INVESTIGATION OF THE BIOPHYSICAL EFFECT OF HYDROPHOBIC NANOPARTICLE EXPOSURE ON PULMONARY SURFACTANT FILMS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MANOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTERS OF SCIENCE

IN

MECHANICAL ENGINEERING

May 2015

By

Russell P. Valle

Thesis Committee:

Yi Zuo, Chairperson
Mehrdad Nejhad
John S. Allen
Acknowledgements

First, I would like to thank my supervisor Dr. Yi Zuo for his assistance and guidance in this work. Of which, I would not have been able to complete the work summarized here. I have learned much under his supervision and I truly appreciate the support he has given me throughout my tenure at the University of Hawaii.

Next, I would like to thank Dr. John Allen and Dr. Mehrdad Nejhad for their time and effort in serving on my thesis committee.

I would like to thank my collaborating authors including Charlotte Liwen Huang and Joachim S.C. Loo. Their contributing efforts in nanoparticle synthesis allowed for my work to continue seamlessly. Additionally, I would like to thank Dr. Walter Klein of ONY Inc. who supplied my research with clinical lung surfactant preparations.

I need to acknowledge my colleagues Rimei Chen, Kyle Yu, Tony Wu, Jinlong Yang and Xinru Zhang. Their support and help throughout my time at the University of Hawaii both professionally and personally were essential in my efforts to conclude this project.

I am truly grateful of my friends and family who supported me during my time at the University of Hawaii.

Finally, I would like to acknowledge the financial support from the NSF under the grant number CBET-1236596.
Abstract

The demand for nanoparticles (NPs) has reached a point where large quantities are being manufactured everyday in nations around the globe. Due to the mass production of NPs, many concerns have arisen about the adverse effects of NPs on human health. An area of focus is the pulmonary system, because many NPs are lightweight and respirable. The first biological barrier with which inhaled NPs come in contact is the pulmonary surfactant (PS) matrix. PS is a lipoprotein complex that lines the entirety of the peripheral lung. The chief functions of the PS matrix are, first, to act in innate immunity for foreign airborne contaminants that may reach the alveoli and second, to reduce alveolar surface tension values which keep the air sacs inflated and maintain normal lung function. Preliminary tests have shown that the biophysical function of PS is inhibited when exposed to NPs. The inhibition potential is dependent on the physicochemical properties of the NPs, such as size, shape, charge, and the agglomeration state. Here we investigate the surface hydrophobicity which is a rarely studied physicochemical property of NPs, but our results demonstrate that it has a significant effect on biophysical inhibition of PS. Being the most commonly used hydrophobic nanomaterials, carbon nanotubes (CNT), and more recently graphene nanoplatelets (GNP), have been extensively manufactured which raises concerns about their impact on occupational inhalation exposure. It is important to simulate the inhalation effect on PS in vitro to understand the biophysical mechanism behind the inhalation toxicity of carbon nanomaterials. Here we developed a novel experimental methodology, called the constrained drop surfactometer (CDS), to perform the first in vitro simulation of nano-bio interactions between airborne carbon nanoaerosols and PS films under physiologically relevant conditions. We found that the doses of both CNT and GNP were within international occupational exposure limits. A large majority of the aerosol aggregates for both CNT and GNP
were sub-micron in size. After exposure to CNT and GNP aerosols we found a dose-dependent surfactant biophysical inhibition. Our *in situ* Langmuir-Blodgett transfer revealed that CNT and GNP aggregates “stick” to the surfactant film where nano-bio interactions induce surfactant inhibition. Our results are promising, demonstrating that both CNT and GNP induce a dose-dependent biophysical inhibition in concentrations comparable to many international occupational exposure limits. All of the results demonstrate that hydrophobicity is a defining pro-inhibitory characteristic of NPs that must be taken into account when studying both nanotoxicology and nanomedicine.
# Table of Contents

Acknowledgements ................................................................................................................... i

Abstract ....................................................................................................................................... ii

List of Tables .................................................................................................................................. ix

List of Figures .................................................................................................................................. x

List of Abbreviations ...................................................................................................................... xiii

Chapter 1. Introduction .................................................................................................................. 1

1.1 Pulmonary surfactant ................................................................................................................. 2

1.1.1 Composition ........................................................................................................................... 3

1.1.1.1 Lipids .................................................................................................................................. 3

1.1.1.2 Surfactant proteins ............................................................................................................. 3

1.1.2 Function ................................................................................................................................. 4

1.1.2.1 Immunological .................................................................................................................. 4

1.1.2.2 Biophysical ...................................................................................................................... 4

1.2 Biophysical inhibition by nanoparticles ..................................................................................... 7

1.2.1 Model membranes ............................................................................................................... 7

1.2.2 Naturally-derived surfactants ............................................................................................... 9

1.2.2.1 Extraction process/composition ....................................................................................... 9

1.2.2.2 Nanoparticle physicochemical characteristic influence ............................................... 10
1.2.3 Aerosolized nanoparticles................................. 13

1.2.3.1 Technical challenges............................................ 13

1.2.3.2 Inhalation simulation............................................ 14

1.3 Inhibition mechanism.................................................. 14

1.3.1 Microscopy investigation......................................... 15

1.3.1.1 Surface visualization........................................... 15

1.3.1.2 Surfactant aqueous ultrastructure........................ 16

1.3.2 Lipoprotein adsorption........................................... 17

1.3.2.1 Hydrophobic pulmonary surfactant component........ 17

1.3.2.2 Hydrophilic pulmonary surfactant component........... 20

1.3.3 Lipoprotein corona................................................ 21

1.4 Cytotoxicity surfactant-coated nanoparticles.................. 22

1.5 Thesis overview....................................................... 24

Chapter 2. Influence of Nanoparticle Hydrophobicity on Pulmonary Surfactant Biophysical Inhibition

Surfactant Biophysical Inhibition........................................ 41

2.1 Background............................................................ 41

2.2 Experimental methods............................................... 42

2.2.1 Pulmonary surfactant.............................................. 42

2.2.2 Nanoparticles....................................................... 43

2.2.3 Rose Bengal partitioning......................................... 44
Chapter 3. Biophysical Influence of Airborne Carbon Nanomaterials on Pulmonary Surfactant Films

3.1 Background .............................................................................................................. 61
3.2 Experimental methods ............................................................................................ 63
   3.2.1 Pulmonary surfactant .................................................................................. 63
   3.2.2 Carbon nanomaterials ................................................................................. 64
   3.2.3 Aerosol concentration determination ......................................................... 64
   3.2.4 Atomic force microscopy ............................................................................ 64
   3.2.5 Transmission electron microscopy ............................................................. 65
   3.2.6 Statistical analyses .................................................................................... 65
3.3 Results and discussion ............................................................................................ 65
   3.3.1 Constrained drop surfactometer development ........................................... 65
3.3.2 Carbonaceous nanomaterial aerosol-induced surfactant inhibition .................. 67
3.3.3 \textit{In situ} Langmuir-Blodgett transfer and film imaging................................. 68
3.3.4 Carbon nanotube occupational exposure limit analysis ........................................ 70
3.3.5 Bridging \textit{in vitro} simulations and \textit{in vivo} inhalation studies .......................... 70
3.4 Conclusions ........................................................................................................ 71

\textbf{Chapter 4. Conclusions}..................................................................................89

4.1 Nanoparticle hydrophobicity effect on pulmonary surfactant biophysics and retention .... 89
4.2 Rose Bengal partitioning assay is an effective method in measuring nanoparticle hydrophobicity ........................................................................................................ 89
4.3 Film compressibility: a more sensitive parameter for measuring surfactant inhibition..... 90
4.4 Further development of the constrained drop surfactometer for studying aerosol-induced surfactant inhibition ........................................................................................................ 91
4.5 Airborne carbonaceous nanomaterial effect on pulmonary surfactant biophysics ........ 91
4.6 \textit{In situ} Langmuir-Blodgett transfer revelation of carbonaceous nanomaterial at air-water interface ........................................................................................................ 92
4.7 Atomic force microscopy structure of adsorbed Infasurf films at core body temperature . 93
4.8 List of publications ................................................................................................ 93

\textbf{Chapter 5. Future Work}..................................................................................96

5.1 Continuous Langmuir-Blodgett transfer ................................................................. 96
5.2 Constrained drop surfactometer aerosol system development ................................ 96
5.3 Difference between carbon nanotube and graphene nanoplatelet inhibition potentials ..... 97

5.4 Comparative study of atomic force microscopy of clinical pulmonary surfactants .......... 97

References ..............................................................................................................................................99
List of Tables

Table 1.1. Biophysical studies of nanoparticle-pulmonary surfactant interactions. .................................................................37

Table 1.2. Immunological studies of nanoparticle-pulmonary surfactant interactions. ...............................................................39

Table 2.1. Morphological and surface characterization of nanoparticles. ...............60

Table 3.1. Carbon nanomaterial aerosol concentration calculations. ......................88
List of Figures

**Figure 1.1.** NP penetration through the lungs and interaction with pulmonary surfactant.................................................................26

**Figure 1.2.** Effect of NPs on synthetic and natural surfactant films.................27

**Figure 1.3.** NP size dependent inhibition on naturally-derived pulmonary surfactant..............................................................................28

**Figure 1.4.** Hydrophobic versus hydrophilic NP effect on naturally-derived pulmonary surfactant..........................................................29

**Figure 1.5.** Effect of NP hydrophobicity on pulmonary surfactant using pulsating bubble surfactometer.................................................30

**Figure 1.6.** Atomic force microscopy imagery and corresponding molecular dynamics simulations of hydrophobic NPs at pulmonary surfactant films........31

**Figure 1.7.** Ultrastructure degradation of pulmonary surfactant vesicles after exposure to titanium dioxide nanoparticles ........................................32

**Figure 1.8.** Molecular dynamics simulation and experimental support for pulmonary surfactant lipid adsorption to carbon nanotubes........33

**Figure 1.9.** Molecular dynamics simulation and experimental support for SP-D adsorption to carbon nanotubes .........................................................34
Figure 1.10. Dynamic behavior of biomolecular coronas ..................................35

Figure 1.11. Cellular nanotoxicity demonstrated by histopathology .................36

Figure 2.1. Hydrophobicity measurements via Rose Bengal partitioning ..........54

Figure 2.2. Comparison of nanoparticle retention at the Infasurf film ..............55

Figure 2.3. Nanoparticle-induced inhibition dependence on time ..................56

Figure 2.4. Comparison of compression-expansion cycles for pure Infasurf and Infasurf mixed with nanoparticles ..............................................................57

Figure 2.5. Statistical analysis of the effect of nanoparticles on surface activity of Infasurf .................................................................58

Figure 2.6. Direct comparison of compressibility and hydrophobicity ............59

Figure 3.1. Schematic of the constrained drop surfactometer for studying biophysical influence of airborne carbon nanomaterials on pulmonary surfactant 73

Figure 3.2. A typical compression-expansion cycle for pure Infasurf at 37°C and 20 cycles/min determined using the constrained drop surfactometer .............74

Figure 3.3. Schematic of constrained drop surfactometer aerosolization system 75

Figure 3.4. Number and size distribution of aggregates during settling ..........76

Figure 3.5. Carbon nanotube and graphene nanoplatelet aerosol size distributions 30 min after initial aerosolization ..................................................77
Figure 3.6. Representative cycles demonstrating carbonaceous nanomaterial dose-dependent inhibition........................................................................................................78

Figure 3.7. Chronological dynamic cycles for carbon nanotube exposure. ..........79

Figure 3.8. Chronological dynamic cycles for graphene nanoplatelet exposure...80

Figure 3.9. Dynamic cycling data for carbon nanotube exposure. ....................81

Figure 3.10. Dynamic cycling data for graphene nanoplatelet exposure. ..........82

Figure 3.11. Statistical analysis of biophysical properties of Infasurf exposed to carbonaceous nanomaterial........................................................................................................83

Figure 3.12. Imaging nano-bio interactions at the pulmonary surfactant film.....84

Figure 3.13. Additional atomic force microscopy images of the Infasurf film exposed to carbon nanotubes. ..........................................................................................85

Figure 3.14. Additional atomic force microscopy images of the Infasurf film exposed to graphene nanoplatelets.................................................................86

Figure 3.15. Transmission electron microscopy images for carbon nanotube and graphene nanoplatelet aerosol................................................................................87

Figure 5.1. Continuous Langmuir-Blodgett transfer calibration .....................98
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
</tr>
<tr>
<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>ADSA</td>
<td>axisymmetric drop shape analysis</td>
</tr>
<tr>
<td>CBS</td>
<td>Captive Bubble Surfactometer</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>CNT</td>
<td>carbon nanotube</td>
</tr>
<tr>
<td>CNM</td>
<td>carbonaceous nanomaterial</td>
</tr>
<tr>
<td>CLD</td>
<td>chronic lung disease</td>
</tr>
<tr>
<td>κ</td>
<td>compressibility</td>
</tr>
<tr>
<td>CDS</td>
<td>Constrained Drop Surfactometer</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>EU</td>
<td>eudragit</td>
</tr>
<tr>
<td>GNP</td>
<td>graphene nanoplatelet</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
</tr>
<tr>
<td>LB</td>
<td>Langmuir-Blodgett</td>
</tr>
<tr>
<td>LDS</td>
<td>laser diffraction spectroscopy</td>
</tr>
<tr>
<td>LE</td>
<td>liquid-expanded</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>NIOSH</td>
<td>National Institute of Occupational Safety and Health</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>OEL</td>
<td>occupational exposure limits</td>
</tr>
<tr>
<td>PA</td>
<td>palmitic acid</td>
</tr>
<tr>
<td>POPG</td>
<td>palmitoyl-oleoyl phosphoglycerol</td>
</tr>
<tr>
<td>PQ</td>
<td>partitioning quotient</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PST</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(D,L-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PS</td>
<td>pulmonary surfactant</td>
</tr>
<tr>
<td>PBS</td>
<td>Pulsating Bubble Surfactometer</td>
</tr>
<tr>
<td>π</td>
<td>surface pressure</td>
</tr>
<tr>
<td>γ</td>
<td>surface tension</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>TC</td>
<td>tilted-condensed</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TM</td>
<td>tubular myelin</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

The multi-disciplinary application of nanotechnology and growth in manufacturing of nanoparticles (NPs) has caused much concern about NP exposure and the associated health risks.\textsuperscript{1,2} Due to their small size (<100 nm) and light weight, NPs are easily airborne and therefore easily inhaled. The pulmonary system has been an area of focus because, as opposed to other environmental exposure, inhaled NPs pose a direct and systemic risk to the human body.\textsuperscript{3} It has been shown that particles smaller than 100 nm penetrate to the alveolar region of the lung and have the potential to deposit in these deep, delicate structures.\textsuperscript{4,5} Meanwhile, discussions in nanomedicinal research have focused on taking advantage of NPs aerodynamics to carry drugs into the pulmonary system for non-invasive, systemic delivery.\textsuperscript{6,7} Whether inhaled intentionally or upon accidental exposure, NPs represent a foreign body in a critical structure for human physiology. For this reason, much research has been focused on the adverse effects of inhaled NPs.

As shown in Figure 1.1 inhaled NPs deposit onto the alveolus, the first biological barrier with which they come in contact is the pulmonary surfactant (PS) matrix.\textsuperscript{8,9} PS is a mixture of phospholipids, neutral lipids and four surfactant proteins (SP-A, B, C, and D) and serves two main purposes in the peripheral lung. The first is to act as a barrier for foreign contaminants aiding in innate immunity. The other is biophysical. Due to its high surface activity PS reduces alveolar surface tension to near-zero levels which prevents smaller alveoli collapsing into larger ones, as predicted by the law of Laplace. Normal tidal breathing contracts and expands the lung ~20\% of its relative surface area. Surface tension reduction to near-zero levels allows for normal breathing cycles to occur with zero mechanical energy expense.\textsuperscript{10} Dysfunctional PS has shown to
lead to serious medical complications including acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and has implications in chronic lung disease (CLD).\textsuperscript{11-13}

After inhalation, NPs will deposit onto the PS film. However, the nano-bio interactions that take place are largely unknown. Despite the unknown mechanisms, preliminary research has shown that these interactions are highly inhibitory to biophysical properties of PS. Over the past two decades much research has been dedicated to understand NP-induced PS inhibition. In this chapter we aim to summarize the nano-bio interactions at PS films, and more importantly the consequences of such interactions.

