ENHANCED PHYSIOLOGICAL MICROENVIRONMENT FOR IMPROVED EVALUATION OF NANOPARTICLE BEHAVIOR

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ABSTRACT

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Due to their distinctive physicochemical properties, nanoparticles (NPs) have proven to be extremely advantageous for product and application development, but are capable of inducing detrimental outcomes in biological systems. Standard \textit{in vitro} methodologies are currently the primary means for evaluating NP safety, as vast quantities of particles exist that require appraisal. Here, we developed an enhanced \textit{in vitro} model that retains the advantages of cell culture, but introduces the key physiological variables of accurate biological fluid and dynamic flow. As NP behavior and subsequent bioresponses are highly dependent upon their surroundings, this developed microenvironment provides a more relevant system to evaluate responses following NP exposure. In this study, the microenvironment is comprised of the A549 lung cell model, artificial alveolar fluid, and dynamic flow at realistic rates; to mimic a NP inhalation exposure. Significant modulations were identified to silver and gold NP characteristics and the nano-cellular interface as a function of particle surface chemistry, fluid composition, and flow condition. More importantly, several of these modifications
were dependent on multiple variables, indicating that these responses were previously unidentifiable in a standard cellular environment. Taken together, this study demonstrates that to fully elucidate the behavior and evaluate the safety of NPs, these assessments need to be carried out in a more complex and physiologically relevant cellular exposure model.
Dedicated to my parents, who always encouraged me in my pursuit of knowledge, and my fiancé, whose never-ending belief in me gave me support and strength.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2D</td>
<td>2-Dimensional</td>
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<tr>
<td>3D</td>
<td>3-Dimensional</td>
</tr>
<tr>
<td>A549</td>
<td>Human Alveolar Epithelial Cell Line</td>
</tr>
<tr>
<td>AAF</td>
<td>Artificial Alveolar Fluid</td>
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<tr>
<td>Ag</td>
<td>Silver</td>
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<tr>
<td>Au</td>
<td>Gold</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>HepG2</td>
<td>Human Liver Carcinoma Cell Line</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Epithelial Cell Line</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTS</td>
<td>CellTiter 96 Aqueous One Solution Assay</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SA/V</td>
<td>Surface Area to Volume Ratio</td>
</tr>
<tr>
<td>TA</td>
<td>Tannic Acid</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>U937</td>
<td>Human Alveolar Macrophage Cell Line</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
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<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible Spectroscopy</td>
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</table>
1.1 – Nanoparticles: Properties and Applications

1.1.1 – The Unique Physicochemical Properties Associated with Nanoparticles.

Nanoparticles (NPs) are defined as any material with at least one dimension measured in the nanoscale range, between 1 nm and 100 nm. In recent years, NPs have played a major role in the development of new and improved technologies within the consumer, industrial, military and medical sectors. NPs are advantageous over other materials for these applications, owing to distinctive traits associated with their physicochemical properties. These characteristics are inherent in nano-sized particles and greatly differentiate NPs from similar bulk counterparts. Some of these unique characteristics include: enhanced strength, plasmonic optical properties, augmented magnetic and electrical behavior, and increased reactivity due to a greater surface area/volume ratio (SA/V).

For example, zinc oxide NPs have been investigated as a nanophotocatalyst in the development of self-cleaning, concrete-based structures. As an additive material, zinc oxide NPs increased the compressive strength of the concrete structures by nearly 15%.
In another study, the high mechanical strength of carbon nanotubes coupled with the electrical resistance of silicon dioxide was utilized to construct a core-shell structure for polymer nanocomposite construction.\textsuperscript{2} This was a unique pairing, as these nanocomposites required the mechanistic stability of carbon nanotubes and low electrical conductivity provided by silicon dioxide. The silicon dioxide was successfully used as an insulating layer around the core carbon nanotube to simultaneously provide mechanical strength and electrical insulation. Unique optical properties, referred to as plasmonic capabilities, are associated with numerous noble metal nanostructures, making them attractive for imaging applications. Plasmonic properties, also referred to as surface plasmon resonance, arise from oscillation of free electrons on the NP surface. This phenomena causes the unique ability associated with NPs for light to be absorbed and reflected at identical wavelengths.\textsuperscript{3} These optical properties also result in the visible color shift for different sizes of spherical gold NPs, as demonstrated in Figure 1.1. Furthermore, it has been identified that some bulk materials retain their ferrous magnetic properties in the nano-size, making them excellent candidates for MRI technologies and separation techniques. Interestingly, this property is not uniform across all NPs, as it has been identified that bismuth ferrite NPs of varying primary particle size display varying degree of magnetic activity.\textsuperscript{4}
NPs can also be specifically designed, engineered, and synthesized to attain desired physical parameters, including, particle size, morphology, core material composition, and surface chemistry (Figure 1.2). With regards to morphology, aside from basic nanospheres, it is now possible to synthesize a wide range of differently shaped materials. Nanorods and nanotubes have become increasingly common in nanotechnology, owing to their greater surface area.\textsuperscript{5,6} However, efforts are continually underway to develop even more atypical nano-morphologies. For example, a recent study developed a method for the synthesis of fuzzy-rods and spindle-like nanoparticles.\textsuperscript{7} Novel synthesis procedures have also been developed for nanoprisms,\textsuperscript{8} hollow nanospheres,\textsuperscript{9} nanowires,\textsuperscript{10} and nanocrystals.\textsuperscript{11}
Figure 1.2: Representation of various tunable NP parameters. NP parameters are easily modified to generate a plethora of available materials. The most commonly modifications that arise include: 1) primary particle size, 2) composition of the NP core material, 3) NP geometric shape, and 4) surface functionalization or chemistry. Previous studies have identified that slight alterations to any of these characteristics cause a significant impact on NP behavior and resultant toxicological outcomes.

The wide variation on NP morphologies generate a vast number of materials for application and product development, however, when tunable particle size is considered, the number of potential NPs exponentially grows. Although synthesis to targeted primary sizes is not possible for all shapes, size tunability has become standard practice for both sphere and rod morphology.12,13 One study even demonstrated the ability to preferentially alter edge lengths of silver nanocubes through hydrothermal synthesis.14

With regards to core compositions, procedures are in place to generate NPs of practically any available material today. Examples of more common core materials include silver, gold, cobalt, iron, titanium dioxide, carbon, and cerium.7-10 Each material that is reduced down to the nanoscale possesses unique properties specific to its composition; making each distinctive and capable of unique behavioral responses. Furthermore, as each of
these core compositions has the potential to be synthesized in a multitude of sizes and shapes, the possibilities are endless for finding the best material fit for nano-based applications.

Furthermore, these NPs can undergo surface modification and be functionalized with either biomolecular, chemical, or protein compounds. The surface chemistry of NPs has been shown to be a critical factor as it serves as a boundary between the NP core and its surrounding environment. Both DNA and proteins, such as antibodies, have been successfully conjugated to nanomaterial surfaces in an effort to detect target molecules or serve as drug delivery devices.\textsuperscript{15,16} Alternatively, chemicals such as tannic acid and Chariot, a reagent based on a synthetic signaling peptide, have been utilized to enhance NP uptake and distribution within cells.\textsuperscript{17} Surface coating is also frequently used to enhance NP stability in solution, through functionalization with chemicals such as polyethylene glycol (PEG), citrate, and polyvinylpyrrolidone (PVP).\textsuperscript{18}

1.1.2 – Current Nanoparticle Applications.

With a wide range of unique physicochemical properties and the ability to specifically tune a number of desired parameters during NP synthesis, it is easy to imagine the vast array of different NPs available for the development and enhancement of nano-based technologies. Currently, NPs have permeated the consumer, industrial, and medical markets in a wide range of products and applications. For example, titanium dioxide NPs have been incorporated into numerous high-technological applications, including as a component of fuel cells, as a photocatalyst for water decontamination, the predominant
player in solar panels, and as UV protection in cosmetics and sunscreens.\textsuperscript{19-21} Similarly, magnetic iron oxide NPs have been approved by the FDA for use as a contrast agent for medical imaging, and are currently being explored for magnetic drug delivery, separation processes, and as reinforcement for concrete steel fibers.\textsuperscript{22,23} Carbon nanotubes are currently utilized in a multitude of applications, such as material additives for improved conductive or strength components, sensors and energy storage devices, water purification systems, and as a drug delivery mechanism.\textsuperscript{24,25}

However, nano-sized silver (Ag NPs) and gold (Au NPs) are still the most prevalent and commonly used NPs to date. Ag NPs are utilized in a wide range of products, including commercial appliances, cosmetics, clothing, personal electronics, and polymer additives.\textsuperscript{26,27} Due to their antimicrobial properties, Ag NPs are frequently found in medical applications such as bandages, bone cement, and antibiotic ointments.\textsuperscript{28,29} Other characteristics, including unique optical properties, allow for potential Ag NP incorporation into electrochemical sensor and catalytic applications.\textsuperscript{30,31}

While Au NPs are utilized nearly as frequently as Ag NPs, their target applications are dramatically different. Due to the general biocompatibility, exceptional plasmonic properties, stability, ease of functionalization, and standardized synthesis procedures Au NPs are highly prevalent in biomedical applications. Current research efforts are underway for implementation of Au NPs for drug delivery techniques, improvement of bio-imaging, cancer therapeutics, and development of vacinnes.\textsuperscript{32,33} Biosensor applications are also emerging as a potential research focus, including the production of a
graphene-gold NP for the detection of glucose oxidase.\textsuperscript{34} However, coinciding with this growth in NP utilization is an increased likelihood of human and environmental exposure to these nano-sized materials, with yet unresolved biological consequences; thus giving rise to the field of nanotoxicology.

1.2 - Nanotoxicology

1.2.1 – Introduction to Nanotoxicology.

In October 2013, the Project on Emerging Nanotechnologies inventoried 1,628 consumer products utilizing NPs that have been introduced into the market since 2005.\textsuperscript{35} This new analysis represents a 24\% increase from the previous inventory in 2010, a mere three year span. Rapid rise of NP incorporation into the consumer market has likewise increased the likelihood of human exposure to these particles, either during production in industry or usage of the product itself. The rapid development of NP exposure in everyday life generated the need to evaluate NP safety, as well as establish recommended exposure limits, leading to the establishment of the field of nanotoxicology.\textsuperscript{36,37}

The overarching goal of the field of nanotoxicology is to evaluate the safety of NPs in a biological setting. Initial efforts were hindered by limitations, including a lack of reproducible synthesis procedures, poor NP characterization, and lack of standardized assessment techniques. NPs behave dramatically different in solution when compared to traditional chemicals, owing to their insoluble nature, ability to agglomeration, lack of typical concentration gradients, and their ability to sediment out of solution. However,
over the years, these issues were worked around and the field now has standardized protocols and procedures in place for synthesis, functionalization, characterization, and assessment of biological targets.

