Biophysical analysis on the interaction of polymeric nanoparticles with biomimetic models of the human lung surfactant

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Biophysical analysis on the interaction of polymeric nanoparticles with biomimetic models of the human lung surfactant

by

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A THESIS
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Abstract

The human body offers many paths that could be used for drug delivery. The pulmonary route, which is delivery through the lungs, provides many advantages such as; 1) direct access to the lungs and blood circulation and 2) large surface area with a thin barrier of about 500 nm thick. These advantages, in addition to increased patient compliance with inhaled medications, have sparked interest in this route in the field of nanomedicine. Nanoparticles are drug delivery vehicles with many advantages over conventional drug delivery methods. These include the high surface area to volume ratio due to their small size and potential for specific targeting.

In the pulmonary route, the air blood barrier is composed of three main layers. The top layer or first point of interaction is through the lung surfactant (LS). This monolayer is composed of 90% lipids and 10% proteins. The lung surfactant’s major role is to reduce surface tension experienced in the lung during breathing cycles in order to prevent lung collapse. Therefore, if nanoparticles are to pass through this monolayer, effects on its stability need to be assessed. In this thesis, a biomimetic model of the LS is developed and its interaction with two biodegradable and biocompatible nanoparticles is tested. Biophysical analysis on the interaction includes the use of Langmuir monolayer pressure-area isotherms, surface potential measurements and visualization through Brewster angle microscopy. Results show that interactions and effects on monolayer elasticity are strongly dependent on electrostatic interactions, charge density of the monolayer, lipid headgroup structure and acyl chain saturation.
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<tr>
<td>BAM</td>
<td>Brewster angle microscopy</td>
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<td>BLES</td>
<td>Bovine lipid extract surfactant</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine</td>
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<tr>
<td>DPPG</td>
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<td>DSC</td>
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<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
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<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid condensed</td>
</tr>
<tr>
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<td>Lα</td>
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<td>Gel phase</td>
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<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(D,L-lactide-co-glycolide)</td>
</tr>
<tr>
<td>POPC</td>
<td>Palmitoyloleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>POPG</td>
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<td>SOPG</td>
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<td>SP-A,B,C,D</td>
<td>Surfactant prote, A,B,C,D</td>
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<tr>
<td>Tc</td>
<td>Crystallization temperature</td>
</tr>
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<td>Tm</td>
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T1/2  Peak width at half height

ΔH  Enthalpy

w/w  Weight per weight ratio

16-1PC  Palmitoylpalmitoleoyl phosphatidylcholine

16-1PG  Palmitoylpalmitoleoyl phosphatidylglycerol
1.1 Nanotechnology and nanomedicine

1.1.1 Nanoparticles as drug delivery vehicles

Richard Feynman, a Nobel laureate in physics, is considered by many as the father of nanotechnology. In his talk in 1959 entitled “There is plenty of room at the bottom” he proposed an idea of manipulating materials at the atomic scale (1). He posed a question to the attendees at his lecture on whether they can miniaturize the Encyclopedia Britannica on the head of a pin (1). This concept of miniaturization has lead others later on to coin the term nanotechnology in 1974 (2). As the name implies, the field of nanotechnology deals with materials at the nanometer scale (1 nm to 1000 nm) in at least one dimension.

Nanotechnology has many applications in science and technology involving medicine and biotechnology. One of the main goals of nanomedicine is to improve and allow targeted drug delivery. The ability to target drugs to a specific location, at the right concentration was envisioned by the German scientist Paul Ehrlich back in the 1890s (3). Even though the field of nanotechnology advanced in the mid-20th century, Ehrlich vision and ideas helped develop the field of nanomedicine. Drugs, albeit being effective for treatment of a certain disease, might have poor physiochemical properties in the body as they circulate the bloodstream before reaching the target location (4). Ehrlich’s vision entailed the ability to fight human disease with a ‘Magic Bullet’ that would allow for targeted drug delivery. The magic bullet idea lead to the
development of nanoparticles; they are particles within the nanometer scale which have the ability to encapsulate, attach, or adsorb drugs or molecules and deliver them to target location (5).

The advantages nanoparticles provide to the field of nanomedicine are numerous and continue to increase. In addition to their ability to protect therapeutic agents until target location is reached, their large surface area to volume ratio means more drugs can be delivered within their small size (6). Furthermore, the small size of nanoparticles allows them to travel through organs, tissues, cells and small blood capillaries (~5 μm) (5, 7, 8). A particular drug’s bioavailability and rate of release can be controlled by manipulating the surface chemistry, shape, and size of the associated nanoparticle (9-12). Drugs that carry harsh side effects such as those in chemotherapy, can be protected within nanoparticles and released only at the target location (13).

Targeting of nanoparticles can be achieved either through passive or active transport. Passive transport of nanoparticles for cancer treatment for example, takes advantage of the enhanced permeability and retention effect associated with the vasculature of cancerous tissues (14). As tumors grow at a fast rate, the need for more blood vessels to deliver oxygen and nutrients to the cancerous cells growth. This results in formation of blood vessels that are leaky and underdeveloped, resulting in increased permeability (15, 16). Active targeting on the other hand involves the use of targeting molecules on the surface of the nanoparticles aimed at
specific cells or even organelles within the cell. These molecules can be antibodies, aptamers, or cell receptor ligands (17). Both targeting techniques are important and useful, the route taken usually depends on the drug dose desired and the target organ/location.

Nanoparticles for drug delivery are synthesized in all sizes and shapes and there are a multitude of materials and formulations that are used to generate those nanoparticles. It is important for drug delivery purposes that the nanoparticle formulation is biodegradable and biocompatible. For example, a study comparing effects of biodegradable (polylactide-co-glycolic acid) and non-biodegradable materials (titanium oxide and polystyrene) found that there was an increase in neutrophil count upon exposure to non-biodegradable nanoparticles where various and comparable sizes were tested (18).

Some of these biodegradable nanoparticle formulations include liposomes, solid lipid nanoparticles, dendrimers, and polymeric nanoparticles. Liposomes are lipid bilayer nanoparticles with a hydrophilic core. They can carry hydrophilic drugs in the core and hydrophobic drugs within the bilayer leaflet (19). Solid lipid nanoparticles are also lipid-based nanoparticles that can carry both hydrophilic and hydrophobic drugs. Unlike liposomes, they have mostly a lipid core region and no outer bilayer structure (20, 21). Dendrimers are a network of highly branched molecules. Drugs can be attached to the branches of dendrimers (22). Polymeric nanoparticles are made from either natural or synthetic polymers. Gelatin (protein based material) and chitosan (polysaccharide based material derived from chitin) are
examples of natural polymers that are used to make nanoparticles. Natural polymers ensure biocompatibility and biodegradability of the nanoparticles. The other class of polymeric nanoparticles are made from synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA), or their copolymer poly(lactic-co-glycolic) acid (PLGA). These polymers are common materials used in the medical field for devices or wound healing due to their biodegradability and biocompatibility (23). They have been approved by the FDA and the European Medicine Agency for use with humans (24, 25). Other synthetic polymers include polybutylcyanoacrylates (PBCA) which are a type of super glue (26). Synthetic polymers are more homogenous in composition and are therefore of a higher purity than natural polymers. This ensures reproducibility in nanoparticle synthesis (27).

