electrostatic parameters as a function of particle size has not been thoroughly investigated.

The final consideration for realistic exposure is to generate representative test environments. Particles that are stable in aqueous media are typically aerosolized using a nebulizer or electrospray (Brandenberger et al., 2010; Kim et al., 2010). Particles which are not stable in aqueous media can be aerosolized from a dry powder (Baron et al.,
2008). Additionally, a wide range of NP materials can be synthesized directly in the gas phase (Biskos et al., 2008; Jaworek and Sobczyk, 2008; Chiang and Sankaran, 2007). Although there are many techniques available for aerosolizing particles in the laboratory, the exposure environment where aerosols are originally generated and emitted will always be the most representative of a realistic exposure because the interaction of particles with gases and other airborne matter can be rapid and dynamic (Lichtveld et al., 2012; Pankow et al., 1997; Warren and Seinfeld, 1985).
Aims of this study

Despite the many challenges remaining for *in vitro* nanotoxicology, there are solutions available to establish this method as an appropriate tool for assessing exposure risk. The key research challenges the present study addresses include (1) elimination of the sedimentation force in submerged exposure to simplify dosimetry, (2) parametric investigation of electrostatic parameters on aerosol dosimetry, and (3) development of a system that can be used to interface *in vitro* aerosol exposure with realistic exposure environments. Addressing these gaps promises to improve repeatability and relevance of *in vitro* studies and produce meaningful data that can be used to identify key exposures that must be mitigated for protecting human health.

The specific aims are as follows:

1. Compare *in vitro* particle dosimetry under traditional static conditions and a novel dynamic condition.
2. Understand the effect of chamber design and process parameters on deposition of aerosol NPs onto cells grown at the ALI.
3. Design a portable scale–up chamber for exposing cells to aerosols generated in a laboratory environment or in a field environment at the site of exposure.
CHAPTER II

THE EFFECT OF SHEAR FLOW ON NANOPARTICLE AGGLOMERATION AND DEPOSITION IN IN VITRO DYNAMIC FLOW MODELS

1. Introduction

Regardless of the exposure route of interest (e.g. inhalation, ingestion, systemic translocation), traditional in vitro cell models for toxicity analyses typically expose cells grown at the bottom of a dish to NPs under static conditions and assume 100% deposition (Teeguarden et al., 2007). This assumption is misleading and does not provide an accurate hazard assessment in terms of dosage. In a static upright system, NPs are deposited onto the cells through a combination of sedimentation (i.e. deposition due to the force of gravity on an object with mass) and Brownian forces (i.e. deposition due to random Brownian motion). The sedimentation force is a function of agglomerate size and density, while the Brownian force only depends on agglomerate size (Hinderliter et al., 2010, Mason and Weaver, 1924). Recent studies have validated methods for measuring the density of NP agglomerates using analytical and volumetric centrifugation (Cohen et al., 2014; DeLoid et al., 2014). Nonetheless, eliminating sedimentation as a factor in dosimetry models would simplify predictions for particle transport.

A recent study comparing cellular orientation (upright versus inverted) in in vitro cultures showed that dosimetry is more predictable in an inverted orientation because NPs deposit onto cells by only Brownian forces (Cho et al., 2011). However, some cell lines do not adhere well when inverted, and standard cell culture plates are not amenable to reproducibly being inverted. Therefore, alternate approaches are required to address
issues related to NP deposition. Recently, Toy and colleagues (2011) demonstrated that NPs, with a larger density than media, carry momentum in the direction of flow and do not readily escape flow due to gravity. Therefore, deposition under dynamic conditions is dominated by diffusion. Therefore, we hypothesize that introducing NPs to cells in a flowing stream of media will reduce the effect of sedimentation during NP exposure, simplifying the dosimetry.

In order to test our hypothesis, we carried out a systematic comparison of NP agglomeration and deposition in static versus dynamic exposure systems. Brain cells were cultured on a porous membrane suspended in a cell culture insert as a representative cell model. We utilized transport modeling to approximate the shear rate experienced by the cells in dynamic conditions to evaluate physiological relevance. We also used transport kinetics to show that NP behavior is governed by both gravity and diffusion forces in static conditions and only diffusion in dynamic conditions. The theory was validated by treating endothelial cells with three different diameters (5, 10, and 30 nm) of gold (Au) NPs under static or dynamic conditions and measuring agglomerate size and deposition after a 24 hr exposure.

2. Materials and Methods

2.1 In vitro model

The in vitro model was developed using 24–well plates containing Transwell™ inserts with 0.4 micron pore polyester membranes and 0.33 cm² growing area (Corning Inc., Corning, NY). Two holes (0.2 cm apart) were drilled in the lid of the plate over each well and connectors were inserted into the holes. Two tubes (internal diameter 0.16 cm,
outer diameter 0.32 cm) were inserted into the luminal side of the transwell at a height of 0.380 cm from the cell layer (Fig. 2.1A). The tubes were connected to a peristaltic pump (Idex Inc., Lake Forest, IL) to circulate flow (Fig. 2.1B, top).

2.2 Shear stress calculations

A three–dimensional geometry of the in vitro model described above was set up in COMSOL (Multiphysics® version 4.2a, Burlington, MA) (Fig. 2.1B, bottom). The computational fluid dynamics module was implemented with laminar flow physics, which was justified by calculating the Reynolds number. The Reynolds number ($Re$) is a dimensionless number that characterizes the flow regime, such as laminar or turbulent (Bird et al., 2002). It is proportional to the ratio of inertial forces to viscous forces in the flowing fluid and was calculated using the following relationship:

$$Re = \frac{\rho L v}{\mu}$$

(2.1)

where $\rho$ is the media density, $L$ is the characteristic length of the system, $v$ is the flow velocity and $\mu$ is the media dynamic viscosity. We used reported values for density and dynamic viscosity for the same cell culture media type used in the present study (RPMI–1640, American Type Culture Collection (ATCC), Manassas, VA) supplemented with 10% fetal bovine serum at 37°C, which were 1.0 g/cm$^3$ and $7.1 \times 10^{-4}$ Pa·s, respectively (Folger et al., 1978). The characteristic length was set equal to the diameter of the transwell. The velocity was set equal to the flow rate divided by the cross sectional area of the inlet tube. Due to the complex nature of the system, the velocity is expected to vary throughout the transwell volume. However, this is not expected to greatly affect the order
of magnitude of the Reynolds number. The Reynolds number at each flow rate is reported in Table 2.1.

A no–slip boundary condition (i.e. zero velocity, no viscous stress) was assumed at the walls in the simulation geometry. The flow rate was specified at the inlet, and the outlet pressure was set to zero to generate flow into and out of the simulated transwell volume. The simulation was completed using the steady state solver. The streamlines were plotted (Fig. 2.2A,C,E).

The shear caused by flow at the cell layer is in the x–y direction (co–planar to the cells). The shear rate at the cell layer ($\dot{\gamma}_{z=0}$) is the rate of deformation and is proportional to the velocity gradient along the cell layer ($\partial v / \partial z|_{z=0}$). The shear stress at the cell layer ($\tau_{z=0}$) depends on the shear rate and the, where $\tau_{z=0} = \mu \dot{\gamma}_{z=0}$. The average, minimum and maximum shear rate at the cell layer output by COMSOL, in addition to the shear stress calculated using media viscosity are reported in Table 2.2. The shear rate gradient at the cell layer (z=0) was plotted (Fig. 2.2B,D,F).

2.3 Gold nanoparticles (Au NPs)

Spherical Au NPs with a reported diameter of 30 nm were purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD), and smaller Au NPs with diameters of close to 5 and 10 nm were synthesized. To synthesize Au NPs, gold (III) chloride trihydrate (HAuCl₄·3H₂O) was purchased from MP Biomedicals (Santa Ana, CA) and trisodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O) and sodium borohydride (NaBH₄) were purchased from Sigma–Aldrich (St. Louis, MO).
Spherical Au NPs with a diameter of around 10 nm were synthesized by a modified version of the Turkevich method (Polavarapu and Xu, 2009). Briefly, 100 mL aqueous solution of 1 mM HAuCl$_4$ was brought to boil with vigorous stirring. Next, 10 mL of 58.2 mM Na$_3$C$_6$H$_5$O$_7$ was added. The solution was boiled for 10 min, and then stirred rapidly for an additional 15 min without heating. The solution was then brought to boil again and 5 mL of additional Na$_3$C$_6$H$_5$O$_7$ solution was added, after which the solution was stirred vigorously for an additional 10 min without heating. The final concentration of Na$_3$C$_6$H$_5$O$_7$ was higher than the published method and the second heating/cooling steps were added in an effort to stabilize the particles more quickly, thus reducing their size from ~13 to ~9–10 nm.

Spherical Au NPs with a diameter close to 5 nm were synthesized as previously described (Murdock, 2010). Briefly, stock solutions of 0.4% w/v (10 mM) HAuCl$_4$·3H$_2$O, 1.0% w/v (34 mM) Na$_3$C$_6$H$_5$O$_7$·2H$_2$O, and 0.5% w/v (132 mM) NaBH$_4$ were freshly prepared and kept on ice. Next, 1.25 mL of 0.4% HAuCl$_4$ was added to 50 mL of de-ionized H$_2$O with vigorous stirring for one minute. Then, 500 μL of 0.5% NaBH$_4$ was added rapidly while continuing to stir vigorously for 1 min. Finally, 200 μL of 1.0% Na$_3$C$_6$H$_5$O$_7$ was added to the solution and stirred for another minute.

NaBH$_4$ was used in the synthesis of 5 nm, but not 10 nm NPs, because it reduces the Au salt faster than Na$_3$C$_6$H$_5$O$_7$ (Polte et al., 2010). However, NaBH$_4$ is known only to reduce the salt and does not act as a capping agent (Seoudi and Said, 2011). On the other hand, citrate both reduces and caps the NPs. We assume that all of the NaBH$_4$ was reacted and then removed during post–synthesis washing steps and did not influence the ensuing cell experiments.
2.4 Characterization of Au NPs

The primary particle diameters and morphology of the Au NPs were characterized using transmission electron microscopy (TEM) on a Hitachi H–7600 (Chiyoda, Tokyo, Japan). NP suspensions were drop cast and dried on formvar/carbon film–coated copper grids (Electron Microscopy Sciences, Hatfield, PA). The NP diameters were determined by measuring at least 100 particles using Imaging Processing and Analysis in Java software (ImageJ, available from the National Institutes of Health, www.imagej.nih.gov). The optical absorbance of the Au NPs which shows the surface plasmon resonance band was obtained with a Varian Cary 300 ultraviolet–visible (UV–vis) absorbance spectrometer (Agilent, Santa Clara, CA). The hydrodynamic diameter \((d_h)\) and polydispersity index (PdI) were measured by dynamic light scattering (DLS), and the zeta (ζ) potential was measured using laser Doppler electrophoresis (LDE), both on a Malvern Zetasizer Nano (Worcestershire, UK).

The extent of agglomeration of Au NPs dispersed in biological media was assessed by DLS. Au NPs at each diameter were dispersed in complete culture medium at a concentration of 5 \(\mu\)g/mL, and the \(d_h\) was either measured immediately at room temperature or after exposure to static, low flow (1.5 mL/min), or high flow (6 mL/min) conditions for 24 hr at 37°C. The \(z\)-average \(d_h\) \((n=6 \text{ for each treatment group})\) for static and low flow conditions were represented as the mean ± standard deviation. The peak \(d_h\) \((n=3 \text{ for each treatment group})\) for all three conditions were plotted. The PdI is calculated based on a Gaussian distribution fit to the correlation data assuming a single particle size mode. Therefore, the PdI value is not relevant for bimodal or polydisperse samples, such as NPs dispersed in biological media, so it was not reported.
2.5 Functionalization of glass coverslips with aminosilane

Glass coverslips were functionalized with aminosilane as previously described (Ungureanu et al., 2010). Briefly, German borosilicate glass coverslips (5 mm diameter, #1 thickness, Warner Instruments, Hamden, CT) were rinsed with 100% ethanol three times, then submerged in 5% solution of (3-aminopropyl) triethoxysilane (Sigma–Aldrich) and ethanol for 15 min. After functionalization, the coverslips were rinsed five times with 100% ethanol. The coverslips were allowed to air dry completely under sterile conditions before being used for deposition experiments.

2.6 Cell lines and culture conditions

Cell lines of the blood brain barrier (astrocytes and endothelial cells) were chosen as a representative in vitro model. NPs exposed via inhalation have been shown to enter blood circulation (Nemmar et al., 2002). Also, Au NPs have been shown to cross the blood brain barrier (Sonavane et al., 2008). Therefore, this model was originally developed to characterize the translocation of NPs across endothelial barriers. Brain astrocytes (C8–D30, CRL–2534, ATCC) and endothelial cells (bEnd.3, CRL–2299, ATCC) were grown according to the supplier’s instructions in Dulbecco’s Modified Eagle’s Medium (ATCC) supplemented with 10% fetal bovine serum (ATCC). Cells were maintained in a humidified cell culture incubator at 37°C and 5% carbon dioxide.

For all experiments, the abluminal (lower) side of the transwell membrane was coated with Matrigel™ (BD, San Jose, CA). Briefly, matrigel was diluted in serum–free media to a concentration of 476 µg/mL. The abluminal side of the transwell membrane was coated with 50 µL of the diluted matrix, and the transwells were incubated for 4 hr at
37°C. Astrocytes were seeded at 2.8×10⁴ cells/membrane on the matrigel–coated abluminal side of the transwell membrane. The inverted transwells were incubated for 3 hr in a humidified cell culture incubator at 37°C and 5% carbon dioxide. The transwells with astrocytes were then transferred to a 24–well plate and incubated. After 24 hr, endothelial cells were seeded on the luminal (upper) side at a density of 1.0×10⁵ cells/membrane. Cell culture media was changed every other day, and Au NP exposure was completed after eight days in culture.

2.7 Conditions for NP exposure

NPs were dispersed in cell media at a concentration of 5 µg/mL and exposed under static and dynamic conditions for 24 hr. The concentration was chosen based on a bio–distribution study by Sonavane et al. (2008) where mice were exposed intravenously to 15 and 50 nm Au NPs. The doses were defined relative to the mass of the mouse. In order to obtain estimates for the mass per volume in the blood, we assumed about 5.5 mL of blood per 100 g mouse (Mouse Genome Database, 2014). The mice were administered about 4000 µg/mL, and at the end of the 24 hr exposure, ~0.2 µg/mL remained in the blood. NPs are cleared in 30–60 min by the liver, kidney, and lung (Niidome et al., 2006). Therefore, we conclude that the 5 µg/mL concentration is within a realistic concentration range for a 24 hr exposure.