Initial nanotoxicological assessments merely answered the question: would exposure to a high dosage of NPs induced cell toxicity? However, over its decade in existence the field has matured and is now focused on evaluation of more in-depth biological mechanisms following NP exposure. Going beyond cytotoxicity, NP evaluation includes assessing more subtle biological responses, including: induction of cellular stress pathways, activation of the immune/inflammatory systems, interference in basal signal transduction, and modulation to gene transcription. Essentially, the goal of nanotoxicology is to identify not only if NPs induce a detrimental response within a biological environment, but to ascertain if NP exposure alters cellular homeostasis.

Furthermore, the large number of tunable NP parameters has resulted in an astounding number of unique NPs that require screening before their release to the public at large. A major focus of nanotoxicology in the past few years has been attempting to link specific physicochemical parameters to nanotoxicological evaluations. While this assessment is far from complete, some general trends have emerged. Firstly, cytotoxicity has been found to increase with decreasing primary particle size. Secondly, spherical morphology tends to be less harmful than fibre/rod shaped particles. Moreover, certain core compositions, such as silver and heavy metals, are innately more toxic than other
materials like gold and silica. Lastly, it is known that surface chemistry plays a predominant role in nano-bioeffects, though a precise correlation has yet to be elucidated.

1.2.2 – *In vitro*/*in vivo* Correlations.

Over the years, the field of nanotoxicology has adopted two main biological mechanisms to carry out assessments: *in vitro* techniques or *in vivo* systems (Figure 1.3). As *in vitro* cell culture methodologies can provide a fast and cost effective option for high throughput NP screening, cell-based models have been predominantly used to date. However, the target model, experimental design, and endpoints selected for evaluation have greatly varied between studies, introducing a sense of inconsistency in current literature. For example, one study examined the effect of hydrogel-carbon nanotube structures on an intestinal cell line,\(^{38}\) while, another study evaluated iron oxide NP effect on HepG2 liver cells.\(^{39}\) Not only did these studies examine different NPs in different cell models, but select dosages, exposure duration, and endpoint analyses varied. In another study, lung cancer cells were used as the choice model to assess silica NP toxicity.\(^{40}\) As these articles indicate, a wide range of different NP/cell line combinations have been the basis of current *in vitro* analysis. While on their own each study has great merit, once compared to one another, a pattern of conflicting data emerges, with limited consistency or correlation. Therefore, although *in vitro* methodologies have the benefit of being both fast and cost effective, the vast amount of data generated does not always allow for corroborating evidence on NP safety.
Furthermore, traditional cell culture systems exhibit the considerable drawback that they are not truly representative of a physiological environment, thereby providing a less realistic exposure scenario. Due to this lack of a realistic environment, any data obtained from such experimentation has poor predictive capabilities when applied to a human exposure scenario. When considered under those circumstances, \textit{in vivo} methodologies not only provide more information, but can be more accurate and have higher predicative capabilities.

For example, following ingestion of silicon dioxide NPs by adult mice, an organism-level safety assessment was carried out; a feat that is not possible with \textit{in vitro} methods.\textsuperscript{41} In this study, the authors discovered toxic effects on multiple organs and tissues, including the liver, kidneys, lung, and testis. Another investigation identified that rats orally administered Au and Ag NPs displayed toxicity in the lungs, myocardial tissue, liver, and kidneys for both particle sets, even though Au NPs are traditionally considered biocompatible.\textsuperscript{42} Aside from rodents, other animal models can be utilized, such as \textit{C. elegans} or \textit{Drosophila}, that may not be as comparable to mammalian models, but have

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & \textbf{In Vitro} & \textbf{In Vivo} \\
\hline
\textbf{Pros} & Fast & Closer correlation to human exposure \\
& Inexpensive & \textbf{} \\
\hline
\textbf{Cons} & Non-realistic exposure routes & Expensive \\
& & Time consuming \\
& & Ethical Issues \\
\hline
\end{tabular}
\caption{Comparison of the advantages and disadvantageous associated with utilization of \textit{in vitro} and \textit{in vivo} methodologies for nanotoxicological evaluation.}
\end{table}
the added benefit of a more rapid experimental time frame. For example, the C. elegans model was utilized to evaluate toxicity, oxidative stress, and DNA damage following exposure to varying Ag NPs, identifying a significant difference in response between PVP coated and uncoated Ag NPs.\textsuperscript{43}

Although in vivo methods allow for the simultaneous evaluation of an entire organism, rather than a single cell line, these studies also suffer from the lack of a standard model for nanotoxicity evaluations, leading to inconclusive results. Another yet unresolved issue pertains to NP dosimetry for in vivo models, with disagreements arising when determining what experimental dosing levels would correlate to equivalent human exposure. Furthermore, animal models in general are hindered by significant ethical, time, regulatory, and financial constraints. When coupled with the vast number of NPs that require evaluation, these hindrances make in vivo systems an unfeasible approach for high throughput nanotoxicology.

Due to inherent differences that exist between in vitro and in vivo systems, NP evaluations have demonstrated poor correlation between cell-based and animal models.\textsuperscript{44-46} To address this limitation, scientists have begun evaluating and comparing both in vitro and in vivo NP bioeffects; in order to assess predictive capabilities of each system. For example, one study evaluated the effect of polymeric nanoparticles on two different in vitro cell lines as well as an in vivo rat model.\textsuperscript{47} Disparate results were obtained, with the in vitro experiments showing a dose-dependent cytotoxic response and the in vivo results demonstrating no cellular disruption. Conversely, two other studies, one involving cobalt
oxide NPs and the other gold NP bioconjugates, identified a high degree of correlation between \textit{in vitro} and \textit{in vivo} testing\textsuperscript{48,49}. Taken together, these results highlight the fact that neither standard \textit{in vitro} systems nor complex \textit{in vivo} models are capable of keeping up with the demanding pace of nanotoxicological safety assessments\textsuperscript{50}. Therefore, there exists a tremendous need for the development of enhanced cellular models that preserve the advantages of \textit{in vitro} models while incorporating key aspects of \textit{in vivo} systems to produce a more realistic NP exposure scenario.

\textbf{1.3 – Enhanced \textit{in vitro} Models.}

Though the field of nanotoxicology has achieved much since it began over a decade ago, there exists a great need for the development of an enhanced cellular model to improve NP evaluation techniques. Current demands include a system that maintains cost effectiveness, has the ability for high-throughput analysis, and provides realistic and accurate safety assessments. Many potential modifications and improvements have been explored for the improvement upon traditional static, 2-dimensional, single cellular \textit{in vitro} analyses. However, what has yet to be accomplished is the merging of these individual improvements into the generation of an “enhanced \textit{in vitro} microenvironment” that retains the advantages of cell culture but is more physiologically relevant. These various enhancements can be broken down into two main categories; modulating the biological system itself or introducing new physical aspects to the cellular system.
1.3.1 – Biological Enhancement: Co-culture, 3-Dimensional.

Enhancing the biological aspect of an *in vitro* system is essential to creating a more realistic environment. Traditional cell-based models consist of a single monolayer of cells adhered to the bottom of a cell culture dish. However, within the human body, cells are found as 3-dimensional (3D) configurations organized into various organs and complex tissues. Furthermore, within these complex tissues, multiple cell types are present, providing specialized functions specific to each cell variety. For example, each pulmonary alveolus of the lung contains three different cell types: Type I cells, Type II cells (aka granular pneumocytes), and macrophages.\(^{51}\) Type I cells are thin and flat and contain the capillaries where oxygen diffusion occurs. The Type II cells secrete lung surfactant, lowering the surface tension of the tissue. Finally, alveolar macrophages are mobile throughout the air cavities and provide an immune function for the system, attacking and engulfing foreign matter than enters the lung. The presence of specialized cells allows for any organ or tissue to function through a wide network of cell-to-cell communication via direct contact between cells and extracellular signaling. A traditional monoculture of a single cell line, grown in a single layer structure cannot mimic this complex, ever changing environment. However, incorporation of multiple cell types through the use of a co- or multi-culture can provide an added layer of realism to the system.

Co-culture models can vary as widely as the cell culture lines themselves, therefore, many different combinations have been explored. One potential co-culture model was constructed utilizing A549 lung epithelial cells and U937 alveolar macrophage immune
cells. Following aluminum NPs exposure to the system, it was found that the co-culture displayed a significant reduction in toxicity compared to an A549 monoculture. This phenomena was presumed to occur due to the ability of the macrophages to performing their effector function and actively seek out and engulf the foreign material. Another potential lung co-culture model centered on the alveolar-capillary interface, utilizing A549 pneumocytes as the lung aspect and human umbilical vein endothelial cells (HUVECs) as the vascular cell line. The cells were grown on opposite sides of a membrane, and exposed to titanium dioxide NPs on the apical (A549) side. A significant increase in adhesion of the A549s to the HUVECs was observed, indicating an activation of the endothelial cells and dramatic modulation to the biological system. Co-culture systems are not limited to lung models, but can also be utilized for other organs and tissues throughout the body. One study focused on an intestinal co-culture model through the use of two different epithelial colon cell lines: one to provide absorption and another to secrete mucus. Alumino-silicate clay nanotubes were exposed to the co-culture and resulted in no loss of viability, despite pro-inflammatory cytokine release. As can be seen in the three representative studies discussed here, a co-culture model can provide more realistic NP evaluation, and bringing about different cellular outcomes than the traditional mono-culture model.