1.1.2 Synthesis methods of nanoparticles

The type of biodegradable and biocompatible materials that could be used for nanoparticle synthesis for drug delivery are variable and continue to increase. There are various synthetic methods associated with each type of material.

For liposomal nanoparticles, synthesis involves drying lipids by using Argon or Nitrogen from an organic solvent and rehydrating with an aqueous solution (28). This results in the formation of multilamellar structures which are composed of several lamellar bilayers just like an onion structure. Liposomes are unilamellar in structure, and methods are available to reduce multilamellar structures into liposomes. The most common methods include sonication and extrusion (28-31). With extrusion, the diameter of liposomes can be controlled by using
polycarbonate filters of various pore size during extrusion. Similarly, the sonication power and time can control the size of liposomes (28, 29).

Polymeric nanoparticles have different synthesis protocols that depend greatly on whether a natural or a synthetic polymer is used (32). Furthermore, the synthesis also will depend on the nature of the drug and the localized environment of the target location.

One of the techniques involved for polymeric nanoparticle synthesis is polymer desolvation. In this method, the polymer aggregates and falls out of solution in a particular solvent usually water which is later changed into another solvent. A stabilizing agent can be added to ensure no further nanoparticle aggregation occurs and thus provide the ability of size control. Other methods include variations of solvent exchange such as oil-in-water or water-in oil emulsification approaches. The resulting emulsions are then stabilized forming nanoparticles. A review on the different common procedures for polymeric nanoparticle synthesis is summarized by our group and can be found here (32).

1.2 Pulmonary drug delivery

1.2.1 Delivery route

The human body offers many routes that could be used for drug delivery. These include oral delivery through gastrointestinal track, transdermal delivery across the skin barrier, intravenous delivery through blood vessels, and pulmonary through the lungs. The advantages provided by the lungs make the pulmonary route one of the preferred delivery methods. This
route is non-invasive and thus generate compliance with patients. The lungs provide a large alveolar surface area of about 43 – 102 m² depending on the age and size of the individual (33, 34). In this route, drugs can be delivered for local and systematic delivery. Local delivery is for lung associated respiratory diseases and infections such as asthma, cystic fibrosis, acute respiratory distress syndrome in infants, pneumonia and tuberculosis among many others. The close proximity to the heart and the large vascularization provide an efficient systematic delivery to the blood that avoids metabolism of the gastrointestinal track (such as digestion by stomach acid) and first pass metabolism associated with oral drug delivery (35).

1.2.2 Lung anatomy and physiology

The lungs are the site of gas exchange between the blood and external environment, where oxygen is inhaled and carbon dioxide is exhaled. The respiratory tract is divided into two parts with the upper respiratory tract composed of the oropharynx, trachea and the large bronchi while the lower tract is composed of the small bronchioles and the alveoli. In a human lung, there are over 300 million alveoli which compromise about 95% of the lung’s total surface area (36-38). The air-blood barrier decreases significantly as you move down the respiratory tract. The upper bronchial region has a barrier with a thickness of about 30-40 μm that decreases to about 500 nm in the alveolar region (39, 40). This thin barrier is one of the main advantages of this delivery route.

The thin alveoli barrier is composed of three layers (Figure 1.1). The first layer is in contact with the blood circulation and is composed of two types of alveolar epithelial cells
called Type I and Type II pneumocytes. Type I pneumocytes are very thin and occupy about 95% of the alveolar epithelial layer while Type II are larger and occupy only 5% of the layer (41, 42).

Above the epithelial cells away from the blood circulation is an aqueous layer that is about 50 – 80 nm thick (43). Type II pneumocytes regulate macrophages that act as a defense mechanism which is localized mostly to the aqueous layer (44). The aqueous layer is covered by a monolayer of lipids and proteins called the lung surfactant. This third layer is also produced by the Type II pneumocytes and its main function is to reduce the surface tension at the air-water interface in order to prevent lung collapse during breathing cycles (38).

Figure 1.1. Schematic representation of the anatomy of 3 layered air blood barrier in the lung’s alveoli.
Lung surfactant lipids and proteins are produced by the endoplasmic reticulum of type II pneumocytes (45). Once produced, the lung surfactant components are assembled together into lamellar bodies (Figure 1.2). These lamellar bodies aid in carrying and secreting the lung surfactant components from type II pneumocytes into the alveolar aqueous layer to be later adsorbed at the air-water interface (46, 47).

Figure 1.2. Schematic representation of the pneumocyte type II production of lung surfactant components through lamellar bodies.
1.2.3 *Inhalable nanoparticles*

In order for nanoparticles to pass through the lung and the alveoli and into the bloodstream, they have to cross two major pulmonary clearance pathways, the mucociliary and the alveolar pathways (48). The mucociliary clearance is localized to the upper airway tracts and is facilitated by ciliated cells and a layer mostly composed of mucus. This mucus layer traps foreign materials and removes them from upper airway either through coughing and/or swallowing into the gastrointestinal tract where they will be broken down and digested (49, 50). The second site of clearance is at the lower airway tract particularly the alveolar region facilitated by macrophages. Nanoparticles smaller than 260 nm in diameter have been found to avoid recognition by macrophages and thus can avoid this clearance and potentially pass through into the bloodstream (43, 51-53).

If particles successfully avoid mucociliary clearance, the next step is deposition into the lower airways of the lungs. There are three main drug and particle deposition mechanisms that can occur in the lungs. These include impaction, sedimentation and diffusion (37). Each of these mechanisms depends greatly on the particle’s characteristics such as weight and aerodynamic diameter. When particles have a diameter greater than 5 μm, they will deposit in the upper airway tract along the oropharynx. On the other hand, smaller particles greater than 1 μm and smaller than 5 μm can reach to the lower airway tract. The efficiency of the sedimentation greatly depends on the breathing pattern since the compression and expansion of the alveoli controls the area available in the lower airways. Particles that are smaller than 1 μm are also
deposited into the alveolar region but are readily cleared by exhalation due to their small size and weight (37, 54).

It is important to ensure that the nanoparticles not only reach but also deposit at the alveolar region for successful pulmonary drug delivery into bloodstream. According to these mechanisms, the small size of nanoparticles will prevent successful deposition at the alveolar region. Therefore, nanoparticles will need to be delivered inside a carrier system that can sediment in the alveolar region yet when released are small enough to avoid recognition by macrophages. Extensive research has been performed in this area and several carrier systems for nanoparticles have been developed. The idea of using a carrier system for nanoparticles was first developed by Tsapis and colleagues in the early 2000s when they incorporated nanoparticles in a lipid-lactose carrier system (55). Sham and colleagues also used lactose based carrier systems for nanoparticle delivery to the lungs using spray drying techniques (56). Once in the alveolar region, the lactose carrier system is expected to dissolve and release the nanoparticle load upon interaction with the aqueous layer as seen with in vitro studies. Mannitol has also been utilized by other groups for the same function (57-59).

1.2.4 Nanotoxicology associated with inhaled nanoparticles

As the fields of nanotechnology and engineering continues to advance, we are continuously being exposed to various forms of nanoparticulate materials. These range from everyday clothing materials to household and sports items. For example, you can currently find clothing items containing silver nanoparticles being sold in stores (60, 61). Even sports
hardware, such as racquets have taken advantage of this field by synthesizing strings using carbon nanotube technology (62). Nanotechnology has greatly advanced the future of drug delivery especially with the use of nanoparticles but more work is needed to identify the mechanisms behind potential associated toxic effects. Nanotoxicology involves studying nanoparticles for toxicity as well as elucidating the mechanisms of toxicity. The current research in this area is mostly in design and enhancement of drug delivery technology but much more work is needed for nanotoxicology.