2.8 Quantification of Au NP deposition and uptake

Functionalized coverslips or co–cultures were exposed to Au NPs of different diameters in static or dynamic conditions for 24 hr (n=3 for each treatment group). For
the deposition assay, the coverslips were removed after the exposure period and transferred to a conical tube. For the cell assay, the cells were washed on the membrane three times with warm 1× phosphate buffered saline (PBS), and then the membrane was cut out of the transwell and transferred to a conical tube containing 500 μL of deionized water. Since the washing steps may not remove all the surface bound NPs, we refer to the results as NPs interacting with the cells rather than internalized by the cells. The conical tube containing the membranes were frozen at –80°C, thawed, and mixed with 75 μL of 1% triton–X 100 (Fisher Scientific, Waltham, MA). For both the deposition and cell interaction assays, aqua regia at a 1:3 v/v ratio of nitric acid (HNO₃, Sigma–Aldrich) to hydrochloric acid (HCl, Fisher Scientific) was added to the tubes. Next, 3 μL of internal standard was added (Perkin Elmer, Waltham, MA), and the volume was brought up to 1.5 mL using deionized water. The Au content in the samples was measured using inductively coupled plasma – mass spectrometry (ICP–MS, Perkin Elmer Nexion 300D). Calibration plots were obtained by injecting Au standard solutions (0.002, 0.050, 0.100, and 0.300 μg/mL in 1% HNO₃ and 3% HCl) at a flow rate of 1.0 mL/min.

2.9 Statistical analysis

Significant differences at p<0.05 were determined by a one–way Analyses of Variance with a Bonferroni's Multiple Comparison Test using GraphPad Prism software (GraphPad Software, La Jolla, CA).
3. Results

3.1 Model setup and shear rate calculation

A dynamic in vitro model was tested by perfusing media through the upper portion of individual transwells in 24–well plates (Fig. 2.1).

The Reynolds number was calculated using equation (2.1). The Reynolds numbers ranged from 57.4 at low flow to 230 at high flow (Table 2.1). In a straight round pipe, the transition from laminar to turbulent flow typically occurs for Reynolds numbers between 1500 and 2100, and creeping flow occurs for Reynolds numbers < 0.1 (Bird et
al., 2002). The transition Reynolds numbers are expected to be unique in our system, since it deviates from a straight round pipe.

Table 2.1. Reynolds Numbers as a Function of Flow Rate

<table>
<thead>
<tr>
<th>Flow rate (mL/min)</th>
<th>1.50</th>
<th>3.00</th>
<th>6.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet velocity (cm/s)</td>
<td>1.26</td>
<td>2.52</td>
<td>5.04</td>
</tr>
<tr>
<td>Reynolds Number</td>
<td>57.4</td>
<td>115</td>
<td>230</td>
</tr>
</tbody>
</table>

The streamlines through the transwell and shear rate at the cell layer were obtained and plotted at low flow (1.5 mL/min), medium flow (3.0 mL/min) and high flow (6.0 mL/min) conditions (Fig. 2.2). The low flow rate condition was the upper limit for disrupting the cell monolayers, and the high flow rate condition was the maximum flow rate deliverable by the peristaltic pump. The medium flow was included to better demonstrate the trend in shear rate as a function of flow rate. The simulated velocity streamlines within the transwells at all flow rates indicate some mixing (Fig. 2.2A,C,E). The maximum shear rate was concentrated around the cells located at the discharge of the inlet for both flow rates (Fig. 2.2B,D,F). Using the simulation, the average shear rate at the cell layer was found to be 3.07, 18.1 and 82.9 s⁻¹ at the low, medium and high flow rates, respectively (Table 2.2).
Figure 2.2. Flow simulation for the dynamic flow model. The velocity streamlines (A,C,E) and shear rate (B,D,F) at the cell layer, z=0 are shown for flow rates of 1.5 mL/min (A–B), 3.0 mL/min (C–D) and 6.0 mL/min (E–F). The units for shear rate are in 1/s and units for the dimensions are in mm.
Table 2.2. Simulation Parameters in the Dynamic Flow Model

<table>
<thead>
<tr>
<th>INPUT</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (mL/min)</td>
<td>1.50</td>
<td>3.00</td>
<td>6.00</td>
</tr>
<tr>
<td>Inlet velocity (cm/s)</td>
<td>1.26</td>
<td>2.52</td>
<td>5.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OUTPUT</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure drop (Pa)</td>
<td>2.11</td>
<td>4.72</td>
<td>11.52</td>
</tr>
<tr>
<td>Shear rate ($\dot{\gamma}_{z=0}, s^{-1}$)</td>
<td>Average</td>
<td>3.07</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>9.50</td>
<td>71.4</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>$3.09 \times 10^{-3}$</td>
<td>$9.73 \times 10^{-3}$</td>
</tr>
<tr>
<td>Shear stress ($\tau_{z=0}, \text{dynes} \cdot \text{cm}^{-2}$)</td>
<td>Average</td>
<td>0.0218</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>Maximum</td>
<td>0.0674</td>
<td>0.507</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
<td>$2.19 \times 10^{-5}$</td>
<td>$6.90 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

3.2 NP characterization

Au NPs dispersed in water were characterized by TEM, UV–vis absorbance spectroscopy, and DLS (Fig. 2.3, Table 2.3). The morphology of the primary particles for all three samples was found to be spherical (Fig. 2.3), and the primary particle diameter ($d_p$) were found to be 4.4, 9.0 and 27.6 nm (Table 2.3). The optical absorbance spectra revealed a peak at ~520 nm for all samples corresponding to the well-known surface plasmon resonance of Au, with a slight shift in the peak towards longer wavelengths as the particle diameter increased, in agreement with previous reports (Hu et al., 2006).
Figure 2.3: Characterization of primary Au NPs. TEM images (A–C) and respective UV–Vis absorption plots (D–F) for Au NPs with sizes of 4.4 nm (A,D); 9.0 nm (B,E); 27.6 nm (C,F).

The size of the NPs in water was assessed by TEM and DLS. The $d_p$ determined via TEM was slightly smaller than the $d_h$ characterized using DLS due to the presence of citrate and formation of a hydration layer in water (Table 2.3). The agglomeration of the NPs in cell culture media was assessed by DLS. We observed a relatively large increase in size for the 5 nm Au NPs in media, followed by 10 nm, then 30 nm (Table 2.3). The $\zeta$ potential for 5, 10, and 30 nm Au NPs exhibited negative values ranging from $-40$ to $-32$ mV when dispersed in water, and $-9$ to $-8$ mV when dispersed in cell culture media. The negative charge for Au NPs stabilized by citrate in water and decrease in the value closer to zero upon dispersion in media supplemented with serum is consistent with previously reported measurements (Mukhopadhyay et al., 2012).
Table 2.3. Characterization of Au NPs in Water and Media

<table>
<thead>
<tr>
<th>$d_p$ (nm)</th>
<th>$d_{h, z-ave}$ (nm)</th>
<th>PdI</th>
<th>$\zeta$ potential (mV)</th>
<th>$d_{h, z-ave}$ (nm)</th>
<th>$\zeta$ potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 ± 0.7</td>
<td>6.1 ± 0.1</td>
<td>0.30 ± 0.01</td>
<td>−32.0 ± 1.6</td>
<td>37.0 ± 0.9</td>
<td>−9.3 ± 1.1</td>
</tr>
<tr>
<td>9.0 ± 0.1</td>
<td>10.8 ± 0.7</td>
<td>0.13 ± 0.01</td>
<td>−32.9 ± 0.2</td>
<td>32.4 ± 0.5</td>
<td>−8.3 ± 0.8</td>
</tr>
<tr>
<td>27.6 ± 2.1</td>
<td>28.3 ± 0.4</td>
<td>0.17 ± 0.01</td>
<td>−40.2 ± 1.8</td>
<td>31.5 ± 0.2</td>
<td>−8.6 ± 0.8</td>
</tr>
</tbody>
</table>

3.3 NP agglomeration in static versus dynamic conditions

A decrease in the average $d_h$ for NP agglomerates was observed under dynamic conditions as compared to static conditions (Table 2.4). This decrease in size was more evident for 5 nm than 10 nm and 30 nm Au NPs. The results here are unique from the results for NPs dispersed in media at room temperature shown in Table 2.3.

Table 2.4. Characterization of Au NPs in Static and Dynamic Conditions

<table>
<thead>
<tr>
<th>$d_p$ (nm)</th>
<th>static</th>
<th>dynamic (1.5 mL/min)</th>
<th>$\Delta d_h$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$d_{h, z-ave}$ (nm)</td>
<td>$d_{h, z-ave}$ (nm)</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>50.2 ± 1.15</td>
<td>29.4 ± 0.7</td>
<td>20.8 ± 0.6 p &lt; 0.01</td>
</tr>
<tr>
<td>9.0</td>
<td>31.9 ± 2.37</td>
<td>25.9 ± 0.5</td>
<td>6.0 ± 1.0 p &lt; 0.01</td>
</tr>
<tr>
<td>27.6</td>
<td>37.6 ± 5.07</td>
<td>30.3 ± 0.5</td>
<td>7.3 ± 2.1 p &lt; 0.01</td>
</tr>
</tbody>
</table>
Given limitations of DLS in accurately assessing the degree of agglomeration for high polydispersity, we also analyzed the peak $d_h$ (Fig. 2.4A). A peak was observed for all samples of NPs in media near 9 nm, which we attribute to the presence of serum proteins. To follow trends in agglomeration, we observed the second peak. The trend was similar to that for average $d_h$, which was smaller under dynamic (1.5 mL/min) than static conditions. The peak $d_h$ continued to decrease when the flow rate was increased from 1.5 to 6.0 mL/min, which is the maximum flow rate allowable in our dynamic setup.

![Diagram of NP erosion under flow conditions](image)

**Figure 2.4. NP erosion under flow conditions.** A. Peak hydrodynamic diameter data for Au NPs dispersed in complete culture medium at a concentration of 5 μg/mL and exposed to static (S), low flow (LF; 1.5 mL/min) or high flow (HF; 6.0 mL/min) for 24 hr. Samples were analyzed immediately after exposure period using DLS. Results are representative of three experiments. A peak was observed for all samples of NPs in media near 9 nm, which we attribute to the presence of serum proteins; B. Schematic depicting erosion of NP agglomerates under flow conditions.
3.4 Dose metrics: mass, number and surface area concentration

We chose to expose cells to three different size NPs at the same mass concentration \(c_M\) measured by ICP–MS. This is one of the most common approaches employed in nanotoxicity investigations, since it is straightforward to measure mass directly. However, data have been presented to argue that surface area and particle number are more relevant metrics for expressing exposure concentrations (Duffin et al., 2007; Oberdörster et al., 2005). Therefore, in order to fully characterize the dose metric, the particle number concentration \(c_N\) and surface area concentration \(c_{SA}\) were calculated based on the following relationships:

\[
c_N = 6c_M / \pi d_p^3 \rho_p \quad (2.2)
\]

\[
c_{SA} = \pi d_p^2 c_N \quad (2.3)
\]

where \(\rho_p\) is the density of Au and \(d_p\) is the primary particle diameter determined using TEM. The concentrations were multiplied by the volume \(V_m\) of media in the transwell and divided by the surface area \(SA\) of the cell layer. It is important to represent the dose per surface area and not volume, since these parameters are not consistent across studies. The results show the drastic difference in particle number and surface area delivered to the cells between the different particle sizes (Table 2.5).

<table>
<thead>
<tr>
<th>(d_p) (nm)</th>
<th>(c_M \times V_m/SA) (µg/cm²)</th>
<th>(c_N \times V_m/SA \times 10^{-10}) (#/cm²)</th>
<th>(c_{SA} \times V_m/SA) (cm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>4.55</td>
<td>528</td>
<td>3.21</td>
</tr>
<tr>
<td>9.0</td>
<td>4.55</td>
<td>61.7</td>
<td>1.57</td>
</tr>
<tr>
<td>27.6</td>
<td>4.55</td>
<td>2.14</td>
<td>0.51</td>
</tr>
</tbody>
</table>
3.5 NP deposition and association with cells in dynamic versus static conditions

Since Au is known to have high affinity for amine groups (–NH₂), the deposition of Au NPs was evaluated using aminosilane–functionalized glass coverslips (Ungureanu et al., 2010). Functionalized coverslips were placed on the upper side of the transwell membrane and exposed to NP dispersions prepared in media at 5 μg/mL for 24 hr under static or low flow dynamic conditions (1.5 mL/min). Au NP deposition was quantified using ICP–MS and found to be greater under static versus dynamic conditions for both the 5 nm and 10 nm NPs with a statistical significance of p<0.05 (Fig. 2.5A). There appeared to be great deposition under static compared to dynamic conditions for the 30 nm NPs, but this difference was not statistically significant.

Acellular conditions do not accurately represent bio–nano interactions, since NP interactions with cells usually result in an energy–dependent internalization processes (Grabinski et al., 2014; Untener et al., 2013; Shukla et al., 2005). Therefore, we treated a co–culture of brain endothelial cells and astrocytes with static or continuous flow of media containing different diameters of Au NPs at 5 μg/mL for 24 hr, and quantified the NP interaction with cells using ICP–MS (Fig. 2.5B). The cellular interactions followed similar trends as the deposition studies. There was a statistically significant increase in interaction for 5 and 10 nm NPs under static versus dynamic conditions. There appeared to be a greater amount of interactions for 5 and 10 nm than 30 nm NPs under static conditions, but this was not statistically significant.
3.6 Theoretical NP deposition

In order to understand the differences between static and dynamic exposures, the deposition of Au NP agglomerates was modeled. For the static case, the transport process was modeled by the following equation which governs steady state transport of small particles (Mason and Weaver, 1924):

\[
\frac{\partial c_N}{\partial t} = D \frac{\partial^2 c_N}{\partial z^2} - v_s \frac{\partial c_N}{\partial z}
\]  \hspace{1cm} (2.4)

where \( D \) is the particle diffusion coefficient, \( v_s \) is the sedimentation velocity, \( t \) is time and \( z \) is vertical direction (see Fig. 2.2B,bottom). This partial differential equation was solved using the theoretical ISDD model (Hinderliter et al., 2010).