1.1 Pulmonary surfactant

The alveoli of the lung are completely lined with a PS matrix atop an aqueous subphase with an average thickness of 0.2 µm.\textsuperscript{14} PS is synthesized in alveolar type II pneumocytes and secreted into the alveolar hyperphase in highly packed lamellar bodies. Upon secretion into the extracellular environment, PS adopts a highly organized structure called tubular myelin (TM). PS within the TM structure subsequently adsorbs to the air-water interface to reduce surface tension. After serving their function, PS components desorb from the surface and are recycled back to the type II pneumocyte, while new surfactant film replaces the desorbed film at the surface. The composition of PS is highly conserved between mammalian species signifying its importance in mammalian evolution.\textsuperscript{15-17} Reconstitution studies have helped evaluate PS and given insight on the specific function for each component.\textsuperscript{18-21}
1.1.1 Composition

1.1.1.1 Lipids

The lipid fraction of PS represents a large majority of its weight (~90%).\textsuperscript{22} Phosphatidylcholines (PC) make up about 70% of the weight of PS and about half of the PC is the disaturated phospholipid, dipalmitoyl phosphatidylcholine (DPPC). Although DPPC is a major contributor to surface tension reduction, its rigidity yields very slow adsorption to the air-water interface. Other than DPPC there are many unsaturated phospholipids that have been shown to contribute to the fluidity of the film.\textsuperscript{15} Neutral lipids including cholesterol, free fatty acids, cholesterol esters, triglycerides, and diglycerides make up a small fraction of PS lipids. Although a small portion, they contribute to the fluidity of the film, especially cholesterol which accounts for a small yet significant, mass fraction of 5-10%.\textsuperscript{22} This amount changes with the physiological conditions within the lung and proves to be important for normal lung mechanics.\textsuperscript{23} Phosphatidylglycerols (PG) and phosphatidylinositols (PI) make up a considerable portion of PS lipids (~10-15%) and are similar in that both have anionic head groups. Reconstitution experiments with DPPC-PG have shown that the fluid-like PG enhances the surface activity and more importantly adsorption of the phospholipid mixture to the air-water interface.\textsuperscript{19} Additionally, the anionic heads of these phospholipids are speculated to interact with cationic, hydrophobic surfactant proteins SP-B and SP-C, via electrostatic interactions.\textsuperscript{21}

1.1.1.2 Surfactant proteins

The four surfactant proteins can be split into two categories: large, hydrophilic (SP-A,D) and small, hydrophobic proteins (SP-B,C).\textsuperscript{22,24} SP-A and SP-D belong to the carbohydrate-binding collectin family. Both reside in the aqueous alveolar subphase and have carbohydrate recognition domains (CRD). The two hydrophobic proteins, SP-B and SP-C, are highly associated with the
pulmonary surfactant film. As stated above these proteins are cationic and coordinate with the anionic phospholipids. SP-C is a transmembrane palmitoylated peptide, giving it an uncharacteristically high hydrophobicity. Due to its hydrophobicity it is strictly found embedded in the phospholipid membrane. SP-B, on the other hand, is not palmityoled and therefore its hydrophobicity is only a direct result from its hydrophobic amino acids residues. The alpha helices of SP-B lie parallel to the lateral dimension of the phospholipid film. Additionally, SP-B helices can transverse multiple layers of the phospholipid membrane.

1.1.2 Function

1.1.2.1 Immunological

The PS film represents a biological barrier exposed to the surrounding environment, but more importantly air contaminants. Therefore, the PS serves an immunological purpose in the peripheral lung. Mainly the two hydrophilic proteins, SP-A and SP-D, contribute to the immune response.\(^{25,26}\) It has been shown that the CRDs of both SP-A and SP-D bind to the carbohydrate chains on pathogens in a Ca\(^{2+}\)-dependent manner.\(^{27}\) However, the CRDs have a non-specific competitive binding which can bind lipids as well as other materials and particles.\(^{28}\) These proteins mark the removal of contaminants by signaling alveolar macrophages for clearance. Although important to the immune response to pulmonary pathogens and contaminants, many studies using mouse knockouts have demonstrated that SP-A and SP-D are not essential for survival. These mice show to be immunocompromised, but survive after birth.\(^{29,30}\)

1.1.2.2 Biophysical

In 1955 a *Nature* publication demonstrated that bubbles created from lung extracts decreased in size until they reached ~50 µm in diameter, relatively the size of a human alveolus.\(^{31}\) The
conclusion made here is that these bubbles must demonstrate low surface tensions ($\gamma \approx 0 \text{ mN/m}$) because according to Equation 1.1, i.e. the Laplace equation of capillarity (shown for spherical bubbles), these bubbles would collapse and be unable to maintain an inflated state. It was not until later that surface tension measurements of lung extracts would be directly measured, but the conclusions drawn from this paper would serve severely important for future work on how PS films affect the biophysical function of the mammalian lung.

$$\Delta P = \frac{2\gamma}{R}$$

Through many composition and biophysical studies it was later found that the high phospholipid content accounts for the high surface activity of PS. The biophysical reduction of surface tension is the major function of PS. DPPC is disaturated and therefore can pack easily under compression which contributes to the low compressibility of surfactant films. During normal tidal breathing the lung surface area expands and contracts only about 20% of its relative area. The low compressibility of PS allows near-zero surface tensions to be reached within the allotted 20% relative area compression.\(^{32}\) Fast adsorption to the surface and dynamic low surface tensions maintain the inflation of the alveoli for normal gas exchange to occur. Unsaturated phospholipids present in the PS complex aid in spreading and respreading to the interface. Surfactant adsorbs to the interface in a matter of milliseconds and reaches the surface tension to an equilibrium value of 25 mN/m in a matter of seconds.\(^{10,33}\) The specific mixture of saturated, unsaturated, anionic, and neutral lipids yields a “soft-yet-strong” matrix that is fluid upon adsorption from the subphase, yet under compression (exhalation) forms a hard film to yield near-zero surface tension. Although lipids make up 90% of PS by weight, it has been shown that
SP-A, SP-B, and SP-C are essential for normal surfactant biophysics and, in turn, normal lung function.

SP-A does have a specific function with the PS lifecycle and structure by maintaining the highly ordered TM. To do so, SP-A CRDs have a non-specific affinity to DPPC molecules in addition to their affinity to bind foreign bodies and pathogens. The stability of the subphase structures allows for more efficient surfactant adsorption as demonstrated. However as stated earlier, SP-A knockout mice survive after birth and pulmonary function is sufficient to breathe. These mice however are more prone to infection, which shows that the primary function of SP-A is in innate immunity but not pulmonary biophysics.

SP-B and SP-C show to be essential for normal lung function, in that SP-B knockout mice do not survive after birth and SP-C knockout mice develop chronic respiratory failure. Due to their hydrophobicity SP-B and SP-C are highly associated with the lipid membrane, both in the subphase and at the alveolar interface. In the subphase, these two peptides aid in the transfer of lipids from vesicular structures to the interfacial film. At the interface SP-B and SP-C are essential in the reversible collapse and respreading of surfactant film upon dynamic compression-expansion cycles. Fluid-like anionic PG and PI are hypothesized to interact with the cationic hydrophobic peptides and this interaction is likely to result in the “squeeze-out” of the anionic lipids from the film under compression. The resulting film is left highly packed and rich in DPPC molecules yielding a hard film at the surface and thus bringing surface tension values to near-zero levels.

Hence the importance of these hydrophobic proteins is marked by two biophysical phenomena. The first is these proteins allow for rapid spontaneous adsorption of PS from the
aqueous phase to the air-water interface to immediately reduce surface tension. The second is that in less than 20% relative area compression, the film is able to reduce the surface tension to nearly zero maintaining normal lung mechanics.

1.2 Biophysical inhibition by nanoparticles

1.2.1 Model membranes

Many NP-PS interaction studies are conducted with lipid mixtures or lipoprotein mixtures that model natural PS. Model membranes give insight on how NP interact with certain components of the PS matrix, however, when interpreting these data caution must be taken as these membranes are not mimicking the lung environment. The PS matrix is a complex mixture where each component plays a very specific role in maintaining normal lung mechanics. Although these model membranes may be conducive in collecting molecular-specific interactions, they cannot reproduce the interactions that would occur within the lung under normal physiological conditions.

Simplistically, NP interactions with PS are modeled with lipid membranes in the absence of protein. In many instances surfactant biophysics has been modeled using a homogenous DPPC monolayer.\textsuperscript{38-43} The fact that roughly half of the lipid weight is DPPC has given researchers reason to study the interaction of DPPC with NPs. Compression isotherms obtained through the use of a classical Langmuir trough of pure DPPC and DPPC mixed with NPs have shown that introduction of NPs cause anywhere from subtle compression isotherm shape changes to major isotherm shifts. These changes in isotherms indicate that there are indeed interactions between lipids and NPs affecting the physical nature of the phospholipid monolayer. Subsequently, many
studies enriched DPPC with other lipids including, but not limited to, palmitoyl-oleoyleolphosphatidyglycerol (POPG), cholesterol, palmitic acid (PA), and dioleoylphosphatidylcholine (DOPC) for the purpose of studying heterogeneous phospholipid mixtures and their interactions with NPs. Not unlike DPPC monolayers, the compression isotherms shift with the addition of NPs marking a non-specific interaction of NPs with the lipid monolayers.

To reiterate, NP interaction with DPPC or phospholipid mixtures is not an accurate representation of the PS system in the lung environment. However, through the use of the captive bubble surfactometer (CBS) Bakshi et al. were able to better simulate the lung environment with model membranes by bringing the films to core body temperature (37°C) and doping phospholipid mixture with SP-B. By incorporating SP-B into a DPPC:POPG phospholipid mixture they were able to obtain dynamic biophysical results that mimicked natural surfactant. After adding gold NPs (Au-NPs) to the mixture they observed an increase in the adsorption surface tension ($\gamma_{ads}$) and minimum surface tension after compression ($\gamma_{min}$); both parameters that have a strong correlation with SP-B function. The biophysical results hinted strongly that Au-NPs interact with not only lipids, but SP-B as well. Although model membranes give an insight into nano-bio interactions at the interfacial films, they do not yield a direct simulation of the nano-bio interactions with PS matrix in the alveoli of the lung. To truly simulate NP-PS interactions natural surfactant must be isolated and in turn tested under physiological conditions.
1.2.2 Naturally-derived surfactants

1.2.2.1 Extraction process/composition

Naturally derived surfactant was first successfully used as a remedy to RDS in human infants in 1980 by Fujiwara et al.\textsuperscript{50} Since then natural surfactant has not only been used extensively in the medicinal field but also in research. Clinical preparations are created by using either a lung lavage or mince of certain terrestrial mammals (usually bovine or porcine samples). These samples go through centrifugation and an organic extraction process to retain all hydrophobic components of PS including phospholipids, neutral lipids, and most of the hydrophobic proteins (SP-B and SP-C). When resuspended in an aqueous buffer, these preparations include all of the surface active components of natural PS and therefore include the entirety of the PS matrix. Experiments conducted with these preparations at 37°C, such as in CBS,\textsuperscript{51} pulsating bubble surfactometer (PBS),\textsuperscript{52-55} or constrained drop surfactometer (CDS)\textsuperscript{56-58} can directly correlate to PS biophysics in the lung environment.

The difference in biophysics between natural PS and model membranes is clear in comparative studies. Beck-Broichsitter et al. demonstrated anionic polystyrene nanoparticle (PST-NP)-induced inhibition on phospholipid mixtures, commercial synthetic surfactant (Venticute), and modified natural surfactant (Alveofact).\textsuperscript{59} The level of inhibition of the naturally-derived Alveofact was significantly lower than both the phospholipid mixtures and the synthetic surfactant (Figure 1.2). This study underlies why extrapolating data from modified naturally-derived surfactant preparations rather than model membranes is paramount when studying nanotoxicology. However, the composition of surfactant preparations is not the major determining factor for NP-induced surfactant inhibition.
1.2.2.2 Nanoparticle physicochemical characteristic influence

Upon further investigation, surfactant biophysical inhibition is critically determined by NP physicochemical characteristics. One of the most influential particle properties is its size. For many years size of inhaled contaminants have been theorized to be a critical determining factor on lung function. When introduced to PS films, in comparison to coarse, micron-sized particles, NPs yield a higher biophysical inhibition at the same mass concentration (Figure 1.3). Both PST-NPs and titanium dioxide NPs (TiO₂-NPs) induced statistically significant increases in both \( \gamma_{\text{ads}} \) and \( \gamma_{\text{min}} \). In contrast, their micron-sized counterparts at the same concentration showed no significant difference in both \( \gamma_{\text{ads}} \) and \( \gamma_{\text{min}} \) from the pure surfactant control. Smaller particles have larger surface area to volume ratios than larger ones. Due to this fact, at the same mass concentration NPs will inherently expose more surface area to surfactant films than coarse particles. Interaction between PS components and the solid particle interface will increase with increasing surface area. A proposed inhibition mechanism directly correlates surfactant dysfunction with PS components adsorbing to NP surfaces and being removed from the interfacial film. However, a more classical theory is that particles will compete with free lipids at the air water interface. Both of these theories associate surfactant dysfunction with high NP surface area, although recent evidence points in favor of the former mechanism.

NP surface charge studies help elaborate on the mechanism for NP-induced surfactant inhibition. When cationic (zeta potential \( \zeta = 59.0 \text{ mV} \)) and anionic (\( \zeta = -46.3 \text{ mV} \)) PST-NPs of the same size and concentration were added to modified natural PS, Alveofact, the anionic particles consistently demonstrated more potent elevation in \( \gamma_{\text{min}} \) when compared to the cationic ones (Figure 1.2). As stated earlier the two hydrophobic proteins associated with PS films, SP-B and SP-C, are essential to surface tension reduction and both are cationic. Due to their net charge
these proteins would demonstrate a strong attraction to negatively-charged NPs. Granted that there is a balance between other forces (e.g. van der Waals forces) that would ultimately govern the adsorption behavior of these proteins, strong attractive electrostatic forces would play a large role in the adsorption of hydrophobic SPs to NPs.

One force that must be taken into consideration is hydrophobic forces. Most recently, a non-traditional physicochemical characteristic that has caught the attention of nanotoxicology researchers is hydrophobicity. Hydrophobic interactions govern the structure of the amphipathic phospholipid film to self-assemble at the air-water interface. Therefore it has been hypothesized that the hydrophobicity of particles would govern their retention at the surface and interaction with the indispensable hydrophobic components of PS.\textsuperscript{55,58} The difficulty in NP hydrophobicity experiments is being able to quantitatively measure the magnitude of hydrophobicity. With bulk materials contact angle experiments are preformed to obtain a relative hydrophobicity measurement. However this procedure to measure hydrophobicity of single particles is technically challenging and is not widely used. To overcome this hurdle the Rose Bengal partitioning method was used to measure the relative hydrophobicity of NPs. This technique employs a hydrophobic dye that adsorbs to NPs in suspension and through the use of UV-Vis spectroscopy the concentration of dye left in solution gives a quantitative assessment of how tight the dye molecules are bound to the surface of the particles, \textit{i.e.} hydrophobic attraction. Through the use of Rose Bengal partitioning the relative hydrophobicity of NPs can be found quickly and easily.

One hydrophobic particle that has been widely used throughout inhibition studies is polystyrene NP (PST-NP). PST-NPs are extensively used for dynamic light scattering calibration due to their stability and monodispersity. However, this particle has been shown to have low
biocompatibility and is hazardous to multiple tissue types. PST-NPs were tested in parallel with hydrophilic hydroxyapatite (HA-NPs) for biophysical inhibition in a classical Langmuir trough. When introduced to a modified natural surfactant, Infasurf, HA-NPs showed an increase in film compressibility which stabilizes after seven hours. Increasing film compressibility correlates with a film that is softer and hence can only reach low surface tensions with a maximum amount of compression. Due to this reason a softer film is considered compromised and inhibited. When compared to HA-NPs, PST-NPs showed increased compressibility to the same extent in only one hour showing a much higher inhibition potential (Figure 1.4).

Another well used hydrophobic NP is poly(D,L-lactide-co-glycolide) (PLGA). PLGA is a polymer that has shown to be biocompatible and used in biomedical applications. PLGA has been shown to be less hydrophobic than PST-NPs. Therefore, PLGA in concert with PST-NPs can generate a group of NPs with a gradient of hydrophobicities. The effect of PST-NPs, PLGA as well as hydrophilic Eudragit (EU-NPs) on Alveofact was studied at physiological conditions through the use of PBS (Figure 1.5). The results showed that increasing hydrophobicity correlated with an increased time to minimize $\gamma_{\text{min}}$ during bubble oscillation. PST-NPs showed the longest time to reduce $\gamma_{\text{min}}$ below 2 mN/m followed by PLGA and the hydrophilic EU-NPs showed little variation from the surfactant control, once again demonstrating the large inhibitory potential associated with hydrophobic NPs.

Our own recent study showed very similar results. Here we introduced two different PLGA particles with different relative hydrophobicities as well as PST-NPs to Infasurf. Through the use of CDS we found that increased film compressibility in physiologically relevant conditions of 37°C and film compression-expansion cycles at tidal breathing speed (20 cycles/min) was attributed to increased particle hydrophobicity. It is clearly demonstrated that relative
hydrophobicity plays an integral role in surfactant inhibition where high hydrophobicity correlates with high inhibitory potential. Our experimental results are presented in details in Chapter 2.