The use of silver nanoparticle incorporated fabrics for antimicrobial effects have been found to potentially cause deleterious health effects linked to silver ions (63). Once internalized, silver was oxidized to Ag-O- which is later changed into Ag-S- as detected through X-ray absorption techniques (64). This form suggest binding of silver to proteins through sulfide bonds and which may disrupt proper protein functions. Furthermore, the Ag-S- form of silver can potentially bind to glutathione resulting in a redox imbalance which can lead to oxidative stress, inflammation, protein denaturation, and even phagocytosis impairment of macrophages (65-69).
1.3 Lung surfactant model systems

1.3.1 Composition

The lung surfactant monolayer in the alveolar region consists of 90% lipids and 10% surfactant proteins produced by type II pneumocytes as mentioned above (70). There are four surfactant proteins (SP) present; SP-A, SP-B, SP-C, and SP-D (71). The main function of the lipid component of the lung surfactant and SP-B and C is to reduce the surface tension during compression and to facilitate the expansion of the alveoli during breathing cycles (72). SP-B (8.75 kDa) is mostly hydrophobic and is thus localized within the lipid monolayer of the lung surfactant (73, 74). SP-B has a net positive charge and therefore it interacts mostly with anionic lipids and enhances the adsorption and respreadability of lung surfactant components (75, 76).

It adopts mainly an alpha-helical structure and is found oriented almost parallel to the monolayer. SP-C (3.7 kDa) is hydrophobic and is primarily in alpha-helical structure. It is a transmembrane protein oriented at a 70 degrees angle from the monolayer interface with a role in maintaining proper dynamics of the film during breathing cycles. SP-A and SP-D on the other hand are hydrophilic and belong to the family of collectins (collagen containing proteins) whose main function is in the immune response (77). With the help of their globular structure, they are involved in binding and eliminating pathogens along with alveolar macrophages (78, 79).

Among the lipid components of the lung surfactant, zwitterionic phosphatidylcholines constitute the majority. The saturated dipalmitoylphosphatidylcholine (DPPC) make up around
50% of the total lung surfactant lipids (80). Unsaturated phosphatidylcholines are also present and constitutes about 20% (80). The second major lipid class is the anionic phosphatidylglycerols and they constitute around 10% (71, 80, 81). Furthermore, neutral lipids such as cholesterol make up 2-8% (71). The different lipid compositions (head group charge and degree of saturation) play important roles in lung surfactant function. For example, anionic lipids are found to interact with cationic surfactant proteins B and C and facilitate adsorption and spreading of the monolayer during breathing cycles (82). Saturated acyl chains such as that of DPPC which constitutes the majority of the lung surfactant composition are vital in achieving low surface tension during exhalation. This is important in order to prevent lung collapse. Since breathing involves exhalation and inhalation, the lung surfactant should be able to compress and reach low surface tension but at the same time be able to respread efficiently for inhalation. This is achieved with the help of the unsaturated lipids in the monolayer which introduce fluidity to the film.

1.4 Research goals and objectives

As mentioned above, the lung surfactant is composed of 90% lipids and 10% proteins. A mass spectrometry study was performed by Postle and colleagues in 2001 on the major lipid classes found in the lung surfactant including phosphatidylcholines and phosphatidylglycerols (83). According to the study, the lipid compositions found in the lung surfactant based on the two lipid classes can be seen in Table 1.1.
Table 1.1. Percentage (mol%) of the major lipid species of phosphatidylcholines (PC) and phosphatidylglycerols (PG) found in the human lung surfactant (83).

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Acyl chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0/16:0</td>
</tr>
<tr>
<td>PC</td>
<td>56.2</td>
</tr>
<tr>
<td>PG</td>
<td>5</td>
</tr>
</tbody>
</table>

The goals of this thesis can be characterized as three fold;

1) Further develop a biomimetic model of the human lung surfactant in order to allow for fast initial *in vitro* studies on nanotoxicology. The biophysical characteristics of this model is compared to commercially available surfactant therapies such as Bovine Lipid Extract Surfactant (BLES). According to Postle’s study (83), the PC 16:0/16:1 and PG 16:0/16:1 are present in the human lung surfactant at a very high concentration. Biophysical characterization of these two lipids is scarce in the literature and their function is not understood. Therefore, extensive biophysical analysis was done on these two lipids to better understand their role in the lung surfactant before incorporating them into the model. Other lipids that were added to the model include PG 18:0/18:1. This lipid is present at a high mol ratio of about 22% of total PGs. The model also contained 2% by weight cholesterol and its effects on the model was assessed.
2) Since the lung surfactant contains both lipids and proteins, SP-B\textsubscript{1-25} was also added to the model. This amino terminal peptide of the SP-B was added since it carries similar surface activity to that of the full protein (84, 85). How this peptide interacts with the individual lipids from different classes with various saturation will be assessed. The contributions to the final model system can be analyzed. This will be done through various surface biophysical analysis techniques.

3) In the light of nanotoxicology and to better understand the mechanism behind nanoparticle toxicity, two different biodegradable, biocompatible polymeric nanoparticles (gelatin and PLGA) effect on the stability of the biomimetic model will be performed. The focus of this thesis is interactions with the deep lower parts of the lungs which constitute an important air-blood barrier. This monolayer is important since any disturbance to its function could be detrimental. The mechanisms of toxicity associated with surface charge and different material composition will be assessed. Effects of these nanoparticles on individual lipids and SP-B\textsubscript{1-25} component of the lung surfactant model functions will be tested. These experiments will help characterize the nanotoxicology associated with the nanoparticles (if present) and which lipid-nanoparticle or peptide interaction are the reason behind potential toxic effect.

The main purpose of this thesis can be simply stated as: “To develop a simple yet effective synthetic biomimetic model of the lung surfactant that could be used as a first line of in vitro
analysis for nanomedicines. Interactions with 2 different polymeric nanoparticles with this system will be assessed.”
Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Lipids, lung surfactant model systems, and chemicals

Dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylglycerol (DPPG), palmitoyloleoyl phosphatidylcholine (POPC), palmitoyloleoyl phosphatidylglycerol (POPG), stearoyloleoyl phosphatidylglycerol (SOPG), palmitoylpalmitoleoyl phosphatidylcholine (16-1PC), palmitoylpalmitoleoyl phosphatidylglycerol (16-1PG), and cholesterol are all purchased from Avanti polar lipids (Alabaster, USA). Chemical structures of lipids used are shown in Figure 2.1. Clinical lung surfactant model Bovine Lung Extract Surfactant (BLES) was a donation from BLES Biochemicals (Ontario, Canada). For HEPES buffer solution, HEPES and sodium chloride were purchased from Sigma Aldrich (Ontario, Canada).
Figure 2.1. Chemical structures of the lipids found in the human lung surfactant used in this study.
2.1.2 Solvents

For monolayer studies, purified ddH$_2$O water from a Millipore Synergy 185 system with a resistivity of 18.2 MΩ.cm purchased from EMD Millipore (Ontario, Canada) was used. For 6:4 Chloroform:Methanol mixtures (v/v), both solvents were HPLC grade purchased from Fisher (Ontario, Canada). For bilayer studies, 20 mM HEPES, 100 mM NaCl with a pH of 7.4 was used. Cleaning solvents; acetone, methanol, hexane and chloroform which are of ACS grade were purchased from Fisher Scientific (Ontario, Canada).