Similarly, the incorporation of a cell scaffold to provide 3-dimensional (3D) structure to the biological model will undoubtedly add a layer of complexity and realism to the in vitro model. As with the co-cultures, various organ and tissue systems have been examined under more controlled environments through the use of 3D in vitro models. For
example, a 3D liver spheroid was generated through the use of a porous hydrogel scaffold and implemented for the evaluation of cadmium telluride and Au NPs. Following exposure to both NP types, greater cytotoxicity was observed for the 2-dimensional (2D) monolayer liver cells versus the enhanced 3D structure. Similarly, a gastrointestinal model of 3D colon cells, evaluated under zinc oxide NP exposure was found to exhibit different modes of cellular death as compared to a 2D system, indicating altered cellular functionality. These studies seem to indicate that the use of 2D monolayer cell cultures for NP safety evaluation might overestimate true NP toxicity in a 3D human system. In any case, the incorporation of biological enhancements, whether additional cell lines or a 3D structure, can create a more realistic model, resulting in different cellular outcomes than a traditional 2D monoculture system. Figure 1.4 represents differences between monoculture, co-culture, and 3D structure incorporating co-culture.

![Monoculture, Co-culture, 3D Co-culture](image)

**Figure 1.4: Representative figure of differences between monoculture, co-culture, and 3D co-culture.** In monoculture techniques, a single cell model is grown over a 2-dimensional growth area. For co-culture models, multiple types and classes of cell lines are grown simultaneously together, forming a symbiotic environment. This system can be further enhanced by the addition of a 3rd spatial dimension, to more closely mimic advanced tissue systems.

1.3.2 – Physical Enhancement: Physiological Fluid, Dynamic Flow, and NP Dosage.

Aside from the biological aspect of the cellular model included within the *in vitro* system, it is also necessary to examine the physical environment of the system itself. Within the
human body, cells and organs systems are surrounded by physiological fluids. Gastric fluid, for example, is prevalent within the gastrointestinal tract and alveolar fluid and lung surfactant within the respiratory tract. Interstitial fluid also surrounds the majority of cells in the body and is utilized to exchange nutrients and waste between the cells and surrounding tissues. However, traditional cell culture media is optimized to promote cell line health and growth in a laboratory setting, and therefore does not represent accurate in vivo conditions.

The incorporation of physiologically relevant fluids into an enhanced in vitro environment can therefore drastically change NP behavior and the nano-bio interface. For example, it was identified that following dispersion in physiological fluids, gold nanorods agglomerated to a large degree, resulting in a substantial loss to the photothermal characteristics during NIR laser irradiation. Similarly, another study investigated the effects of multiple physiological fluids, such as lysosomal, alveolar, and interstitial fluids, on hydrocarbon and polysaccharide coated Ag NPs. Significant alterations to NP morphology, agglomeration, and reactivity were observed following exposure to the physiologically relevant environments, resulting in increased cytotoxicity of an alveolar macrophage cell line (as compared to treatment with NPs in traditional cell culture media).

Furthermore, the physiological fluid within the human body is dynamic and constantly moving, due to the cardiovascular system, effecting both cellular morphology and function. Therefore, it logically follows that an in vitro system that incorporates fluid
movement would better predict a true response. When NPs are introduced into a
dynamic system, the competing forces of sedimentation and diffusion would be affected,
and likely overpowered, by convective forces, altering the ratio of NPs that actually reach
and interact with the cells (Figure 1.5). Additionally, NP properties themselves, such as
agglomeration tendencies and ionic dissolution rates, can be altered due to NP presence
in a dynamic flow system. The addition of this dynamic motion aspect into an otherwise
static *in vitro* system can, therefore, have drastic effects on both cellular and NP
behavior, leading to a more accurate and realistic environment.

**Figure 1.5:** Representation of the competing forces of sedimentation, diffusion, and
lateral flow in a dynamic flow NP exposure system. The AuNPs are subject to the
influence of multiple transport fluid flow forces, including diffusion, sedimentation, and
lateral flow. NPs typically migrate through a system solely by a combination of
sedimentation and diffusion, however, the introduction of dynamic flow initiates
convective forces as well.

Two methods are commonly utilized for dynamic flow evaluations: bioreactors or
microfluidics. In one study, a bioreactor flow model was used to evaluate the effect of six
different NP types on HUVEC cells under both static and dynamic conditions. Results
demonstrated increased cytotoxicity following Ag NP introduction under dynamic flow.60
Similarly, experimentation completed using a microfluidic device successfully evaluated
cell cycle progression following exposure to Ag NPs. Under shear stress induced by
dynamic flow, the authors identified accelerated G2 phase arrest and subsequent cytotoxicity when compared against static conditions. In this instance, the introduction of dynamic flow increased the delivered NP dose, providing a rationale to the increased cytotoxicity. In contrast, a second microfluidic model that assessed AuNP exposure to endothelial cells observed decreased NP sedimentation under dynamic flow, resulting in a correlative decrease in cytotoxicity.

These studies highlight the critical need to incorporate dynamic flow into *in vitro* models for increased accuracy during assessment. Moreover, these conflicting reports demonstrate the need to standardize testing procedures, as these results were observed with different models and flow rates. Furthermore, none of the previously discussed dynamic flow experiments conducted static evaluations in identical vessel/cell culture environments as the dynamic flow experiments. As the nano-bio interface is highly dependent upon environmental variations, this experimental design flaw provides a likely cause for these discrepancies.

Lastly, both the accurate mode and level of realistic human exposure to NPs must be considered. In the early days of the field of nanotoxicology, most experimentation was conducted with acute NP concentrations at much higher levels than a human could realistically be expected to encounter in daily life. Furthermore, little care was taken to choose a cell line for experimentation that coincided with common routes of NP exposure in humans: inhalation, dermal contact, and ingestion. More recently, this phenomena has trended toward more realistic NP exposure models and careful consideration of coupling
NPs with likely biological targets. For example, one study evaluated the cellular effect of low level Ag NP dosing via a chronic exposure metric of 8hr/day, 5 day/week for 3 months. Significant cellular functionality differences were observed for the chronic exposure as compared to acute high dosages of the same NP, including augmented stress, actin disorganization, and gene modification. Furthermore, various review articles have discussed the importance of relevant NP dosages, including understanding the relationship between concentration-, surface area-, and particle number-based dosing. In recent years, steps have been taken to more accurately mimic relevant exposure routes and NP concentrations; however, much can still be examined to improve experimental designs.

1.4 – Conclusions

The capabilities of nanomaterial synthesis have advanced dramatically in recent years, allowing for the precise tunability of various NP parameters. Coupled with the vast number of unique physicochemical properties inherent with NPs, little doubt exists as to why NPs have become commonplace for the enhancement of novel products and applications. However, before incorporation into everyday life progresses too far, NP safety must be thoroughly evaluated. Though the field of nanotoxicology has diligently assessed NP bioeffects through various methodologies throughout the years, limited correlations exist between in vitro and in vivo studies, resulting in inconclusive results and an inability to definitively determine NP exposure guidelines. This shortcoming indicates the strong need for an enhanced in vitro system, capable of providing timely, cost effective, realistic NP assessments. Considerations for this system must include
enhancements to both the biological and physical aspects of the model, such as multiple cell types (co-culture) oriented into a 3D structure, relevant physiological fluid under dynamic motion, and realistic NP exposure levels. Through the incorporation of these enhancements, an in vitro system could be designed that would have the potential to provide more accurate correlation to human biology, while still allowing for high throughput NP screenings.

1.5 – References


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CHAPTER 2
EXPERIMENTAL PROCEDURES

2.1 – Introduction

The research performed and presented in this thesis incorporated a large number of procedures and protocols from a diverse background. This is one of the many challenges of a multidisciplinary field, such as bioengineering. Research topics and procedures throughout this thesis include standard techniques from the fields of: biology, chemical engineering, material science, toxicology, and chemistry. What follows in this chapter is an itemized list of all experimental procedures utilized for this research, including an explanation of each experimental goal as it related to the overall research and an in depth description of the experimental steps for each protocol.

2.2 – Nanoparticle Characterization

The field of nanotechnology has recently seen exponential growth, driven by the continuous discovery of new and novel nanomaterials, useful for a wide range of products and applications. With this growth follows the vital need for full characterization of nanoparticle (NP) properties and behaviors for any NP investigation.
2.2.1 – Transmission Electron Microscopy

Experimental Goal: Transmission electron microscopy (TEM) imaging was an imperative NP characterization assessment step utilized to identify unique physicochemical parameters of the NPs. TEM images allowed for verification of NP morphology and the determination of primary NP size.

Procedure:

1) The NP stocks were removed from proper storage (dark and 4°C) and vortexed to ensure a homogeneous, dispersed particle solution.

2) 1 drop of NP solution (approx. 25µl) was placed onto a copper TEM grid (Electron Microscopy Sciences) and allowed to air dry.

3) The grid was imaged on a Hitachi H-7600 microscope (Figure 2.1) using standard TEM protocols.

4) NP morphology was verified using representative TEM images, spherical in Figure 2.1.

5) Using Image J software, which measures the size of individual particles through comparison of pixel numbers, representative images were used to determine primary particles size for each NP set.

Figure 2.1: Representative TEM image of 60nm tannic acid coated gold NPs.
2.2.2 – Dynamic Light Scattering

**Experimental Goal:** Dynamic light scattering (DLS) was an important characterization assessment for identifying unique physicochemical parameters of NPs in solution. It is known that all NPs will agglomerate to some degree in solution, but full characterization included evaluating the extent of aggregate formation. DLS was utilized to determine the hydrodynamic diameter of each NP set, providing insight into the agglomeration patterns of the NPs within each environment.\(^1\)

**Procedure:**

1) The NP stocks were removed from storage and vortexed to ensure a homogeneous, dispersed particle solution.

2) The NP stocks were then diluted to 25µg/ml in either cell culture media or artificial alveolar fluid and incubated for 24 hours at 37°C under static or dynamic flow conditions. (Initial characterization was conducted in water with no incubation period.)

3) 1ml samples were then removed to an assay cuvette and imaged with a standard digital camera to capture any visual indications of agglomeration (color shifts).

4) The assay cuvette was then placed into the analysis chamber of a Malvern Zetasizer Nano ZS.

5) Dynamic light scattering analysis was performed on each sample twice (using settings specific to the NP core composition) and the average diameter was recorded in nanometers.
6) Steps 2-4 were performed in triplicate to ensure statistically accurate results.

2.2.3 – Zeta Potential

**Experimental Goal:** Zeta potential was also a critical characterization evaluation for identifying the surface charge of each NP set. As NP surface charge can be altered by the physical environment surrounding the NPs, zeta potential was utilized to determine surface charge following dispersion in solution.

**Procedure:**

1) The NP stocks were removed from storage and vortexed to ensure a homogeneous, dispersed particle solution.