1.2.3 Aerosolized nanoparticles

Existing data on NP-induced PS inhibition give great insight into how NP physicochemical characteristics affect PS film biophysics. However, all of these studies with naturally-derived clinical surfactants have a technical limitation that keeps them from simulating inhalation toxicology. This limitation lies inherent in the method of introducing particles to PS in the liquid phase. When particles are inhaled they interact with the PS matrix from the air side of the air-water interface. All of these previous studies either spread a NP suspension atop a PS film or mix aqueous suspensions of NPs and PS, which does not truly simulate NP inhalation. To truly simulate inhalation airborne NPs must be introduced to the PS films in vitro.

1.2.3.1 Technical challenges

Introducing controlled aerosol to PS films using existing in vitro biophysical methodologies is technically challenging. The classical Langmuir trough is usually exposed to an open environment and if it were to be enclosed would account for a large airspace to fill with aerosol, not to mention that this technique neither has temperature nor humidity control and therefore cannot simulate the dynamic nature of the lung. Both PBS and CBS employ a small bubble surrounded by a large volume of surfactant. In the PBS a small air bubble is connected to a series of tubing and the bubble size is varied by controlling the air pressure. However, this technique already has difficulty in surfactant leakage and is not ideal for aerosol testing. CBS utilizes hydraulic pressure to control the size of a captured air bubble and therefore presents a challenge
of introducing aerosol to a small volume of air within a chamber of aqueous surfactant. Putting all these aside, the CDS is a biophysical in vitro technique that shows promise with aerosol experimentation.

1.2.3.2 Inhalation simulation

Physiologically relevant inhalation simulations can be effectively created though the use of the CDS as shown later in Chapter 3 of this thesis. The CDS uses a droplet contained in a chamber therefore aerosol can easily be introduced to the chamber and subsequently interact with the surfactant film adsorbed at the air-water interface of the constrained droplet. With this technique surface tension measurements can be accurately recorded remotely in the presence of nanoaerosols. More importantly, the concentration of such aerosols can be recorded after each exposure. In this setup inhalation is simulated by acute exposure to nanoaerosols. Meanwhile breathing is simulated by oscillating a surfactant droplet at the rate of tidal breathing (3 seconds/cycle) while maintaining the droplet at core body temperature (37±0.1 °C). Our results show that there is a concentration-dependent inhibition to both airborne carbon nanotubes (CNT) and graphene nanoplatelets (GNP) at physiologically relevant airborne concentrations (4-70 µg/m³). To date, this setup demonstrates the most accurate model for inhalation simulations, in vitro.

1.3 Inhibition mechanism

Despite limitations of current in vitro work, much knowledge has been accumulated about the mechanism of NP-induced surfactant inhibition. Many biophysical tests point toward the mechanism involving lipid and protein adsorption onto NPs upon contact with PS films.
However, simply testing surfactant biophysics is not sufficient to prove this theory. Other methodologies including microscopy and chemical analyses must be explored.

### 1.3.1 Microscopy investigation

#### 1.3.1.1 Surface visualization

Visualizing the air-water interface with submicron resolution is one method into probing nano-bio interactions. This can be done via Langmuir-Blodgett or Langmuir-Schaefer film transfer in concert with atomic force microscopy (AFM). This technique immobilizes the fluid membrane onto a solid substrate. The topography of the immobilized sample can then be visualized using AFM. Introducing hydrophobic NPs (*i.e.* polyorganosiloxane and alkylated Au-NPs) to DPPC membranes proved that NPs remained at the interface where nano-bio interactions most likely take place. After enhancing model membranes to include heterogeneous phospholipid mixtures and SPs, hydrophobic NPs were seen interacting within film multilayer structures under high resolution AFM. Tatur *et al.* demonstrated that at the interface of a naturally-derived surfactant, Survanta, NPs lied in the fluid phase of the surfactant monolayer around the large solid-like lipid domains. By immobilizing the fluid surface and subsequent AFM scanning, a snapshot of the NP-PS interactions can be visualized and therefore analyzed with submicron resolution.

Particles are not always found at the surface of transferred films. When hydrophilic particles were exposed to naturally-derived surfactant no protrusions were found upon AFM analysis although biophysical tests showed surfactant inhibition had occurred. However, in agreement with model membrane studies AFM revealed that hydrophobic PLGA and PST-NPs were retained at the surfactant film. Figure 1.6a shows the AFM images of Infasurf films exposed to
NPs with increasing hydrophobicities. It can be seen that increasing hydrophobicity of NPs increased the frequency of such particles retained at the interfacial surface. Additionally, in good agreement with the model membranes, these hydrophobic NPs were exclusively found embedded in the multilayer structures at high surface pressures above 50 mN/m (i.e. low surface tensions). Regardless of the extent hydrophobicity/hydrophilicity, the lateral film structure was compromised after incubation with NPs as can be seen from the AFM images of Infasurf treated with hydrophilic HA-NPs. Uniform multilayer structures degenerated after HA-NP and PST-NPs exposure indicating that the incubation with both hydrophilic and hydrophobic NPs inhibited the structure and therefore function of the film. These data strongly suggest that the retention of inhaled NPs at the alveolar surface highly depend on their hydrophobicity.

1.3.1.2 Surfactant aqueous ultrastructure

AFM results established that the fate of hydrophilic NPs does not end at the interfacial film, but that these particles can penetrate the PS and enter the aqueous subphase where more interactions may take place. The PS matrix relies on the structure of the vesicles in the aqueous subphase in order for correct biophysical function and to replenish surfactant at the interface. Transmission electron microscopy (TEM) has been valuable in the study of the ultrastructure of surfactant vesicles after NP exposure. TEM results exhibited that hydrophilic NPs indeed interact with the surfactant ultrastructures (no such aqueous vesicular studies have been done with hydrophobic NPs, however their high retention at PS films demonstrates that these particles will not penetrate the surfactant film and will remain at the alveolar surface until cleared). TiO$_2$-NPs introduced to a modified naturally-derived surfactant, Curosurf, induced large, structured lamellar bodies to degrade into smaller less ordered ones and even create unilamellar vesicles (Figure 1.7). The transformation of large surfactant aggregates to small ones is a sign of PS
degradation.\textsuperscript{62,63} Hydrophilic NPs of a different material (HA-NPs) also demonstrated the same phenomenon where large aqueous surfactant vesicles dissociate into small unilamellar vesicles upon NP exposure. High magnification images of the unilamellar vesicles in both studies reveal NP aggregates associated with the small vesicular structures. The strong association of hydrophilic NPs with aqueous surfactant vesicles and of hydrophobic NPs with the interfacial surfactant film demonstrate strong evidence that NPs, regardless of their hydrophobicity, bind and adsorb to the PS matrix.

1.3.2 Lipoprotein adsorption

Biophysical investigations of NP-induced surfactant inhibition highlight differential inhibitory potentials for certain characteristics of NPs. On the other hand, these results give nearly no information on the NP mechanism after introduction to PS. Microscopy investigations take one step further, in which surfactant visualization can determine where certain NPs interact with which PS structures. Whether they bind at the interfacial film or in the subphase is dependent on NP physicochemical characteristics. Although important investigative techniques, neither biophysical nor microscopy studies can shed any light on biophysicochemical molecular mechanisms. Therefore chemical assays and \textit{in silico} methods have been utilized to probe the molecular interactions between NPs and PS components.

1.3.2.1 Hydrophobic pulmonary surfactant component

Hydrophobic components of PS, including phospholipids, neutral lipids and SP-B/C, are responsible for its high surface activity and biophysical function. Hence, many studies have delved into the interactions between these components and NPs to explain NP-induced surfactant biophysical inhibition. Many studies have focused on how NPs affect homogenous DPPC
monolayers through molecular dynamics (MD) simulations. Simplistically carbonaceous fullerene spheres were simulated to interact with a DPPC monolayer.\textsuperscript{64} Upon lateral compression of the DPPC monolayer, all sizes of the fullerene spheres preferentially collapsed into the bicelle folds. In comparison, \textit{in vitro} AFM imaging of hydrophobic particles upon model membranes also showed to preferentially deposit in multilayer structures upon lateral compression.\textsuperscript{48} At lower surface pressures dendrimer particles were simulated during phase transition of a DPPC monolayer. All but the smallest dendrimer particle showed to induce structural changes within the lipid monolayer.\textsuperscript{65} These differences inhibited the lipid phase transition from the fluid-like liquid-expanded (LE) phase to the solid-like tilted-condensed (TC) phase. The TC phase of DPPC results from compressing the monolayer. When this phase is present all DPPC molecules are packed tightly which yields a decrease in surface tension. Therefore this transition is relevant to the essential biophysical function of DPPC in PS.

Other MD simulation studies focus on the adsorption patterns of specific PS biomolecules to NPs at a molecular level. Due to the high prevalence in carbonaceous nanomaterial (CNM) in the nanotechnology industry, many studies focus on CNM toxicity.\textsuperscript{66} In addition, CNM show unprecedentedly high hydrophobicities, and based on biophysical data pose a higher inhibitory potential than most other NPs. The idea of DPPC adsorption to CNT has been addressed multiple times in MD simulations.\textsuperscript{67,68} As shown in Figure 1.8 these studies have shown that DPPC binds tightly to CNT, in a non-specific reversible fashion. Therefore, a dynamic adsorption and desorption of DPPC molecules is expected as long as there exists an energetically favorable coating on the pristine CNT. Based on MD simulation results Obata \textit{et al.} hypothesized that when these particles are exposed to a phospholipid mixture, as found in PS, there would be an exchange to a more favorable NP-phospholipid complex.\textsuperscript{67}
A year following this study Kapralov et al. simulated the adsorption pattern of multiple PS phospholipids onto bare CNTs (Figure 1.8).\(^6\) In this study phospholipid binding was a result from the alkyl chains’ hydrophobic attraction to the hydrophobic CNT. Their simulation of PG coated CNT striation patterns were in good agreement with the AFM results of phospholipid coated CNT. Finally, liquid chromatography in combination with mass spectroscopy results of PS phospholipid-coated CNT indicated that PC and PG dominated the adsorbed species (Fig. 1.8b). This is in good agreement with both chemical compositions where PC and PG are present in the highest ratios as well as adsorption energetics dictated by MD simulations.

Although PS is composed mostly of phospholipids (\(\sim 90\% \, w/w\)) it has been proven that the presence of surfactant proteins are crucial for its biophysical function. Therefore MD studies have been conducted to establish the interaction between SPs and NPs. Preliminary MD simulations with SP-B included carbonaceous NP interacting with a DPPC monolayer doped with SP-B.\(^6\) The interactions between SP-B and carbonaceous NPs were shown to be repulsive. The claim for surfactant inhibition in this study was the change in tilt angle of SP-B molecules proximal to NPs which led to an incorrect binding to surrounding phospholipids. After a more comprehensive simulation our results showed that anionic NPs will adsorb precious SP-B due to electrostatic attraction.\(^5\) Both Figure 1.1d and Figure 1.6b demonstrate simulations of anionic particles. Notice that in each case SP-B (purple) is adsorbed to the NP surface. After anionic hydrophilic NPs (Fig. 1.1d, black) showed to adsorb SP-B and drag it through a model surfactant membrane into the aqueous subphase, whereas anionic hydrophobic NPs (Fig 1.1d, blue) are retained at the interface and adsorb SP-B. Neutral and cationic particles showed no affinity to SP-B. This finding explains the non-attractive behavior of the neutral carbonaceous NP in the preliminary findings.
These electrostatic attraction results obtained through MD simulations are in good agreement with chemical analyses of SP-B adsorption. In one study, many anionic NPs composed of multiple different materials were tested for peptide adsorption upon contact with PS. All NPs tested had different surface chemistry, hydrophobicity, and size. This study noted that “Pulmonary surfactant-associated protein B was among the major attached proteins on the surface of engineered nanomaterials”. Recently SP-B and SP-C adhesion to polymeric NPs has been correlated with biophysical inhibition in model membranes. Supplemented SP-B and recombinant SP-C to phospholipid mixtures showed a significant and dose dependent decrease in concentration after exposed to anionic, polymeric-NPs. Biophysical surfactant dysfunction was measured in parallel with SP depletion. These experimental results in coordination with MD simulation results give a strong argument that electrostatic interaction is the major determining factor in SP-B adsorption, in which the resulting consequence is biophysical surfactant dysfunction.

### 1.3.2.2 Hydrophilic pulmonary surfactant component

Many clinical preparations of surfactant use an organic extraction process to eliminate hydrophilic components of the natural pulmonary surfactant. However, upon NP interaction with complete intact PS hydrophilic SP-A and SP-D also demonstrate adsorption behavior. Knowing that the main function for the hydrophilic SP-A and SP-D is immunological means that their binding will not have a catastrophic effect on surfactant biophysics like the adsorption of SP-B and SP-C.

As stated earlier this chapter, SP-A and SP-D both contain CRDs which bind to a wide range of contaminants, not only carbohydrates contrary to what the name suggests. MD simulations of SP-D have demonstrated that these binding sites adhere both CNT as well as PS phospholipids.
Additionally, when these particles are coated with phospholipids, the SP-D shows affinity to the hydrophilic head groups oriented facing away from the CNT surface. SP-D is not limited to binding carbon, though. SP-D has also shown to bind spherical PST-NPs. The binding of SP-D as well as SP-A to CNT was confirmed with TEM, which visualized SP-A and SP-D multimers bound to the surface of double-walled CNT (Fig. 1.9b). Chemical analyses also proved that SP-A and SP-D bind NPs after exposure to bronchoalveolar lavage fluids. Unlike SP-B/C adsorption to NPs which is strictly inhibitory, hydrophilic SP binding serves an immunological purpose. SP-A interaction with NPs appears to trigger alveolar macrophage (AM) clearance of NPs in the peripheral lung. When compared to NPs treated with albumin, NPs pretreated with SP-A induced a significant increase in AM uptake regardless of NP material or physicochemical properties. Unlike the inhibitory effect that NPs take on the biophysical function of PS, SP-A and SP-D are components that readily adsorb to NPs to remove the foreign bodies from the alveoli. The innate immunological function of PS is directly related to the nano-bio interactions of the hydrophilic SPs associated with PS.

1.3.3 Lipoprotein corona

Upon analyzing all of the molecular mechanism data, one outcome is apparent. This is that when NPs come in contact with PS, both lipids and proteins will adsorb to the particle surface and create a lipoprotein “corona” that coats the bare NP. Biomolecular coronas have been outlined in many studies and reviews demonstrating that in any biological environment almost immediate adsorption of biomolecules will occur at the nano-bio interface. The PS lining of the peripheral lung is no exception. In fact, because the PS matrix represents a biological barrier separating the atmospheric environment and the rest of the body, the initial biomolecular corona
established at the surface of airborne nanoparticles is composed of PS lipids and proteins. The same dynamic adsorption-desorption behavior that was stated above about DPPC onto CNT surfaces has been shown to apply to all biomolecular coronas where energetics and chemical reactions dictate the composition and structure of the corona (Figure 1.10). 

Once the bimolecular corona is established in the biological environment it is hypothesized that further interactions with the NP will be determined by the biomolecules presented at the surface of the corona rather than the physicochemical properties of the pristine NP (Figure 1.10). This theory has been tested in the case of the alveolar PS matrix where NPs of different hydrophobicities both found an increase in AM clearance after exposure to PS lipids and proteins. AM recognized the adsorbed PS components on both NPs. Animal models have demonstrated that inhaled NPs are not always cleared by AMs and can translocate through the pulmonary epithelium and enter the blood stream. The migratory NPs therefore have the opportunity to present biomolecules adsorbed at the alveolar surface to cells throughout the body. It is not surprising that inhaled NPs cause adverse effects to a wide range of organ systems in multiple studies. Although chemical functionalization of NPs can help mitigate some nano-bio interactions, a PS corona alters the adsorption of certain blood plasma proteins as well. Therefore, the adsorption of PS lipids and proteins to NPs not only affect the pulmonary system directly, but can potentially affect their fate in the rest of the body.

1.4 Cytotoxicity surfactant-coated nanoparticles

Toxicology studies have focused on the outcomes of inhaled NPs on a cellular level within the lung. Certain studies have demonstrated that NPs induce carcinomas, increase oxidative
stress, as well as increase inflammation.\textsuperscript{2} Just as in biophysical PS inhibition, the extent of nanotoxicity is also determined by the physicochemical characteristics of NPs. In the past few years hydrophobicity has proved to be a key contributing factor in biophysical PS inhibition, and cytotoxic potential proves to be no exception. Recently it has been determined, at least to some extent that toxicity of inhaled NPs depends on hydrophobicity. Jones \textit{et al.} recently studied pulmonary inflammation and the resulting cellular destruction caused by NP inhalation (Figure 1.11).\textsuperscript{80} Hydrophilic NPs demonstrated minimal to no inflammation at both low and high doses. Meanwhile, hydrophobic NPs elevated inflammatory markers, induced neutrophil penetration, and demonstrated cell structural damage upon histology assessment. With this said, hydrophobicity is not the only physicochemical characteristic that showed to be a factor in pulmonary cytotoxicity. Size has shown to influence PS biophysical inhibition dramatically; however agglomeration state is one characteristic that has not been as widely viewed as pro-inhibitory. Nevertheless, inhaled TiO\textsubscript{2}-NPs of larger and smaller agglomerates resulted in different toxic potentials.\textsuperscript{81} Aggregates with a total aerodynamic radius of less than 100 nm increased oxidative stress in rat models. Once again the cellular and biochemical pathways which induce inflammation and oxidative stress are still unknown, but evidence points that the physicochemical characteristics of the NPs plays a major role.