Based on equation (2.4), it is evident that the transport of particles depends on both diffusion and sedimentation. The former is a function of agglomerate size, while the latter is a function of both agglomerate size and density. The agglomerate density was
calculated as a function of fractal dimension using a packing factor of 0.634 for random packing (Cohen et al., 2014).

For the dynamic condition, the critical force allowing NPs to escape the flow and deposit onto the cells is diffusion (Toy et al., 2011). Sedimentation is not considered because a particle with sufficient mass experiences momentum due to the flow of media, and gravity does not overcome this force.

The Péclet number \( (Pe) \) is a dimensionless number that indicates the ability for a particle to deviate from a streamline, i.e. whether diffusion is a probable mechanism for deposition (Davis and Davis, 2003). It is defined as the ratio of the rate of convection to the rate of diffusion and was calculated using the following relationship:

\[
Pe = \frac{vd_h}{D}
\]  

We used the peak \( d_h \) values for this calculation. A summary of the calculated \( Pe \) are shown in Table 2.6. The results indicate that diffusion dominates for the primary 4.4 nm particles and convection dominates for primary 9.0 and 27.6 nm particles, in addition to all of the particle agglomerates. However, this calculation is approximate, and literature results have shown that diffusive transport could still be important even when \( Pe \) is up to two orders of magnitude greater than those calculated in the present study (Toy et al., 2011). We note that another potential mechanism for particle deposition is interception, which occurs when a particle following a streamline comes within one particle radius of the substrate (Xie et al., 2013). The Au NPs used in the present study are spherical and on the order of 25–35 nm in diameter, so this would require streamlines to flow within nanometers of the cell layer. Therefore, it is unclear that interception
contributes because the size of the transwell is large, and wall effects should not be as important as diffusion.

Table 2.6. Péclet Number as a Function of Particle Size at 1.5 mL/min

<table>
<thead>
<tr>
<th>$d_p$ (nm)</th>
<th>4.4</th>
<th>9.0</th>
<th>27.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Pe$ for $d_p$</td>
<td>0.38</td>
<td>1.59</td>
<td>15.0</td>
</tr>
<tr>
<td>peak $d_h$ (nm)</td>
<td>54.8</td>
<td>63.2</td>
<td>70.2</td>
</tr>
<tr>
<td>$Pe$ for peak $d_h$</td>
<td>59.1</td>
<td>78.6</td>
<td>96.9</td>
</tr>
</tbody>
</table>

The flux of NPs was estimated using a simplified mass balance equation, assuming that the NP flux ($J$) occurs only in the vertical $z$–direction and particle concentration does not change in the direction of flow (Cussler, 1997):

$$- \frac{\partial J}{\partial z} = D \frac{\partial^2 c_M}{\partial z^2} = 0$$  \hspace{1cm} (2.6)

Equation (2.6) can be solved analytically by applying boundary conditions. Assuming that at the center of the parabolic flow, 0.5$h$, the concentration is equal to that of the bulk, and, at the cell layer, the concentration is zero (i.e. NPs bind to the cells quickly), the appropriate boundary conditions are:

At $z=0$, $c_M = 0$ \hspace{1cm} (2.7)

At $z=0.5h$, $c_M = 5 \mu g/cm^3$ \hspace{1cm} (2.8)

By applying these boundary conditions, the flux at the cell layer, $J_{z=0}$, or concentration per surface area per time, can be calculated using the following equation:

$$J_{z=0} = \frac{D}{0.5h} \left( c_{M,z=0} - c_{M,z=h} \right)$$  \hspace{1cm} (2.9)
The deposition mass per surface area for the static condition was calculated using the ISDD model and is shown as a function of effective density, with a horizontal line indicating the value for average deposition measured using ICP–MS at each NP diameter (Fig 2.6A–C). For the dynamic condition, the flux was determined using equation (2.9) multiplied by the 24 hr exposure time for each NP diameter (Fig. 2.6D). A horizontal line is included to note the average value measured using ICP–MS. The analytical results show excellent correlation with the experiments for the dynamic condition.

**Figure 2.6.** Theoretical NP deposition for static and dynamic conditions using peak hydrodynamic diameter. A–C. Theoretical deposition after static exposure for 5 nm (A), 10 nm (B) and 30 nm Au NPs (C) as a function of effective density; D. Theoretical deposition after dynamic exposure. The measured deposition is represented by a horizontal line for reference. The dotted lines indicate standard deviation of measured deposition data.
4. Discussion

The shear rates at the cell layer ranged from $3.09 \times 10^{-3}$ to 9.50 s$^{-1}$ at low flow rates (1.5 mL/min), to $3.14 \times 10^{-2}$ to 376 s$^{-1}$ at high flow rates (see Table 2.2). To test whether the shear in our system is representative of shear related to changes in cell phenotype in vitro or physiological values, we compared the shear rate in the dynamic model to published results. In vitro changes to endothelial cell phenotype mimicking physiological conditions, including cell alignment with flow and formation of tight junctions, were reported at 0.2–5.0 Pa (shear rates 67–1667 s$^{-1}$) (Seebach et al., 2000).

To compare to physiologically relevant values, the viscosity for blood was used to convert between shear rates and shear stress. The viscosity of blood varies with shear rate, but is on the order of 3 mPa·s, at shear rates greater than 10 s$^{-1}$ (Rosencranz et al., 2006). The reported range for physiologically relevant shear stress is 0.3–10.5 Pa. Specifically, physiological values were reported to be 1.5–2.0 Pa (shear rate ~500–667 s$^{-1}$) for wall shear stress in human arteries, 0.2 Pa (shear rate ~50 s$^{-1}$) for higher diameter post–capillary venules, and 4.8 Pa (shear rate ~1600 s$^{-1}$) for small capillaries (Koutsiaris et al., 2007; Ku, 1997). Another study reported physiological shear stress from about 1.7 to 10.5 Pa (shear rates 587–3515 s$^{-1}$) in human arterioles (Koutsiaris et al., 2013).

Therefore, we conclude that the shear rates achieved using the transwell based dynamic setup were not likely to induce significant biological response from cells in culture. Hence, this system does not mimic physiological conditions of blood flow, but instead suggests that a broad range of adherent cell types can be treated using a dynamic exposure condition to simplify dosimetry.
Au NPs were chosen for this study because they are relatively stable and inert in a biological environment, i.e. the primary particles retain their morphology and size distribution and do not release ions (Auffan et al., 2009; Daniel and Astruc, 2004). The NPs used in this study exhibited spherical morphology and uniform size distribution (Fig. 2.3). The $d_h$ for NPs diluted in water was slightly larger than the primary size measured using TEM due to the formation of a hydration layer. When dispersed in media, the NP agglomerate size was found to increase with decreasing primary particle size (Table 2.3). The change in ζ potential observed when the NPs were dispersed in water versus media indicates adsorption of serum proteins on the NP surface and has been previously observed for Au NPs in culture media supplemented with serum proteins (Mukhopadhyay et al., 2012; Alkilany et al., 2010). NPs are known to agglomerate in the presence of salts due to a reduction in the electronic double layer around each particle, allowing for adhesion through van der Waals forces (Lyklema et al., 1999). In culture media containing serum proteins, NP agglomerates are stabilized by adsorbing serum proteins, forming a ‘protein corona’ (Arvizo et al., 2012; Nel et al., 2009; Lundqvist et al., 2008).

The NP agglomeration was measured using DLS after 24 hr at each condition. The results indicated a reduction in agglomerate size with increasing flow rate (Table 2.4, Fig. 2.4A). This can be attributed to the shear forces experienced by NPs under flow and subsequent erosion (depicted in Fig. 2.4B), which has been previously observed in mixing applications and is characterized by the detachment of small fragments from the outer surface of the agglomerates (Scurati et al., 2005). Erosion increases with shear until
a minimum particle size is achieved. Velocity streamlines within the transwells indicate mixing, which may play a role in the agglomeration behavior of the NPs (Fig. 2.2A,C,E).

At higher shear rates, as reported for physiological conditions, NP agglomeration is likely impacted by erosion to a much greater extent. Thus, erosion may play a role in the dose rate and toxicity of NPs \textit{in vivo} and should be considered in future \textit{in vitro} studies investigating the interaction of NPs with endothelial cells. The agglomeration and biomolecule adsorption kinetics will be further altered due to the differences in formulation of blood \textit{versus} cell culture media. This complicates the ability to mimic \textit{in vivo} results using \textit{in vitro} systems, which is also a topic of interest for future investigations.

The deposition and cell interaction of NPs was consistently lower in dynamic \textit{versus} static conditions (Fig. 2.5). Transport equations were used to elucidate the mechanism by which differences in deposition occurred for the static \textit{versus} dynamic conditions (Fig. 2.6). For the static condition, theoretical predictions are highly dependent on agglomerate density, and without a direct measurement, it is difficult to predict dose. For the dynamic condition, the theoretical predictions and empirical data were strongly correlated, indicating that the assumptions used to simplify the transport equations were valid. Under flow conditions, deposition occurs almost exclusively by diffusion, which is a function of only particle agglomerate size distribution. Therefore, the deposition in the dynamic condition is more predictable. Further, although the deposition and dose under the dynamic condition appear to be relatively low, the values are likely more predictive of realistic dose rate in an \textit{in vivo} condition, especially for the case of environmental exposures, where material tends to accumulate slowly over time.
5. Conclusions

In conclusion, we showed that treating an *in vitro* cell culture with NPs under flow conditions reduced the size of NP agglomerates *versus* static conditions. We also showed that NP deposition and cellular interaction were reduced under flow conditions, due to the near elimination of the sedimentation force. Therefore, the dose under flow condition can be estimated from NP agglomerate size, which greatly simplifies the dosimetry. Simplified dosimetry has significant implications for improving repeatability in nanotoxicity studies. The results shown here were also published in Nanotoxicology (Grabinski *et al.*, 2015).
CHAPTER III
SIMULATIONS OF SUBMICRON AEROSOL DEPOSITION AT AN ALI FOR IN VITRO TOXICOLOGY

1. Introduction

Exposure of cells grown at the ALI to aerosolized particles has been found to be a more accurate model than liquid dispersions, particularly in the assessment of toxicity resulting from inhalation (Lichtveld et al., 2012; Lenz et al., 2009; Shvedova et al., 2008). Further, lung cells prefer to grow at the ALI and certain cells display differentiated features when grown using this approach, making it necessary to bring in particles from the gas phase (Huh et al., 2010; Wengst and Reichl, 2010). An additional advantage of aerosol studies is that on line measurements via electrical mobility allow aerosol particle size distributions to be monitored in parallel to cell exposure (Comouth et al., 2013). Therefore, it is possible to predict the deposition rate more accurately for aerosol than traditional static exposure to aqueous dispersions.

Experimental systems for exposing cells at an ALI to aerosol particles have been designed and reported over the last decade (Raemy et al., 2011; Brandenberger et al., 2010; Müller et al., 2010; Lenz et al., 2009; Paur et al., 2008; Aufderheide, 2005; Knebel et al., 2002). In general, these systems rely on sedimentation and diffusion forces for deposition, which are insufficient to efficiently deposit submicron sized particles from a gas flow (Melling, 1997). Parametric aerosol dosimetry has been investigated for sedimentation and diffusion forces (Fujitani et al., 2014; Comouth et al., 2013; Desantes
et al., 2006; Tippe et al., 2002). Results showed that deposition of particles in the submicron size range deposit with efficiency of a maximum of 10%, with the majority of deposition occurring at less than 2% efficiency (DeLoid et al., 2014; Comouth et al., 2013; Desantes et al., 2006). Several recent studies addressed the low deposition efficiency by incorporating electric fields to enhance deposition (Saffari et al., 2012; Aufderheide et al., 2011; Grigg et al., 2009; Volckens et al., 2009; Savi et al., 2008; Stevens et al., 2008). However, a systematic study of deposition as a function of electrostatic parameters has not yet been carried out.

The objective of this study is to investigate the deposition of submicron particles in the presence of electric fields in an in vitro aerosol exposure system using simulations and experiments. We developed a simulation using multiphysics software to model deposition as a function of various parameters. The simulation was validated by comparing results with previously reported analytical solutions. Experiments were then carried out in a chamber constructed with a similar geometry to the simulations. Overall, the goal is to combine modeling with controlled experiments to address challenges in developing in vitro models for aerosol exposure at an ALI and provide preliminary guidelines for controlling the dose of submicron aerosols which has been a significant challenge to date.

2. Materials and Methods

2.1 In vitro aerosol deposition chamber

An in vitro aerosol deposition chamber was designed to expose cells at an ALI to aerosol particles (Fig. 3.1A). The chamber was constructed by modifying a commercial
horizontal diffusion chamber (Harvard Apparatus, Holliston, MA). Modifications were supported by Frazer–Nash USA (Lowell, IN). A stainless steel inlet tube was inserted at the center of the apparatus, and the space between the tube discharge end and ALI could be varied between 0.1–0.8 cm. The diameter of the inlet tube was designed to flare towards the end, in order to provide a more homogenous distribution of flow across the cell layer (Mülhopt et al., 2009; Aufderheide, 2008; Savi et al., 2008; Bitterle et al., 2006). A conductive screen could be optionally inserted at the end of the inlet tube. The diameter of the screen was designed to be close to that of the cell layer to ensure a uniform parallel electric field across the ALI (Savi et al., 2008). The region between the ALI and end of the inlet tube was the focus of the simulations.

A cell culture insert containing a porous membrane with a surface area of 1.12 cm$^2$ was positioned below the inlet tube at the base of the apparatus (Fig. 3.1A). For deposition experiments, the cell culture membrane was replaced with a glass coverslip, which was sealed in place using clear nail polish. A stainless steel plate was placed below the insert, 0.085 cm below the ALI and electrically grounded. The space between the electrode and ALI was perfused with cell culture media.

2.2 Simulations

Finite element modeling was performed to study the transport and deposition of aerosol particles using COMSOL multiphysics software. A two–dimensional mesh of the in vitro chamber was rendered in Cartesian coordinates (Fig. 3.1B). The mesh sequence was physics–controlled with extra fine element size. The simulations were carried out in two steps. In the first step, the laminar flow and AC/DC modules were used to establish
the air flow and electric field conditions in the chamber. In the second step, particle tracking with the fluid flow module was implemented to obtain particle trajectories under the established conditions. The trajectory calculations were based on a force balance on each particle using local continuous phase conditions as the particle moved through the flow. If a particle contacted the ALI, it was assumed to deposit.