2) The NP stocks were then diluted to 25µg/ml in either cell culture media or artificial alveolar fluid and incubated for 24 hours at 37°C under static or dynamic flow conditions. (Initial characterization was conducted in water with no incubation period.)

3) 1ml samples were then removed to an assay cuvette, the electrode probe was inserted, and the entire apparatus was placed into the analysis chamber of a Malvern Zetasizer Nano ZS.

4) Zeta Potential analysis was performed on each sample twice (using settings specific to the NP core composition) and the average diameter was recorded in nanometers.

5) Steps 2-4 were performed in triplicate to ensure statistically accurate results.
2.2.4 – UV-Vis

**Experimental Goal:** Ultraviolet-visible spectroscopy (UV-Vis) was another characterization method useful for identifying changes in NP spectral profiles and optical properties. UV-Vis was furthermore, utilized to verify agglomeration tendencies and NP quality for each NP set, through the analysis of NP light absorbance.

**Procedure:**

1) NP stocks were removed from storage and vortexed to ensure a homogeneous, dispersed particle solution.

2) The NP stocks were then diluted to 25µg/ml in either cell culture media or artificial alveolar fluid (AAF). (Water was utilized for initial characterization.)

3) 100µl samples of the diluted NP stocks were placed into individual wells of a 96-well plate.

4) The well plate was inserted into a SpectraMAX Plus 190 microplate reader and the samples were analyzed for absorbance over a variety of wavelengths, from 400 to 700nm.

5) The spectral profiles and agglomeration patterns were observed via representative absorbance vs. wavelength graphs (Figure 2.2).
2.2.5 – Ionic Dissolution

**Experimental Goal:** Ionic dissolution, or the ability of NPs to undergo dissolution into ions, has recently become the focus of many NP toxicity mechanism investigation, as it is currently inconclusive what role ions play in these mechanisms. Therefore, ionic dissolution is an important characterization assessment for any NP investigation. Furthermore, as the kinetic rate of ionic dissolution can be modified by local environmental factors, characterizing this NP parameter was important for comparison between NP sets in this research.²

**Procedure:**

1) The NP stocks were removed from storage and vortexed to ensure a homogeneous, dispersed particle solution.

2) The NP stocks were diluted to 0, 25, 50, and 100µg/ml concentrations in water, cell culture media, and artificial alveolar fluid.

3) 100µl samples were then placed into a 96-well plate (in triplicate) and analyzed for absorbance via a SpectraMAX Plus 190 microplate reader to generate standard
curves of absorbance as a function of NP concentration for each NP/fluid combination.

4) NP stocks were then diluted for each experiment to 25µg/ml in water, cell culture media, or AAF.

5) 100µl samples were removed and analyzed (in triplicate) in a 96-well plate to determine initial t=0 absorbance values.

6) The remaining 25µg/ml solutions were then incubated for 24 hours at 37°C under either static or dynamic flow conditions.

7) Following incubation, 100µl samples were removed and analyzed (in triplicate) in a 96-well plate to determine final t=24 absorbance values.

8) Using the previously determined standard curves for each NP/fluid combination, the absorbance values of both the initial and final readings were directly correlated to NP concentration.

9) Finally, ionic dissolution was calculated by determining the percent mass lost during the incubation period via the following equation: \( \%\ Dissolution = 100 \times \frac{(NP\ mass_{t=0} - NP\ mass_{t=24})}{NP\ mass_{t=0}} \).

10) Steps 4-8 were conducted 3 separate times to determine standard error of the mean for the final values presented.

2.3 – In vitro Responses

Currently in the field of nanotoxicology, in vitro methodologies are the predominant means of assessing the safety and toxicity mechanisms of NP interactions within a
biological system. This evaluation metric allows for rapid NP screenings for the wide range of different NPs with relative ease and speed.

2.3.1 – Cell Culture

Experimental Goal: In order to accurately analyze cellular bioeffects, it was necessary to utilized aseptic mammalian cellular culture and maintenance techniques as well as appropriate materials throughout experimentation.

Procedure:

1) The A549 human alveolar epithelial cell culture was purchased from ATCC (American Type Cell Culture).

2) Cultures were grown on tissue culture treated petri dishes (BD Falcon) in RPMI 1640 (Life Technologies) medium supplemented with 1% penicillin/streptomycin (Life Technologies) and 10% fetal bovine serum (Life Technologies) in a 5% CO₂ incubator at 37°C.

3) When cells were approximately 80-90% confluent (as determined visually via a light microscope), the media was removed.

4) The culture was then washed with 5ml of phosphate buffered saline (PBS) (Life Technologies).

5) Following the wash, 3ml of .25% trypsin (Life Technologies) was added and the culture was incubated for 5-10 minutes until the cells had detached from the bottom of the petri dish, as verified through visualization with light microscopy.
6) 5ml of fresh media was then added to deactivate the trypsin and the cells were thoroughly mixed via pipetting.

7) 2ml of the cell mixture was transferred to a new petri dish containing 8ml of fresh media and the new petri dish was then returned to the incubator.

8) Steps 3-7 were repeated every 3-4 days, as needed, for normal cell maintenance.

2.3.2 – Cell Counting/Plating

Experimental Goal: Cell counting and plating was a vital procedure required as a preliminary step before further biological in vitro assays could be performed. As it was imperative that each well contained an equal, optimized number of cells, great care was taken with cell counting and plating to ensure accurate assay results.

Procedure:

1) A nearly confluent petri dish of A549 cells was removed from the incubator, washed with PBS, and incubated with 3ml of trypsin.

2) Following cell detachment from the bottom of the dish, 5ml of fresh media was added.

3) 10µl of the cell solution was then mixed with 10µl of 0.4% trypan blue stain (Invitrogen) in a single well of a 96-well plate.

4) 10µl of this mixture was then pipetted into one side of the counting chamber slide.
5) The slide was then inserted into a Countess Cell Counter (Invitrogen) and analyzed to determine cell concentration. The Countess functions by counting the number of cells with stained exterior (dead) and subtracting the dead number from the total number of detected cells.

6) The cell mixture was then diluted to the desired plating concentration with fresh media and plated into the desired well plate for further experimentation. (Well plate size and plating concentration were dependent on which assay was to follow the plating.)

7) The well plate was then returned to the incubator and allowed to equilibrate and grow for 24 hours.

2.3.3 – Cell Viability

Experimental Goal: Cell viability was a critical in vitro cellular bioeffect technique for identifying cellular responses to the varying NP sets. In order to determine a proper NP concentration for further experimentation, it was necessary to evaluate cellular viability when exposed to each NP over a range of concentrations.

Procedure:

1) A549 cells were plated in a 96-well plate at 2x10^5 cells/ml following the procedure outlined in 2.3.2.

2) NP exposure solutions were created by diluting vortexed NP stock solutions to 0, 5, 25, and 50µg/ml in either cell culture media or artificial alveolar fluid
3) Following the 24 hour growth period, the cells were washed with PBS, 100µl of NP exposure solution was added, and the well plate was returned to the incubator for 24 hours.

4) NP exposure solutions were then removed and the cells were washed with PBS.

5) 120µl of a 5:1 mixture of cell culture media to CellTiter 96 Aqueous One Solution (Promega) was then added to each well and incubated for 2 hours at 37°C.

6) Following the incubation, 100µl of the solution was removed from each well and placed into a new 96-well plate to ensure no interference from any remaining NPs.

7) The 96-well plate was then analyzed for absorbance at 490nm using a SpectraMAX Plus 190 microplate reader.

8) Cellular viability was calculated as percent control of untreated cell wells from the same plate via the following equation: 

\[
\text{% Viability} = 100 \times \left( \frac{\text{Absorbance}_{\text{treated}}}{\text{Absorbance}_{\text{control}}} \right)
\]

2.3.4 – Lactate Dehydrogenase (LDH) Release

Experimental Goal: A second biological assay was also an important cellular bioeffect technique for determining cell death through the release of lactate dehydrogenase. This procedure provided an alternative method for determining cell viability through the measurement of LDH released by cells with a loss in membrane integrity, typically following cellular death.
Procedure:

1) A549 cells were plated in a 96-well plate at 2x10^5 cells/ml following the procedure outlined in 2.3.2.

2) NP exposure solutions were created by diluting vortexed NP stock solutions to 0, 1, 5, 15, and 25µg/ml in either cell culture media or artificial alveolar fluid.

3) Following the 24 hour growth period, the cells were washed with PBS, 100µl of NP exposure solution was added, and the well plate was returned to the incubator for 24 hours.

4) For each well designated as the positive control, 10µl of Lysis Buffer from the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) kit were added and the plate was allowed to incubate for another 45 minutes.

5) 50µl of solution from each well was then removed and placed into a new 96-well plate in the same order.

6) Following the preparation of the assay buffer per the provided protocol, 50µl was then added and allowed to incubate at room temperature, protected from light, for 30 minutes.

7) 50µl of Stop solution provided in the kit was then added to each well and the entire plate was read for absorbance using the SpectraMAX Plus 190 microplate reader at 490nm.

8) LDH release was calculated as a percent of negative control wells from the same plate via the following equation: __% LDH Release = 100 * (Absorbance_{treated} / Absorbance_{control}).__
2.3.5 – Reactive Oxygen Species (ROS) Production

**Experimental Goal:** ROS production was also a central cellular bioeffect technique for determining levels of cellular stress via reactive oxygen species, a recognized predictor of cellular death. This procedure provided a means for real time analysis of the ROS production at various time intervals following exposure.

**Procedure:**

1) A549 cells were plated in a 96-well plate at $2 \times 10^5$ cells/ml following the procedure outlined in 2.3.2.

2) Dry DCFDA (2',7'-dichlorodihydrofluorescein diacetate) powder (Invitrogen) was rehydrated in ultrapure dimethyl sulfoxide (DMSO) (Fisher) to a concentration of 1mM.

3) The DCFDA solution was then diluted to a concentration of 100µM in PBS.

4) Following the 24 hour growth period, cells were washed with PBS and 100µl of the diluted PBS/DCFDA mixture was added to each well.

5) The well plate was then returned to the incubator for 30 minutes.

6) NP exposure solutions were created by diluting vortexed NP stock solutions to 0, 1, 5, 15, and 25µg/ml in either cell culture media or artificial alveolar fluid.

7) Following the PBS/DCFDA incubation, the cells were washed with PBS and 100µl of NP exposure solution was added to each well.