Because these pathways are unknown, \textit{in vitro} methods have been employed to further study the effect of NP-induced cellular responses. As demonstrated above, the lipoprotein corona originating from the PS film upon inhalation will play a crucial role with the cellular and biological interactions with NPs in the alveoli. For this reason NPs pretreated in naturally-derived PS preparations have been tested with alveolar cell types. The preliminary data from these studies show that one clinical preparation, Curosurf, induced mild toxicity.\textsuperscript{82} Another
surfactant preparation, Survanta, showed no adverse effects, but did not mitigate the toxic effects of single-walled CNTs. Animal-derived surfactant preparations do differ slightly in their composition and therefore have the potential to cause slightly different lipoprotein coronas. Due to this fact the effects of pretreatment of different clinical surfactants may differ slightly. However, to fully understand how PS coating affects cellular mechanisms much more testing must be conducted.

1.5 Thesis overview

In summary, the respiratory system represents a direct and systemic portal of entry for airborne contaminants. When investigating the most crucial biological structure in this organ system, the alveolus, the PS matrix at the air-water interface shows to be crucially important to the normal function of the mammalian lung both in lung mechanics and immune defense. However when respirable NPs penetrate to the terminal alveolar structures, they have the potential to compromise the PS matrix. The inhibitory nano-bio interactions that occur at the PS film are largely determined by the physicochemical properties at the NP surface including, size, shape, charge, agglomeration state, and hydrophobicity. In this thesis the effect of hydrophobicity of NPs will be discussed.

This thesis is organized as the following. Chapter 2 will demonstrate the inhibitory effect of hydrophobic NPs on naturally-derived PS films. This chapter will outline not only how hydrophobic NPs inhibit the PS film, but also how increasing hydrophobicity ultimately increases the inhibitory potential of NPs. Chapter 3 further explains how hydrophobic NPs inhibit the PS matrix by closely resembling inhalation. Introducing CNM from the airside to
naturally-derived PS allows us to study the inhibitory potential of airborne NPs in close simulation to what happens in the lung. This chapter will discuss the development of the CDS with its novel aerosol generation and analysis system. Chapter 4 summarizes the conclusions of this thesis work. Chapter 5 outlines the prospective future work that has stemmed from this thesis.
Figure 1.1. NP penetration through the lungs and interaction with pulmonary surfactant. (a) NPs are lightweight and respirable which allows them to reach the alveoli. (b) At the alveolus the first biological barrier that NPs contact is the pulmonary surfactant (PS). NPs will frustrate alveolar macrophages (AM) and inhibit the pulmonary surfactant film. (c) Under magnification NPs adhere to the surfactant at the air-water interface and cause lamellar bodies to degrade into unilamellar vesicles. (d) The molecular interactions of NPs and PS components have been modeled with molecular dynamics simulations. Anionic particles (black and blue) will adsorb essential SP-B and remove it from the film. Hydrophilic particles (black) adsorb SP-B and translocate the film and drag SP-B into the aqueous subphase. Hydrophobic particles (blue) adsorb SP-B at the surface and are retained within the surfactant film at the interface.
Figure 1.2. Effect of NPs on synthetic and natural surfactant films.\textsuperscript{59} Anionic polystyrene nanoparticles (PST-NPs) (open squares) and cationic PST-NPs (closed squares) were introduced to an (A) phospholipid mixture, (B) phospholipid mixture supplemented with SP-B, (C) Venticute, (D) Alveofact, and (E) large surfactant aggregates from rabbit bronchoalveolar lavage fluid. Biophysical inhibition demonstrated as an increase in $\gamma_{\text{min}}$ was most potent with the phospholipid mixture followed by the phospholipid mixture with SP-B, Venticute, Alveofact, and finally large aggregates at arbitrary NP concentrations. For all surfactant models anionic PST-NPs induced a higher inhibition than cationic PST-NPs.
Titanium dioxide nanoparticles (TiO₂-NPs) and (B, D) TiO₂ micron-sized particles (MSP) were introduced to a naturally derived surfactant, Curosurf. Inhibition was demonstrated by (A-B) $\gamma_{ads}$ and (C-D) $\gamma_{min}$. Both of these inhibition parameters demonstrated that NPs are more inhibitory than MSP.
Figure 1.4. Hydrophobic versus hydrophilic NP effect on naturally-derived pulmonary surfactant. Compression isotherms of (a) pure Infasurf, (b) Infasurf mixed with HA-NPs, and (c) Infasurf mixed with PST-NPs were taken every half hour until isotherm shifting reached equilibrium. Shifting to the left represents surfactant inhibition by an increase of film compressibility. Both HA-NPs and PST-NPs reached a similar magnitude of inhibition, however PST-NPs reached inhibition equilibrium in one hour whereas HA-NPs required 7 hours to reach inhibition equilibrium. The pure Infasurf control showed no increase in compressibility even after one day.
Figure 1.5. Effect of NP hydrophobicity on pulmonary surfactant using pulsating bubble surfactometer. Continuous oscillation of an air bubble in Alveofact mixed with (A) PST-NPs, (B) PLGA-NPs, and (C) EU-NPs demonstrated the time it took to reduce $\gamma_{\text{min}}$ below 5 mN/m. Increasing dynamic surface tension lowering time is consistent with increasing NP hydrophobicity. (D) The final $\gamma_{\text{min}}$ reported after 300 seconds seems to have no correlation with hydrophobicity.
Figure 1.6. Atomic force microscopy imagery and corresponding molecular dynamics simulations of hydrophobic NPs at pulmonary surfactant films.\textsuperscript{56-58} (a) AFM images of Infasurf alone and Infasurf mixed with four NPs HA-NPs, P02A, P103E, and PST-NPs which increase in surface hydrophobicity, respectively. Lateral images show Infasurf films at surface pressures ($\pi$) of 20, 30, and 40 mN/m which all correspond to a monolayer. Increasing hydrophobicity of NPs directly results in a high frequency of NPs retained at the surface film. Three-dimensional AFM images demonstrate the film at $\pi = 50$ mN/m which corresponds to a film with multilayer protrusions as shown by the pure Infasurf control. The sole hydrophilic NP (HA) does not show any NPs at the interface. However, all three other hydrophobic particles are visualized at the surface within the multilayer folds. (b) MD simulations of hydrophobic particles upon compression support the experimental data that hydrophobic particles remain at the surface and preferentially sit in multilayer protrusions upon $\pi > 50$ mN/m.
Figure 1.7. Ultrastructure degradation of pulmonary surfactant vesicles after exposure to titanium dioxide nanoparticles.\textsuperscript{52} (A-B) Untreated surfactant vesicles form well structured and highly organized lamellar body like (LBL) structures in the alveolar hypophase. (C) After TiO\textsubscript{2}-NPs are introduced to the surfactant, these LBL structures deform into globular less structured LBL and even degrade into unilamellar vesicles (UV). (D) NP aggregates can be visualized residing within the surfactant structures with red circles.
Figure 1.8. Molecular dynamics simulation and experimental support for pulmonary surfactant lipid adsorption to carbon nanotubes. (a) Molecular dynamics simulations demonstrate that all phospholipids of pulmonary surfactant can adsorb to a CNT surface. (b) Experimental data proves that many different phospholipids do indeed adsorb to the surface of CNTs. Additionally, the proportions of the lipids are in agreement with the concentration of PS and the binding affinity found via simulations.
Figure 1.9. Molecular dynamics simulation and experimental support for SP-D adsorption to carbon nanotubes.\textsuperscript{68,72} (a) SP-D binding domain recognizes the terminal end of CNT. (b) TEM results validate this claim where SP-D multimers were found adsorbed and bound to the surface of CNT.
Figure 1.10. Dynamic behavior of biomolecular coronas. (a) After coming in contact with PS, NPs adsorb a corona complex and have the potential to translocate the PS film and enter the subphase. These NPs can then interact with cells and their surface presenting membranes. (b) The corona is dynamic in nature where biomolecules are constantly adsorbing and desorbing to the surface. It is the biomolecules on the surface of the NP that will govern its interactions with cell surface receptors.
Figure 1.11. Cellular nanotoxicity demonstrated by histopathology.\textsuperscript{80} (A) Normal lung samples demonstrate open, healthy alveolar structures. (B-C) Histopathology results for lung tissue exposed to NPs with low surface hydrophobicity showed minor adverse effects to the alveolar structure. (D-E) Whereas, severe adverse effects and alveolar collapse was demonstrated when high hydrophobic NPs were exposed to mouse models.
Table 1.1. Biophysical studies of nanoparticle-pulmonary surfactant interactions.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Nanoparticle</th>
<th>Methodologies</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Material</td>
<td>Size</td>
<td>Charge</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>DPPC</td>
<td>Gelatin</td>
<td>136-287 nm</td>
<td>+</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>DPPC</td>
<td>PST-COOH</td>
<td>200 nm</td>
<td>-</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>DPPC Survanta</td>
<td>C16-S-Au-NPs</td>
<td>~2 nm</td>
<td>n.d.</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>DPPC</td>
<td>Fullerene</td>
<td>~1 nm</td>
<td>n.d.</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>DPPC DPPC/PA</td>
<td>SiO2-NPs</td>
<td>30 nm</td>
<td>-</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>DPPC DPPC/DOPC</td>
<td>Carbon Black</td>
<td>15-20 nm</td>
<td>n.d.</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>DPPC DPPC/SP-C</td>
<td>AmorSil20-NPs</td>
<td>~22 nm</td>
<td>n.d.</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>DPPC DPPC/POPG/SP-B</td>
<td>capped Au-NPs</td>
<td>~15 nm</td>
<td>n.d.</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Curosurf</td>
<td>TiO2-NPs</td>
<td>~5 nm</td>
<td>n.d.</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Alveofact</td>
<td>PST-NPs</td>
<td>100 nm</td>
<td>-</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>Infasurf</td>
<td>HA-NPs</td>
<td>~100 nm</td>
<td>-</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>DPPC</td>
<td>Eicosane</td>
<td>N/A</td>
<td>n.d.</td>
<td>Hydrophilic</td>
</tr>
</tbody>
</table>

Mouse lung lavage
<table>
<thead>
<tr>
<th>Product</th>
<th>Material</th>
<th>Diameter</th>
<th>Nature</th>
<th>PBS</th>
<th>LOD</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curosurf</td>
<td>Au-NPs</td>
<td>~5nm</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infasurf</td>
<td>PLGA</td>
<td>~200-300</td>
<td>Hydrophobic</td>
<td>LB + AFM</td>
<td>Increasing hydrophobicity increases inhibition and retention at surface</td>
<td></td>
</tr>
<tr>
<td>PST</td>
<td>~100</td>
<td>-</td>
<td>Hydrophobic</td>
<td>CDS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveofact</td>
<td>PST(+)NPs</td>
<td>100 nm</td>
<td>Hydrophobic</td>
<td>PBS</td>
<td>Anionic NPs cause more inhibition; natural surfactant is inhibited less than model membranes</td>
<td></td>
</tr>
<tr>
<td>Venticute</td>
<td>PST(-)NPs</td>
<td>100 nm</td>
<td>Hydrophobic</td>
<td>PBS</td>
<td>NPs adsorb SPs and inhibit film biophysics</td>
<td></td>
</tr>
<tr>
<td>DPPC/POPG/SP-B</td>
<td>PLA-NPs</td>
<td>&lt; 100 nm</td>
<td>Hydrophobic</td>
<td>PBS</td>
<td>NPs repel SP-B and affect interaction geometry</td>
<td></td>
</tr>
<tr>
<td>DPPC/POPG/PA</td>
<td>DPPC/POPG/SP-B/C</td>
<td>PLA-NPs</td>
<td>&lt; 100 nm</td>
<td>Hydrophobic</td>
<td>MD</td>
<td>NPs repel SP-B and affect interaction geometry</td>
</tr>
<tr>
<td>Infasurf</td>
<td>HA-NPs</td>
<td>~100 nm</td>
<td>Hydrophilic</td>
<td>LB + AFM</td>
<td>Hydrophobic NPs retain at surface and inhibit more than hydrophilic</td>
<td></td>
</tr>
<tr>
<td>PST-NPs</td>
<td>~100 nm</td>
<td>-</td>
<td>Hydrophobic</td>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>Carbon</td>
<td>0.17 nm</td>
<td>Neutral</td>
<td>PBS</td>
<td>Energetics determines DPPC adsorption orientation and behavior</td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>swCNT</td>
<td>1.36 nm</td>
<td>Hydrophobic</td>
<td>MD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>mwCNT</td>
<td>50 nm</td>
<td>Hydrophobic</td>
<td>MD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>Dendrimer</td>
<td>Hydrophilic</td>
<td>MD</td>
<td>Dendrimers affect structure of monolayer and phase transition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>Fullerene</td>
<td>Hydrophobic</td>
<td>MD</td>
<td>NPs deposit into monolayers; under compression in protrusions</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2. Immunological studies of nanoparticle-pulmonary surfactant interactions.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Nanoparticle</th>
<th>Material</th>
<th>Size</th>
<th>Charge</th>
<th>Charge</th>
<th>Methodologies</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DPPC/POPG/PA/SP-A,D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Starch-NPs</td>
<td></td>
<td>~150 nm</td>
<td></td>
<td></td>
<td>Hydrophilic</td>
<td>FC, CM, SDS-PAGE</td>
<td>SP-A signals hydrophobic clearance, SP-D signals hydrophilic clearance</td>
</tr>
<tr>
<td></td>
<td>Lipid-NPs</td>
<td></td>
<td>~125 nm</td>
<td></td>
<td></td>
<td>Hydrophobic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porcine BALF</td>
<td>BaSO4</td>
<td></td>
<td>38 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td>SDS-PAGE, Western Blot, TEM</td>
<td>SP-A binds metal oxides with different adsorption patterns.</td>
</tr>
<tr>
<td></td>
<td>AIOOH</td>
<td></td>
<td>40 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TiO2</td>
<td></td>
<td>20-30 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CeO2</td>
<td></td>
<td>14-70 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CB</td>
<td></td>
<td>16 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated SP-A</td>
<td>Starch-NPs</td>
<td></td>
<td>140 nm</td>
<td></td>
<td></td>
<td>Hydrophilic</td>
<td>FC, SDS-PAGE, CM</td>
<td>NPs induce unique protein priming after SP-A adsorption.</td>
</tr>
<tr>
<td></td>
<td>Chitosan</td>
<td></td>
<td>165 nm</td>
<td></td>
<td></td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carboxymethyl dextran</td>
<td></td>
<td>180 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly-maleic-oleic acid</td>
<td></td>
<td>115 nm</td>
<td></td>
<td>+</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td></td>
<td>130 nm</td>
<td></td>
<td></td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human BALF</td>
<td>dwCNT</td>
<td></td>
<td>3 nm x 500 nm</td>
<td>n.d.</td>
<td></td>
<td>Hydrophilic</td>
<td>SDS-PAGE, Western Blot, ELISA, IR</td>
<td>dwCNT sequester SP-A,D through adsorption.</td>
</tr>
<tr>
<td>Curosurf</td>
<td>Metal Oxides</td>
<td></td>
<td>10 nm - 5 um</td>
<td>-</td>
<td>n.d.</td>
<td>Hydrophilic</td>
<td>SDS-PAGE, LCMS</td>
<td>SP-B major protein adsorbed to all NPs</td>
</tr>
<tr>
<td></td>
<td>mwCNT</td>
<td></td>
<td>10 - 30 nm x 1 - 2 um</td>
<td>-</td>
<td>n.d.</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>swCNT</td>
<td></td>
<td>&lt; 2 nm x 1 - 5 um</td>
<td>-</td>
<td>n.d.</td>
<td>Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quartz</td>
<td></td>
<td>&lt; 5 um</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse BALF</td>
<td>swCNT</td>
<td>1-4 nm x 700 nm</td>
<td>Hydrophobic</td>
<td>LCMS, AFM, FC, SDS-PAGE</td>
<td>Lipids and SPs adsorb with same ratios as PS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------</td>
<td>----------------</td>
<td>-------------</td>
<td>------------------------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curosurf</td>
<td>mwCNT</td>
<td>30-40 nm x 500-2000 um</td>
<td>-</td>
<td>Hydrophobic</td>
<td>LCMS, TLC, SDS-PAGE</td>
<td>PS adsorbs immediately to NPs changing surface characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mwCNT-COOH</td>
<td>30-40 nm x 500-2000 um</td>
<td>-</td>
<td>Hydrophobic</td>
<td>LCMS, TLC, SDS-PAGE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mwCNT-NH2</td>
<td>30-40 nm x 500-2000 um</td>
<td>+</td>
<td>Hydrophobic</td>
<td>LCMS, TLC, SDS-PAGE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat BALF</td>
<td>TiO2</td>
<td>5 nm</td>
<td>n.d.</td>
<td>Hydrophilic</td>
<td>XRD, Agglomeration state affects toxicity</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Page 40
Chapter 2. Influence of Nanoparticle Hydrophobicity on Pulmonary Surfactant Biophysical Inhibition

2.1 Background

Interest in nanoscience and nanotechnology has grown immensely, particularly for its applications in nanomedicine and nanotoxicology.\textsuperscript{3,6} Much emphasis has been put on the pulmonary system as a portal of entry for nanoparticles (NPs) due to its administration ease and direct systemic access.\textsuperscript{5,84} When NPs are inhaled the first biological barrier they interact with is the native pulmonary surfactant (PS) system.\textsuperscript{8,77} The PS has several physiological and biophysical functions that are essential for normal lung function and immune health.\textsuperscript{10} Lack or dysfunction of PS can cause pathological pulmonary conditions related to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS),\textsuperscript{12} so it is crucial to preserve functional PS to maintain pulmonary health.