The laminar flow and direct current (DC) conditions were established using the stationary solver, while alternating current (AC) conditions and particle tracking were established using the time–dependent solver. The time step was set to 0.005 s, and the simulation was run for 25 s to allow for all of the particles to travel through the system. The generalized alpha solver was used with default settings and free time stepping in all cases except for the time dependent solver used to generate AC fields in which case the time stepping was set to intermediate rather than free. Intermediate time stepping is required for simulations with a time–dependent boundary condition. Additional details for the simulated flow and electric field, in addition to particle trajectory calculations can be found in Appendix A (Figs. A.1 and A.2).

Figure 3.1. Schematic and simulation geometry for aerosol exposure at ALI. A. Schematic of the chamber and deposition interface; B. Multiphysics model simulation geometry. The units in (B) are in mm.
2.3 Particles

Surface enhanced fluorescence (SEF) particles with manufacturer reported size of 90 nm silver (Ag) core and a 15–20 nm silica shell encapsulating rhodamine fluorophore (excitation/emission 555/578 nm) were purchased from nanoComposix (San Diego, CA). The plasmon resonance of the Ag core matched the excitation wavelength of the fluorophore to enable imaging of individual particles by fluorescence microscopy. The particles were diluted from their original concentration of 1 mg/mL to 0.1 mg/mL in isopropyl alcohol for electrospraying (discussed in section 2.4).

As it is critical to characterize primary and dispersed particles used for biological experiments, the morphology and primary particle size distributions of the primary SEF particles in solution were characterized using a Hitachi H–7600 TEM at the Nanoscale Engineering Science and Technology Laboratory, University of Dayton. Samples were prepared by pipetting particle dispersions onto formvar/carbon film–coated copper grids (Electron Microscopy Sciences) and drying in air. A representative image and histogram for particle size distribution are shown in Appendix B (Fig. B.1). The average \( d_p \) was determined by measuring 100 particles using ImageJ. The core and shell diameters were also measured and averaged for 100 particles using ImageJ and found to be 96 nm and 44 nm, respectively, 6 nm and 24 nm larger, respectively, than reported by the manufacturer. The overall particle density was estimated by a weighted average of the densities based on the volume occupied by silica and silver with respect to the overall volume of the particle (Table 3.1).

The \( d_h \) of the particle dispersion was measured in triplicate by DLS, and the conductivity and \( \zeta \) potential were measured in triplicate using LDE (Zetasizer Nano ZS).
The value for $d_h$ was 36 nm larger than the $d_p$, indicating the formation of a liquid layer around the particles. The PdI was 0.21, which is at the upper end of the range indicates a dispersion is monodisperse (Cho et al., 2013). The $\zeta$ potential was close to zero, indicating that the particles carried a neutral charge. The conductivity was three orders of magnitude lower than the conductivity of buffers commonly used for electrospray, but this did not affect the ability to aerosolize the particles.

Table 3.1. Characterization of SEF Particles

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average primary diameter (nm)</td>
<td>140.2 ± 7.04</td>
</tr>
<tr>
<td>Average primary core diameter (nm)</td>
<td>96.0 ± 7.38</td>
</tr>
<tr>
<td>Approximate particle density (g/cm$^3$)</td>
<td>5.17</td>
</tr>
<tr>
<td>Hydrodynamic diameter (nm)</td>
<td>176.7 ± 1.80</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.21 ± 0.003</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td>Conductivity (mS/cm)</td>
<td>0.0087 ± 0.0002</td>
</tr>
<tr>
<td>Aerosol count mean diameter (nm)</td>
<td>148.7 ± 2.86</td>
</tr>
</tbody>
</table>

2.4 Aerosolization and Deposition Experiments

A flow diagram of the overall experimental setup is schematically shown in Figure 3.2. The particles were aerosolized from a liquid dispersion by electrospray (Electrospray Aerosol Generator 3480, TSI, Shoreview, MN). The applied voltage was
adjusted to develop a steady cone jet (Chen et al., 1995). The flow rate was a function of the capillary inner diameter (0.004 cm), length of the capillary (25 cm), pressure drop across the capillary (3.7 psi) and viscosity of the particle dispersion (0.02 poise) and was estimated to be 193 nL/min. The highly charged droplets released from the cone jet were dried by a 1 L/min flow of 95% air and 5% carbon dioxide, and neutralized by a sealed Polonium–210 radioactive source. The final aerosol flow was distributed in three ways: (1) to a scanning mobility particle sizer (SMPS, NanoScan 3910, TSI, Shoreview, MN)) to measure the particle size distribution on–line, (2) to the in vitro chamber to deposit the particles, and (3) to a high efficiency particle air (HEPA) filter to relieve excess pressure.

The electric field was applied with a function generator (BK Precision, Yorba Linda, CA) in series with a high voltage power amplifier (Trek, Medina, NY). The voltage was continuously monitored using an oscilloscope. Aerosol particles were introduced into the in vitro chamber at a flow rate of 20 mL/min using a vacuum pump (GilAir Plus, Sensidyne, Mühlheim, Germany). The flow rate was calibrated before and after each experiment using a digital bubble flow meter (Gilian Gilibrator II, Sensidyne). The space between the electrode and ALI was perfused with cell culture media, which was maintained at 37°C using an inline heater at a flow rate of 370 mL/min. The chamber was also heated by contacting the base with an aluminum plate heated to 37°C. The temperature for both the in–line heater and heat plate was controlled using a standard temperature controller (Warner Instruments, Hamden, CT).
Since the SEF particles were diluted in isopropyl alcohol which can be toxic to cells, we also separately measured the concentration of the isopropyl alcohol entering the \textit{in vitro} chamber using a photoionization detector (MultiRae, Honeywell, San Jose, CA).

After each deposition experiment, fluorescence microscopy was used to acquire a minimum of 12 images per sample at the center, middle, and edge of the substrate. The average particle count in each micrograph was divided by the surface area of the micrograph and exposure time to determine the experimental particle flux. The experimental deposition efficiency calculations are described in detail in Appendix B.
2.5 Cell Culture and Assays

Human lung cells (A549, CCL–185, ATCC, Manassas, VA) were cultured according to manufacturer instructions. Cells were seeded onto Snapwell cell culture inserts with a 12 mm diameter and 0.4 µm pore polyester membrane (Corning, Corning, NY), at a density of $5.0 \times 10^4$ cells/cm$^2$. The basal and apical media was replaced on day two, and the apical media was removed and not replaced on day four to place the cells at the ALI. On day five, the insert was removed from the holder and placed in the base of the chamber with fresh media. The incubator control was fed fresh media to coordinate with the time the insert was transferred to the chamber.

After exposure for 1 hr in the chamber to air or particles, the cell insert was transferred back into the holder. The Alamar Blue Cell Viability Assay (Life Technologies, Carlsbad, CA) was completed according to manufacturer instructions. Exposures to air and particles were each completed twice and results were compared to individual incubator controls. After exposure to Alamar Blue, the same membranes were washed with warm PBS, treated with 0.16 µM AlexaFluor® 350 Phalloidin (Life Technologies) diluted in PBS to stain the actin, rinsed with PBS, and then treated with 1 µM SYTOX Green Nucleic Acid Stain (Life Technologies) diluted in PBS to stain the nuclei. The membranes were washed again with PBS, cut out of the cell culture insert, and then mounted onto a glass slide using Prolong Gold (Life Technologies). The membranes were imaged the next day using a standard inverted fluorescence microscope (IX71, Olympus, Center Valley, PA), and images were captured using QCapture Pro software (QIMAGING, Surrey, BC, Canada).
2.6 Relevance of deposition to dose

In order for the *in vitro* chamber to be used as a model for assessing toxicity of particles, the deposition should represent a realistic dose for the cells or organ of interest. The Multiple Path Particle Dosimetry (MPPD) model is a computational model (version 2.1), which can be used to calculate theoretical deposition efficiencies for aerosols in the respiratory tract (Anjilvel and Asgharian, 1995). In this model, deposition occurs by diffusion, sedimentation, and impaction (Asgharian *et al.*, 2001).

Here, the human Yeh/Schum symmetric model was used with default conditions, which is based on morphological data for humans published by Yeh and Schum (1980). Properties for the SEF particles were included as input parameters, including aerodynamic diameter and density. Default conditions for the MPPD model included: Human Yeh/Schum symmetrical model; uniform expansion; functional residual capacity = 3300 mL; upper respiratory tract volume = 50 mL; constant exposure; upright body orientation; breathing frequency = 12 breaths per min; tidal volume = 625 mL; nasal breathing scenario. The values used for regional surface area and volume are shown in Table 3.2. The most recent version of the software is available for download (www.ara.com/products/mppd.htm).

Table 3.2. Lung Regional Dimension Values

<table>
<thead>
<tr>
<th>Region</th>
<th>Surface Area, $SA_R$ (cm$^2$)</th>
<th>Volume, $V_R$ (cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheobronchial</td>
<td>3,451</td>
<td>161</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>650,000</td>
<td>4,710</td>
</tr>
</tbody>
</table>
3. Results and Discussion

3.1 Deposition of particles without electric field

The velocity profile obtained from simulations is shown in Appendix A (Fig. A.1). Particle tracking simulation results as a function of particle size at a flow rate of 20 mL/min are shown in Fig. 3.3A. Here, we assumed only diffusion and sedimentation forces and no electric field. The results indicate that 10 nm particles are deposited with an efficiency of 1% while 60 nm particles follow the air flow and do not deposit. The larger 1000 nm and 6000 nm particles deposit more effectively with efficiencies of 10% and 100%, respectively.

In order to better understand the particle–size–dependent simulation results, we developed an analytical model based on a simple mass balance assuming a semi–closed chamber and well–dispersed particles and obtained the following relationship for the deposition efficiency, $\epsilon_{theor}$:

$$
\epsilon_{theor} = 1 - \exp\left(-\frac{y}{h}\right)
$$

(3.1)

where $y$ is the negative vertical distance traveled by the particle due to diffusion or gravity over a residence time, $\tau$, and $h$ is the height of the inlet tube discharge above the ALI. The derivation for equation (3.1) is detailed in Appendix C.

The distance $y$ is given by $\sqrt{2D\tau}$ for diffusion and $v_s\tau$ for gravity where $D$ is the particle diffusion coefficient and $v_s$ is the sedimentation velocity. The equations used to calculate $D$ and $v_s$ are well–known and are described in Appendix A. In brief, diffusion increases with decreasing particle size and sedimentation increases with particle size and density.
The residence time, $\tau$, was obtained by dividing the volume of the chamber, $V_{ch}$, by the volumetric flow rate. The chamber volume was estimated by assuming a truncated cone between the tube end and the deposition area, $V_{ch} = \frac{1}{3} \cdot \pi \cdot (r_0^2 + r_0r + r^2) \cdot h$, where $r_0$ is the radius of the inlet tube discharge and $r$ is the deposition radius (see Fig. 3.1). Dimensions and residence times for published results were used to generate Figure 3.3C (Fujitani et al., 2014).

The analytical solution shows excellent correlation with the simulation results for deposition (Fig. 3.3B). The simulations were limited to particles more than 10 nm in size because of the increasingly small time step required in COMSOL to accommodate the rapidly increasing diffusion force (note that diffusion is inversely proportional to particle size and directly proportional to the Cunningham correction factor), which became computationally extensive. Regardless, the agreement demonstrated in Figure 3.3B confirms the validity of the simulations and suggests that either approach can be used to predict particle deposition with no external force, depending on which is easiest, for example based on the chamber configuration.

Further analysis of the simulation and analytical results indicates that there is no region where diffusion and gravity forces are both important, and the deposition efficiency for particles in the range of 10–200 nm is <1%. Overall, these results are in agreement with previously reported calculations by Comouth and colleagues (2013), who reported that several days of continuous exposure would be required to achieve lowest–observed–adverse–effect–levels for submicron silica particles. It is important to note that our results are for Ag particles and particles with lower density or fractal agglomerates will exhibit much lower deposition by sedimentation (Comouth et al., 2013).
An analytical model was again developed to validate the simulation results (Fig. 3.5C,D). For the case with the screen, the assumptions of a semi–closed chamber and well–dispersed particles remain valid and equation (3.1) was used. However, the distance $y$ is now equal to $v_E \tau$, where $v_E$ is the electrical drift velocity. The electrical drift velocity is equal to the product of the electrical mobility, $Z_p$, and electric potential, $V$, divided by the discharge height, $h$. The expression for $Z_p$ is included in Appendix A. Briefly, $Z_p$ increases with decreasing particle size and increases with the number of charges carried by the particle.

For the case without a screen, the assumption of a semi–closed chamber is no longer valid. Therefore, the deposition efficiency varies linearly with electrical drift (see Appendix C for derivation). In addition, $h$ may be larger than with a screen because the distance from the deposition interface where particles begin to respond to the electric field extends inside the inlet tube. We found that the best fit to the simulations was obtained with a factor of 1.35 in the expression for the deposition efficiency:

$$\epsilon_{\text{ theor}} = \frac{y}{1.35h}$$  \hspace{1cm} (3.2)

Note that the value for $\tau$ is also affected by assuming a larger chamber volume for the case with no screen. Since the factor 1.35 was added to fit the analytical model to the simulation results, equation (3.2) is technically a semi-empirical model.

The simulation and analytical results were found to correlate well for both the conditions with and without the screen. This is important because to our knowledge, the literature has not yet provided a strategy for choosing a voltage and geometry to achieve optimal deposition of aerosols at an ALI.
Figure 3.3. Theoretical effect of size on particle deposition efficiency with no external force. A. Particle tracking simulations for 10 nm, 60 nm, 1000 nm and 6000 nm Ag particles; B. Deposition efficiency for simulations compared to the analytical solution for Ag particles in the present study; C. Deposition efficiency for simulations and experiments from Fujitani et al. (2014) compared to the analytical solution derived in the present study using the density for polystyrene particles.

Deposition of submicron particles with no electric field has been reported in some aerosol exposure studies (Kim et al., 2013; Diabaté et al., 2008; Cheng et al., 2003). In some cases, the particle dose was not measured, so chemical vapors present in the aerosolized material could have caused observed toxicity, leading to the assumption of particle deposition. In the cases where particle dose was measured, one explanation for the discrepancy may be that particle deposition measurements by mass or fluorescence intensity were skewed by larger size fractions present in the aerosol. Another explanation
may be humidity which can lead to hygroscopic growth of the particles and increase the diameter sufficiently for sedimentation to occur (Biskos et al., 2006).