2.1.3 Lung surfactant model systems

All lipid systems tested were weighed using a Sartorius MC 5 microbalance (Goettingen, Germany) to a final 1 mM concentration with 6:4 chloroform:methanol. The addition of SP-B$_{1-25}$ at a 10% weight ratio, gelatin nanoparticles at a 10:1 lipid:nanoparticle weight ratio, and PLGA nanoparticles of 10:1 and 1:1 weight ratios are tested with all the systems.

Table 2.1 below shows the molar concentrations of the major lipid classes found in the human lung surfactant based on published data (83). These include phosphatidylcholines (PCs) and phosphatidylglycerols (PGs). The PC lipids have phosphocholine as the head group and thus are zwitterionic with an overall charge of 0. This is due to the negative charge of the phosphate group canceling the positive charge of the choline group (Figure 2.1). DPPC being fully saturated with two 16 carbon acyl chain tails, constitutes the major component of the lung surfactant with about 56.2 mol% of the total PCs. The unsaturated POPC, is about 10 mol% of the total PC lipids. This lipid has one fully saturated 16 carbon acyl chain while the other is an
18 carbon with a double bond at carbon number 9. The other unsaturated lipid is 16-1PC which also constitutes about 10% of the PC lipids. In this lipid, one of the acyl chains is a fully saturated 16 carbon while the other is 16 carbon in length with a double bond at carbon number 9.

Another of lipid found in the human lung surfactant is the PGs. These lipids have glycerol as the headgroup and thus an overall charge of -1. Like the PCs, the lung surfactant contains saturated PG lipids such as DPPG which contains two 16 carbon acyl chains. It constitutes about 5 mol% of the total PG lipids. Unsaturated PGs such as POPG which contains one saturated 16 carbon acyl chain and the other is 18 carbon with a double bond at carbon number 9 is also present at 30 mol% of total PGs. 16-1PG which is present at about 10 mol% contains two 16 carbon acyl chains with one of them having a double bond at carbon 9. There exists another unsaturated PG lipid, SOPG, at a high molar concentration of about 22 mol%. This lipid has two 18 carbon acyl chains with one of them having a double bond at carbon 9 (Figure 2.1).

The overall percentage of PCs found in the lung surfactant has been found to be four times that of the PGs (83, 86-89). This was considered when building up the more complex lung surfactant model systems as can be seen in Table 2.2. The effects of the headgroup charge and chain structure on interaction with the nanoparticles are studied here in details starting with individual lipid to more complex systems.
Table 2.1. Molar percentage of the two major lipid classes found in the human lung surfactant.

These ratios are adapted from (83).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Mol % ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>56.2</td>
</tr>
<tr>
<td>POPC</td>
<td>10</td>
</tr>
<tr>
<td>DPPG</td>
<td>5</td>
</tr>
<tr>
<td>POPG</td>
<td>30</td>
</tr>
<tr>
<td>SOPG</td>
<td>22</td>
</tr>
<tr>
<td>16-1 PC</td>
<td>10</td>
</tr>
<tr>
<td>16-1 PG</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 2.2. The various model systems tested and developed along with the molar ratio of the lipids used. Each system was made to a final 1 mM concentration.

<table>
<thead>
<tr>
<th>System</th>
<th>Lipids</th>
<th>Mol % ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>DPPC</td>
<td>-</td>
</tr>
<tr>
<td>POPC</td>
<td>POPC</td>
<td>-</td>
</tr>
<tr>
<td>DPPG</td>
<td>DPPG</td>
<td>-</td>
</tr>
<tr>
<td>POPG</td>
<td>POPG</td>
<td>-</td>
</tr>
<tr>
<td>SOPG</td>
<td>SOPG</td>
<td>-</td>
</tr>
<tr>
<td>16-1PC</td>
<td>16-0/16-1 PC</td>
<td>-</td>
</tr>
<tr>
<td>16-1PG</td>
<td>16-0/16-1 PG</td>
<td>-</td>
</tr>
<tr>
<td>Binary PC</td>
<td>DPPC:POPC</td>
<td>5.6:1</td>
</tr>
<tr>
<td>Binary PG</td>
<td>DPPG:POPG</td>
<td>1:6</td>
</tr>
<tr>
<td>4 lipid</td>
<td>DPPC:POPC:DPPG:POPG</td>
<td>45:8:1:6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45:8:1:6 (+ 2wt% cholesterol)</td>
</tr>
<tr>
<td>5 lipid</td>
<td>DPPC:POPC:DPPG:POPG:</td>
<td>45:8:1:6</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>45:8:1:6:4.4:8:2</td>
</tr>
<tr>
<td></td>
<td>16-1PC:16-1PG</td>
<td>(+ 2wt% cholesterol)</td>
</tr>
<tr>
<td></td>
<td>16-1PC:16-1PG: Cholesterol</td>
<td>(+ 2wt% cholesterol)</td>
</tr>
</tbody>
</table>
2.1.4 **Surfactant protein B peptide, SP-B1-25**

SP-B1-25 peptide was synthesized by AAPPTec (Kentucky, USA) to a 97.52% purity (HPLC) with the following sequence:

\[
\text{NH}_2\text{-FPIPLPYCWLCRALIKRIQAMIPKG-COOH}
\]

This is the N-terminal portion of the full SP-B1-79. It has been shown in the literature that the N-terminal sequence of the protein mimics many of the activities of the full protein (84, 90, 91). It was added to lipid systems at a 10% wt ratio of the lipids in 6:4 chloroform:methanol.

2.1.5 **Nanoparticles**

Gelatin type B (bloom of 225), 25% grade II glutaraldehyde, 75:25 (lactide:glycolide) MW 75,000 – 120,000 poly(D,L-lactide-co-glycolide) (PLGA), and ethyl acetate were purchased from Sigma Aldrich (Ontario, Canada). Hydrochloric acid purchased from EMD Millipore (Ontario, Canada). Pluronic F-68 was a donation to the lab from collaborators at the University of Alberta (Dr. Löbenberg’s Lab).

2.2 **Nanoparticle synthesis**

2.2.1 **Gelatin nanoparticles**

For gelatin nanoparticle synthesis, the optimized method by Azarmi S. and colleagues which was further improved by a previous lab member Dr. Lai was used (92). 1.25 g of gelatin type B (bloom 225) was added to 25 mL of ddH\(_2\)O in an Erlenmeyer flask. The solution was then heated to 40°C using a hot plate to dissolve the gelatin of high and low molecular weights. In
order to separate high molecular weight gelatin for nanoparticle synthesis, 21 mL of acetone was added dropwise while stirring in order to precipitate high molecular weight gelatin. The sample was left standing for 20 minutes in order to ensure full precipitation of high molecular weight gelatin. The supernatant containing low molecular weight was discarded and flask was rinsed gently with ddH₂O while not disturbing the pellet. Another 25 mL of ddH₂O was added to the flask and the sample was heated to 40°C in order to dissolve the high molecular weight gelatin. The sample’s pH was dropped down to 2.5 using hydrochloric acid. Acetone was then added dropwise for nanoparticle formation until the solution turned cloudy with an optical density of 0.08 at 600 nm (75 mL of acetone). In order to crosslink and stabilize the nanoparticles, 250 μL of 25% glutaraldehyde was added and the sample was stirred for 16 hours at 600 rpm in the dark. Using a rotary evaporator with a bath temperature set to 40°C, acetone was evaporated from the acetone-water mixture. The nanoparticles were stored in water at 4°C until further use.