The PS is a complex mixture of mostly lipids (~90\% by weight) and four surfactant proteins (SP-A, B, C, and D, ~10\% by weight) that lines the entire alveolar surface as a thin film.\textsuperscript{10} A closer evaluation of the lipid portion of PS reveals that the majority of the lipids are phospholipids that contribute to its high surface activity (\textit{i.e.}, the ability to reduce alveolar surface tension to near-zero). SP-A and SP-D are hydrophilic proteins that assist in macrophage clearance and other innate immune responses.\textsuperscript{74} The other two proteins, SP-B and SP-C, are hydrophobic proteins embedded in the phospholipid matrix that work in coordination with phospholipids to achieve low surface tension during respiration cycles.\textsuperscript{22}

The interactions of NPs and PS films are largely not understood, but it has been shown that the physicochemical characteristics of NPs are ultimately a determining factor in their interaction
behavior.\textsuperscript{48,49,52,56,57} One defining characteristic that has been shown to be integral in nano-bio interaction is the hydrophobicity of NPs.\textsuperscript{41,55,57,59,85} Reports have shown that hydrophobic NPs induce PS inhibition much more readily and to a higher degree than hydrophilic NPs.\textsuperscript{55,57} In addition to surfactant inhibition, recent molecular dynamics simulations have predicted that hydrophobicity also governs the NP translocation behavior through PS monolayer.\textsuperscript{57} The translocation behavior ultimately determines if a particle can quickly reach the pulmonary epithelia or if it will be retained at the air-water interface of the lung for a prolonged period of time. However, experimental correlation between particle retention and surfactant inhibition due to hydrophobic NPs has not been established.

In this chapter, we experimentally studied how three polymeric NPs with varying relative hydrophobicities affect, \textit{in vitro}, biophysical properties of an animal-derived surfactant preparation, Infasurf. We first characterized the hydrophobicity of these three NPs using the Rose Bengal partitioning method. Subsequently, we studied the translocation/retention behavior of these NPs at the Infasurf monolayer using the combination of a classical Langmuir-Blodgett trough and Atomic Force Microscopy (AFM). Finally, we evaluated surfactant inhibition caused by these three NPs under physiologically relevant conditions using a newly developed experimental method called the Constrained Drop Surfactometer (CDS). All of these experiments showed consistent results and indicated that increasing surface hydrophobicity of NPs provokes their retention at the surfactant monolayer and deteriorates surface activity of PS.

2.2 Experimental methods

2.2.1 Pulmonary surfactant

Infasurf\textsuperscript{®} (calfactant) is a gift from ONY, Inc. (Amherst, NY). It is a modified natural surfactant prepared from lung lavage of newborn calves by centrifugation and organic solvent
extraction. Infasurf contains all hydrophobic components of the bovine natural surfactant. Hydrophilic surfactant proteins (SP-A and SP-D), however, were removed during the extraction process. Infasurf was stored frozen in sterilized vials with an initial concentration of 35 mg total phospholipids per milliliter. On the day of the experiment, it was diluted by a saline buffer of 0.9% NaCl, 1.5 mM CaCl₂, and 2.5 mM HEPES, adjusted to pH 7.0.

### 2.2.2 Nanoparticles

Poly(D,L-lactide-co-glycolide) 53/47 with an intrinsic viscosity of 0.2 dL/g (acid-terminated PLGA; abbreviated P02A) and 1.03 dL/g (ester-terminated PLGA; abbreviated P103E) were purchased from Purac (Gorinchem, Netherlands). P02A and P103E nanoparticles were synthesized by a water-in-oil emulsion solvent evaporation technique and stabilized with PVA. Polystyrene (PST) nanoparticles were purchased from Thermo Scientific (3090A Nanosphere Size Standards, Fremont, CA). All NPs were characterized for their primary size, hydrodynamic size, surface charge, and surface hydrophobicity. The primary size was determined by analyzing FESEM and TEM (Hitachi HT7700) images with the ImageJ software (n=20). The hydrodynamic size and zeta potential of the NPs were determined at a dilute particle concentration of 0.01 mg/mL under the same buffering condition of the natural PS, i.e., 0.9% NaCl, 1.5 mM CaCl₂, and 2.5 mM HEPES, at pH 7.0. The hydrodynamic size was measured using a Brookhaven 90Plus/BI-MAS particle sizer (Holtsville, NY, USA). Zeta potential was determined using a Brookhaven Zetaplus zeta potential analyzer. The surface hydrophobicity was measured with the Rose Bengal partitioning method.
2.2.3 Rose Bengal partitioning

Hydrophobicity of the NPs was studied using the Rose Bengal partitioning method as previously described. A solution of Rose Bengal (RB) reagent (Sigma-Aldrich, St. Louis, MO) in 0.1 M phosphate buffer (pH 7.4) was diluted to 20 μg/mL. Various concentrations of nanoparticles were added to the solution to create a wide array of RB-NP suspensions. Each sample was incubated at 25°C for 3 hours. Suspensions were subsequently centrifuged at 16,000 g for 1 hour. The supernatant was collected and absorbance was read using a UV/Vis spectrometer at 543 nm (Epoch, BioTek, Winooski, VT). Partitioning Quotient (PQ) was determined as the ratio of RB bound onto the particle surface to free RB in the liquid phase, i.e.,

\[
PQ = \frac{RB_{\text{bound}}}{RB_{\text{free}}}.
\]

A plot of PQ versus surface area was made. The slope of the linear regression line can be used as an accurate representation of relative surface hydrophobicity, where increasing slope correlates with increasing surface hydrophobicity.

2.2.4 Langmuir-Blodgett trough and atomic force microscopy

Spread, compression and Langmuir-Blodgett (LB) transfer of surfactant films were conducted with a LB trough (KSV Nima, Coventry, UK) at room temperature (20 ± 1°C). Detailed experimental protocol can be found elsewhere. Briefly, all three NPs were diluted down to 50 μg/mL with a 5 mg/mL Infasurf suspension, i.e., 1% NP to surfactant by weight. This NP-PS suspension was spread atop a Milli-Q water subphase. The spread films were compressed at a rate of 40 cm²/min, namely 0.2% initial surface area per second. For AFM imaging, spread films at the air-water interface were transferred to the surface of freshly cleaved mica using the LB technique. Transferred films were scanned by an Innova AFM (Bruker, Santa Barbara, CA)
in air with the contact mode. Each sample was characterized at multiple locations with various scan areas to ensure the detection of representative structures.

### 2.2.5 Constrained drop surfactometer

*In vitro* simulations of surfactant activity and inhibition under physiologically relevant conditions were studied with a Constrained Drop Surfactometer (CDS). The CDS is a droplet-based surface tensiometer, newly developed for *in vitro* assessing biophysical properties of lung surfactant. The CDS uses the air-water interface of a sessile drop (~3 mm), constrained on a carefully machined drop pedestal with a sharp knife-edge, to accommodate the adsorbed surfactant films. Hence, the CDS requires only a minute sample size of about 10 µL for studying PS. Also owing to system miniaturization, the CDS permits a precise control of physiological conditions using an environmental control chamber. To simulate breathing, the droplet can be compressed and expanded at a physiologically relevant rate using a motorized syringe. The surface tension and surface area are determined photographically from the shape of the droplet using Axisymmetric Drop Shape Analysis (ADSA). Compared to the Wilhelmy plate method used in the classical Langmuir balance, ADSA measures surface tension accurately and remotely, thus minimizing potential sample contamination and facilitating undisturbed drop oscillation.

Specifically, Infasurf was diluted to a concentration of 1 mg/mL and mixed with NPs to a final NP concentration of 10 µg/mL, *i.e.*, 1% NP to surfactant by weight. This NP concentration was selected corresponding to the lower end of NP concentrations tested in previous studies. The NPs were incubated with Infasurf at 37 °C for 1 hour before cycling trials commenced. A droplet (~10 µL) of the NP-Infasurf mixture was dispensed onto the CDS drop
pedestal. After drop formation, the surface tension was recorded and found to quickly decrease to an equilibrium value of approximately 22-25 mN/m. Once equilibrium was established, the droplet was compressed and expanded at a rate of 3 seconds per cycle with a compression ratio controlled to be less than 50% of the initial surface area, to simulate normal tidal breathing. At least five continuous compression-expansion cycles were studied for each droplet. It was found that the cycles became repeatable right after the first cycle, similar to surfactant behavior in a captive bubble surfactometer (CBS). Surface activity was quantified with several parameters: the minimum surface tension ($\gamma_{\text{min}}$) at the end of compression, the maximum surface tension ($\gamma_{\text{max}}$) at the end of expansion, and the film compressibility, $\kappa = \frac{1}{A} \left( \frac{\partial A}{\partial \gamma} \right)$, during both the compression and expansion processes. Given the fact that $\kappa$ varies during the compression and expansion processes, an averaged $\kappa$ value was compared for each process. All simulations were conducted under well-controlled physiological conditions (37 °C and 100% relative humidity).

2.2.6 Statistical analysis

Statistical data were represented by the mean ± SEM. The measurements were based on dynamic cycling data for Infasurf (n=9), Infasurf + P02A (n=7), Infasurf + P103E (n=7), and Infasurf + PST (n=7). One-way ANOVA was used for statistical calculations (OriginPro, Northampton, MA). Tukey and Bonferroni means comparison tests were used and a probability value of $p < 0.05$ was considered statistically significant.
2.3 Results and discussion

2.3.1 Characterization of nanoparticles

We first characterized morphology and surface properties of the three NPs in study, i.e., P02A, P103E, and PST. As can be seen from Table 2.1, all NPs were spherical in shape and had mean primary sizes of 231, 264, and 84 nm, respectively. The mean hydrodynamic sizes of these NPs in buffer were measured to be 260, 350, and 95 nm, respectively, thus indicating no significant particle aggregation. Surface charge measurement by ζ-potential showed that all NPs were negatively charged to a similar degree. Surface hydrophobicity was measured by comparing the adsorption of Rose Bengal, a hydrophobic dye, to the NPs. A plot of Partitioning Quotient (PQ) versus NP surface area per milliliter was constructed in Figure 2.1. The slope of this plot has been used recurrently as an accurate representation of surface hydrophobicity, where increasing slope is proportional to increasing hydrophobicity of NPs.\(^{55,86,93,94}\) PST (0.069 \(\times10^{-9}\) mL/\(\mu\text{m}^2\)) yielded the highest slope, followed by P103E (0.025 \(\times10^{-9}\) mL/\(\mu\text{m}^2\)), and P02A (0.012 \(\times10^{-9}\) mL/\(\mu\text{m}^2\)) showed the lowest slope. These results indicate that relative to one another, PST is the most hydrophobic, followed by P103E, and P02A is the least hydrophobic of the three.

2.3.2 Particle retention at surfactant monolayer

We first studied NP-PS interaction and particle retention using the combination of LB trough and AFM. Compression isotherms were measured for pure Infasurf, Infasurf + P02A, Infasurf + P103E, and Infasurf + PST (Figure 2.2a). The exposure of all three NPs shifted the compression isotherm of Infasurf to the left. This shift indicates the compressibility (κ) of the film is increased when NPs are introduced, i.e., more area compression is necessary for the Infasurf + NP films to attain the same arbitrary surface pressure compared to pure Infasurf. Figure 2.3 demonstrates the
kinetics of the inhibition. Exposure of each particle induces compressibility to the same magnitude within 1 hour. Note that the surface pressure ($\pi$) of a monolayer is inversely related to its surface tension ($\gamma$) by $\pi = \gamma_0 - \gamma$, in which $\gamma_0$ is the surface tension of pure water. Hence, the shift of the compression isotherm to the lower surface area region indicates that all three NPs inhibited the surface activity of Infasurf, i.e., decreased its ability to reduce surface tension upon film compression.

AFM topographical images of the surfactant film for pure Infasurf as well as Infasurf mixed with NPs at four characteristic surface pressures were taken to examine lateral film structures (Figure 2.2b). It is found that the addition of NPs inhibited phospholipid phase transitions, as revealed by reduced phospholipid domains at the surfactant monolayer. At $\pi$ of 50 mN/m, the Infasurf monolayer was transformed into a multilayered structure with uniformly distributed lipid protrusions closely attached to the interfacial surfactant monolayer. However, at 50 mN/m (which is also the collapse pressure of the Infasurf film exposed to NPs), addition of all NPs disturbed this normal conformational monolayer-to-multilayer transformation by forming non-uniform large protrusions comparable to the hydrodynamic size of the respective NPs.

Similar disturbance of surfactant monolayer has been found with other NPs. Phospholipid phase transitions are considered to be necessary for normal biophysical function of lung surfactant, at least under in vitro conditions. Meanwhile, uniform monolayer-to-multilayer transformations have been proven to be a necessity for lung surfactant to reach physiologically relevant low surface tensions (or, high surface pressures). Disturbance of phospholipid phase transition and monolayer-to-multilayer transformation by the addition of NPs appears to cause surfactant inhibition shown in Figure 2.2a.
More interestingly, AFM revealed that with increasing surface hydrophobicity the frequency of NP aggregates visualized at the surfactant monolayer also increased. While the least hydrophobic NPs, P02A, only appeared at the surface at 50 mN/m, the most hydrophobic NPs, PST, were found at much lower surface pressures (20 mN/m), indicating a strong correlation between surface hydrophobicity of NPs and their retention/translocation behavior at the surfactant monolayer. It should be noted that in addition to surface hydrophobicity, hydrodynamic sizes of these three NPs are also different. Many studies have shown that size and shape of NPs determine their translocation behavior across lipid monolayers/bilayers or even cell membranes, with larger particles encountering a higher energy barrier for translocation. However, the size of PST used in this study is smaller than the other two NPs. Hence, the higher retention rate of PST at the Infasurf monolayer can be only related to its higher hydrophobicity.

This finding is in good agreement with our recent molecular dynamics simulations in which we found that hydrophobicity is the determining factor for NP translocation/retention at the surfactant monolayer. Previous molecular dynamics simulations further predicted that hydrophobic NPs can be encapsulated by a surfactant lipoprotein corona and trapped at the surfactant monolayer upon compression. These in silico results are also consistent with our current in vitro measurements presented in chapter 2 (Figure 2.2b), which show that the height of large protrusions formed at the surfactant monolayer is comparable to the actual hydrodynamic size of the respective NPs.

Our present in vitro findings in the retention of hydrophobic PST NPs at the surfactant monolayer may be related to previous animal trials. Both ex vivo and in vivo experiments demonstrated that intratracheally instilled PST NPs, in a size range comparable to what we studied here, showed very limited translocation from the lungs. Prolonged retention of NPs
in the lungs can have a significant toxicological effect due to increased production of reactive oxygen species (ROS), and in turn, increased inflammatory potential.

### 2.3.3 Physiologically relevant study of surfactant inhibition

Self-assembled monolayer at the air-water interface of a Langmuir trough is a commonly used *in vitro* model for studying lung surfactant. However, data obtained from such experiments have only limited physiological relevance due to commonly uncontrolled environmental conditions, relatively slow rate of film oscillation, and other design limitations. In order to conduct physiologically relevant *in vitro* simulations, we studied surfactant inhibition using the Constrained Drop Surfactometer (CDS), in which a surfactant droplet is oscillated at a physiologically relevant rate and the environment is manipulated to mimic physiological conditions.

Figure 2.4 compares typical compression-expansion cycles of pure Infasurf, and Infasurf mixed with P02A, P103E, and PST nanoparticles. It can be seen that all three NPs significantly increased the hysteresis area of the compression-expansion loop. More importantly, the hysteresis area increased with increasing surface hydrophobicity of the NPs. Large hysteresis areas of dynamic cycling loops are a strong indication of film instability and surfactant inhibition. Therefore, our *in vitro* simulations with the CDS not only demonstrated a NP-induced surfactant inhibition, which is consistent with our Langmuir trough studies (Figure 2.2a), but also revealed a NP hydrophobicity-dependent inhibitory potency, which the Langmuir trough was not sensitive enough to uncover.