8) A t=0 initial timepoint was then recorded using the SpectraMAX Plus 190 microplate reader on the fluorescence setting with an excitation wavelength of
485nm and an emission wavelength of 538nm and the plate was returned to the incubator.

9) At each subsequent timepoint desired (24 hours in this case), the process for step 8 was repeated.

10) ROS production was calculated as a percent of negative control from untreated wells on the same plate following a subtraction of the t=0 background using the following equation:

\[
\text{% ROS production} = 100 \times \left( \frac{\text{Fluorescence}_{\text{treated}}}{\text{Fluorescence}_{\text{control}}} \right).
\]

2.4 – Evaluating the Nano-Cellular Interface

Within the field of nanotoxicology, the nano-cellular or nano-bio interface, where the NPs physically encounter the cell or biological surface, is an important evaluation metric for examining both NP behaviors and cellular responses. This interface has the potential to provide significant information to determine NP toxicity mechanisms, as the nano-cellular interface is the initial interaction point between the NP and the cell.

2.4.1 – Deposition Efficiency

**Experimental Goal:** Deposition efficiency was an essential nano-cellular interface assessment that provided the means for determining the fraction of dosed NPs that actually reached the cell surface. As NP applications generally require high numbers of NPs to reach the target area, deposition efficiency was an important NP behavior for investigation.

**Procedure:**
1) A549 cells were plated in a 24-well plate at 1.6x10^5 cells/well following the procedure outlined in 2.3.2.

2) NP stocks were removed from storage, vortexed, and diluted to 10µg/ml in cell culture media or AAF to create the NP exposure solutions.

3) The A549 cells were washed with PBS and dosed with varying NP exposure solutions, with the volume of exposure solutions depending on flow condition (2ml for static, 3ml for dynamic).

4) 100µl samples were taken (in triplicate) from each well without disturbing the cells, placed into a 96-well plate, and analyzed for absorbance spectra via the SpectraMAX Plus 190 microplate reader to obtain an initial t=0 timepoint.

5) The 24-well plates were then returned to the incubator for 24 hours under either static or dynamic flow conditions.

6) Following the 24 hour incubation, 100µl samples were again taken (in triplicate) without disturbing the cells and analyzed for absorbance spectra to obtain the final t=24 timepoint.

7) Using the previously determined UV-Vis standard curves from 2.2.5, the absorbance values were converted to NP concentrations.

8) Deposition efficiency was calculated as a percent change in NP concentration following cellular exposure via the following equation: 
\[
\% \text{Deposition} = 100 \times \frac{(NP \ mass_{t=0} - NP \ mass_{t=24})}{NP \ mass_{t=0}}.
\]
2.4.2 – Cellular Internalization via TEM Imaging

Experimental Goal: Determining the cellular internalization of NPs was also an important tool to confirm and corroborate deposition efficiency and visualize uptake patterns for specific NP sets. This was conducted via biological preparation and TEM imagery.³

Procedure:

1) A549 cells were plated in a 24-well plate at 1.6x10⁵ cells/well following the procedure outlined in 2.3.2.

2) NP stocks were removed from storage, vortexed, and diluted to 10µg/ml in cell culture media or AAF to create the NP exposure solutions.

3) Following the 24 hour growth period, the cells were washed with PBS and exposed to 2ml or 3ml of NP exposure solution under either static or dynamic flow conditions, respectively, for 24 hours within the incubator.

4) After 24 hours, the plate was removed and the cells were washed with PBS.

5) 500µl of 0.25% trypsin (Life Technologies) was then added to each well and the plate was returned to the incubator until the cells detached from the bottom.

6) 1ml of fresh media was added to each well and the contents were then transferred to individual centrifuge tubes.

7) The tubes were spun down at 10,000 rcf for 10 minutes until the cells suspended in the media were pelleted at the bottom.

8) Media was then removed and the cell pellets were fixed in a 2% glutaraldehyde/2% paraformaldehyde (Electron Microscopy Sciences) solution for 2 hours at room temperature.
9) The glutaraldehyde/paraformaldehyde solution was then removed and the cell pellets were washed with PBS.

10) A 1% osmium tetroxide (Electron Microscopy Sciences) solution was then added to the cell pellets for approximately 2 hours until the stain had visibly penetrated the entire cell pellet.

11) The cell pellets were then washed with PBS and removed to beam capsules.

12) Increasing concentrations of ethanol were then used to dehydrate the cell pellets, with sequential 30 minute incubations at 50, 70, 90, and 100%.

13) LR White Resin (Electron Microscopy Sciences) was then added and each beam capsule was cured overnight in a 60°C vacuum oven.

14) Following curing, cell pellets were removed from the beam capsule and thinly sectioned to 70nm using an ultramicrotome (Model EM UC7, Leica).

15) Sections were then placed onto copper TEM grids (Electron Microscopy Sciences), allowed to dry, and imaged via a Hitachi H-7600 microscope (Figure 2.3).

Figure 2.3: Representative TEM image of A549 cell exposed to tannic acid coated Au NPs in AAF under dynamic flow conditions.
2.4.3 – High Resolution Fluorescence and Darkfield Microscopy

**Experimental Goal:** High resolution fluorescence microscopy was also utilized in tandem with darkfield microscopy to visualize fluorescently stained A549 cell morphology simultaneously with exposed NPs. 4

**Procedure:**

1) A549 cells were plated at 1.5x10^5 cells per chamber on a Nunc Lab-Tek 2-chambered slide (Thermo Scientific) following the procedure outlined in 2.3.2.

2) NP stocks were removed from storage, vortexed, and diluted to 25µg/ml in cell culture media or AAF to create the NP exposure solutions.

3) Following the 24 hour growth period, the cells were washed with PBS and exposed to either 2ml or 3ml of NP exposure solution under either static or dynamic flow conditions, respectively, for 24 hours within the incubator.

4) After the 24 hour exposure, the cells were washed with PBS and fixed with 500µl of 4% paraformaldehyde (Electron Microscopy Sciences) per chamber for 10 minutes.

5) Following another PBS wash, the cells were permeablilized with 0.1% Triton X-100 (Sigma Aldrich) for 20 minutes.

6) Cells were then washed again with PBS and incubated for 45 minutes with 1% bovine serum albumin (BSA) (Sigma Aldrich) as a non-specific binding blocker.
7) Alexa Fluor 555-phalloidin (Invitrogen) was then mixed with 1% BSA at a 1:40 ratio and incubated with the cells at room temperature, protected from light for 30 minutes.

8) Cells were then washed with PBS once more, the chamber was removed from the slide, and the slides were allowed to dry.

9) 1 drop of Prolong Gold Antifade Reagent plus 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) was then added to each side of the slide, a cover slip was placed on top, and the slide was sealed with clear nail polish for preservation.

10) The slides were then imaged using a CytoViva 150 ultraresolution attachment to an Olympus BX41 microscope (Aetos Technologies) via QCapture Pro Imaging Software (Figure 2.4).

![Image](image.png)

**Figure 2.4:** Representative high resolution fluorescence and darkfield microscopy of A549 cell exposed to 75nm PVP coated Au NPs. In this image, cell nuclei and actin are colored blue and red, respectively and NPs are colored white.

2.5 – *Variations on in vitro Systems*

To construct a more realistic *in vitro* microenvironment for NP behavior and cellular bioeffect evaluations, specific aspects of the system were targeted for variation and
enhancement. Both relevant physiological fluid and dynamic motion were incorporated to achieve this goal.

2.5.1 – Artificial Alveolar Fluid

Experimental Goal: As the A549 cell line selected was a human alveolar epithelial line, the relevant physiological fluid incorporated into the microenvironment was selected to be artificial alveolar fluid. The following procedure was based on the method published by Stopford et. al.5

Procedure:

1) 500ml of distilled water was measured out and added to a sterile bottle.

2) A stir bar was added and the bottle was placed onto a stir plate.

3) The following chemical powders were then added in the order and amounts indicated in the table below while the solution remained stirring on the stir plate:

<table>
<thead>
<tr>
<th>Chemical Powder</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>0.2033g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6.0193g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2982g</td>
</tr>
<tr>
<td>Dibasic sodium phosphate</td>
<td>0.1420g</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>0.0710g</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>0.3676g</td>
</tr>
<tr>
<td>Sodium acetate trihydrate</td>
<td>0.9526g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>2.6043g</td>
</tr>
<tr>
<td>Sodium citrate dihydrate</td>
<td>0.0970g</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>0.1000g</td>
</tr>
</tbody>
</table>
4) Following the additions, the solution was brought to a final volume of 1L and allowed to thoroughly mix until all ingredients were incorporated.

2.5.2 – Dynamic Flow Exposure System

**Experimental Goal:** The dynamic flow enhancement to the *in vitro* system was achieved through the use of a peristaltic pump. Multiple channels allowed for the simultaneous evaluation of different samples, while the use of a 24-well plate allowed for consistent comparison between static and dynamic conditions.

**Procedure:**

1) A 24-channel peristaltic pump (Ismatec, model #ISM939D) was selected to provide the flow with each channel exclusively connected to a single well of a 24-well plate.

2) 1/16 inch (inner diameter) tubing from each channel was secured through the lid of the 24-well plate to ensure that the inlet and outlet tubing for each channel remained at opposite ends of each well, providing unidirectional flow (Figure 2.5).

3) For each experimentation, the tubing was primed with exposure fluid to ensure that the liquid height remained the same for both static and dynamic conditions.

4) During experimentation, the pump, tubing, and 24-well plate were stored within the incubator to maintain optimal cell culture conditions of 5% CO₂ and 37°C.
5) Calibration was conducted to correlate the pump settings to volumetric flow rates (in ml/min) as follows:

a. The inlet end of tubing was placed into a reservoir of water and the outlet into a graduated cylinder.

b. The length of tubing was primed with water from the reservoir to ensure measurement would be immediate and accurate.

c. A pump setting of interest was selected and the pump was run for exactly 30 seconds.

d. Following the 30 second run, the volume of water in the graduated cylinder was measured and recorded.

e. This process was repeated for a wide range of pump settings, from the maximum of 11.8 to the minimum of 0.98.

f. A calibration curve was created with this data for interpolation of any pump setting desired.