2.2.2 PLGA nanoparticles

PLGA nanoparticles were synthesized using the emulsification-diffusion method by Song K.C (93). 100 mg of PLGA was dissolved in 10 mL of ethyl acetate constituting the organic phase. Into a 20 mL of ddH₂O, 1% (w/v) of Pluronic F68 stabilizer was added. The organic phase is then added to the aqueous phase. The mixture is emulsified using a probe sonicator (QSonica LLC, USA) at 10W for 1 min. To this, 80 mL of ddH₂O is added while stirring at 600 rpm in order to allow for the organic solvent to diffuse from the particles out into the aqueous phase leading to
the formation of PLGA nanoparticles. The nanoparticles were stored in water at 4°C until further use.

2.3 Dynamic Light Scattering (DLS)

2.3.1 Principle

This is a technique that enables the measurement of the hydrodynamic diameter of particles in solution based on their Brownian motion. A HeNe laser with a wavelength of 633 nm is focused into a cuvette and the scattered light at 173° is collected by a detector. The intensity of the scattered light is plotted versus time with larger particles diffusing through the solution slower than smaller particles. From this data, an autocorrelation function is calculated according to the following equation:

\[
g^2(\tau) = \frac{\langle I(t) \times I(t + \tau) \rangle}{\langle I \rangle^2}
\]

(1)

Where \( g^2(\tau) \) is the autocorrelation function, \( I \) is the intensity, \( t \) is time and \( \tau \) is a time lag. The \( g^2(\tau) \) from equation 1 was then related to the diffusion coefficient using the expanded Siegert equation:
\[ g^2(\tau) = 1 + \beta \left( \exp(-D \left( \frac{4\pi n}{\lambda_0} \sin \left( \frac{\theta}{2} \right) \right)^2 \tau) \right)^2 \]

(2)

Where \( \beta \) is an instrumental parameter, \( D \) is the diffusion coefficient, \( n \) is a refractive index, and \( \lambda \) is the wavelength of light. Once the diffusion coefficient is obtained, it can be fitted into the Einstein-Stokes equation to calculate the radius (\( R \)) where \( k \) is the Boltzmann’s constant, \( T \) is temperature, and \( \mu \) is viscosity:

\[ R = \frac{kT}{6\pi\mu D} \]

(3)

All nanoparticles were sized using a Malvern Zetasizer Nano-ZS from Malvern Panalytical (Quebec, Canada).

2.3.2 Protocol for nanoparticle sizing

Gelatin and PLGA nanoparticles were sized according to the same protocol. 1 mL of the nanoparticle suspended in ddH\(_2\)O was added to a 4xOptical polystyrene cuvette from Sarstedt (Nümbrecht, Germany) for size and polydispersity analysis with Malvern Zetasizer Nano-ZS from Malvern Panalytical (Quebec, Canada). Each sample was an average of at least 3 measurements.
2.4 Zeta potential

2.4.1 Principle

Zeta potential is a measurement of the electrostatic repulsion or attraction between particles in solution. Depending on the surface groups of nanoparticles, under different solvents or pH, the nanoparticles can either display a charge or no charge. Charged particles will repel each other while neutral or uncharged particles will aggregate out of solution. Therefore, zeta potential is an important measurement for determination of particle stability in suspension.

An important distinction is that the zeta potential is not the same as the surface potential. Charged nanoparticles as they diffuse through a media due to Brownian motion, will always have a layer of oppositely charged ions surrounding them. These ions are strongly bound to the nanoparticle and they constitute the Stern layer. Surrounding this tightly bound layer, is another layer which is bound less tightly but still diffuse around along with the nanoparticle as one entity. It is at the interface region between the bound double layer and surrounding ions, called the Slipping plane, that the electrical potential or zeta potential is measured (Figure 2.2). The thickness of the bound layer will depend greatly on the media and therefore, the surface potential will not be the same as the zeta potential.

An electrical field is applied between two oppositely charged electrodes in a capillary cell. The charged nanoparticles are then attracted to the oppositely charged electrode. The change in the frequency of the scattered light due to nanoparticle movement is used to
calculate the particle’s electrophoretic mobility ($U_E$). From the $U_E$, the zeta potential can be calculated through the following equation;

$$U_E = \frac{2\varepsilon \zeta f(\kappa a)}{3\mu}$$

(4)

Where, $\varepsilon$ is the dielectric constant of the medium, $\zeta$ is zeta potential, $f(\kappa a)$ is the Henry function which depends on the medium’s polarity, and $\mu$ is the medium’s viscosity.

**Figure 2.2.** A schematic diagram of a negatively charged particle along with the surrounding double layer.
2.4.2 Protocol for nanoparticles zeta potential

Nanoparticles were centrifuged and resuspended in a 10 mM NaCl solution in order to increase conductivity for zeta potential measurements and accommodate for effects of pH adjustments. 1 mL at pH 7 was added using a syringe into a folded capillary cell from Malvern (Quebec, Canada). These cells have two electrodes to measure the nanoparticles electrophoretic mobility in an electric field of 150 mV (92). Malvern Zetasizer Nano-ZS from Malvern Panalytical (Quebec, Canada).

2.5 Langmuir Trough measurements

2.5.1 Principle

This technique facilitates the study of insoluble monolayer films at the air-water interface. The films usually consist of molecules such as lipids or amphiphiles which have both a hydrophilic (head) and a hydrophobic (tails) region. It was around 1920 that Irving Langmuir presented evidence for monolayers and their characterization and thus the name Langmuir monolayers (94). The instrument used to characterize and study Langmuir monolayers is called Langmuir trough. It is a Teflon based trough that contains water or subphase onto which the monolayer of study is deposited. It also contains one or two movable barriers to compress and/or expand the monolayer. A schematic of the Langmuir trough set up can be seen in figure 2.3.
Figure 2.3. Schematic diagram of a Langmuir trough with a pressure sensor and Wilhelmy plate for pressure-area isotherms and a surface potential probe for surface potential-area isotherms.

There are multiple techniques available to study Langmuir monolayers (95), but for the purposes of this thesis, we use surface-pressure area isotherms and surface potential-area isotherms. As the name implies, pressure isotherms are used to characterize the change in surface pressure in response to the area available per molecule (Å²/molecule) at the air-water interface. Changes to the surface pressure are recorded using a wetted Wilhelmy plate that is partially submerged into the subphase (about 1 mm). There are multiple forces acting on the plate that are recorded by a pressure sensor. The weight of the Wilhelmy plate (gravity) and surface tension pull the plate with a downward force, while buoyancy pulls in the opposite direction upwards. Surface tension ($\gamma$) is calculated as;
\[ \gamma = \frac{F}{2(w + d)\cos\theta} \]  

(5)

Where \( F \) is the sum of all the forces acting on the plate, \( w \) is the width of the plate, \( d \) is the thickness of the plate, and \( \theta \) is the contact angle which should be zero if the plate is positioned perpendicularly to the subphase. The relationship between surface pressure and surface tension is given by the following equation;

\[ \pi = \gamma_0 - \gamma \]  

(6)

Here, \( \gamma_0 \) is the surface tension in the absence of a monolayer at the air-water interface which is equal to 72 mN/m for water. We can obtain from this equation the relationship between surface pressure (\( \pi \)) and surface tension (\( \gamma \)) in the presence of monolayers.