To completely understand the effects of the three NPs on dynamic surface activity of Infasurf, statistical analyses of the data were performed (Figures 2.5). Figure 2.5a shows the
comparison of minimum surface tension ($\gamma_{\text{min}}$) at the end of compression and maximum surface tension ($\gamma_{\text{max}}$) at the end of expansion. It can be seen that the $\gamma_{\text{min}}$ shows a statistically significant increase due to exposure to all three NPs, although a very low particle concentration (i.e., 10 $\mu$g/mL) was used. However, with all NPs, Infasurf was still able to achieve a $\gamma_{\text{min}}$ below 3 mN/m. Hence, the variations in $\gamma_{\text{min}}$ due to exposure to these low-concentration NPs can be considered physiologically immaterial. Similarly, the $\gamma_{\text{max}}$ shows no statistically significant changes with the addition of NPs. The lack of a physiologically significant alteration of surface tension is consistent with Beck-Broichsitter et. al., who demonstrated that $\gamma_{\text{min}}$ only showed a significant increase when exposed to PST NP concentrations greater than 1 mg/mL, i.e., NP weight greater than 50% of surfactant weight in their experiments.\(^{59}\)

Figure 2.5b shows the comparison of film compressibility ($\kappa$) during both compression and expansion processes. Addition of all three NPs, at a very low concentration of 10 $\mu$g/mL, significantly increased the $\kappa$ of Infasurf during compression ($\kappa_{\text{comp}}$) and decreased the $\kappa$ during expansion ($\kappa_{\text{exp}}$). The increase in $\kappa_{\text{comp}}$ is clearly proportional to hydrophobicity of the NPs. It should be noted that differences in $\kappa_{\text{comp}}$ were statistically significant between all four groups, indicating that surface hydrophobicity of NPs plays a significant role in affecting surfactant inhibition. Figure 2.6 demonstrates the significant differences in $\kappa_{\text{comp}}$ with respect to their relative hydrophobicities, further demonstrating the effect of hydrophobicity.

It is known that the $\kappa$ is a much more sensitive parameter than $\gamma_{\text{min}}$ to evaluate surface activity and surfactant inhibition.\(^{88,101}\) Compressibility of a monolayer can be loosely related to its “hardness”. A low $\kappa$ indicates a strong film while a high $\kappa$ indicates a soft film. A good lung surfactant film should have a soft-yet-strong attribute.\(^{10}\) Upon film compression during exhalation, the surfactant film should have a low $\kappa$, thus decreasing alveolar $\gamma$ (equivalent to
increasing $\pi$) to near-zero with less than 20% area compression.$^{22,35}$ Upon film expansion during inhalation, the surfactant film should have a high $\kappa$, thus only increasing $\gamma$ to a limited value. Although still unclear in detailed mechanisms, lung surfactant appears to achieve this soft-yet-strong attribute by selective compositional and/or conformational variations during the compression-expansion cycles.$^{96,97,102}$ It appears that addition of NPs inhibits surface activity of lung surfactant by increasing $\kappa_{\text{comp}}$ and decreasing $\kappa_{\text{exp}}$ (Figure 2.5b), thus increasing hysteresis area of the compression-expansion loop (Figure 2.4).

Previous studies suggested that NPs inhibit lung surfactant by adsorbing surfactant proteins (such as SP-B and SP-C), i.e., through the formation of native surfactant lipoprotein corona.$^{55-57,70}$ After binding to solid particles, surfactant proteins can be denatured and/or depleted from the surfactant system, thereby causing surfactant inhibition. More hydrophobic NPs have a higher retention rate at the surfactant monolayer (Figure 2.2) and hence a higher chance of adsorbing surfactant proteins. Consequently, more hydrophobic NPs appear to have a higher inhibitory potential to lung surfactant (Figures 2.4-2.6).

In addition to surfactant inhibition, prolonged retention of hydrophobic NPs at the surfactant monolayer may have a significant toxicological effect. A major contributor in NP-associated pulmonary nanotoxicology is the increase of reactive oxygen species (ROS), and in turn inflammation.$^{82,103}$ It has been reported that more hydrophobic NPs are associated with a larger magnitude of ROS production and inflammation.$^{82}$ Dailey et. al. demonstrated that PST NPs showed considerably higher nanotoxicological effects than PLGA at the same size, surface area, and concentration.$^{103}$ Very recently, Dailey and coworkers established the direct correlation between NP hydrophobicity and pulmonary toxicity.$^{80}$ They found that intratracheal administration of more hydrophobic NPs (with PST NPs being the most hydrophobic) to mice
induced acute respiratory toxicity revealed by neutrophil infiltration, elevation of pro-
inflammatory cytokines and adverse histopathology findings; while less hydrophobic NPs caused
little or no inflammatory response or tissue damage. Although the underlying relationship
between the pulmonary toxicity of hydrophobic NPs and their inhibitory potential on lung
surfactant is yet to be developed, our current *in vitro* data clearly support such correlations.

2.4 Conclusions

We found that hydrophobicity of NPs plays an important role in affecting their retention at
the surfactant monolayer and their inhibitory potential on surface activity of pulmonary
surfactant. Through the use of AFM imaging our results found that increasing hydrophobicity
ultimately increases NP retention at the surfactant film. Biophysical data obtained from CDS
demonstrated that hydrophobicity does exacerbate PS inhibition. The CDS was able to detect
subtle compressibility inhibition which other techniques are not sensitive enough to capture.
Thus, the CDS developed in our laboratory can be used as a sensitive precautionary tool for
probing surfactant inhibition by NPs.
Figure 2.1. Hydrophobicity measurements via Rose Bengal partitioning. Relative hydrophobicity was measured using the Rose Bengal partitioning method. The three particles’ linear relationships of partitioning quotient (PQ) versus surface area were plotted. Linear regression lines were then calculated where the slopes are proportional to the surface hydrophobicity of the NPs. These data show that the particles’ hydrophobicity increases in such a manner that P02A is the least hydrophobic, P103E has a moderate hydrophobicity and PST is the most hydrophobic.
Figure 2.2. Comparison of nanoparticle retention at the Infasurf film. (a) Effect of 50 µg/mL NPs (i.e., 1% w/w of surfactant phospholipids) on the compression isotherm of Infasurf. (b) Lateral film structures of pure Infasurf and Infasurf mixed with P02A, P103E, and PST at four characteristic surface pressures (20, 30, 40, and 50 mN/m). All monolayer images (20, 30, and 40 mN/m) are shown at a resolution of 50µm × 50µm and have a z-range of 5 nm. The multilayer structures are all shown at a resolution of 20µm × 20µm and their z-ranges are dependent on the size of the particle (Infasurf: 20 nm, Infasurf + P02A: 250 nm, Infasurf + P103E: 350 nm, and Infasurf + PST: 120 nm). These high pressure images are shown in 3D to capture the topographic contrast between the particles and multilayer structures. The presence of NPs, denoted by white arrows, increases with increasing hydrophobicity. After the monolayer-to-multilayer transition (at 50 mN/m) all three NPs are found at the surface and embedded in the large hydrophobic multilayer protrusions. In contrast, pure Infasurf shows uniform protrusions that line the entire film surface.
Figure 2.3. Nanoparticle-induced inhibition dependence on time. Pure Infasurf (a) and all three hydrophobic NPs + Infasurf (b-c) compression isotherms. All three NPs induce surfactant inhibition shown by a shift in the Infasurf isotherm to the left. In contrast Infasurf alone maintains the same isotherm shown by the superimposable paths. The inhibitory shift indicates that more compression is necessary to reach an arbitrary surface pressure when compared to the pure Infasurf isotherm. All three particles induce a shift in the isotherm within 1 hour after exposure. The magnitude of inhibition is relatively the same for all particles as well.
Figure 2.4. Comparison of compression-expansion cycles for pure Infasurf and Infasurf mixed with nanoparticles. Cycling data for pure Infasurf and Infasurf mixed with P02A, P103E, and PST are represented by overlying surface tension versus surface area plots. Infasurf was diluted to a concentration of 1 mg/mL and mixed with NPs to 10 μg/mL, *i.e.*, 1% NP:Surfactant (w/w). Dynamic cycling was conducted with the CDS under physiological temperature (37°C) and cycling speed (3 sec/cycle). It can be seen that the hysteresis area of the compression-expansion loop increases significantly with increasing NP hydrophobicity.
Figure 2.5. Statistical analysis of the effect of nanoparticles on surface activity of Infasurf. (a) Minimum surface tension ($\gamma_{\text{min}}$) at the end of compression and maximum surface tension ($\gamma_{\text{max}}$) at the end of expansion for Infasurf and mixed Infasurf + NP suspensions. (b) Film compressibility during compression ($\kappa_{\text{comp}}$) and expansion ($\kappa_{\text{exp}}$) processes for Infasurf and mixed Infasurf + NP suspensions. Each trial was conducted with the CDS at 37°C and cycled at a physiologically relevant rate (3 sec/cycle). * $p < 0.05$ for comparison to pure Infasurf.
**Figure 2.6.** Direct comparison of compressibility and hydrophobicity. These data show the significant increase in compressibility ($\kappa_{\text{comp}}$) resulting from increasing surface hydrophobicity. Error bars represent the standard deviation hydrophobicity (horizontal) and film compressibility (vertical). The red line demonstrates the linear regression line of the three data points.
Table 2.1. Morphological and surface characterization of nanoparticles.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Acid-terminated PLGA (P02A)</th>
<th>Ester-terminated PLGA (P103E)</th>
<th>Polystyrene (PST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Microscopy</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Primary Size (nm)</td>
<td>231 ± 41</td>
<td>264 ± 55</td>
<td>84 ± 13</td>
</tr>
<tr>
<td>Hydrodynamic Size (nm)</td>
<td>260 ± 8</td>
<td>350 ± 7</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>ζ-Potential (mV)</td>
<td>-17 ± 0.5</td>
<td>-15 ± 0.1</td>
<td>-27 ± 0.6</td>
</tr>
<tr>
<td>Hydrophobicity (10^-9 mL/µm^2)</td>
<td>0.012 ± 0.002</td>
<td>0.025 ± 0.003</td>
<td>0.069 ± 0.009</td>
</tr>
</tbody>
</table>
Chapter 3. Biophysical Influence of Airborne Carbon Nanomaterials on Pulmonary Surfactant Films

3.1 Background

The development of engineered nanomaterials has flourished in recent decades. Carbonaceous nanomaterials (CNM), including carbon nanotubes (CNT) and graphene nanoplatelets (GNP), have unique characteristics that have helped innovate modern technology.\(^1\) Their unique aspect ratios, conductivity, and physicochemical properties have brought carbon to the forefront of nanotechnology. Most notably, their sizes and aspect ratios give them unprecedentedly large surface area to volume ratios. This property among others of CNM has allowed researchers to push the boundaries of electronics, biomedicine, and many other applications.\(^1\) However, the same attributes of CNM that give them the large range of applications also pose as potential health hazards to those who handle them.\(^66^{,104}\)

Both CNT and GNP are lightweight and easily airborne. One of the serious risks when working with nanomaterials is particle inhalation. Inhaled nanoparticles (NP) have been reported to cause damage to respiratory, cardiovascular, neurological, hepatic, and gastrointestinal tissues.\(^2\) Due to their small size, when inhaled, NP penetrate deep and deposit primarily in the alveolar region of the lung.\(^3\) Studies have shown that inhaled NP, including CNM, disrupt lung function by increasing oxidative stress, inflammation and can ultimately cause carcinomas.\(^2,3,78\)

The entirety of the peripheral lung is lined with a lipid-protein pulmonary surfactant (PS) complex.\(^10\) PS serves two major functions in the lung. The first is innate immunity, \(i.e.,\) being the initial biological barrier for any air contaminants that may reach the deep lung. The second is its biophysical function that allows for normal lung mechanics and prevents alveolar collapse by reducing the surface tension. Its ability in reducing surface tension is attributed to the high
phospholipid content as well as the two hydrophobic surfactant proteins associated with PS: SP-B and SP-C.\textsuperscript{105} By reducing the alveolar surface tension to near-zero levels, inhalation-exhalation cycles can occur at minimum mechanical energy expense.\textsuperscript{10} When PS is inhibited, its biophysical function deteriorates leading to respiratory ailments including acute lung injury (ALI) and even acute respiratory distress syndrome (ARDS).\textsuperscript{12}

A wealth of \textit{in vitro} studies, including our own, have demonstrated that the biophysical function of PS can be inhibited by NP\textsuperscript{41,47-49,51,52,55-59} The degree of biophysical inhibition depends on the physicochemical characteristics of the NP, such as their size, shape, charge, hydrophobicity, and agglomeration state.\textsuperscript{41,47-49,51,52,55-59} Although providing certain physiological insight into the interaction between PS and inhaled NP, all previous \textit{in vitro} studies suffer from the technical limitation that the NP must be brought into contact with PS from a liquid phase. When studying hydrophilic NP, the NP are usually dispersed in an aqueous buffer and then mixed with the PS suspension.\textsuperscript{52,55-57} When studying hydrophobic NP, the NP are commonly dispersed in an organic solvent and subsequently spread atop the PS suspension, or co-spread with organic-extracted PS.\textsuperscript{41,47,48}

These NP handling techniques are largely limited by the \textit{in vitro} experimental methodologies used in previous studies, including the Langmuir trough,\textsuperscript{41,47,48,56,57} pulsating bubble surfactometer,\textsuperscript{52,55,59} and captive bubble surfactometer.\textsuperscript{49,51} These \textit{in vitro} methods fail to mimic the true physiological conditions of NP-PS interactions, where the adsorbed PS films at the alveolar interface interact with NP deposited from the air. Consequently, extrapolating available \textit{in vitro} data to \textit{in vivo} tests has achieved only limited success. Physiologically irrelevant large NP concentrations are usually needed to induce \textit{in vitro} surfactant inhibition.
We have developed a novel experimental methodology called the constrained drop surfactometer (CDS) to fully simulate nano-bio interactions between natural PS and airborne CNM. We will show that the CDS can be used as an ideal *in vitro* biophysical model to mimic the physiological condition of respiration. The CDS offers, for the first time, quantitative correlations between airborne CNM and aerosol-induced PS inhibition under physiologically relevant conditions. With a novel *in situ* Langmuir-Blodgett (LB) transfer technique, the CDS permits direct visualization of nano-bio interaction at the PS interface, thus allowing for mechanism studies of surfactant inhibition by airborne CNM. The development of the CDS has made it possible to better understand how submicron airborne nanomaterials affect the PS lining of the lung.

### 3.2 Experimental methods

#### 3.2.1 Pulmonary surfactant

For these studies we used an animal-derived PS, Infasurf (Calfactant), which was a gift from ONY Inc. (Amherst, MA). Infasurf was purified from whole-lung bronchopulmonary lavage of newborn calves. Through an extraction process, Infasurf retained all of the hydrophobic components of bovine endogenous surfactant including phospholipids, cholesterol, and most hydrophobic surfactant proteins (SP-B and SP-C). For all trials Infasurf was diluted to a phospholipid concentration of 0.5 mg/mL with a saline buffer of 0.9% NaCl, 1.5 mM CaCl$_2$, and 2.5 mM HEPES, adjusted to pH 7.0. The saline buffer was made with Milli-Q ultrapure water (Millipore, Billerica, MA) with a resistivity greater than 18 MΩ·cm at room temperature.
3.2.2 Carbon nanomaterials

Multi-walled CNT with a length of 1-5 µm and diameter of 30±15 nm was purchased from NanoLab Inc. (Waltham, MA). GNP with a sheet length of 5 µm and a thickness of 6-8 nm was purchased from Strem Chemicals Inc. (Newburyport, MA). All CNM were thoroughly characterized for purity and morphology before use.

3.2.3 Aerosol concentration determination

The airborne particle number concentration in the chamber was determined with laser diffraction spectroscopy (LDS) using an AeroTrak airborne particle counter (TSI Inc., Shoreview, MN). The particle number concentration was subsequently converted to the particle mass concentration using a standard approach,\textsuperscript{106} by assuming a spherical shape and an aerosol density of 0.07 g/cm\textsuperscript{3}, which is much less than the density of bulk CNM.\textsuperscript{107} Low, moderate, and high exposure levels were produced in experiments by loading and in turn aerosolizing the CNM multiple times. Concentration for each exposure level was measured in triplicate. Detailed conversion and reproducibility are summarized in Table 3.1.

3.2.4 Atomic force microscopy

Topographical images were obtained using an Innova AFM (Bruker, Santa Barbara, CA). Samples were scanned in air where both tapping mode and contact mode gave similar results. A silicon nitride cantilever with a spring constant of 0.12 N/m and a tip radius of 2 nm was used in contact mode. In tapping mode a resonance frequency of 300 kHz and spring constant of 40 N/m was used. Lateral structures were analyzed and 3-dimensional rendering were produced using Nanoscope Analysis (version 1.5). Images were taken at multiple positions to ensure reproducibility.
3.2.5 Transmission electron microscopy

Both CNT and GNP were characterized with TEM (Hitachi HT7700). To find aerosol aggregate size and morphology, TEM grids were placed at the purging port of the CDS chamber prior to insufflations. Once in place, the CNM was aerosolized in the chamber and allotted 30 minutes to allow larger aggregates to settle. The chamber was purged and these grids were subsequently visualized via TEM within 24 hours of aggregate collection.