6) Volumetric flow rates were subsequently converted to linear velocity (cm/sec) through the following steps:

a. The tubing inner diameter of 1.52mm was utilized to determine the cross sectional area of the tubing via the equation $A = \pi \times r^2$ where $A$ is the cross sectional area, $\pi=3.14$, and $r$=tubing inner radius=0.076cm, providing a cross sectional area of $A=0.0182cm^2$.

b. Using the cross sectional area, each volumetric flow rate (ml/min) was converted to linear velocity (cm/sec) via the equation $Q = V \times A$, where $V$=linear velocity and $Q$=volumetric flow rate. For example, the pump
setting 1.48 corresponding to a volumetric flow rate of 0.75 ml/min, was converted to a linear velocity of \( Q = 0.689 \text{ cm/sec} \) via \( Q = 0.75 \text{ ml/min} \times \frac{0.0182 \text{ cm}^2}{1.48} \).

7) Once the pump settings were converted to linear velocities, a pump setting was selected (1.48), which correlated to a linear velocity between 0.2 and 1.0 cm/sec, the standard linear velocity range for arteries, arterioles, capillaries, and venules based on Table 2.1, as adapted from *Introduction to Bioengineering, 1996*.

### Table 2.1: Blood circulation properties, adapted from *Introduction to Bioengineering, 1996*

<table>
<thead>
<tr>
<th>Vessels</th>
<th>Diameter (mm)</th>
<th>Length (cm)</th>
<th>Wall Thickness (mm)</th>
<th>Contained Volume (mL)</th>
<th>Mean Pressure (mmHg)</th>
<th>Average Velocity (cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>25.0</td>
<td>40.0</td>
<td>2.0</td>
<td>100</td>
<td>100 (av.)</td>
<td>40 (av.)</td>
</tr>
<tr>
<td>Arteries</td>
<td>15-0.15</td>
<td>15.0</td>
<td>0.8</td>
<td>350</td>
<td>90 (av.)</td>
<td>40-10</td>
</tr>
<tr>
<td>Arterioles</td>
<td>0.14-0.01</td>
<td>0.2</td>
<td>0.02</td>
<td>50</td>
<td>60</td>
<td>10-0.1</td>
</tr>
<tr>
<td>Capillaries</td>
<td>0.008</td>
<td>0.05</td>
<td>0.001</td>
<td>300</td>
<td>30-20</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Venules</td>
<td>0.01-0.14</td>
<td>0.2</td>
<td>0.002</td>
<td>300</td>
<td>20</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Veins</td>
<td>0.15-15</td>
<td>18.0</td>
<td>0.6</td>
<td>2500</td>
<td>15-10</td>
<td>0.3-5</td>
</tr>
<tr>
<td>Vena Cava</td>
<td>30.0</td>
<td>40.0</td>
<td>1.5</td>
<td>300</td>
<td>10-5</td>
<td>5-30</td>
</tr>
</tbody>
</table>
Figure 2.5: Image of peristaltic pump inlet and outlet secured inside the lid for a single well.

2.6 - References


CHAPTER 3
RESULTS, DISCUSSION, AND CONCLUSIONS

3.1 – Introduction
In recent years, nanoparticle (NP) prevalence throughout the consumer, medical, and industrial markets has exponentially increased. This phenomena is due to the various advantages provided by the unique physicochemical properties inherent in nano-sized particles that differ from their bulk counterparts. By taking advantage of distinctive NP characteristics, such as enhanced optical properties, improved magnetic features, and amplified reactivity due to an increased surface area to volume ratio, a wide range of products and treatments have been developed for biotechnology applications.\textsuperscript{1,2} For example, both silver (Ag) and gold (Au) NPs are highly utilized due to antimicrobial and plasmonic properties, respectively.\textsuperscript{3-5}

However, this rapid incorporation of NP products into daily life also increases the likelihood of human exposure to these particles, during either production in industry or during product usage itself. With increased exposure, the need for nanomaterial toxicology investigations arose, with the difficult goal of ascertaining the impact of NPs on a biological system and establishing recommended exposure limits.\textsuperscript{6,7} Further complicating this endeavor are the vast number of different NPs that require screening,
arising from various tunable NP parameters, including size, shape, surface modification, and composition, that allow for the discovery of novel NP combinations.\textsuperscript{8}

\textit{In vitro} methodologies are widely used within the field of nanotoxicology, as a fast and cost effective option for high throughput NP screenings.\textsuperscript{9} However, traditional monolayer cell culture possesses a considerable drawback, as these models are not truly representative of an \textit{in vivo} human system. Conversely, \textit{in vivo} animal models can provide much more accurate predictions of human environments and exposure routes, but are less utilized due to significance expense and time constraints. Furthermore, a lack of correlation exists between these two methodologies due to the innate differences between them.\textsuperscript{10-12} To that end, the nanotoxicology community would benefit greatly from the development of a cellular model that retains the advantages of an \textit{in vitro} system while incorporating \textit{in vivo} enhancements, to provide more realistic NP exposure scenarios.

In order to address the aforementioned issues, and to attempt to bridge the gap between current \textit{in vitro} and \textit{in vivo} systems, the goal of this research was to construct and implement a more realistic microenvironment for improved evaluation of NP behavior and cellular responses within the nano-bio interface (Figure 3.1). As inhalation has been identified as a primary exposure route,\textsuperscript{13} the A549 human lung alveolar epithelial cell line was utilized within the constructed system and paired with artificial alveolar fluid. Dynamic flow was also incorporated at relevant physiological flow rates within the human circulatory system. Furthermore, multiple NPs of varying core compositions and surface coatings were selected in this proof-of-concept study to fully elucidate any
interactions and behaviors within the system. The following chapter enumerates the various results and findings of this research utilizing the protocols and procedures explained in Chapter 2.

![Objective 1: Establishment of Enhanced Microenvironment](image1)

**Objective 1**
- Dynamic flow system
- Physiologic Fluid
- Relevant Cell Line

![Objective 2: NP Characterization](image2)

**Objective 2**
- NP Characterization: (DLS, TEM, UV-Vis, etc.)
  1. Pre-microenvironment
     - Initial
  2. Post-microenvironment
     - Fluid or flow dependency

![Objective 3: Evaluation of Nano-Bio Interface](image3)

**Objective 3**
- NP Deoposition Efficiency
- NP Internalization
- Cell Morphology

**Figure 3.1: Overview of the three main objectives for this research.**

3.2 – Results

3.2.1 – Generation of Enhanced *in vitro* Microenvironment

In order to more accurately mimic an *in vivo* NP exposure scenario, the enhanced microenvironment incorporated three critical design elements: physiologically accurate dynamic flow rates, a cell line representative of common NP exposure routes, and an artificial physiological fluid corresponding to the selected cell line (Objective 1).

Dynamic flow was established through the use of a multi-channel peristaltic pump, which allowed for the simultaneous evaluation of independent samples. The inlet and outlet tubing of each channel was secured through the lid of a 24-well plate (Figure 3.2), to provide unidirectional flow across the bottom of each well. Following calibration of the
peristaltic pump as explained in 2.5.2, a pump setting was determined that allowed for the linear velocity to fall within the standard physiological range for veins and arterioles as shown in Table 3.1, adapted from Introduction to Bioengineering, 1996.\textsuperscript{14}

![Peristaltic Pump Diagram]

\textbf{Figure 3.2: Representation of single independent channel/well microenvironment with arrows indicating directionality of flow.}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Vessels & Diameter (mm) & Length (cm) & Wall Thickness (mm) & Contained Volume (mL) & Mean Pressure (mmHg) & Average Velocity (cm/sec) \\
\hline
Aorta & 25.0 & 40.0 & 2.0 & 100 & 100 (av.) & 40 (av.) \\
Arteries & 15-0.15 & 15.0 & 0.8 & 350 & 90 (av.) & 40-10 \\
Arterioles & 0.14-0.01 & 0.2 & 0.02 & 50 & 60 & 10-0.1 \\
Capillaries & 0.008 & 0.05 & 0.001 & 300 & 30-20 & <0.1 \\
Venules & 0.01-0.14 & 0.2 & 0.002 & 300 & 20 & <0.3 \\
Veins & 0.15-15 & 18.0 & 0.6 & 2500 & 15-10 & 0.3-5 \\
Vena Cava & 30.0 & 40.0 & 1.5 & 300 & 10-5 & 5-30 \\
\hline
\end{tabular}
\caption{Blood circulation properties, adapted from Introduction to Bioengineering 1996.\textsuperscript{14}}
\end{table}

One major advantage of the microenvironment design is the ability for customization to a specific cellular organ or location, simply through the selection of a relevant cell type and physiological fluid. For this proof-of-concept study, a lung system was modeled, as
inhalation is a primary route of NP exposure in humans. The A549 cell line, human alveolar basal epithelial, was specifically selected for inclusion within the microenvironment due to its well documented use in NP exposure studies. Furthermore, in support of the lung exposure route, artificial alveolar fluid was incorporated into the system as a more accurate mimic of the fluid found within the alveolar region of the lungs in the human body.

3.2.2 – NP Selection and Initial Characterization

In order to evaluate the improved functionality of the enhanced in vitro microenvironment, several NPs, of varying core composition and surface modification, were selected for assessment. Due to a high rate of utilization, increased likelihood of human exposure, and well established response in biological systems, both gold (Au) and silver (Ag) NPs were selected for use in this study. Furthermore, as surface chemistry has been shown to have a significant effect on cellular interactions, Au NPs were selected with two different surface modifications to evaluate the influence of surface functionalization on NP behavior and the nano-cellular interface within the enhanced microenvironment. To this end, the three NP sets purchased from nanoComposix as concentrated liquid stock solutions for experimentation were: 75nm polyvinylpyrrolidone (PVP) coated Au NPs (Au PVP), 75nm PVP coated Ag NPs (Ag PVP), and 60nm tannic acid (TA) coated Au NPs (Au TA). PVP surface modification was chosen due to its well documented stabilizing effect, whereas TA was selected to promote NP-NP and NP-protein interactions.
With the rapid growth and development of nanotechnologies, full NP characterization has become a vital aspect of any NP investigation.\(^8\) To that end, extensive NP characterization was conducted on each NP stock to determine a baseline of key parameters for comparison and to verify NP quality. TEM imaging (Figure 3.3: A-C) was performed as described in section 2.2.1 to allow for visualization of NP morphology and determination of primary particle size. Spherical morphology was confirmed for all NP stocks and primary particle size was determined to be approximately 80 nm for PVP coated NPs and 65nm for the Au TA set (Table 3.2). As all NPs agglomerate to some degree, depending on NP properties and environmental variables,\(^{21,22}\) dynamic light scattering (DLS) was performed, as described in 2.2.2. As shown in Table 3.2, all NP stocks showed minimal agglomeration in water.