During compression isotherms, the lipid monolayer undergoes multiple phase transitions that are characteristic to each lipid or lipid mixture. Before compressing the barriers, the lipids are far apart with minimum to no interaction between the lipids and thus are said to be in the ‘gaseous’ phase. No surface pressure is recorded. With further compression, the lipids are getting closer to each other and as a result, van der Waals interactions increases. This results in a significant increase in the recorded surface pressure (decrease in surface tension). At this stage, the lipids are said to enter the liquid expanded, LE phase. The lipids in the LE
monolayer are more closely packed and thus more ordered compared to the lipids in the gaseous, G phase. Further compression of the monolayer forces the transition into the liquid condensed LC phase where the lipids display higher rigidity and ordering compared to the LE phase. Some lipids or lipid combination will display a plateau region in the pressure-area isotherm that correspond to phase coexistence between the LE and LC phases. Eventually further compression will result in monolayer collapse where the formation of multilamellar structures is seen. Figure 2.4 below shows a pressure-area isotherm of DPPC which displays the plateau region of phase coexistence (Figure 2.4) (95).

**Figure 2.4.** A schematic representation of a pressure-area isotherm of DPPC lipid showing the different lipid phases the monolayer undergoes during compression from large to small molecular areas. LE denotes Liquid Expanded; LC denotes liquid condensed.
For surface potential measurements, the monolayer’s surface potential ($\Delta V$) can be represented by the following equation;

$$\Delta V = V - V_0$$

(7)

Where $V$ is the potential in the presence of a monolayer, and $V_0$ is the potential in the absence of a monolayer (clean subphase). The $\Delta V$ of the monolayer is the sum of multiple dipole moments as depicted by the Demchak and Fort model (96) (Figure 2.5) through the following equation;

$$\Delta V = \frac{\left(\frac{\mu_1}{\varepsilon_1} + \frac{\mu_2}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3}\right)}{A \varepsilon_0}$$

(8)

Where $\mu_1$, $\mu_2$, and $\mu_3$ are the three dipole moments contributing to $\Delta V$, $\varepsilon_0$ is the permittivity of free space while $\varepsilon_1$, $\varepsilon_2$, and $\varepsilon_3$ are the effective relative permittivities of free space taking into account the induced polarization of neighbouring molecules of the three different regions contributing to the three different dipoles (Figure 2.5). $A$ is average area per molecule.

The surface potential is measured through the vibrating capacitor technique. The potential difference between two parallel electrode plates is measured. One electrode, made of platinum, is in the aqueous subphase while the second oscillating electrode is about 2-3 mm positioned above the aqueous phase. The oscillating upper electrode creates a displacement current that is nulled through an external bias until the potential difference between the two
electrodes is zero. As the lipid monolayer is being compressed, the lipids headgroup and tails change orientation and dipole moment and thus $\Delta V$ is collected per area.

Figure 2.5. Schematic representation of the various dipole moments and permittivities ($\mu$ and $\varepsilon$) experienced in a lipid monolayer based on the Demchak and Fort model (96). The bottom region denotes the first region and it is of the dipole moment of the lipid headgroup as it interacts with the surrounding aqueous subphase. The middle polar region is for the dipole moment of the head group, and the third region is due to the dipole moment of the hydrophobic tails (terminal CH$_3$).
2.5.2 Protocol for surface pressure-area isotherms

For pressure-area isotherms, two troughs of different sizes were used depending on the method of nanoparticle introduction. This is done in order to minimize the amount of nanoparticles required. For experiments involving gelatin nanoparticles, which were deposited along with the lipids at the air-water interface, a large trough with an area of 720 cm\(^2\) was used. While for PLGA nanoparticles experiments, the particles were added to the subphase and therefore, a smaller trough with an area of 200 cm\(^2\) was used. This is because PLGA nanoparticles are not stable in organic solvents and thus have to be introduced with the aqueous subphase.

Troughs were 4 solvent (acetone, methanol, hexane and chloroform) cleaned before each trial. Once cleaned, for the large trough, 400 mL of ddH\(_2\)O was added as the subphase. About 70 μL of a 1 mM lipid sample of the system understudy, was deposited dropwise at the air-water interface. For systems with gelatin nanoparticles, a weight ratio of 10:1 lipid to nanoparticle was tested. After deposition, a 10 min wait period was used in order to allow for organic solvent evaporation. The monolayer film was then compressed at a speed of 100 cm\(^2\)/min at room temperature until a maximum surface pressure or collapse was reached. Each reported pressure-area isotherm is an average of at least 3 isotherms.

For the smaller trough, similar cleaning procedures were used. The volume of the aqueous subphase for the smaller trough is 120 mL. In systems where PLGA nanoparticles were added, a weight ratio of 10:1 (lipid to nanoparticle) was used. Since it is a large subphase
volume, two concentrations of PLGA nanoparticles are added in the subphase and their surface characteristics were determined using pressure-area isotherms. A volume of about 20 μL of a 1 mM sample was deposited dropwise at the air-water interface. Similarly, after a wait period of 10 mins, the monolayer was compressed at a speed of 97 cm²/min at room temperature until a maximum surface pressure or collapse is reached. Each reported pressure-area isotherm is an average of at least 3 isotherms. The pressure-area isotherms for both troughs were recorded using a KSV Nima Pressure Sensor (Coventry, England).

2.5.3 Protocol for surface potential-area isotherms

The surface potential-area isotherms were collected in a similar protocol as the pressure-area isotherms. As seen in Figure 2.3, A KSV Nima Surface Potential Sensor (SPOT) from Biolin Scientific (Espoo, Finland) with the two electrodes was added so that surface potential can be collected.

2.5.4 Compression modulus profile and protocol

This technique was used to assess the interfacial elasticity or fluidity of the monolayer under study. It is calculated from the pressure-area isotherms through the following equation:

\[ C_s = -\frac{1}{A} \frac{dA}{d\pi} \]  (9)
Where $C_s$ is the lateral compression modulus (mN/m), $A$ is the molecular area ($\text{Å}^2$/mol), and $\pi$ as the surface pressure (mN/m). The derivative was calculated as the slope between the molecular area and surface pressure. The compression modulus is the inverse of compressibility. In this thesis, the compression modulus is reported as opposed to the compressibility due to values generated in the same magnitude as the pressure-area isotherms. From the compression modulus profile and values, the monolayer’s phases can be characterized (97). Values under 12 mN/m correspond to the gas phase, values between 12 mN/m and 100 mN/m correspond to a LE phase, and values above correspond to a LC phase (97-99). The higher the compression modulus value, the more rigid the monolayer is.

2.6 Brewster angle microscopy (BAM)

2.6.1 Principle

Brewster angle microscopy (BAM) is an imaging technique that allows visualization of the lateral organization of monolayers in real time without the use of exogenous probes or dyes (100). It was developed simultaneously and independently by two separate groups in 1990s (101, 102).