3.2 *Simulated electric field*

An electric field was applied between the inlet tube and the ALI to enhance the deposition of submicron aerosol particles. The shape of the electric field is important as it can affect the particle trajectories. In the past, studies have incorporated a screen at the end of the inlet tube to produce a uniform parallel electric field (Savi et al., 2008). We carried out simulations of an electric field both with and without a wire screen (Fig. 3.4). As expected, a uniform parallel electric field was generated when a screen was used (Fig. 3.4A). In this case, the desired electric field is roughly equal to the applied voltage divided by the tube height (Appendix A). When the screen was removed, the electric field extended into the inlet tube (Fig. 3.4B). In this scenario, the relationship between applied voltage and electric field is more complicated and difficult to model. Therefore, applied voltage and tube height were reported rather than electric field strength.

![Figure 3.4. Simulated electric field with or without a wire screen across the discharge at an applied voltage of 0.4 kV. A. Electric field with a screen; B. Electric field without a screen. The units are in kV/cm.](image-url)
3.3 Deposition of charged particles in an electric field

The introduction of electric fields to deposit particles electrostatically, i.e. electrostatic precipitation, can overcome the low deposition of submicron particles by diffusion and sedimentation (Dixkens and Fissan, 1999). Electrostatic precipitation is based on the principle that charged particles are attracted and migrate to a substrate with an opposite bias. The tendency of the particles to move in the electric field, their electrical mobility, is a function of particle size and number of charges, but not density, so the calculations here are applicable to a range of particle compositions.

The simulation was performed for 60 nm particles, each carrying 1 charge, as a function of applied voltage with or without a screen (Fig. 3.5). The results confirm the expected result that deposition efficiency increases with increasing voltage (Dixkens and Fissan, 1999). Interestingly, the presence of the screen produced gaps in deposition at the ALI, which is not desired when dosing cells for toxicology (Fig. 3.5A). The spacing between particles was less apparent when the screen was removed (Fig. 3.5B). Notably, the voltage potential required to achieve 100% deposition of charged particles was much lower without the screen, which we suggest is caused by penetration of the electric field into the inlet tube.
Figure 3.5. Theoretical effect of applied voltage on particle (60 nm Ag) deposition with or without a wire screen across the discharge at \( h = 0.4 \text{ cm} \). A. Particle tracking simulation with a screen for 0.4 kV (left) and 0.7 kV (right); B. Particle tracking simulation without a screen at 0.16 kV (left) and 0.32 kV (right); C. Deposition efficiency with a screen; D. Deposition efficiency without a screen.

To compare modeling results with experiments, SEF particles were aerosolized by electrospray and characterized using a SMPS. The average aerosol diameter was about 150 nm (Table 3.1). The aerosolized SEF particles were then deposited onto 12 mm glass coverslips at different DC potentials both with and without the screen. The deposited particles were analyzed by fluorescence microscopy for each condition. Representative micrographs taken at the center of the coverslip from lowest to highest voltage (–0.3 kV, 0.9 kV and –1.5 kV) with the screen removed are shown in Appendix B (Fig. B.1).
The experimentally-derived deposition efficiencies were compared to those obtained from the analytical model by using equation (3.1) or (3.2) for the condition with or without the screen, respectively. In the simulations and analytical solutions, we assumed that 100% of the particles carried one or two charges. In experiments, particles were neutralized by a bipolar charger, so the size–dependent charging probabilities based were included in the analytical models (Appendix D, Fig D.5).

With a screen, the experimental results demonstrated a very slight (2%) increase in deposition efficiency in the presence of an electric field, although the increase was not statistically significant using a t–test with a two–tailed distribution (p=0.2). However, comparing to the analytical model, it becomes apparent that the deposition efficiencies are much lower than predicted (Fig. 3.6A). This may be due to particles being trapped by the screen. When the screen was removed, there was good agreement between the experimental and analytical results (Fig. 3.6B). As expected, the amount of particles deposited increased with increasing voltage. The large standard deviation at the highest voltage (~1.5 kV) is due to focusing, which will be described in section 3.4.

![Figure 3.6](image.png)

*Figure 3.6. Experimental deposition efficiency of SEF particles. A. Deposition efficiency with a screen; B. Deposition efficiency without a screen. The error bars represent the standard deviation between 12 micrographs.*
3.4 Deposition of charged particles in an electric field – focusing

Focusing is the tendency of particles to electrostatically deposit towards the center of the deposition interface with increasing voltage. This is a concern in cell studies because uneven deposition will affect dose uniformity. A representative simulation output at high deposition voltage demonstrated focusing for 60 nm particles each carrying two charges (Fig. 3.7A).

Figure 3.7B shows simulation results for the deposition radius as a function of applied voltage. The deposition radius decreased with increasing voltage. The deposition radius decreased at a slower rate for the condition with a screen versus without a screen. Therefore, when the screen is removed, extra care must be taken to avoid focusing. In order to describe this analytically, we compared our results the model previously developed by Camata (1998): 

\[
V = \frac{Qh}{2\pi Z_p r(r - r_0)}
\]

for a simple outward radial flow where \(Q\) is flow rate and \(r\) is the deposition radius. We did not see correlation, which is likely because this equation assumes the deposition radius is greater than the radius of the inlet tube, whereas our simulation results suggest that the deposition radius could be less than that of the inlet tube discharge radius.

To verify the simulation results, experiments were carried out using the 150 nm SEF particles (Fig. 3.7C). The deposition was characterized by counting the number of particles deposited at \(V = 0.4, 1.2\) or \(2.0\) kV in different radial zones. As expected, the difference between the number of particles in zones 1 (center of the substrate) and 3 (near the edge of the substrate) was greatest at the highest applied voltage.
Figure 3.7. Focusing and deposition radius. A. Particle tracking simulation for 60 nm particles carrying two charges with no screen at 0.34 kV; B. Theoretical deposition radius as a function of applied voltage for 60 nm particles carrying two charges with or without a screen; C. Experimental deposition flux for 150 nm particles as a function of applied voltage. The percent difference between the outermost and innermost zones are noted above the bars.

3.5 Effect of inlet tube height and flow rate on particle deposition

Other important experimental design features of the in vitro exposure system include the inlet tube height and flow rate. The effect of inlet tube height without an electric field was previously demonstrated by Fujitani et al. (2014), where deposition efficiency increased with decreased distance between the inlet tube discharge and ALI. In
the presence of an electric field, the analytical deposition calculated using equation (3.1) was not affected by inlet tube discharge height (Fig. 3.8A). However, the simulation suggests a slight decrease in efficiency with decreasing height (Fig. 3.8B). This may be related to the slight decrease in electric field strength with decreasing height that we observed in the simulations (see Fig. A.2).

![Figure 3.8. Theoretical effect of inlet tube height on particle deposition with electrostatics at a flow rate of 20 mL/min and target electric field of 1.75 kV/cm; A. Theoretical deposition efficiency as a function of particle size and inlet tube height; B. Particle tracking simulation for 60 nm particles at $V = 0.35$ kV; $h = 0.2$ cm (left) and $V = 1.4$ kV; $h = 0.8$ cm (right).]

As expected, deposition efficiency was found to increase with decreasing flow rate both with and without an electric field ($V = 0.1$ kV), respectively (Fig. 3.9A,B). This was due to an increase in the residence time of the particles in the chamber. A consequence of decreasing the flow rate is that the flux of particles to the interface will also decrease. Based on a constant aerosol concentration of $1.0 \times 10^5$ particles/cm$^3$, we calculated the flux of particles as a function of flow rate and obtained the overall deposition rate by multiplying the deposition efficiency and normalizing to the deposition surface area (Fig. 3.9C,D).
The results highlight that the particle flux and deposition efficiency counteract one another. In fact, the highest flow rate studied here produced a marginally higher particle flux when no external electric field was applied and a much greater particle flux in the presence of an electric field. Consequently, the deposition efficiency may not be the best metric for an in vitro aerosol exposure system. Instead, it is more appropriate to choose a value close to the maximum flow rate that the cells can tolerate for the desired exposure time to maximize the amount of articles available for deposition.
3.6 Deposition of charged particles in the presence of an electric field – AC frequency

In addition to particle deposition by DC electric fields which have been discussed up to now, we also investigated the application of AC electric fields. AC fields have been previously reported to avoid ion migration in the culture medium and cells (Savi et al., 2008). In addition, the switching field could enable both negatively and positively charged particles to be deposited, increasing efficiency. Particle tracking simulations indicate that at low frequencies (1 Hz), 100% deposition efficiency occurs, but at higher frequencies (> 5 Hz), the trajectories follow the air stream lines and there is no deposition (Fig. 3.10A–D). At low frequencies, a focusing effect is also observed. This has been previously observed for deposition of 200 nm polystyrene particles with a 1 Hz square wave in a chamber with a similar geometry to that presented here (Savi et al., 2008).

The deposition efficiency is shown as a function of frequency in Fig. 3.10E. The deposition is highest at a frequency of 1 Hz and rapidly decreases with increasing frequency. A rigorous analytical model to validate simulation results for the AC condition is more complicated than the case of DC due to the time dependent variation in the electric field. For this reason, we performed a scaling analysis to support our results. We assumed that the deposition efficiency is likely related to the number of cycles occurring during the residence time, \( \tau \). During one cycle, the particle experiences motion towards the ALI for half of the time, followed by motion away from the interface for the other half. In the case of an electric field that switches from positive to negative, the frequency and residence time must have time scales such that the attracting force on the particles is present long enough for particles to deposit onto the substrate. Therefore, it is expected that the deposition efficiency would be optimal when there is one complete
cycle during the residence time. For our geometry and conditions, we estimate a residence time of 1.07 s which corresponds to an AC frequency of 1 Hz, consistent with the observations.

In order to confirm this trend experimentally, SEF particles were deposited at frequencies of 0, 1, and 5 Hz at a constant applied voltage of 0.9 kV (Fig. 3.10F). The experiments showed the highest deposition efficiency at 1 Hz, followed by 0 Hz, then 5 Hz, in good agreement with the simulation results. Importantly, the higher deposition efficiency at 1 Hz as compared to the DC condition of 0 Hz is related to both positively and negatively–charged particles depositing in a switching field. We note that the error bars are large due to focusing effects (the deposition efficiency in the center was 0.26 ± 0.05, while near the edges it was 0.08 ± 0.03).

Figure 3.10. Effect of AC frequency on particle deposition. A–D. Particle tracking simulation for 1 Hz (A); 5 Hz (B); 15 Hz (C); 60 Hz (D); E. Simulated deposition efficiency; F. Empirical deposition efficiency.
These results are promising for improving deposition efficiency of bipolar charged particles. However, there are still challenges, including focusing and the sharp drop in efficiency with increasing frequency. Additionally, the effect of the frequency wave shape was not investigated, which could play another role in deposition. Further, the deposition in an AC field with the screen removed would be an interesting topic for future study.

3.7 Dose relevance

The goal in cell culture studies is not to maximize deposition efficiency, but to deposit close to realistic conditions. The deposition flux was calculated using a dosimetry model for inhalation exposure to demonstrate the relevance of these data in the respiratory tract. The exposure concentration was set to values ranging from 0.01–1.0 mg/m$^3$ for 150 nm particles with a calculated effective density of 5.17 g/cm$^3$ (Table 3.1). The values for regional deposition fraction ($\chi_R$) in the pulmonary and tracheobronchial regions were found to be 0.151 and 0.09, respectively. The deposition flux ($J_R$) in each region was found by assuming monodisperse spherical particles using the following equation: 

$$ J_R = \chi_R \cdot C_{exp} \cdot V_R / S A_R \cdot 12 / \rho_p / V_p $$

where $C_{exp}$ is the exposure concentration in mass per volume, $V_R$ is regional volume, $S A_R$ is the regional surface area, $\rho_p$ is the particle density, and $V_p$ is the particle volume. The fraction of inhaled mass deposited is per breath and multiplied by 12 for the number of breaths per minute. The $S A_R$ and $V_R$ for the tracheobronchial and pulmonary regions are shown in Table 3.2.

The results show that the regional deposition flux is within an order of magnitude of deposition conditions in the chamber (Table 3.3). The minimum and maximum
deposition flux in the present study was $4.0 \times 10^5$ #/cm$^2$/min ($V = 0.3$ kV, $h = 0.4$ cm, with screen) and $3.1 \times 10^6$ #/cm$^2$/min ($V = 1.5$ kV, $h = 0.4$ cm, no screen), respectively. Therefore, the flux achieved in the chamber is greater than that predicted for realistic exposures. In most *in vitro* studies, the cumulative dose can be compared to a realistic exposure, but not the dose rate. The cumulative dose was calculated by multiplying the flux by a time period corresponding to a work day (Table 3.3). *In vitro* exposure times required to achieve a work day dose were also calculated based on a dose rate of $1.0 \times 10^6$ particles/cm$^2$/min and found to be reasonable (~0.006–23 min). Pulsed fields may be useful for achieving realistic dose rates over the course of several days to months, which would be possible using differentiated *in vitro* models that are stable over long periods of time and produce a mucus layer to protect them from shear produced by exposure to air (Geiser and Lang, 2007).

Table 3.3. MPPD Output and Exposure Calculations for SEF Particles

<table>
<thead>
<tr>
<th>Exposure concentration (mg/m$^3$)</th>
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<tbody>
<tr>
<td></td>
<td>0.01</td>
<td>10.0</td>
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<tr>
<td><strong>Region</strong></td>
<td></td>
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<tr>
<td>Pulmonary</td>
<td>Flux (#/cm$^2$/min)</td>
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<tr>
<td></td>
<td>13</td>
<td>49</td>
</tr>
<tr>
<td>Tracheo-bronchial</td>
<td></td>
<td></td>
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<tr>
<td>Pulmonary</td>
<td>Cumulative deposition, 8 hr (#/cm$^2$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.0×10$^3$</td>
<td>2.4×10$^4$</td>
</tr>
<tr>
<td>Tracheo-bronchial</td>
<td>Required <em>in vitro</em> exposure (min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.006</td>
<td>0.02</td>
</tr>
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</table>
3.8 Deposition of charged particles onto cells

To test the in vitro exposure system for cell studies, we exposed cells to clean air flow (no aerosol particles) of 20 mL/min (18 mL/min/cm²) with a sinusoidal AC electric field at 1 Hz for 1 hr, and assessed cell viability immediately after exposure (Fig. 3.11). No effect on cell viability was observed compared to a control that was kept in a standard incubator. In support, results from a previous study using a co–culture comprised of a bronchial cell–line and differentiated monocytes observed a reduction in viability for 64 mL/min/cm², but not 21 mL/min/cm² after 1 hr (Diabaté et al., 2008). Therefore, a target of 10–20 mL/min/cm² should be safe for short–term aerosol exposure (≤ 1 h).