![Representative TEM images of (A) Ag PVP, (B) Au PVP, and (C) Au TA NP stock solutions.](image)

**Figure 3.3:** Representative TEM images of (A) Ag PVP, (B) Au PVP, and (C) Au TA NP stock solutions. TEM images of (D) Ag PVP, (E) Au PVP, and (F) Au TA NPs are following 24 hour incubation in AAF.
Furthermore, through the use of UV-Vis (Ultraviolet-Visible spectroscopy) analysis (2.2.4), the spectral profile of each NP stock was determined (Figure 3.4) for use as a baseline comparison for future experimentation. The spectra of all three particle types in water show well-defined peaks at the proper composition specific wavelengths (approximately 450nm for Ag NPs and 550nm for Au NPs) further verifying the minimal agglomeration determined via DLS. The surface charge of each NP set was also analyzed via the protocol described in 2.2.3. As seen in Table 3.2, negative zeta potentials were observed for all NP sets, in accordance with the charge of their respective surface coatings. Finally, the rate of ionic dissolution was determined for each NP set following a 24 hour incubation at 37°C in water. Ag PVP showed significantly higher dissolution than the Au NP sets, in agreement with current literature.23

Table 3.2: Initial characterization of NP stock solutions in water.

<table>
<thead>
<tr>
<th>NP Type</th>
<th>Primary Size (nm)</th>
<th>Agglomerate Size (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Dissolution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag PVP</td>
<td>82.4 ± 6.5</td>
<td>93.6 ± 1.6</td>
<td>-18.3 ± 2.9</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Au PVP</td>
<td>84.3 ± 11.0</td>
<td>106.7 ± 1.2</td>
<td>-15.0 ± 1.2</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Au TA</td>
<td>65.1 ± 5.3</td>
<td>74.8 ± 1.0</td>
<td>-31.8 ± 0.9</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>
3.2.3 – NP Characterization under the Influence of Microenvironment Variables

As recent studies have identified an intrinsic link between local environmental factors, NP behavior, and resultant cellular bioeffects,\textsuperscript{24,25} it was essential to elucidate the influence of the microenvironment physiological variables on key NP parameters. Specifically, it was important to isolate and identify if NP behavior within the microenvironment was a function of either/both dynamic flow and/or environmental composition (physiological fluid). To that end, initial experimental efforts centered on NP characterization within the microenvironment under acellular conditions (Objective 2 in Figure 3.1).

First, the spectral profile of each NP set was evaluated following dispersion in media and AAF as described in 2.2.4, and compared against the stock spectra (Figure 3.4). Alterations were seen to the UV-Vis profiles dependent on NP surface coating and fluid type. Both Au PVP and Ag PVP displayed negligible alterations to their spectral signatures (Figures 3.4A and 3.4B). However, a significant loss in maximum absorbance and peak broadening was associated with the Au TA NPs in AAF (Figure 3.4C). These modifications are indicative of excessive NP agglomeration, resulting in a loss of

![Figure 3.4: UV-Vis spectral profiles of (A) Ag PVP, (B) Au PVP, and (C) Au TA NPs following dispersion in water, media, and AAF.](image-url)
stability.\textsuperscript{26} Furthermore, the spectral alterations for Au TA in AAF were verified via inspection: with a visible color shift from red to purple demonstrating the change in absorbance (Figure 3.5).

![Figure 3.5: Images of NP solutions following dispersion in either water, media, or AAF. (A) Ag PVP in water, (B) Ag PVP in media, (C) Ag PVP in AAF, (D) Au PVP in water, (E) Au PVP in media, (F) Au PVP in AAF, (G) Au TA in water, (H) Au TA in media, (I) Au TA in AAF.](image)

To further confirm and quantify the extent of NP agglomeration, DLS was performed on all NP sets (Figure 3.6). The agglomerate sizes showed excellent correlation with the previously determined spectral profiles. Minor alterations in agglomerate size were identified for PVP coated particles (Figures 3.6A and 3.6B), as expected due to the stabilizing effect of PVP surface modification. In contrast, Au TA NPs exhibited significant agglomeration in AAF, with final aggregate sizes of approximately 260 nm (Figure 3.6C). The extensive agglomeration of Au TA NPs in AAF led to a loss of
particle stability, producing the shift in the spectral profile. In the absence of a protein rich media, the TA surface modification likely increased the number of NP-NP interactions, leading to the increased agglomerate size.

Agglomeration patterns were further visualized via TEM imagery (Figure 3.3D-F). No significant changes were observed in agglomerate size for either PVP coated NP set, in agreement with UV-Vis and DLS data. Additionally, increased particle interactions were observed for Au TA in AAF (Figure 3.3F), further confirming the previous observations.

![Figure 3.6](image)

**Figure 3.6:** Agglomerate size of (A) Ag PVP, (B) Au PVP, and (C) Au TA NPs following a 24 hour incubation in either media or AAF under either static or dynamic conditions. (represents three independent trials, * denotes statistical significance between media and AAF samples under the same flow condition, p<0.05)

This analysis demonstrated that the NP spectral profiles and degree of agglomeration were dependent on both environmental composition and surface chemistry. Next, it was then necessary to ascertain if exposure to dynamic flow modified these results. As seen in Figure 3.6, the introduction of shear stress brought on by dynamic flow did not alter the agglomerate size of any of the NP sets. This result was not surprising, as the flow was set to low, physiologically relevant rates and introduced very low shear into the system. To
further explore the stability of the aggregates under dynamic flow, the 24 hour exposure analysis was repeated at a significantly higher flow rate, comparable to the vena cava, of 6 ml/min (a linear velocity of 5.5 cm/sec). Even following this increase, of nearly one order of magnitude, the extent of NP agglomeration remained unaltered (Figure 3.7).

Next, the surface charge (2.2.3) of each NP set was evaluated following dispersion in either media or AAF under either static or dynamic conditions (Figure 3.8). For the PVP coated NPs, no significant change was observed, as all NP sets showed a negative surface charge around -10 mV, very similar to the original values (approximately -15 mV) dispersed in water. The Au TA NPs, however, exhibited a slight change in surface charge following dispersion in media and AAF (from approximately -30 mV to -10 mV), though still remaining negative. Similarly, dynamic flow appeared to have no effect on NP surface charge (Table 3.2).

Figure 3.7: Evaluation of NP agglomerate size under low and high volumetric flow rates in water, media, and AAF. Final agglomerate size after a 24 hour exposure are show for (A) Ag PVP, (B) Au PVP, and (C) Au TA.
Finally, the rate of NP ionic dissolution was evaluated as a function of environmental composition and dynamic flow (Figure 3.9). Under static conditions, a fluid-specific effect was observed, with a drop in the rate of ion formation being seen in AAF versus media. In particular, Au TA NPs exhibited a significant decrease in dissolution rate in AAF. Due to the extensive agglomeration in AAF, the Au TA NPs have a smaller surface area to volume ratio, resulting in lower NP reactivity. As previous reports have identified higher dissolution rates associated with smaller NPs, it therefore follows that lower dissolution rates would be seen in the Au TA NPs with high agglomeration. The impact of dynamic flow on ionic dissolution rates, however, was not as straightforward a physiological fluids. For Ag PVP NPs in media, a slight drop in the rate of ion formation was observed. However, for both Au NP sets in AAF ionic dissolution exhibited an increase from static to dynamic conditions. This observation is, hypothetically, due in part to the fact that Au NPs have the unique, complex ability that following release, a gold ion is capable of re-depositing elsewhere. Essentially this means that Au ionic dissolution is, in part, reversible, providing a potential explanation for the unpredicted behavior and larger error associated with both Au NP sets. No other statistically significant alterations were noted for other NP/fluid/flow combinations, therefore, while
the presence of shear stress does have the potential to modulate kinetic rates of
dissolution, there doesn’t appear to be a consensus as to how the response varies.

Figure 3.9: Ionic dissolution rates on a per mass basis of (A) Ag PVP, (B) Au PVP, and (C) Au TA NPs after a 24 hour incubation in media or AAF under either static or dynamic conditions. (represents four independent trials, * denotes statistical significance between media and AAF samples under the same flow conditions, † denotes significance between static and dynamic flow conditions for the same fluid environment, p<0.05)

3.2.4 – Biological Responses

After determining that physiological fluid and dynamic flow were capable of modulating
NP behaviors, the next desired endpoint was an examination of the nano-bio interface. In
order to achieve this, however, basic biological responses to the various
microenvironmental conditions (AAF and NPs) needed to be evaluated to ensure cellular
compatibility throughout the experimentation. To this end, basic cell viability was
assessed for both media and AAF exposure for the Ag PVP and Au PVP NP sets via
MTS (CellTiter 96 Aqueous One Solution) analysis (Figure 3.10) as described in 2.3.3.
The Au TA NP set was not included as gold NPs are generally considered biocompatible,
and only one NP set of that core composition was required. As expected, Ag PVP
displayed statistically significant viability loss in media while Au PVP remained
generally biocompatible.
However, in the presence of AAF, both NP sets under all concentrations exhibited a consistent and significant loss in viability. It was hypothesized that this anomaly was due to an interference of the AAF with the cellular viability assay, based on the phospholipid component of the AAF itself. As the viability assay was a mitochondrial function assay, and therefore a measure of live cells, it was possible that the AAF interfered with the reagent permeability of the membrane.

![Figure 3.10](image)

**Figure 3.10: Cellular viability for (A) Ag PVP and (B) Au PVP NPs in either media or AAF.** (represents three independent trials, * denotes statistical significance from control of the same fluid, p<0.05)

To remedy the situation, a second biological assay was chosen, that determined the cellular viability through the release of lactate dehydrogenase (LDH) from disrupted cell membranes (2.3.4), thus measuring cytotoxicity based off the number of dead cells. As this assay was not dependent on the permeability of the reagent to the cell membrane, greater success was achieved. Both the Ag PVP and Au PVP sets were again analyzed, as shown in Figure 3.11. As expected, the Ag PVP NPs exhibited a higher degree of dose dependent LDH leakage than the Au PVP NPs, indicating a greater loss of cellular viability. Furthermore, statistically significant differences were observed between the
media and AAF conditions for the Ag PVP NPs, with greater LDH leakage present for the media condition, indicating a less toxic response in AAF. The Au PVP NPs show a slight dose dependent increase in LDH release, with the media condition, again, showing greater toxicity than the corresponding AAF. This differential response to the physiological fluids, represented altered cellular or NP behaviors that warranted further investigation.