3.2.6 Statistical analyses

Statistical data are represented by the mean ± standard deviation. The measurements are based on dynamic cycling data for ambient air control and three increasing concentrations all with a sample size of n=3. One-way ANOVA was used for statistical calculations (OriginPro, Northampton, MA). Tukey means comparison test was used, and a probability value of $p < 0.05$ was considered statistically significant.

3.3 Results and discussion

3.3.1 Constrained drop surfactometer development

Figure 3.1 shows a schematic of the CDS setup. The CDS simulates the air-water interface of the alveolar lining using a surfactant droplet (∼10 µL), on which a surfactant film is quickly formed by natural adsorption, indicated by reaching the equilibrium surface tension of ∼25 mN/m in a few seconds. The droplet is constrained onto a 3-mm hydrophilic pedestal with a knife-sharp edge, which prevents leakage of the surfactant film even at substantially low, near-zero surface tensions upon compression. Formation, oscillation and removal of the droplet are controlled by a programmable motorized syringe. This droplet is illuminated by a backlight while a high-definition camera continuously records images of the droplet profile. Drop images
are processed using an upgraded axisymmetric drop shape analysis (ADSA) algorithm that automatically determines the surface tension, surface area, and drop volume, from the shape of the droplet.\textsuperscript{58,89} To mimic pulmonary conditions, the surfactant droplet is enclosed in an environmental control chamber, which maintains core body temperature (37.0±0.1°C). To simulate respiration, the adsorbed surfactant film is periodically compressed and expanded, by precisely controlling liquid flow into and out of the droplet, at a rate of 20 cycles per minute with area variations no more than 20%, all mimicking normal tidal breathing.\textsuperscript{90}

Figure 3.2 shows a surface tension ($\gamma$) versus relative surface area (A) plot of a typical compression-expansion cycle of an adsorbed Infasurf film, recorded in ambient air as a control. The inserts show representative droplet images at various surface tensions. It can be seen that the surface tension reaches a value lower than 5 mN/m with less than 20% film compression, indicating a "strong" surfactant film with a low compressibility. When the surfactant film is expanded, the surface tension gradually increases to follow the path of the compression branch, thus resulting in a minimum hysteresis loop. The moderate increase in surface tension indicates a "soft" film that is efficiently replenished by surfactant readsorption during expansion. Hence, the CDS successfully simulates the soft-yet-strong biophysical property of natural PS film under physiologically relevant conditions.\textsuperscript{10,105}

To introduce CNM aerosols, a miniscule amount of CNT or GNP (~0.1 mg) is loaded into a dry powder insufflator (Penn-Century Inc., Glenside, PA) and subsequently insufflated into the environmental control chamber. The airborne particle size distribution in the chamber is determined with laser diffraction spectroscopy (LDS). After insufflation, the airborne CNM are allowed 30 min to settle naturally before forming the surfactant droplet. Figure 3.3 is a schematic of the aerosol system integrated into the CDS. As shown in Figure 3.4, this settling process
eliminates most airborne particles larger than 2.5 µm, thus forming a relatively uniform aerosol distribution in the chamber. Figure 3.5 shows the stabilized aerosol distributions of CNT and GNP. 99% of airborne CNT and 98% of airborne GNP particles have an aerodynamic size of less than 2.5 µm. A large majority (84% and 63%) of these airborne particles lie in the submicron (300-500 nm) range. To facilitate comparison with existing in vivo and occupational safety data, the particle number concentration determined by LDS is converted to particle mass concentration (µg/m³) using a standard approach.106

3.3.2 Carbonaceous nanomaterial aerosol-induced surfactant inhibition

Once the airborne particle distribution is stabilized (Figure 3.5), an Infasurf droplet is formed atop the CDS pedestal, incubated in the aerosols for 10 minutes, and then subjected to compression-expansion cycles to simulate respiration in the polluted environment. Figure 3.6 shows representative cycles for Infasurf exposed to ambient air and three increasing concentrations of the CNM, i.e., 4.5±0.4, 60±18, and 70±18 µg/m³ for CNT and 8.0±1.7, 15±3, and 25±6 µg/m³ for GNP. Figures 3.7 and 3.8 demonstrate the first 40 cycles for both CNT and GNP, respectively. It takes about 40 cycles for the inhibition to reach equilibrium. Reproducibility of these cycles can be found in Figures 3.9 and 3.10. It is clear that the biophysical properties of Infasurf are inhibited by the airborne CNM in a concentration dependent manner, as indicated by the inability of reaching low surface tension upon 20% relative area compression and the increasing hysteresis area of the compression-expansion loops. (Note that when exposing to CNM aerosols, we increased the amount of film compression to 30% to examine the extent of surfactant inhibition.)

To gain a statistical understanding of surfactant inhibition caused by the CNM aerosols, Figure 3.11 shows four statistical parameters to quantify surfactant biophysics and inhibition.
These are the minimum surface tension after compression ($\gamma_{\text{min}}$), maximum surface tension after expansion ($\gamma_{\text{max}}$), film compressibility ($\kappa_{\text{comp}}$), and film expandability ($\kappa_{\text{exp}}$) with and without exposure to CNM aerosols. A good surfactant should reduce surface tension below 5 mN/m with under 20% relative area compression,\textsuperscript{90,91} as shown by the ambient air control (Figure 3.2). Upon CNM exposure, $\gamma_{\text{min}}$ rises considerably in a dose dependent manner from ~2 mN/m when exposed to the ambient air, to >10 mN/m after exposure to higher than 20 µg/m\textsuperscript{3} for both CNT and GNP.

In contrast to $\gamma_{\text{min}}$, the compressibility ($\kappa_{\text{comp}}$) and expandability ($\kappa_{\text{exp}}$) of surfactant films, defined as $\kappa = (1/A)(\partial A/\partial \gamma)$ during the compression and expansion processes, are more sensitive parameters for measuring surfactant inhibition.\textsuperscript{58,91} Increasing $\kappa_{\text{comp}}$ correlates with a softer film, which requires more compression to ultimately reach the same $\gamma_{\text{min}}$, \textit{i.e.}, surfactant inhibition. As shown by both CNM, $\kappa_{\text{comp}}$ increases with increasing aerosol concentration. Additionally, the hysteresis loop associated with surfactant inhibition is caused by the difference between $\kappa_{\text{comp}}$ and $\kappa_{\text{exp}}$. When these two values are identical it yields zero hysteresis, \textit{i.e.}, expansion path retraces compression path as shown by the ambient air control, indicating no mechanical energy expense per respiration cycle.\textsuperscript{58,91} However, after exposure to the highest exposure of CNM, $\kappa_{\text{comp}}$ and $\kappa_{\text{exp}}$ show large differences (Figure 3.11) and thus increasing hysteresis area (Figure 3.6), indicating loss of mechanical energy which is ultimately unsustainable and will lead to respiratory failure.

\textbf{3.3.3 In situ Langmuir-Blodgett transfer and film imaging}

To explore the mechanism of surfactant inhibition, we have developed a novel \textit{in situ} Langmuir-Blodgett (LB) transfer technique that allows us to immobilize the surfactant film at the droplet surface under controlled environment, thus probing the nano-bio interactions with
submicron resolution using atomic force microscopy (AFM). Before transferring the surfactant film, we first wash away surfactant vesicles in the droplet with buffer using a subphase replacement technique. Subsequently, the surfactant film at the air-water interface of the droplet is transferred onto a small piece of freshly peeled mica sheet, under controlled environmental conditions in the chamber (see Figure 3.12a for schematic).

As shown in Figure 3.12b, pure Infasurf after de nova adsorption shows uniform multilayer protrusions of ~20 nm, which is in good agreement with the microstructure of spread Infasurf film around the equilibrium spreading pressure. However after exposure to CNT or GNP, large aggregates appear on the surfactant film (Figures 3.12c-d) within the aerosol size range reported by LDS (Figure 3.5). To further scrutinize these aggregates, we have directly studied the morphology of CNM aerosols collected from the environmental control chamber, using TEM. As shown in Figure 3.12e, CNT form fibrous or isometric aerosols where all tubes entangle with each other, very similar to the CNT “birdnest” structures after aerosolization, reported by others. GNP form large platelet aggregates with multiple sheets stacked onto each other as shown in Figure 3.12f. (See Figures 3.13-3.15 for additional AFM and TEM images)

It is clear that both size and morphology of these CNM aerosols are comparable to the aggregates found at the surfactant film (Figure 3.12c-f), which confirms that nano- and micron-sized airborne CNM adsorb to the surfactant film where nano-bio interactions take place inducing surfactant inhibition. These results are in good agreement with many in vitro and in silico studies that show hydrophobic NP adsorb to the surfactant film where the nano-bio interactions govern the surfactant inhibition.
3.3.4 Carbon nanotube occupational exposure limit analysis

The CDS proved to be a sensitive tool for studying aerosol-induced surfactant inhibition. More importantly, it demonstrates the potential as an *in vitro* model for studying inhalation toxicology with controlled aerosol dosimetry. Worldwide airborne CNT occupational exposure limits (OELs) range from 7-50 μg/m\(^3\).\(^{109}\) In Japan several studies using mice models showed no-observed adverse effect level (NOAEL) at 65 μg/m\(^3\).\(^{110,111}\) However, after adjusting to human exposure they determined the OEL for multi-walled CNT to be 30 μg/m\(^3\). In contrast, the National Institute of Occupational Safety and Health (NIOSH) has recommended OEL CNT standards to a more conservative 7 μg/m\(^3\) in the United States based on chronic inhalation studies.\(^{112,113}\) Although there are differences between international OELs, they are around 10-50 μg/m\(^3\). Previous PS-NP *in vitro* studies recorded PS inhibition from 10 μg/mL to 2 mg/mL,\(^{41,47-49,51,52,55-59}\) which are astronomically larger than these international standards. In contrast, these international OELs fall directly in the detection range of the CDS.

In agreement with NIOSH, we found negligible adverse biophysical influences for GNP and CNT below 8 μg/m\(^3\). Slightly higher concentrations show minor inhibition patterns, *i.e.* increases in \(\kappa_{\text{comp}}\) (Figures 3.6 and 3.9), associated with doses below 20 μg/m\(^3\) for both CNT and GNP. Concentrations higher than 20 μg/m\(^3\) yielded a more severe surfactant inhibition signified by an increase \(\gamma_{\text{min}}\) above 5 mN/m as well as a continued increase in \(\kappa_{\text{comp}}\). Hence, not only is the CDS able to detect airborne concentrations in the range of international OELs, but it can also monitor the dose-dependent biophysical inhibition associated with CNM aerosols.

3.3.5 Bridging *in vitro* simulations and *in vivo* inhalation studies

In addition to agreeing with international standards, our *in vitro* findings also provide insight into occupational safety. Erdely *et al.* recently toured several U.S. manufacturing facilities and
tested the airborne CNT exposure to workers.\textsuperscript{114} The goal of their study was to provide information on human occupational exposure limits and ultimately correlate existing \textit{in vivo} data to day-to-day workplace exposures. The average respirable CNT concentration they found was 2.65 $\mu$g/m\textsuperscript{3}, which is similar to the lower end of the CDS detection range. With these data, they were able to link worker exposure to results obtained from \textit{in vivo} mouse inhalation models.\textsuperscript{112,113,115-119} With our \textit{in vitro} results we can conclude that acute exposure to this concentration of CNT will have minimal inhibitory affect on the PS system. Hence, the CDS provides a promising model for correlating \textit{in vitro} aerosol data with \textit{in vivo} exposure and even human occupational safety data.

Compared to CNT, much less work has been focused on the inhalation toxicology of GNP. However, the unique platelet shape of GNP has researchers asking how its inhalation toxicological potential differs from that of the fibrous CNT.\textsuperscript{120} In a comparative CNM inhalation study, Ma-Hock \textit{et al.} demonstrated that CNT and GNP showed pulmonary toxicity at different potentials; but the origin of this difference was still unknown.\textsuperscript{119} We also found that although CNT and GNP both inhibit PS at increasing concentration and both adsorb to the PS film, their inhibition potentials were not identical. We are currently investigating this difference.

### 3.4 Conclusions

We have developed a novel technique to bridge the gap of knowledge between \textit{in vitro} NP-PS tests and \textit{in vivo} inhalation toxicology studies. The CDS is the first \textit{in vitro} model capable of quantitatively evaluating surfactant inhibition caused by airborne nanomaterials. Both CNM tested, CNT and GNP, demonstrated a concentration dependent surfactant inhibition under physiologically relevant conditions. With a unique \textit{in situ} LB transfer technique, the CDS
demonstrated that surfactant inhibition was caused by the adsorption of CNM aerosols onto the surfactant film, which disturbed molecular conformation and film structure of PS. The CDS exhibits promise to be developed into a precautionary tool for evaluating surfactant inhibition and ultimately for study inhalation toxicology due to airborne nanomaterials.
Figure 3.1. Schematic of the constrained drop surfactometer for studying biophysical influence of airborne carbon nanomaterials on pulmonary surfactant. A PS droplet is formed on a 3-mm hydrophilic knife-sharp pedestal using a programmable motorized syringe. This droplet is enclosed in an environmental control chamber where core body temperature is maintained with a thermoelectric heater. The drop profile is illuminated with a monochromatic parallel backlight and is recorded continuously with a high-definition camera. The profile images are sent directly to the axisymmetric drop shape analysis (ADSA) software and processed to yield real-time surface tension measurements. Carbon nanomaterials are aerosolized into the chamber using a particle insufflator. The airborne particle size distribution is determined with laser diffraction spectroscopy using a particle counter. The environmental control chamber and all tubing are airtight to ensure no particle leakage.
Figure 3.2. A typical compression-expansion cycle for pure Infasurf at 37°C and 20 cycles/min determined using the constrained drop surfactometer (CDS). Infasurf reaches a minimum surface tension below 5 mN/m within 20% relative area compression, indicating high surface activity. The compression-expansion hysteresis loop is narrow stemming from the compressibility of the film being almost equivalent to its expandability. It means that during each compression-expansion cycle there is a minimal mechanical energy expense, which is crucial for maintaining the normal respiratory mechanics. Surface tension was determined using axisymmetric drop shape analysis (ADSA). The drop images along the cycle path are demonstrative of how surface tension is a function of the drop shape.
Figure 3.3. Schematic of constrained drop surfactometer aerosolization system. Initially dry particles are insufflated into the sealed chamber and allowed to interact with the surfactant droplet. After biophysical tests are run, the two valves on each side of the chamber are opened and the particle counter begins to purge the chamber and collect data on particle count and size distribution using LDS. The zero filter on the left allows air flow through the chamber to ensure complete particle clearance. The zero filter on the right (just before the particle counter) dilutes the particles to ensure the particles are not purged in a concentration above the capacity of the particle counter.
Figure 3.4. Number and size distribution of aggregates during settling. Multiple settlement tests were run with the lowest exposure limit of CNT and GNP (n=3) (a). Larger sized aggregates decrease to nearly zero after 10 minutes for CNT and 20 minutes for GNP. The percentage of these size distributions were calculated after initial insufflation and waiting the appropriate settlement time (b). For both CNT and GNP, a higher percentage of the aerosol are aggregates smaller than 2.5 µm after appropriate settling time (>99%, 98%) than the initial aerosol (>98%, 90%).
Figure 3.5. Carbon nanotube and graphene nanoplatelet aerosol size distributions 30 min after initial aerosolization. A large majority of both (a) CNT (99%) and (b) GNP (>98%) aerosol aggregates are less than 2.5 μm. Most of the aerosols less than 2.5 μm for CNT (84%) and GNP (63%) are in the submicron range (300-500 nm). Larger, coarse aggregates make up less than 1% and 2% of the aerosols in the chamber for CNT and GNP, respectively.
Figure 3.6. Representative cycles demonstrating carbonaceous nanomaterial dose-dependent inhibition. Comparison of dynamic cycling of Infasurf exposed to CNT (a) or GNP (b) with increasing aerosol concentration. Each plot demonstrates a representative compression-expansion cycle of Infasurf exposed to ambient air and increasing concentration of CNM aerosols. For both cases increasing CNM aerosol concentration increased the minimum surface tension upon compression and increased the hysteresis area of the compression-expansion loops, indicating surfactant inhibition.
Figure 3.7. Chronological dynamic cycles for carbon nanotube exposure. The first 40 cycles are shown for pure Infasurf and Infasurf exposed to 4.5, 59, and 70 µg/m³ of carbon nanotube (CNT) aerosol. Cycling was conducted at physiological speed (3 s/cycle) and temperature (37°C). It takes about 30-40 cycles for the cycling to become consistent and reach equilibrium. Therefore after 30 cycles these data were used for analysis.
Figure 3.8. Chronological dynamic cycles for graphene nanoplatelet exposure. The first 40 cycles are shown for pure Infasurf and Infasurf exposed to 8, 16, and 24 µg/m³ of graphene nanoplatelet (GNP) aerosol. Cycling was conducted at physiological speed (3 s/cycle) and temperature (37°C). It takes about 30-40 cycles for the cycling to become consistent and reach equilibrium. Therefore after 30 cycles these data were used for analysis.
<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (μg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>Run 1</td>
<td><img src="image1" alt="Graph 1" /></td>
</tr>
<tr>
<td>Run 2</td>
<td><img src="image4" alt="Graph 4" /></td>
</tr>
<tr>
<td>Run 3</td>
<td><img src="image7" alt="Graph 7" /></td>
</tr>
</tbody>
</table>