We then exposed cells to SEF particles in an air flow of 18 mL/min/cm² with a sinusoidal AC electric field at 1 Hz for 1 hr, and similarly assessed cell viability immediately after exposure (Fig. 3.11B). The results indicate no difference from the incubator control. The concentration of isopropyl alcohol in the aerosol was measured to be 1.1 parts per million, which is two orders of magnitude lower than the NIOSH recommended short–term exposure limit of 500 parts per million. The actin and nuclei were stained blue and green respectively for the chamber control and SEF exposed cells (Fig. 3.11C–D). Red SEF particles are shown in the cytoplasm of the latter.
Figure 3.11. Cell exposure to filtered air or SEF particles with electrostatics. A. Cell viability after 1 hr in the exposure chamber exposed to air flow (18 mL/cm$^2$) compared to control maintained in the incubator, measured using the Alamar Blue assay (n=3); B. Fluorescence micrograph of cells exposed to 1 hr air flow (18 mL/cm$^2$); Fluorescence micrograph of cells exposed for 1h to SEF particles at 1 Hz at 1.2 kV and 0.4 cm discharge height. The actin is stained blue using AlexaFluor 350–phalloidin and Nuclei were stained green using SYTOX green.

From a biological perspective, one might be suspicious of incorporating electric fields in an in vitro exposure system. However, the high voltage and low current required for electrostatic precipitation has been reported to have no effect on cell behavior (Savi et al., 2008). Regardless, as advanced in vitro models and exposure systems continue to develop, it would be interesting to complete more extensive studies to investigate the effect of these fields on cellular response.
Another approach to deposition is thermophoresis, where particles migrate from higher to lower temperatures. Since the sample plate must be maintained near 37°C, the inlet air must be heated. In a previous study, a temperature difference of 40°C yielded very little improvement in deposition for particles from 1 nm to about 50 nm, and no improvement for particles >50 nm at air flow velocities near the deposition interface similar to those used in the current study (Dixkens and Fissan, 1999). Increasing the inlet air temperature further may affect the aerosol stability, leading to chemical reactions and evaporation, and affect the cells.

Alternatively, deposition of sub–200 nm by diffusion may be considered as a more relevant approach because of the similarity with physiological exposure. The key feature of such a system would be the surface to volume ratio to mimic the respiratory system. This may be feasible by exploiting microfabrication, also known as organ–on–a–chip (Huh et al., 2010). Aerosol exposure has not yet been extensively explored using these devices, as the volume is many orders of magnitude smaller than conventional cell culture systems, so many challenges remain.

4. Conclusions

Finite element modeling was carried out and validated by analytical models and experiments to predict deposition efficiency in an in vitro aerosol exposure chamber. Results showed that an electric field is essential for efficiently depositing particles in the range of 10–200 nm. When using an electric field, there are many parameters to consider, including chamber configuration, electric field strength, electric field frequency, electric field frequency and flow rate. The introduction of a screen at the discharge of the inlet
tube which is used to make the electric field uniform is found to require higher voltages, and is also detrimental, as it appears to trap the particles, preventing them from depositing.

The implications of this study are guidelines for choosing chamber configuration and deposition parameters for achieving the desired dose and dose uniformity for in vitro aerosol exposures. Although the models were shown to agree reasonably well with experimental data, further research to identify discrepancies from the theory is also required. Further, the approaches used in this study assumed uniform spherical particles. Future work should include correction factors for size distribution and morphology, in addition to the effect of humidity on particle growth. Furthermore, additional studies on the effect of AC field on particle behavior would be beneficial, such as optimization of electric field strength, frequency and shape to minimize focusing.
CHAPTER IV

SCALED–UP PORTABLE MULTI–WELL AEROSOL EXPOSURE CHAMBER DESIGN
AND VALIDATION FOR DEPOSITING AEROSOLIZED PARTICLES ONTO CELLS
GROWN AT THE ALI

1. Introduction

Several devices for exposing aerosols to cells grown at an ALI have now been reported in the literature, including a few commercially available models (Jeannet et al., 2014; Holder et al., 2013; Kim et al., 2013; Aufderheide et al., 2011; Raemy et al., 2011; Müller et al., 2010; Grigg et al., 2009; Lenz et al., 2009; Volckens et al., 2009; Paur et al., 2008; Bitterle et al., 2006). For all of the reported systems, access to a biological safety cabinet is required for assaying cells after exposure. Due to the dynamic nature of particles, it is challenging to collect and re–disperse for cell exposure while preserving the original properties (Lichtveld et al., 2012). Consequently, an aerosol exposure system designed for assessing toxicity directly at the site of exposure in a field environment is desired.

Several recent studies have shown the physical limitations of achieving efficient particle deposition without the use of an external force such as electrophoresis (see Chapter III). Therefore, an aerosol exposure system must include electrically biased and insulated components for electrostatic deposition of submicron particles, in addition to features, such as cell culture insert holders and temperature control, for maintaining cells at the ALI. The objective of this study was to design and construct a portable multi–well aerosol exposure chamber (PM–AEC) incorporating the necessary features
described, validate the chamber for maintaining cells and depositing particles at the ALI, and demonstrate the portability of the chamber by transporting cells and exposing them to particles generated in a real world environment where exposure is a concern.

2. Materials and Methods

2.1 Chamber Development

The following criteria were considered for chamber development:

- Radially arranged wells with uniform flow paths for control and exposed cells in a minimum of triplicate each
- Chamber geometry to adapt multiple insert types
- Integral heating
- Adjustable inlet discharge to membrane distance
- Integrated electrically biased and insulated components
- Materials of construction to support autoclave sterilization
- Portable for transport between laboratories and field environments
- Integral chamber leveling
- Cell media access in a field environment without compromising sterility

2.2 Chamber Validation

The aerosol exposure system consisted of filtered dry air at 20 psi and carbon dioxide at 10 psi supplied to an electrospray (TSI), or filtered dry air at 20 psi supplied to a powder aerosol generator (Vilnius Aerosol Generator, CH Technologies), to generate an aerosol stream. The aerosol was then distributed to (1) a particle recovery stage
consisting of a 0.8 micron mixed cellulose ester filter in a sealed filter holder and (2) a soft X–ray ionizing source (Advanced Aerosol Neutralizer, TSI) followed by a regulator, which distributed controlled flow to (2a) a scanning mobility particle sizer (SMPS, NanoScan 3910, TSI) to monitor the particle size distribution, (2b) the PM–AEC, and (2c) a HEPA filter to relieve excess pressure. A detailed schematic of the exposure system is shown in Fig. 4.1.

Figure 4.1. Aerosol exposure flow schematic; Note: T = temperature and RH = relative humidity.

Ag NPs coated with citrate and dispersed in water (60 nm, Ted Pella) were aerosolized using an electrospray. Ag NPs coated with polyaromatic pyrolyzed hydrocarbons (25 nm, Novacentrix) were aerosolized from a dry powder using the Vilnius Aerosol Generator. To deposit and characterize the aerosolized Ag NPs, a formvar–carbon coated copper grid was placed at the ALI in one well of the chamber at 0.6 cm distance between the cell layer and discharge end of the inlet tube and electrically biased at –3 kV for 50 min. A total of 24 images were acquired at 4,000 times magnification using a TEM (Hitachi® H–7600, Hitachi). Particles in each image of
565.25 square microns were counted, averaged and divided by the surface area of the micrograph (see Appendix B).

A human type II pneumocyte cell line (A549, ATCC, Manassas, VA) was cultured at 37°C and 100% humidity in RPMI–1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were seeded onto polyester membranes (10 microns thick, 0.4 micron pores) with a growing area of 0.33 cm² at a concentration of 5.0×10⁴ cells/cm². The cells were allowed to grow to confluence for 2–3 days, then the basal media was replaced with fresh media, and the apical media was removed to place the cells at the ALI. Using transepithelial electrical resistance measurements, the cells were shown to maintain the monolayer after six more days at the ALI. To ensure cell integrity during the course of the experiments, cells were transferred to the PM–AEC for exposure after 24–48 hrs at the ALI. The cells were exposed to an electric field and either filtered air or Ag aerosols for 15 min. Post–exposure, the basal medial was assayed for lactate dehydrogenase using the CytoTox–ONE™ Homogeneous Membrane Integrity Assay (Promega). Cells were also stained with phalloidin–Alexa Fluor® 555 (Life Technologies) to label the actin and ProLong Gold Antifade Mountant with DAPI (Life Technologies) to label the nuclei, and then imaged using fluorescence microscopy.

2.3 Chamber Deployment

A human bronchiolar–type cell line (H441, ATCC) was cultured at 37°C and 100% humidity in RPMI–1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were seeded onto polyester membranes (10 microns thick,
0.4 micron pores) with a growing area of 0.33 cm$^2$ at a concentration of $1.0 \times 10^5$ cells/cm$^2$. The cells were allowed to grow to confluence for 2–3 days, then the basal media was replaced with fresh media, and the apical media was removed to place the cells at the ALI. After 24–48 hrs, the cells were transferred to the PM–AEC in a biological safety cabinet, which was then sealed and transferred over 700 miles to a munitions laboratory conducting impact testing of explosives. The internal temperature was maintained at 37°C, and cell culture media was replaced every 12 hrs using a syringe inserted into the silicone sleeves surrounding each chamber well. No change in flow rate was observed after penetration of the syringe into the silicone sleeves.

Lines leading to three control wells were sealed using silicone plugs, and the impaction testing exposure environment was drawn through the remaining lines at a rate of 19 mL/min/cm$^2$. Post–exposure, the basal media was removed and stored on dry ice and cells were fixed in 4% paraformaldehyde. Once the chamber was transported back to the home laboratory, the basal media was tested for interleukin 8 release using an enzyme–linked immunosorbent assay (Bio–Rad). Fixed cells were stained, and then examined using fluorescence microscopy and TEM.

Impact testing is a standard method for testing the sensitivity of explosive materials and is conducted by dropping a weight from a fixed height onto a measured mass of explosive. In this study, 24 samples of a reference explosive each weighing approximately 35 mg material were impacted at a height of 200 cm. Nano–sized materials were not used in the fabrication of the reference explosive. The ventilation fan was turned off for the first 12 impact trials, and then turned on for the second 12 to assess the effect of the engineering control on mitigating particle exposure. The aerosol
sampling equipment and PM–AEC was located approximately 1 m from the impaction test.

3. Results

The final configuration of the aerosol exposure chamber is shown in Fig. 4.2. The device includes eight wells for simultaneous replicate exposures and incorporates electrical biasing and insulating components for depositing submicron particles at the ALI by electrostatic precipitation. Each well is designed to hold a porous membrane insert, with cell culture media being maintained on the basal (lower) surface to generate the ALI. The heating mechanism comprises a heater–stirrer plate with temperature feedback, which stirs a stainless steel lined water bath inside the base of the chamber to maintain even internal chamber heating. The distance between the inlet tube and the cell layer is adjustable by turning a wheel that raises or lowers the plate containing the inlet tubes. The media dishes in the cell culture wells were manufactured using medical grade polyether ether ketone, and the sidewalls of the cell culture wells were composed of compressed silicone sleeves to allow for access to the cell culture media outside of a controlled sterile environment. Components in contact with the flow path were constructed using stainless steel, which is corrosion resistant and does not charge. Components that were not in contact with the cells or flow path were constructed using polycarbonate, which is low cost and light weight. The chamber is easily disassembled, and all of the components are autoclavable and compatible with alcohol based solvents.
The aerosolized Ag particles were characterized on-line using a SMPS. The median size of the Ag NPs aerosolized from a liquid dispersion (Ag (L)) and a powder (Ag (P)) were about 90 nm and 80 nm, respectively (Fig. 4.3).
Figure 4.3. Characterization of aerosolized and deposited Ag NPs. A. SMPS results for Ag particles electrosprayed from liquid dispersion; B. SMPS results for Ag aerosol generated from a powder.

The mass of Ag particles deposited in all eight wells was measured using ICP–MS after a 1 hr exposure to Ag (L) or a 15 min exposure to Ag (P) (Fig. 4.4). The mass of Ag (L) was not detectable by ICP–MS. Therefore, we instead counted particles from TEM images. We found that the particle flux was $1.0 \times 10^5 \pm 2.4 \times 10^4$ #/cm$^2$/min. For the Ag (P), the deposited mass was 34 times higher in the presence of an electric field. The percent difference among individual wells was 23% on average. Variation among chamber wells was previously reported where well to well deposition was reported to vary by 30% in an electrostatic aerosol in vitro exposure system (Hawley et al., 2014; Volckens et al., 2010). Representative images of deposited particles are shown in Fig. 4.3. The Ag (L) were well–dispersed, while the Ag (P) suffered from severe agglomeration.
To assess cell viability, lactate dehydrogenase release was compared for cells grown in a standard incubator (Incubator Control) to cells in the chamber exposed to air flow (19 mL/cm²/min) and an electric field of 5 kV/cm for 1 hr (Chamber Control). The cells were then exposed to Ag (P) was exposed once for 15 min, yielding an average concentration of 5 µg/cm². No difference in cell viability was observed for cells exposed to air flow with electrostatics or Ag particles with electrostatics compared to the incubator control (Fig. 4.5). The cell morphology was examined in the Chamber Control and the cells exposed to Ag NPs by staining the actin and nuclei. The morphology of the cells was not significantly affected and appeared as a confluent layer.
To demonstrate the portability of the PM−AEC, we grew a human bronchiolar-type cell line (H441) in the chamber and transported it over 700 miles to a laboratory where munition testing was being performed. The cells were maintained in the chamber at the ALI under standard cell culture conditions (37°C, 100% humidity) for several days without electric field or air flow. The media was replaced every 12 hr through the resealable silicone chamber walls.

Room air was drawn through the chamber during impaction tests for a period of about 40 min. A picture of the test setup is shown in Fig. 4.6A. The impaction test demonstrated emission of submicron aerosols ranging from 20,000 to >100,000 #/cm³ without the ventilation system as measured by a condensation particle counter (Fig. 4.6B). Post–exposure, basal media was removed and stored on dry ice and a fixative was added to the cells to preserve them for imaging. The basal media was assessed for the inflammatory marker interleukin–8, which increased after exposure to the impaction environment versus control wells (Fig. 4.6C). Cells stained for actin (red) and nuclei

\[ \text{Percent Dead Cells} \times 100 \]

\begin{tabular}{c c c c}
\hline
Group & Percent Dead Cells \\
\hline
Positive Control & 0 & 20 & 40 & 60 & 80 & 100 & 120 \\
Incubator Control & 0 & 20 & 40 & 60 & 80 & 100 & 120 \\
Chamber Control & 0 & 20 & 40 & 60 & 80 & 100 & 120 \\
Ag(P) 5 μg/cm² & 0 & 20 & 40 & 60 & 80 & 100 & 120 \\
\hline
\end{tabular}

\* Indicates significant difference from control (P < 0.05)

**Figure 4.5** Cell exposures to filtered air or Ag (P)s with electrostatics. A. LDH release; B. Cell morphology. White arrows indicate particle agglomerates.
(blue) appeared as a confluent layer on the cell culture inserts for both unexposed and exposed cells (Fig. 4.6D).