![Figure 3.11](image)

**Figure 3.11**: LDH release of A549 cells following 24 hour exposure to (A) Ag PVP and (B) Au PVP NPs in either media or AAF. (represents three independent trials, * denotes statistical significance between media and AAF samples under the same flow conditions, † denotes significance between static and dynamic flow conditions for the same fluid environment, p<0.05)

The final biological assay, ROS (reactive oxygen species) production was also evaluated for both Ag PVP and Au PVP NPs (Figure 3.12) to evaluate cell stress following NP exposure. In agreement with the previous assay, ROS levels were shown to increase following 24 hour exposure in both media and AAF for the Ag PVP NPs, indicating an increase in stress levels within the cells. Furthermore, ROS levels were significantly higher under media exposure as compared to AAF, representing greater cellular stress in traditional cell media. This observations further confirms the increased toxicity observed for the Ag PVP NPs in media via the LDH leakage (Figure 3.11A). The Au PVP NPs
exhibited a slight increase in ROS levels corresponding to the LDH increase previously observed, with higher levels observed for media exposure. Additionally, the Au PVP NPs under both fluid conditions, produced a dose dependent ROS response, with ROS levels decreasing with increasing NP concentration. Previous studies have shown that Au NPs can serve as an antioxidant and reduce free radical formation (ROS), providing support for this observation.

Figure 3.12: ROS production of A549 cells following 24 hour exposure to (A) Ag PVP and (B) Au PVP NPs in either media or AAF. (represents three independent trials, * denotes statistical significance between media and AAF samples under the same flow conditions, † denotes significance between static and dynamic flow conditions for the same fluid environment, p<0.05)

3.2.5 – NP Deposition in Microenvironment

Following the determination that physiological variables were capable of altering key NP parameters and behaviors as well as modulating basic biological responses, the next step was to examine if these modifications translated to an altered nano-bio interface. First, the fraction of the dosed NPs that actually reached the nano-cellular interface, known as the deposition efficiency, was determined (Figure 3.13). Under static conditions, incubation in AAF produced a slight deposition increase for PVP coated NPs. This negligible modification can be attributed to the stability and lack of agglomeration
brought on by the PVP functionalization. However, for Au TA NPs the extensive agglomeration in AAF induced significant amounts of particle sedimentation, causing the deposition rate to more than double.

After the introduction of dynamic flow, the Au TA NPs deposition efficiency was unchanged, again due to the strong sedimentation force present with extensively agglomerated NPs. Furthermore, TA forms a tight bond with the cellular membrane,\textsuperscript{19} which is unlikely to be disturbed by the low flow rates present within the microenvironment, allowing for an increased deposition efficiency. In contrast, the PVP coated NPs displayed substantially lower rates of cellular deposition under dynamic conditions, for both media and AAF. As the PVP NPs remained as relatively small agglomerates under all NP/fluid/flow combinations, the rates of NP sedimentation were relatively constant. Therefore, it is likely that the flowing system was able to disrupt the internalization process through the breakage of the weak PVP-cellular bonds and promote lateral movement throughout the system, resulting in lower deposition efficiencies.

Figure 3.13: NP deposition efficiency associated with the A549 cell line of (A) Ag PVP, (B) Au PVP, and (C) Au TA NPs. Evaluation of NP deposition was carried out in both media and AAF environments under either static or dynamic flow conditions. (represents four independent trials, * denotes statistical significance between media and AAF samples under the same flow conditions, † denotes significance between static and dynamic flow conditions for the same fluid environment, p<0.05)
3.2.6 – Nano-cellular Interface

As significant alterations were observed in NP deposition efficiency as a function of both dynamic flow and physiological composition, visualization of the nano-cellular interface was desired. First, TEM images were obtained to allow for visualization of NP uptake patterns within the A549 model under the various experimental conditions as described in 2.4.2. As the Au TA NPs exhibited the highest deposition efficiency, these particles were selected for TEM uptake analysis (Figure 3.14 and 3.15). TEM imagery further confirmed the deposition pattern previously observed, showing greater NP-cell association in AAF as compared to media. Under static conditions (Figure 3.15A and 3.15B) NPs with increased agglomerate size in AAF were identified in both intracellular vacuoles and bound to the cell surface. Under media exposure regardless of flow condition, no differences were discernable in NP presence or degree of uptake, in agreement with the deposition data (Figure 3.13). Interestingly, under dynamic flow in AAF (Figure 3.15D), the majority of Au TA NPs were discovered bound mainly to the cell surface rather than internalized. These images seem to support the hypothesis that the greater deposition of Au TA NPs occurs in the AAF due to the high energy bonding between the TA surface modification and the proteins on the cellular surface.
Figure 3.14: Representative control images for TEM evaluation of (A) static media, (B) static AAF, (C) dynamic media, and (D) dynamic AAF.

Figure 3.15: TEM imaging was used to visualize and verify deposition patterns following a 24 hour exposure to 10 µg/mL Au TA NPs. Representative images are shown of A549 exposure under the following conditions: (A) static media, (B) static AAF, (C) dynamic media, and (D) dynamic AAF. The area within the black box is enlarged within the inset for each condition.

The nano-cellular interface of all NP/fluid combinations was then visualized and examined through the use of high fluorescence and darkfield microscopy (Figures 3.16 and 3.17) via the protocol described in 2.4.3. These images provided a number of critical pieces of information regarding cellular behavior and NP interaction with the A549 cells within the microenvironment. Firstly, the images show a large degree of association
between the NPs and the cells. Furthermore, the NPs can be seen in a wide variety of agglomerate sizes, in agreement with the varying degree of agglomeration determined during NP characterization. Of greater interest, however, were the observations of altered cellular morphology dependent on microenvironmental variables. Under dynamic conditions, the actin, and thus the A549 cells themselves, elongated in the directionality of flow; severely disrupting the normal cellular morphology and behavior. When incubated in AAF, under either static or dynamic conditions, the cells displayed a high degree of curvature that is extremely atypical for this cell model, suggesting further disruption of normal cell behavior. Consequently, when exposed to both AAF and dynamic flow, the cells were observed to be elongated and curved into nearly a half circle shape, even in the absence of NPs entirely (Figure 3.16F).

Figure 3.16: Fluorescence imaging utilized to view the nano-cellular interface within the microenvironment under the influence of select variables. Representative images are shown for: (A) static media control (B) static AAF control (C) static media Au PVP (D) static AAF Au PVP (E) dynamic media control (F) dynamic AAF control (G) dynamic media Au PVP (H) dynamic AAF Au PVP. In these images, actin and nuclei are stained red and blue, respectively, with the NPs appearing as white.
Figure 3.17: Fluorescence imaging utilized to view the nano-cellular interface within the microenvironment under the influence of select variables. Representative images are shown for: (A) static media Ag PVP (B) static AAF Ag PVP (C) static media Au TA (D) static AAF Au TA (E) dynamic media Ag PVP (F) dynamic AAF Ag PVP (G) dynamic media Au TA (H) dynamic AAF Au TA. In these images, actin and nuclei are stained red and blue, respectively, with the NPs appearing as white.

Finally, standard light microscopy was utilized to further verify the cell morphological changes as observed via fluorescent microscopy (Figure 3.18). As shown in Figure 3.18B, curvature was seen, to some degree, in nearly all cells, indicating a common effect across the entire cell population. Cellular elongation under dynamic flow was similarly supported through this methodology (Figure 3.18C & D).
3.3 – Discussion

Through the inclusion of relevant physiological variables, this research successfully generated an enhanced *in vitro* system that more closely mimics an *in vivo* model. As it is well documented that NP behavior depends strongly on both key physicochemical traits and the surrounding environment, it logically followed that unique behavioral endpoints could be identified through utilization of the constructed microenvironment. Based on proof-of-concept experimentation that incorporated three NP sets and a cell model that targeted a lung exposure system, modified particle and cellular performance was demonstrated that varied as a function of NP surface chemistry, fluid composition, and flow condition.

For example, the combination of tannic acid surface coating and an AAF environment resulted in significant NP agglomeration, a loss of particle stability, decreased ionic
dissolution, and greater cellular deposition. However, none of these responses existed in a standard media environment. Furthermore, experimentation in AAF not only identified modified particle response, but altered cellular morphology as well. Incubation in AAF produced a clearly-recognizable curved pattern in the A549 cellular morphology. One potential explanation is that these alterations arise from the fact that along with numerous salts, the AAF also includes the lipid phosphatidylcholine, a major component of the cellular membrane. Taken together, this research highlights how conducting NP evaluation in a physiological fluid generates a novel set of results that cannot be captured through standard methodologies.

Furthermore, a flow-dependent response was also identified that arose in conjunction with PVP surface coating. Under dynamic flow, Ag and Au PVP NPs demonstrated altered rates of ionic dissolution and a significant decrease in deposition efficiency. While the dissolution was also dependent on fluid, the disruption to the nano-bio interface occurred in both media and AAF, indicating a significant dependence on dynamic flow. The introduction of dynamic movement also caused substantial cellular morphological changes, with the A549 cells becoming elongated in the direction of flow. Once you consider that a true in vivo model is under constant circulation, and observe the degree of alteration to the nano-cellular interface under flow conditions described in this study, the necessity of evaluating NP safety under shear stress becomes evident.
3.4 - Conclusions

The goal of this study was to construct and implement an enhanced in vitro model that incorporated relevant physiological fluids and dynamic flow, in an effort to generate a means of rapid NP evaluation in a more realistic model. Through the selection of an alveolar cell line and use of AAF, the constructed microenvironment more accurately mimicked a lung NP exposure scenario than standard in vitro techniques. Through rigorous experimentation, significant changes were identified to NP characteristics and the nano-cellular interface as a function of NP surface chemistry, fluid environment, and the presence of dynamic flow. Furthermore, NP exposure within the microenvironment generated novel cellular and NP responses that were previously unattainable in a standard in vitro system. These results support the emerging idea that numerous environmental variables can influence NP properties and behaviors, with the potential to affect downstream bioeffects and overall safety evaluations. To that end, the utilization of a complex and physiologically relevant model, such as the one devised and implemented in this study, is necessary to accurately and effectively evaluate the safety and performance of NPs in nano-based applications.

3.5 – References