In the absence of a monolayer, when a plane polarized light (light parallel to the plane of incidence) is directed onto the air-water interface at the Brewster angle of water (~53°) no light is reflected (Figure 2.6). In the presence of a monolayer film, at the same Brewster angle, light will be reflected. This is due to the additional refractive index of the monolayer. The
reflected light is then analyzed for grayscale intensity and collected by a CCD camera. The grayscale intensity of the reflected light is dependent on the ordering of the lipid phases with tightly packed lipids displaying a higher intensity than less ordered lipids. This is due to increased van der Waals interactions between the hydrocarbon acyl chains which is a characteristic of the LC phase. Lipids in this phase are more ordered with extended chains that protrude farther into the air. These monolayer protrusions or domains, reflect more light off the monolayer and thus they appear brighter on the greyscale of a BAM image.

This technique is very useful for viewing the various lipid phases and phase coexistence and to investigate the formation of domains. The formation and shape of the domains are greatly dependent on the line tension and dipole electrostatics between the two coexisting phases (103-105).
Figure 2.6. Schematic of the instrument set up and principle of Brewster angle microscopy.

2.6.2 Protocol for BAM images

A similar protocol for collecting pressure-area isotherms was used. The trough is four solvent cleaned and ddH$_2$O was added as the subphase. Both troughs were used for BAM imaging depending whether gelatin or PLGA nanoparticles were tested. After depositing the lipid sample dropwise, a wait period of 10 min was used to allow for solvent evaporation before
the onset of compression. This process was stopped at various specific pressures for images to be collected. The images were obtained using an EP3 imaging ellipsometer from Accurion (Goettingen, Germany) and EP3 view software. Images are collected with at least 3 replicates at each surface pressure. Furthermore, the EP3 software allows for 3D image analysis. This is a qualitative analysis of the image’s grayscale intensity of the monolayer that can be used to characterize lipid nanoparticle interactions on the lateral organization.

2.7 Differential Scanning Calorimetry (DSC)

2.7.1 Principle

Differential Scanning Calorimetry (DSC) is a calorimetric technique that can be used to determine thermodynamic parameters of molecules during phase transitions without the use of exogenous probes (106-108). When a sample undergoes a temperature increase, it will undergo a phase transition from gel phase ($L_{\beta}$) to a liquid crystalline phase ($L_{\alpha}$). In the gel phase, lipids are more rigid and highly ordered due to tight packing of the headgroups and the acyl chains. In the liquid crystalline phase, lipids are more fluid and less ordered (106). The temperature at which this phase transition occurs is greatly dependent on the nature of the headgroup in terms of size and charge, as well as the length and saturation of the acyl chains. This temperature is called the phase transition ($T_m$) and it is greatly dependent on the nature of the lipid (Figure 2.7).
Figure 2.7. Schematic of the thermograph of a gel to liquid crystalline phase transition. $T_m$ is the transition temperature at which lipids exist at equal concentrations of the gel and liquid crystalline lipid phases. $T_{1/2}$ is the full width at half height and $\Delta H$ denotes the enthalpy of the transition which is the integration of the area under the curve.

Some lipids will display a pre-transition temperature which corresponds to the phase change from the gel phase to the ripple phase (108). As the name suggests, it is a phase characterized by undulations in the membrane before entering the liquid crystalline phase. Another useful parameter that can be obtained from DSC data is the $T_{1/2}$, full width at half height of the thermograph. This is the cooperativity of phase transition which is a representation of how well the lipids melt together. Therefore, a low $T_{1/2}$ value indicates high
cooperativity and a high $T_{1/2}$ value indicates poor cooperativity of the phase transition. The $\Delta H_{\text{cal}}$ is the enthalpy associated with the phase transition and it is the integration of the area under the curve. A positive $\Delta H_{\text{cal}}$ indicates an endothermic reaction while a negative $\Delta H_{\text{cal}}$ indicates an exothermic reaction.

In a typical DSC instrument, there are two cells (sample cell and a reference cell) where the sample cell will contain the lipids (multilamellar vesicles) in buffer and the reference cell will contain the same buffer solution with no lipids. Both cells are enclosed in an adiabatic chamber so that no heat is transferred in or out of the cells. As the instrument scans a certain temperature range, the sample cell will generate a heat change due to the phase transition of lipids that are either endothermic or exothermic. The instrument will then try to maintain the same temperature between the sample cell and the reference cell by either providing or withdrawing heat. This thermal energy required is characterized by the heat capacity or $C_p$. It is simply the amount of energy required to raise the system’s temperature by 1°C. Graphing the $C_p$ as a function of temperature generates a thermograph as seen in Figure 2.7.

2.7.2 Protocol for Multilamellar vesicle preparation

Multilamellar vesicles or MLVs are vesicles composed of multiple bilayers. They are prepared by weighing a 1 mM lipid sample dissolved with 6:4 chloroform:methanol. The organic solvent was then evaporated using argon in order to form a lipid film in a glass vials. To insure complete evaporation of the organic solvent, the vials were kept in a vacuum oven overnight and stored in -20°C until ready to be used. The vials were then hydrated with a 20 mM HEPES,
100mM NaCl pH 7.4 buffer. The films were vortexed and undergo 4 freeze-thaw cycles in order to ensure complete hydration of the film from the glass vial into solution.

2.7.3 Protocol for DSC experiment

Once the sample MLVs were prepared, they were degassed for 10 mins in order to ensure no air bubbles were in sample which can interfere with the DSC experiment. 250 μL of the 20 mM HEPES, 100 mM NaCl buffer was loaded with a Hamilton syringe into the reference cell. Similarly, 250 μL of the MLV sample is added to the sample cell. The instrument was set to scan from 20°C to 60°C with a scan rate of 10°C/hr. For each sample, 4 up-scans (heating scans) and 4 down-scans (cooling scans) were performed with at least 2 replicates.
Chapter Three: **Biophysical characterization of 16:1PC/PG lipid containing systems**

### 3.1 Background and Objectives

The objective of this chapter was to biophysically analyze 16:0/16:1 PC and PG lipids (referred to hereon as 16-1PC and 16-1PG). These two lipids constitute a major component of the human lung surfactant at about 10% for each lipid class. In regards to the 8 lipid system, together they constitute about 13.5% mol ratio (83). Biophysical characterizations of these two lipids are scarce in the literature, and thus it is important to better understand the behaviour of these lipids and their contribution to the function of lung surfactant.

Pressure-area isotherms on a Langmuir trough as well as lateral monolayer organization using Brewster angle microscopy were used. Bilayer studies were also implemented using differential scanning calorimetry (DSC) in order to assess the thermotropic properties of these lipids such as the melting temperature and phase transition cooperativity from the gel phase ($L_\beta$) to the liquid crystalline phase ($L_\alpha$). These experiments were done by comparing POPC and 16-1PC and similarly POPG and 16-1PG (Table 3.1 and 3.2). Structurally, the PO- lipids differ from 16-1 lipids through the extra two carbons at the sn-2 position (18:1 vs 16:1).

The purpose of this chapter is to investigate the physical behaviour and characteristics of these previously uncharacterized lipids. This is important to be done before incorporation into the lung surfactant studies in order to better characterize their contribution to the human lung surfactant.
Table 3.1. List of PC systems used in monolayer and bilayer analysis in molar ratios.