![Image](image1)

**A.** Image of testing scenario (arrow indicates impaction test apparatus); **B.** Particle count data for the duration of the impaction test; **C.** Interleukin–8 release after exposure; **D.** Fluorescence micrographs of chamber control cells (left) and chamber exposed cells (right).

**Figure 4.6.** Cell exposure in a field environment. A. Image of testing scenario (arrow indicates impaction test apparatus); B. Particle count data for the duration of the impaction test; C. Interleukin–8 release after exposure; D. Fluorescence micrographs of chamber control cells (left) and chamber exposed cells (right).

### 4. Discussion

A PM–AEC was developed for depositing submicron particles onto cells grown at the ALI in any environment (Fig. 4.2). One unique feature of the PM–AEC that was not highlighted in the validation steps is that the height of the inlet tubes can be adjusted to increase or decrease the distance from the cells, which has been shown to affect particle deposition, with deposition efficiency slightly improving at smaller distances (Fujitani et al., 2014). In consideration of electrophoresis, a larger distance requires higher applied
voltage to achieve equivalent electric field strength, which may be undesirable, especially in a high humidity environment. However, smaller distances will produce a higher shear force at the cell layer due to air flow, which can affect cell viability. Shear force is a function of both the inlet tube height and the flow rate. On the other hand, higher flow rates will increase the deposition flux, which can make up for reduced deposition efficiency. Therefore, the distance must be optimized to balance deposition flux and efficiency. The ability to adjust the inlet tube height enables an optimal distance to be obtained in cases where certain cell types are more sensitive to shear, applied voltage requirements are particularly high (as for larger particles), or to control deposition rates.

The deposition of Ag (L) particles in the PM–AEC was not detectable by mass (Fig. 4.4). In order to assess whether the deposition was still relevant, we compared the deposition flux to that predicted by the MPPD model for 90 nm Ag particles (see Chapter III, section 2.6 for details). The deposition flux for Ag (L) was about $1 \times 10^5$ #/cm$^2$/min, which is comparable for an exposure concentration of $> 10$ mg/m$^3$ (Table 4.1). The current exposure limit for Ag dust is 0.01 mg/m$^3$ (NIOSH, 2010). Therefore, the flux in the chamber is much higher than a realistic scenario.

### Table 4.1. MPPD Output and Exposure Calculations for Ag Particles (90 nm)

<table>
<thead>
<tr>
<th>Exposure limit concentration (mg/m$^3$)</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deposition flux (#/cm$^2$/min)</td>
<td>$1.3 \times 10^2$</td>
<td>$1.3 \times 10^3$</td>
<td>$1.3 \times 10^4$</td>
</tr>
<tr>
<td>Deposition flux after 8 hr exposure</td>
<td>$6.2 \times 10^5$</td>
<td>$6.2 \times 10^7$</td>
<td>$6.2 \times 10^7$</td>
</tr>
</tbody>
</table>
However, for *in vitro* studies, the overall concentration at different time scales rather than the flux is the figure of merit, and in this case, the exposure would be equivalent to an 8 hr exposure at 0.1 mg/m³. Ag agglomerates were deposited from a powder at a much higher concentration, which is typical for acute *in vitro* toxicity investigations (Carlson *et al.*, 2008). Therefore, monodisperse Ag NPs were deposited at the ALI in the PM–AEC at deposition rates, which are relevant for *in vitro* toxicity investigations.

We found the percent difference in deposition to vary from well to well by an average of 23%. Variation among chamber wells was previously reported where well to well deposition was reported to vary by 30% in an electrostatic aerosol *in vitro* exposure system (EAVES) (Hawley *et al.*, 2014; Volckens *et al.*, 2010). Variances amongst the radial chamber wells was anticipated, so special care was taken for precise machining of the radial features for complete uniformity. To reduce variability, we propose a design modification to control the flow rate at the outlet of each well individually rather than pulling from a common outlet. Deposition efficiency is highly dependent on flow rate (Chapter III), so any slight change in resistance in one well *versus* another will strongly affect the deposition.

The cell viability was similar for cells maintained in an incubator or in the chamber in the presence of an electric field and air flow at 19 mL/min.cm² (Fig. 4.5), which is in agreement with results from previous studies. A reduction in viability at higher flow rates of 64 mL/min/cm², but not 21 mL/min/cm², after 1 hr with no electric field was observed for a co–culture comprised of a bronchial cell–line and differentiated monocytes (Diabaté *et al.*, 2008). No effect on cell viability was observed for air flow of
11 mL/cm²/min and a square wave 1 Hz electric field and inlet tube height of 0.4 cm for one hour (Savi et al., 2008). Therefore, the PM–AEC is capable of maintaining cells under the stress of air flow. Although the high voltage and low current required for electrostatic precipitation has been reported to have no effect on cell viability in the present study and in published literature (Hawley et al., 2014; Savi et al., 2008), it will be critical to complete a comprehensive investigation of the effect of the electric field on molecular pathways in the cells.

Finally, the chamber was transported and exposed to submicron particles emitted in a field environment (Fig. 4.6). Cells were maintained in the chamber for several days of transport and were exposed to submicron particles. Post-exposure, basal media was assayed for the inflammatory marker interleukin–8 and cells were successfully imaged using fluorescence microscopy. These results are very promising for assessing toxicity of aerosols in relevant environments. However, the results also highlight some future requirements for generating meaningful data. First, since field environments are not well-characterized, it may be desirable to carefully characterize the environmental aerosol, including the presence of volatile or semi-volatile chemicals, biological material and particulate properties, including size, morphology, composition, and endotoxin content. Additionally it would be useful to integrate real-time measurements of cell response using endothelial resistance measurements for integrity of the cell layer and continuous perfusion of media from the chamber to an analysis station for assessing key biomarkers.
5. Conclusions

The PM–AEC incorporated most of the desired features, allowing for maintenance of cells grown at the ALI and efficient deposition of NPs. One key feature of the chamber is the ability for cell culture media to be replenished or replaced at any time, which may be particularly useful for maintaining and assaying cells at the site of exposure. The chamber has proven as a useful tool for relevant exposure of aerosolized particles to cells cultured at the ALI and shows promise for assessing particle toxicity in realistic field environments.
CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The global in vitro toxicology testing market has been estimated to reach $9.9 billion by 2017 for a compound annual growth rate of 14.7% from 2012–2017 (BBC Research, 2014). The growth of the market is largely attributed to the high cost, time requirements and failure rates associated with animal studies (Hartung, 2013). However, there are still shortcomings with current in vitro models, especially for particle toxicity, where the dose is not necessarily proportional to the exposure (Teeguarden et al., 2007). Therefore, it is increasingly important to address challenges related to repeatability and relevance of in vitro toxicology for screening particle toxicity. Key parameters of in vitro particle toxicity investigations that require deeper understanding include dosimetry and realistic exposure.

In order to address particle dosimetry, we reviewed current models for treating cells under the traditional static condition and introduced a new model for treating cells under a dynamic flow condition (Chapter II). An interesting result was that particle agglomerates reduced in size in the dynamic flow condition versus the static condition due to erosion, which could be useful for controlling agglomeration of certain particle types. More importantly, there were significant differences in dosimetry. In the static system, agglomerates were transported to the cell layer via both diffusion and sedimentation forces. The former force is a function of agglomerate diameter, while the latter is a function of both agglomerate diameter and density. In the dynamic system,
agglomerate deposition and cellular interaction were reduced compared to the static system due to the elimination of the sedimentation force. Therefore, the dose under the dynamic condition could be estimated from agglomerate size alone, which greatly simplified the dosimetry. Notably, shear stress at the cell layer was found to be negligible at the flow rate used, allowing this method to applicable for a wide range of adherent cell types. The results from this study have significant implications for improving repeatability across nanotoxicity assessments.

Recommended follow–up studies include investigating the behavior of a large panel of particles at a range of concentrations. Important properties may include agglomerate size, shape and density. For example, it is unclear how flow may affect erosion of metal oxide NPs that can agglomerate up to several hundreds of nanometers in size (Murdock et al., 2008). Further, unagglomerated rod–shaped NPs adhered to a channel surface more efficiently than spherical particles under flow (Toy et al., 2011). Therefore, different shaped particles would be interesting to test in a dynamic flow model, although it may not always be the case that the shape is maintained in the agglomerated state. Finally, density can affect the propensity for particles to escape flow (Toy et al., 2011). However, the velocity was an order of magnitude greater than that used in the present study. Since momentum is a product of velocity and mass, it would be valuable to further investigate the effect of agglomerate density in the context of low shear rates used for dosimetry where particle momentum is smaller.

Particle dosimetry is further complicated by a subset of situations, which must be considered when planning in vitro particle toxicity investigations. First, approaches to understand exposure methodology and dose characterization require modification when
cells are growing in suspension. Also, additional characterization is necessary for the toxicity testing of dispersions containing soluble/semi–soluble NPs, such as copper and silver, which readily oxidize and release ions over time (Maurer et al., 2014; Zhang et al., 2011; Studer et al., 2010). When testing the toxicity of such materials, the rate of ion release must be characterized. Further, when correlating in vitro to in vivo responses, differences in agglomeration behavior in cell culture media versus physiologically relevant media and scaling differences for exposure will affect the rate that particles reach the target tissue or organ, and all of these parameters must be considered (Han et al., 2012).

To further address particle dosimetry in the context of relevant exposure to represent inhalation, we investigated aerosol dosimetry (Chapter III). Finite element modeling was carried out and validated by analytical models and experiments to predict deposition efficiency in an in vitro aerosol exposure device. Results showed that an electric field is essential for efficiently depositing particles in the range of 10–200 nm. Analytical models were validated against simulations as straightforward approaches for estimating field strength and flow rate as a function of particle size to achieve optimal deposition. The introduction of a screen at the discharge of the inlet tube, which is often done to make the electric field in the chamber uniform, required higher voltages to achieve equivalent deposition efficiency as when the screen was removed. Further, the screen appeared to trap the particles, preventing them from depositing, suggesting that removal of the screen is ideal. AC fields at a frequency closely matched to the residence time in the chamber increased particle deposition above DC fields at similar strengths. The key outcome of this study is set of guidelines for choosing chamber configuration
and deposition parameters for achieving the desired dose and dose uniformity for \textit{in vitro} aerosol exposures.

Future work should investigate the effect of humidity on particle growth, as cells are generally maintained in a humid environment, and deposition is a function of particle size. Additional studies investigating the effect of AC field on particle behavior would be beneficial, such as optimization of electric field strength, frequency and shape to minimize focusing and maximize deposition. Furthermore, production of a large data set for deposition in an AC field would support the development of an analytical model to allow for facile estimation of deposition parameters.

Based on the success of the aerosol dosimetry investigation, the aerosol exposure device used to validate simulations was scaled-up and validated for maintaining cell cultures and exposing aerosols at the ALI (Chapter IV). The device included eight wells for simultaneous replicate exposures and incorporated electrostatics for depositing submicron particles. The cell culture media was maintained at 37 °C using a stirred water bath. The sidewalls of the cell culture wells were composed of compressed silicone sleeves to allow for access to the cell culture media using a sterile syringe outside of a controlled biohood environment. A human alveolar cell–line was successfully maintained in the chamber with the presence of air flow (19 mL/cm\(^2\)/min) and electric field (5 kV/cm) for one hour. Ag particles in two different forms, including aqueous dispersion and powder, were aerosolized using an electrospray and powder generator, respectively. Both were shown to successfully deposit at the ALI in the chamber in the presence of an electric field. To demonstrate the portability of the scaled-up aerosol exposure device, we grew a human pulmonary epithelial cell–line in the chamber and transported it over 700
miles to a laboratory where munition testing was being performed. Without air flow, the cell monolayer was maintained in the chamber at the ALI for several days with periodic media changes to allow for transporting the chamber. The inflammatory marker interleukin–8 increased after exposure to the impaction environment versus control wells.

Future alterations to the existing chamber may include incorporating continuous media perfusion rather than interval media exchanges, so the media is continually buffered. Also, the flow rate should be ideally controlled at the outlet of each well individually rather than pulling from a common outlet. Any slight change in resistance in one well versus another will strongly affect particle deposition. Additionally, a filtration unit at the inlet to deactivate bioaerosol contaminants and modifying the chamber to allow for on–site or real–time investigation of particle deposition and cytotoxicity in the chamber would advance this technology to a more practical state. Also, although the high voltage, low current electric fields required for electrostatic precipitation have been reported to have no effect on cell viability (Savi et al., 2008), it would be interesting to complete a comprehensive investigation of the effect of the electric field on molecular pathways in the cells.

Overall, the results of the compilation of dosimetry and exposure studies presented in this thesis highlight key points to facilitate more thoughtful study planning for in vitro toxicology experiments considering the many facets of dosimetry and exposure methodology. They also contribute to the justification for retiring outdated traditional exposure methodologies where dosimetry is difficult to predict and exposure is not relevant to a realistic scenario.
Beyond the consideration of dosimetry and exposure methodology, \textit{in vitro} studies are further lacking in the complexity of cell models and system microstructure to mimic realistic conditions. It has been demonstrated and is generally accepted that co-cultures are better than monocultures and that primary cells are more useful than cell lines for predicting whole organ responses (Hartung \textit{et al.}, 2013; Rothen–Rutishauser \textit{et al.}, 2005). More recently, microfluidic devices have emerged as a successful \textit{in vitro} platform for generating whole organ responses in response to disease and insult (Huh \textit{et al.}, 2012; Huh \textit{et al.}, 2010). Multiple cell types can be incorporated to more closely mimic physiological conditions (Huh \textit{et al.}, 2013; Sivagnanam \textit{et al.}, 2013). Also, the microstructural dimensions and shear rates can be designed to match those of the targeted organ or organ system, which can stabilize primary cells for more realistic response \textit{versus} commonly used immortalized cell lines (Sivagnanam \textit{et al.}, 2013; Huh \textit{et al.}, 2010; Seebach \textit{et al.}, 2000). In next generation \textit{in vitro} toxicology studies, complex \textit{in vitro} models must be combined with well-characterized dosimetry and relevant exposure methodology to achieve the final goal for \textit{in vitro} particle toxicity results to be comparable across laboratories and meaningful to the risk assessment community.
A.1 Simulation – velocity profile

The laminar flow module was used to simulate the velocity profile as described in the manuscript, chapter III, section 2.1. For all simulations, a flow rate of 20 mL/min with a parabolic inflow was used. The resulting profile can be divided into two regions (Fig. A.1A). In region I, the velocity smoothly decreases from the tube end towards the ALI. At the ALI, the flow is guided along the interface with a stagnation region near the center. In region II, the flow is guided up the edges of the cell culture insert, and the velocity increases substantially as the flow is forced out of the chamber through the annular space around the outside of the inlet tube. The velocity near the interface is dictated by both the flow rate and chamber dimensions, and it is important to control for two reasons: the first is to optimize residence time for deposition to occur, and the second is to regulate stress experienced by the cells at the ALI.