**Figure 3.9.** Dynamic cycling data for carbon nanotube exposure. An Infasurf droplet was exposed to a chamber containing 4.5, 60, and 70 μg/m³ of carbon nanotube (CNT) aerosol. This droplet was cycled after 10 minutes of exposure. Inhibition parameters $\gamma_{\text{min}}$, $\gamma_{\text{max}}$, $\kappa_{\text{comp}}$, and $\kappa_{\text{exp}}$ were extrapolated through these data and statistical analyses were run (n=3) from these data.
<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (μg/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Run 1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GNP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Run 3</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.10. Dynamic cycling data for graphene nanoplatelet exposure. An Infasurf droplet was exposed to a chamber containing 8, 15, and 25 μg/m$^3$ of graphene nanoplatelets (GNP). This droplet was cycled after 10 minutes of exposure. Inhibition parameters $\gamma_{\text{min}}$, $\gamma_{\text{max}}$, $\kappa_{\text{comp}}$, and $\kappa_{\text{exp}}$ were extrapolated through these data and statistical analyses were run (n=3) from these data.
Figure 3.11. Statistical analysis of biophysical properties of Infasurf exposed to carbonaceous nanomaterial. (a, c) Carbon nanotubes (CNT) or (b, d) graphene nanoplatelets (GNP) induced biophysical inhibition demonstrated with increasing aerosol concentration. These four biophysical properties are: the minimum surface tension at the end of compression ($\gamma_{\text{min}}$), maximum surface tension at the end of expansion ($\gamma_{\text{max}}$), film compressibility ($\kappa_{\text{comp}}$), and film expandability ($\kappa_{\text{exp}}$). All cycles were produced at 37 °C and 3 seconds/cycle to mimic respiration. There is a statistically significant increase in both $\gamma_{\text{min}}$ and $\kappa_{\text{comp}}$ after exposure to aerosol concentrations greater than 20 $\mu$g/m$^3$, indicating surfactant inhibition. *$p < 0.05$ of comparison to ambient air control.
Figure 3.12. Imaging nano-bio interactions at the pulmonary surfactant film. (a) Schematic of the *in situ* Langmuir-Blodgett (LB) transfer technique integrated into the CDS. The surfactant film at the air-water interface of the droplet is transferred onto a small piece of freshly peeled mica sheet at a lifting speed of 1 mm/min, under controlled environmental conditions in the chamber. (b) AFM topographic image (20×20 μm) of a pure Infasurf film that shows uniformly distributed multilayer structures. (c-d) AFM topographic images of the Infasurf film exposed to CNT (10×10 μm) and GNP (20×20 μm) aerosols, respectively. Both images show the adsorption of large aggregates onto the surfactant film. (e-f) TEM images of CNT and GNP aerosols recovered from the chamber. Both size and morphology of these CNM aerosols match the large aggregates found at the surfactant film.
**Figure 3.13.** Additional atomic force microscopy images of the Infasurf film exposed to carbon nanotubes. Low and high resolution images show multiple particles per image noted by the white arrows. 3D images are given beneath each lateral structure image to give a contrast perspective of the height difference.
### Table 3.14

<table>
<thead>
<tr>
<th>Lateral</th>
<th>100×100 μm</th>
<th>20×20 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-D</td>
<td><img src="image1.png" alt="3D Image" /></td>
<td><img src="image2.png" alt="3D Image" /></td>
</tr>
<tr>
<td>Lateral</td>
<td><img src="image3.png" alt="Lateral Image" /></td>
<td><img src="image4.png" alt="Lateral Image" /></td>
</tr>
<tr>
<td>3-D</td>
<td><img src="image5.png" alt="3D Image" /></td>
<td><img src="image6.png" alt="3D Image" /></td>
</tr>
</tbody>
</table>

**Figure 3.14.** Additional atomic force microscopy images of the Infasurf film exposed to graphene nanoplatelets. Images show two positions where GNP particles were found. Both have similar morphology and have similar heights of ~1-1.5 μm. The low resolution image shows multiple particles on the same image noted by white arrows. 3D images are given beneath each lateral structure image to give a contrast perspective of the height difference.
Figure 3.15. Transmission electron microscopy images for carbon nanotube and graphene nanoplatelet aerosol. Prior to insufflating the carbonaceous nanomaterial (CNM), transmission electron microscopy (TEM) grids were positioned within the purging port of the chamber. Carbon nanotubes (CNT) and graphene nanoplatelets (GNP) were aerosolized and waited for large aggregates to settle before being purged and caught on TEM grids. These grids were analyzed to produce the images above.
Table 3.1. Carbon nanomaterial aerosol concentration calculations. Following the literature we used the following calculation to convert particle number concentration to mass concentration outlined in $C = N \rho r V^{-1}$, where $C$ is the mass concentration, $N$ is the particle count, $r$ is the average of size range, $\rho$ is the particle density, and $V$ is the total volume of the CDS chamber. The table outlines the mass contributions from three particle size ranges, measured with the laser diffraction spectroscopy.

<table>
<thead>
<tr>
<th>Exposure Level</th>
<th>Trial</th>
<th>Particle Count</th>
<th>Mass Concentration Contribution (µg/m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>300 nm - 500 nm</td>
<td>500 nm - 2.5 µm</td>
</tr>
<tr>
<td>CNT Low</td>
<td>1</td>
<td>191058</td>
<td>38171</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>224837</td>
<td>44914</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>208851</td>
<td>41720</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>3358589</td>
<td>670912</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2210592</td>
<td>441588</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2526103</td>
<td>504615</td>
</tr>
<tr>
<td>High</td>
<td>1</td>
<td>2285104</td>
<td>456473</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3766302</td>
<td>752357</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3634564</td>
<td>726041</td>
</tr>
<tr>
<td>GNP Low</td>
<td>1</td>
<td>441803</td>
<td>238940</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>425178</td>
<td>229949</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>287896</td>
<td>155703</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>674253</td>
<td>364657</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>718141</td>
<td>388393</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>924058</td>
<td>499760</td>
</tr>
<tr>
<td>High</td>
<td>1</td>
<td>974891</td>
<td>527252</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1499618</td>
<td>811040</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>968330</td>
<td>523703</td>
</tr>
</tbody>
</table>
Chapter 4. Conclusions

4.1 Nanoparticle hydrophobicity effect on pulmonary surfactant biophysics and retention

Through the use of three different types of hydrophobic nanoparticles (NPs) we were able to show that hydrophobicity does play a significant role on surfactant inhibition. Our constrained drop surfactometer (CDS) results demonstrated that increasing hydrophobicity of the NPs increased their pro-inhibitory potential. Pulmonary surfactant (PS) biophysical inhibition was marked by an increase in compressibility of the film. Film visualization with atomic force microscopy (AFM) revealed that an increase in hydrophobicity of NPs increased the frequency of NPs found at the surface of the PS film. It had already been shown that hydrophobic NPs remain at the surface where hydrophilic particles penetrate the PS film,57 but in this work we demonstrate that the magnitude of hydrophobicity determines the frequency of retention at the surface. In conclusion, these data show that hydrophobicity is an important factor in NP-induced PS inhibition and must be taken into consideration when researching nanotoxicology or nanomedicine.

4.2 Rose Bengal partitioning assay is an effective method in measuring nanoparticle hydrophobicity

Classical methods for measuring hydrophobicity of bulk materials, such as contact angle measurements, are technically difficult and impractical for measuring the hydrophobicity of single NPs. In this thesis we adopted the Rose Bengal partitioning method to measure the relative hydrophobicities of NPs. This method uses a hydrophobic dye, Rose Bengal, and uses the
attractive hydrophobic force to determine relative hydrophobicities of certain NPs. This method was proven to be useful for measuring the magnitude of hydrophobicity of polystyrene (PST-NPs), and poly(D,L-lactide-co-glycolide) (PLGA) NPs. It is concluded that the Rose Bengal partitioning method is an effective and quick measurement of relative hydrophobicity of NPs.

4.3 Film compressibility: a more sensitive parameter for measuring surfactant inhibition

Increased minimum surface tension after compression ($\gamma_{\text{min}}$) is a classical parameter to measure surfactant inhibition under dynamic compression-expansion cycling. Many studies demonstrate a rise in $\gamma_{\text{min}}$ after exposure to NPs.$^{49,52,55,59}$ However, these measurements were done with unrealistically and unreasonable large NP concentrations. In our study we did not measure concentration dependence of PST-NPs and PLGA due to the fact that unrealistic NP concentrations give zero insight on physiologically relevant cases. In our results the exposure of these NPs at low, physiologically-relevant concentrations did not increase $\gamma_{\text{min}}$. This is in good agreement with previous studies. However, we found that simply because $\gamma_{\text{min}}$ stayed below 5 mN/m does not mean inhibition is not induced. Another parameter, film compressibility ($\kappa$), proved to be a much more sensitive indication of surfactant inhibition. An increase in $\kappa$ represents a softer film that requires more area compression to reach an arbitrary $\gamma_{\text{min}}$. $\kappa$ increased significantly with the low NP concentrations we used when $\gamma_{\text{min}}$ remained unchanged. Ultimately, $\kappa$ can indicate subtle PS biophysical inhibition.
4.4 Further development of the constrained drop surfactometer for studying aerosol-induced surfactant inhibition

There is a gap of knowledge with in vitro PS-NP studies in that all existing work handles NPs in a liquid suspension. We recognized this problem with the in vitro studies (including our own) and developed a method to deliver NP aerosol to a surfactant droplet. The most promising available in vitro technique to accommodate nanoaerosol is the constrained drop surfactometer (CDS). A droplet of surfactant is enclosed in a stainless steel chamber to maintain core body temperature (37±0.1 °C). We drilled two ports on opposite ends of the CDS chamber for aerosolization and aerosol purging. Our unique and novel setup proved to deliver sub-micron carbonaceous nanomaterial (CNM) aerosols. A particle counter purges the chamber of sub-micron aerosol. Meanwhile, it uses laser diffraction spectroscopy (LDS) to count the number of airborne particles as well as simultaneously measuring the size distribution. With this setup we are the first group in the world to introduce sub-micron aerosol to a pulmonary surfactant film and quantitatively measure surfactant biophysics as well as aerosol characterization.

4.5 Airborne carbonaceous nanomaterial effect on pulmonary surfactant biophysics

Airborne CNM showed to induce a distinctive dose-dependent inhibition when exposed to a naturally-derived surfactant, Infasurf. As shown for both CNM, carbon nanotube (CNT) and graphene nanoplatelet (GNP) concentrations below 20 µg/m³ induced slight inhibition by increasing compressibility (κ) of the surfactant film. This is consistent with the effect of low concentrations of other hydrophobic NPs demonstrated in Chapter 2. Concentrations above 20
\(\mu g/m^3\) showed a stronger inhibition where minimum surface tension (\(\gamma_{min}\)) rose above 5 mN/m. All of these data are consistent with international standards of airborne CNT exposure limits. The direct comparison with the detection limit of the CDS and international CNT standards is important because it demonstrates the accuracy of our unique setup.

4.6 In situ Langmuir-Blodgett transfer revelation of carbonaceous nanomaterial at air-water interface

We were able to probe NP-PS interactions at the air-water interface at physiologically relevant conditions with the development of a novel in situ Langmuir-Blodgett transfer technique. With this technique we were able to transfer and immobilize a surfactant film at 37°C after exposure to CNM aerosol onto a mica substrate. This substrate was allowed to dry at 37°C and subsequently visualized via AFM. Our results demonstrated that after exposure to CNM, aerosol aggregates adsorbed to the film surface and remained there due to their high hydrophobicity. These AFM images were compared to TEM images of aerosol aggregates where morphology of CNT and GNP aggregates were strikingly similar to the aggregates found via AFM. Through this technique we were able to conclude that sub-micron sized aggregates "stick" to the surfactant film where nano-bio interactions take place and induce the biophysical inhibition observed by surface tension measurements. These results in combination with our own previous studies on inhibition mechanism,\(^{57}\) as well as results from other published studies,\(^{54,68}\) surfactant protein (SP) adsorption to the nanoparticle surface occurs after aerosol aggregate deposition onto the surfactant film. These interactions most likely deplete the essential SP-B and SP-C from the interface therefore inducing biophysical inhibition.
4.7 Atomic force microscopy structure of adsorbed Infasurf films at core body temperature

This work delivered key information on PS structure. AFM images of adsorbed PS have not been published. In our aerosol study we developed a subphase replacement technique in coordination with the in situ Langmuir-Blodgett transfer to allow us to visualize the interface of adsorbed surfactant films with AFM. The subphase of adsorbed films must be replaced with clean buffer because large (~10-100µm) surfactant vesicles create a topographical contrast that makes it impossible to visualize the surfactant film structures (~1-20 nm). We showed the structure of Infasurf at 37°C with AFM in which uniform multilayer protrusions reside at the surface which is in good agreement with the spread film structure.96,97 These results not only represent unpublished data matching theory of how adsorbed film is constructed, but matches previous data of spread films demonstrating the accuracy of the our new technique.

4.8 List of publications

The following is a list of peer-reviewed journal papers, conference posters and presentations published or in preparation based on the work of this thesis.

Papers published in refereed journals or under review for publication


**Conference Presentations and Posters**


**Papers in preparation**


3. Lin X.; Valle R. P.; Zuo Y. Y.; Gu N. Experiments and simulations of the interactions between PAMAM dendrimer and DPPC monolayer.
Chapter 5. Future Work

5.1 Continuous Langmuir-Blodgett transfer

Figure 2.2a was created with the use of a classical Langmuir trough. The classical Langmuir-Blodgett technique transfers a surfactant film at constant surface pressure ($\pi$). This means that one film transfer correlates to one surface pressure. This technique is time consuming, which led us to develop a continuous Langmuir-Blodgett transfer technique. This technique simplistically withdraws the mica perpendicularly to the water surface meanwhile compressing the surfactant film varying the surface pressure. By calibrating the withdrawing and compressing speeds, we were able to correlate distance on the mica with a specific surface pressure (Figure 5.1). Although this technique is already a success, future work can be done to perfect this technique and develop a standard procedure.

5.2 Constrained drop surfactometer aerosol system development

Although the existing model of the constrained drop surfactometer (CDS) is able to measure both pulmonary surfactant (PS) biophysical inhibition and nanoparticle (NP) aerosol concentrations effectively, there is room for improvement. The aerosol delivery system is composed of a dry powder insufflator. The aerosol is insufflated into the CDS chamber, biophysical tests are run and then the aerosol is finally counted. Due to this procedure, aerosol concentrations cannot be effectively controlled within the CDS chamber. Future work on this design will compose of delivering aerosol while continuously monitoring its concentration.
5.3 Difference between carbon nanotube and graphene nanoplatelet inhibition potentials

Our preliminary results with carbonaceous nanomaterial (CNM) aerosols demonstrated that carbon nanotubes (CNT) and graphene nanoplatelets (GNP) yield different inhibition potentials as shown by lower doses of GNP inhibit film more readily. This would indicate that GNP are more inhibitory than CNT. There are several reasons why this could be so including aerosol aggregation state, chemical purity, primary NP size, surface area exposed, etc. Others working on inhalation toxicology have found that CNT and GNP demonstrate different cytotoxic potentials as well. Further studies with these aerosols will help analyze why these two different CNM give different PS inhibition potentials.

5.4 Comparative study of atomic force microscopy of clinical pulmonary surfactants

In our investigation with CNM aerosols we developed a technique that combined subphase replacement with in situ Langmuir-Blodgett transfer. This technique allowed us to visualize adsorbed films at core body temperature (37°C). Surfactant structures under these conditions have never been published with atomic force microscopy (AFM). This technique can lead us into a whole new realm of PS structure studies via AFM. Infasurf only represents one of many clinically available naturally-derived PS preparations. In future work we can compare the adsorbed structure of each surfactant film at 37°C, which directly simulates how these films are formed in the lung environment.
**Figure 5.1.** Continuous Langmuir-Blodgett transfer calibration. (a) A calibration plot for the continuous transfer technique showing surface pressure as a function of distance from the top of the cleaved mica sheet. (b) A schematic of a graduated mica sheet indicating the withdrawing transfer direction. The arrows point to where the corresponding surface pressures would be visualized on the mica sheet.
References