The formation of a low velocity region near the cell layer is important for particle deposition (Dixkens and Fissan, 1999). According to our simulation, at a discharge distance of 0.4 cm from the ALI, the velocity decreases to 0.1 cm/s (Fig. A.1B). This velocity is similar to that used in an electrostatic precipitator to achieve optimal deposition efficiency for off-line particle analysis (Dixkens and Fissan, 1999). The key when choosing a flow rate is to balance deposition efficiency and number of particles that
can be passed through the chamber during a given exposure period, which was described in the manuscript and demonstrated in Figure 3.8.

Air flow can cause stress to the cells by creating shear forces at the deposition interface and if the air is dry, it can also cause evaporation of the cell culture media. A shear stress at the ALI for this flow rate was estimated from simulations to be 0.00035, 0.00054 and 0.00240 dynes/cm² at inlet tube discharge heights of 0.8, 0.4 and 0.2 cm, respectively. Measured values range from 0.5–3.0 dynes/cm² at rest breathing and up to 1700 dynes/cm² with cough (Sidhaye et al., 2007). Therefore, the shear stress is 2–3 orders of magnitude less in the chamber than in the physiological system.

![Simulation of air velocity profile](image)

**Figure A.1. Simulation of air velocity profile.** A. Flow regions: I. Low velocity deposition region, II. High velocity exit region; B. Contour lines with values for velocity plotted. Units are in cm/s.

**A.2 Simulation – electrostatics module**

The electrostatics module was used to calculate the electric field within the chamber volume as described in the manuscript, chapter III, section 2.1. The electrode
was selected as the ground, and the inlet tube with or without the screen were selected to apply a voltage potential. The electric field generated in the chamber is approximately equal to the voltage applied to the inlet tube divided by the distance of the inlet tube discharge end to the ground electrode. In the real scenario, the electric field will be affected by the presence of the 0.086 cm thick media layer between the grounded electrode and deposition interface.

The potential \( V \) applied to the inlet tube to generate the desired electric field \( E \) was determined using the following equation to account for the presence of a dielectric material (cell layer, culture membrane and culture media):

\[
V = E \cdot \left( h + \frac{h_m}{\sigma_m} \right)
\]  

(A.1)

where \( h \) is the distance from the discharge end of the inlet tube to the deposition interface, \( h_m \) is the distance from the deposition interface to the ground electrode (i.e. thickness of the media layer) and \( \sigma_m \) is the relative permittivity of media. The relative permittivity of air is approximately 1, while the relative permittivity of media was approximated to be about 75, which is the value for water at 37°C (Uematsu and Franck, 1980). The presence of salts would also affect the relative permittivity (Wang and Anderko, 2001). We did not account for this in our study, as a difference in relative permittivity by a value up to 20 changed the applied voltage by less than 1%.

The effect of dielectric thickness on voltage potential required to achieve the desired electric field was calculated using equation (A.1) at \( h = 4 \) cm (Fig. A.2A). The percent difference from no dielectric thickness was 0.3, 3.3 and 29% for \( h_m = 0.1, 1.0 \) and 10 cm, respectively. The value for \( h_m \) in the present studies is 0.086 cm, so can be neglected (percent difference \( \{(value1 - value2)/(value1 + value2)/2 \cdot 100\} < 0.3\% \).
Next, the effect of $h$ on the electric field was calculated using equation (A.1) with $h_m = 0.086$ cm and $\sigma_m = 75$, and then compared to values measured using the simulation (Fig. A.2B). The electric field strength in the simulation at the midway line from the ALI to the inlet tube discharge was measured in COMSOL for each condition. Deviation of simulated electric field strength from analytical was observed for the condition of screen included in the discharge with percent difference was $-4.0$, $-2.0$, $-0.6$ and $3.1\%$ for $h = 0.2$, 0.4, 0.6 and 0.8 cm, respectively. This difference is likely due to differences in the shape of the area between the inlet tube discharge and deposition interface with increasing values for $h$, which is apparent in the simulation results (Fig. A.2C). The electric field strength in the case of the no screen condition could not be estimated using equation (A.1).

Figure A.2. Simulation of electric field strength properties (for condition with screen). A. Effect of dielectric thickness on applied voltage potential required to achieve desired electric field; B. Deviation of simulated electric field strength from analytically predicted; C. Simulated electric field strength for a common applied voltage of 0.4 kV.
A.3 Simulation – particle trajectory module

The velocity and electric field distributions developed in study 1 were used to solve for the Lagrangian particle trajectories in the time dependent domain. The particle phase was assumed sufficiently dilute that particle–particle interactions are negligible. For particle tracing, the particle inertia was equated with the net forces on the particle \((\sum \vec{F})\).

\[
m_p \frac{d\vec{v}_p}{dt} = \sum \vec{F}
\]

where \(\vec{v}_p\) is the particle velocity, \(m_p\) is the particle mass.

Forces included in the simulation included drag, gravity, Brownian motion and electric force. The drag force \((\vec{F}_d)\) is derived using Stoke’s law, which is that the resisting force exerted by air on the particle is equal to:

\[
\vec{F}_d = -(3\pi \mu d_p / C_C)\vec{v}_p
\]

where \(d_p\) is the particle diameter, \(\mu\) is air viscosity, and \(C_C\) is the Cunningham Correction Factor. For particles on the same order of magnitude as the mean free path of air (about 68 nm), the particle motion is affected by collisions with gas molecules. This is accounted for using the Cunningham Correction Factor:

\[
C_C = 1 + \left( \frac{\lambda}{d_p} \right) \left( 2.514 + 0.8 e^{-0.55d_p/\lambda} \right)
\]

where \(\lambda\) is the mean free path of air. We used 68.2 nm for this value, which was calculated based on the following equation:

\[
\lambda = 1/(\sqrt{2} n_{mol} \pi d_a^2)
\]
where \( n_{mol} \) is the number of molecules per volume (approximated using the ideal gas law at atmospheric pressure and room temperature) and \( d_a \) is the collision diameter for air, which is 0.37 nm (Vincent, 1995; Jennings, 1988).

Stokes law applies only for Reynolds numbers below 1. The particle Reynolds number \((Re_p)\) is calculated using the following equation:

\[
Re_p = d_p \rho_a v_s / \mu
\]

where \( \rho_a \) is the density of air and \( v_s \) is the sedimentation velocity. The gravity force \((\vec{F}_g)\) describes the effect of gravity on an object with mass:

\[
\vec{F}_g = m_p g
\]

where \( g \) is acceleration due to gravity (0.098 cm/s). The sedimentation force is derived by equating the gravity force with the drag force:

\[
v_s = (\rho_p d_p^2 g C_C) / (18 \mu)
\]

where \( \rho_p \) is particle density and \( d_p \) is the particle diameter. If we set \( Re_p \) to 1, then we can estimate that for Ag NPs in air, the upper size limit for applying Stokes law to predict deposition in the chamber is about 37 microns. This is above the particle size that we would be interested for biological exposure studies, since inhalable particles are considered to be 50% cut–off point of 10 microns (EPA, 2013).

The diffusivity \((D)\) is equal to:

\[
D = C_C k_B T / 3 \pi \mu d_p
\]

where \( k_B \) is Boltzmann’s constant and \( T \) is temperature. The Brownian force \((\vec{F}_{B,i})\) was approximated at each time step \((\Delta t)\) using the following relationship:

\[
\vec{F}_{B,i} = G_i \sqrt{6 \pi d_p \mu k_B T / C_C \Delta t}
\]
where $G_i$ is a Gaussian random number with zero mean and unit variance, which was varied randomly at each time step (Kim and Zydney, 2004; Li and Ahmadi, 1992). This equation can be derived by assuming that the root square distance $y$ moved by a particle in one direction in a given amount of time ($\Delta t$) is equal to: $y = \sqrt{2D\Delta t}$ and that $v_p = y/\Delta t$, and then substituting for $v_p$ in the drag force equation.

When applicable, the electric force ($\vec{F}_E$) was calculated:

$$\vec{F}_E = EZ_p$$

where $Z_p$ is the electrical mobility:

$$Z_p = qn_eeC_c/3\pi\mu d_p$$

where $q$ is the electron charge constant ($1.6 \times 10^{-19}$ $C$) and $n_e$ is equal to the number of charges carried by the particle.
APPENDIX B
PARTICLE CHARACTERIZATION AND DEPOSITION

The characterization data for SEF particles are shown in Fig. B.1. The particles were dropped onto a copper grid, allowed to dry, and then imaged using TEM (Fig. B.1A). The particles appeared spherical in morphology with a core–shell structure. The overall primary diameter was measured for 100 particles using ImageJ software, and a histogram for the results is shown in Fig. B.1B. The core and shell were also measured separately, and the results were used to calculate the effective density of the particles (Chapter III, Table 3.1).

![Image A](image1.png)

![Image B](image2.png)

Figure B.1. Characterization of primary SEF particles. A. Electron micrograph of surface enhanced fluorescence particles; B. Primary particle size distribution.

The SEF particles were aerosolized using electrospray and deposited onto glass coverslips placed at the ALI within the chamber (Chapter III). After each experiment, the coverslip was removed and transferred to a glass slide, which was imaged using a TRITC
filter on an inverted fluorescence microscope (Olympus IX–71). Representative fluorescence micrographs are shown in Figure B.2.

![Representative fluorescence micrographs of SEF particles deposited onto a coverslip at 0.3, 0.9 and 1.5 kV with no screen.](image)

*Figure B.2. Representative fluorescence micrographs of SEF particles deposited onto a coverslip at 0.3, 0.9 and 1.5 kV with no screen.*

The flux at the cell layer \((J_y=0)\) was calculated using the following relationship:

\[
J_y=0 = \left( \sum N_p / SA_{micrograph} \right) / (N_{micrographs} \cdot t)
\]

where \(N_p\) is the number of particles counted per micrograph, \(SA_{micrograph}\) is the surface area of the micrograph, \(N_{micrographs}\) is the number of micrographs, and \(t\) is the exposure time. Next, the experimental deposition efficiency \((\epsilon_{exp})\) was calculated using the following relationship:

\[
\epsilon_{exp} = J_y=0 / (C_{SMPS} Q / SA_{ALI})
\]

where \(C_{SMPS}\) is the integrated particle number concentration in particles per volume measured by the SMPS, \(Q\) is the flow rate through the system and \(SA_{ALI}\) is the surface area of the ALI, or surface area available for deposition.
The analytical equation (3.1) and semi-empirical equation (3.2) displayed in chapter III were based on the following derivations.

*C.1 Derivation of equation (3.1)*

Mass balance for a control volume within the chamber:

\[
\text{\{ particles in\} } - \text{\{ particles out\}} = \text{\{ particles consumed (i.e. deposited)\}}
\]

\[N_{p,\text{in}} - N_{p,\text{out}} = (v_p/v_a)N_p\]

where the fraction of particles removed from the control volume due to a force on the particle is equal to the ratio of particle velocity to air flow velocity and \(N_p\) is the number of particles in the control volume.

Rearrange:

\[- \int_{N_{p,\text{in}}}^{N_{p,\text{out}}} \left(\frac{dN_p}{N_p}\right) = \frac{v_p}{v_a}\]

Integrate:

\[\ln\left(\frac{N_{p,\text{out}}}{N_{p,\text{in}}}\right) = -\frac{v_p}{v_a}\]

Define deposition efficiency:

\[\epsilon_{\text{theor}} = 1 - \frac{N_{p,\text{out}}}{N_{p,\text{in}}}\]

Substitute:

\[\epsilon_{\text{theor}} = 1 - \exp\left(-\frac{v_p}{v_a}\right)\]
\[ v_p = \frac{y}{\tau} \]
\[ v_a = \frac{h}{\tau} \]
\[ \frac{v_p}{v_a} = \frac{y}{h} \]
\[ \epsilon_{theor} = 1 - \exp(-y/h) \]

C.2 Derivation of equation (3.2)

When the chamber is not semi–closed, the number of particles in the control volume is equal to the number that enters the chamber.

\[ N_{p,in} - N_{p,out} = \frac{y}{h} N_{p,in} \]
\[ 1 - \frac{N_{p,out}}{N_{p,in}} = \frac{y}{h} \]
\[ \epsilon_{theor} = \frac{y}{h} \]

In this case the value for \( h \) is unique from the previous case because the distance from the deposition interface where particles begin to respond to the electric field extends above the inlet tube discharge. By fitting to the simulation, we accommodated for this by multiplying by 1.35.

\[ \epsilon_{theor} = 1.35 \frac{y}{h} \]
APPENDIX D

BIPOLAR CHARGING PROBABILITY

Bipolar charge distribution varies with particle size according to the Fuchs and Gunn models (Wiedensohler, 1988). The Fuchs model is valid for particles carrying one or two elementary charges, which is accurate for particles < 1 micron. The Gunn model is valid for particles carrying three elementary charges, which is accurate for particles > 50 nm. Values for charging probability were calculated using these models and plotted (Fig. D.1). There are slightly more particles carrying negative than positive charges.

Figure D.1. Particle bipolar charging probability. A. Charging probability as a function of diameter and number of charges; B. Maximum negative, positive and total charging probability as a function of diameter.

To adjust for charging probability in Chapter III Figure 3.6, the analytically derived deposition efficiency for each particle charge number was multiplied by the known charging probability, and the resulting adjusted efficiencies for each particle charge number were summed.
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