<table>
<thead>
<tr>
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<th>DPPC</th>
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<tbody>
<tr>
<td>75 DPPC:25 POPC</td>
<td>75 DPPC:25 16-1PC</td>
</tr>
<tr>
<td>50 DPPC:50 POPC</td>
<td>50 DPPC:50 16-1PC</td>
</tr>
<tr>
<td>25 DPPC:75 POPC</td>
<td>25 DPPC:75 16-1PC</td>
</tr>
<tr>
<td>POPC</td>
<td>16-1PC</td>
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Table 3.2. List of PG systems used in monolayer and bilayer analysis in molar ratios.

<table>
<thead>
<tr>
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<th>DPPG</th>
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<tr>
<td>75 DPPG:25 POPG</td>
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<tr>
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<td>50 DPPG:50 16-1PG</td>
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<tr>
<td>25 DPPG:75 POPG</td>
<td>25 DPPG:75 16-1PG</td>
</tr>
<tr>
<td>POPG</td>
<td>16-1PG</td>
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3.2 Pressure-Area isotherm analysis

DPPC was used as the medium to study the effects of 16-1PC at various ratios when compared to POPC. DPPC is composed of two saturated 16:0 acyl chains. During compression this lipid forms LC domains that are extensively characterized in literature. Therefore, any changes to the isotherm and LC domains due to fluid lipids such as 16-1PC can be better
characterized. Figure 3.1A below shows the pressure-area isotherm of DPPC which entered the LE phase at a molecular area of around $98 \text{ Å}^2/\text{mol}$ and exhibited a plateau at surface pressure of $7 \text{ mN/m}$ that was maintained between $77$ and $57 \text{ Å}^2/\text{mol}$. This characteristic feature indicated LE-LC phase coexistence, which also led to the formation of distinct lateral domains. Further compression resulted in monolayer collapse at $57 \text{ mN/m}$ and $37 \text{ Å}^2/\text{mol}$ (Figure 3.1A). This surface pressure was lower than that reported in the literature of about $72 \text{ mN/m}$ (109) which is ultimately due to the size of the Langmuir trough used and subphase composition. A similar collapse pressure of $60 \text{ mN/m}$ was obtained with a $500 \text{ cm}^2$ sized trough (In our case, size is $720 \text{ cm}^2$) (110). Nonetheless, the overall shape and surface pressures of significant regions in the isotherm obtained here were consistent with literature values.

POPC contains one 16:0 acyl chain at sn-1 position and another 18:1 monounsaturated acyl chain at sn-2 with a double bond at carbon number 9. POPC’s isotherm lifted off from the gas phase at around $125 \text{ Å}^2/\text{mol}$ and collapsed at $48 \text{ mN/m}$ at a molecular area of $58.6 \text{ Å}^2/\text{mol}$ (Figure 3.1A). The earlier entrance into the LE phase when compared to DPPC indicates less packing and more fluid films due solely to the difference in the acyl chain. Saturated lipids pack tighter than their unsaturated counterparts. The pressure-area isotherm of 16:1PC was between these two lipids but closer to POPC. 16:1PC entered the LE phase at around $118 \text{ Å}^2/\text{mol}$ and collapsed at a surface pressure of $45 \text{ mN/m}$. This indicates that 16:1PC is slightly less fluid when compared to POPC due to its shorter unsaturated acyl chain (16 vs. 18).
To further identify the behaviour of 16-1PC, other ratios were compared including those representing lung surfactant systems. In the 50:50 system between DPPC and POPC or 16-1PC, a trend was seen similar to that of pure fluid lipids (POPC and 16-1PC) (Figure 3.1A). Systems that include 16-1PC lifted off from the gas phase at a smaller molecular area when compared to systems including POPC. This late entry into the LE phase further in compression indicates decreased fluidity (increased rigidity). Rigid lipids enter the LE phase later in compression compared to fluid lipids. Due to the presence of 50% POPC, the plateau of DPPC was present but reduced to a shoulder at higher surface pressure. For 50% POPC containing systems, the plateau occurred at 22.5 mN/m, while for 50% 16-1PC systems it was slightly reduced and occurred at 21 mN/m (Figure 3.1A, insets). The onset of collapse for the 50 DPPC:50 POPC was similar to that of pure POPC at 48 mN/m and reached a final collapse pressure of 51.5 mN/m at 37 Å²/mol. Likewise, 50 DPPC:50 16-1PC also collapsed at a similar surface pressure to that of pure 16-1PC at 45 mN/m (Figure 3.1A, arrows pointing to start of collapse for both POPC and 16-1PC).

It is established in the literature for fully saturated lipids that longer acyl chains will occupy smaller molecular areas at a certain surface pressure when compared to shorter chains. For example, DSPC which contains two 18:0 acyl chains lifts off from the gas phase at smaller molecular areas when compared to shorter DMPC lipid with 14:0 acyl chains (111-113). This is due to increased attractive van der Waals interactions between the acyl chains as their length increase (114). Nonetheless, when unsaturation is involved, the lipid’s behaviour is different.
When comparing POPC to DPPC, lift off from the gas phase occurred earlier in compression with POPC. This literature trend (115-118) was also seen throughout in this thesis. In this case, the effect of unsaturation was stronger than the longer acyl chain effect where a stronger shift is seen when a double bond in present in one of the lipid’s acyl chains.

Since 16-1PC lipid was custom made by Avanti Polar Lipids Inc for this thesis, there is limited data on its behaviour. From the pressure-area isotherms, 16-1PC systems were found to be more rigid than POPC. The results showed that a 16:1 acyl chain at sn-2 position was more rigid compared to longer 18:1 acyl chains. This is expected since the unsaturation effect from the POPC were stronger due to longer chain than 16-1PC.

Next, the 16-1PG containing systems were assessed. 16-1PG is another important component of the lung surfactant without significant analysis in the literature. In order to characterize 16-1PG’s behaviour, pressure-area isotherms of systems similar to 16-1PC were conducted (Figure 3.1B). The saturated 16:0/16:0 PG lipid (DPPG) was also used as a matrix to study 16-1PG in order to allow direct comparison between the two lipid classes. The main difference is the negative charge of the PG lipids versus zwitterionic PCs.
Figure 3.1. Pressure-area isotherms of A) DPPC to POPC and 16-1 PC, B) DPPG to POPG and 16-1PG at various ratios. Each isotherm is an average of various replicates (n ≥ 3).
The saturated DPPG lifted off from the gas phase and entered the LE phase at a molecular area of 55 Å²/mol (Figure 3.1B). It remained in the LE phase for a short compression range until a surface pressure of 4 mN/m at 45 Å²/mol. Further compression caused a sharp increase in the slope of the isotherm until film collapse at 52 mN/m and 33 Å²/mol. For similar reasons discussed above, the monounsaturated POPG lifted off at a higher molecular area earlier in compression and entered the LE phase at 112 Å²/mol (Figure 3.1B). A constant slope in the isotherm was maintained up to a surface pressure of 25 mN/m. Above this surface pressure, POPG entered the LC phase characterized by tighter packing whereby the phase change was indicated by a kink in the isotherm (76) (Figure 3.1B, red arrow). POPG collapsed at 47 mN/m at a molecular area of 28 Å²/mol. 16-1PG entered the LE phase at a similar molecular area of POPG and overlapped with the isotherm up to a surface pressure of 15 mN/m after which it shifted to smaller areas with further compression. Pure 16-1PG collapsed at a surface pressure of 44 mN/m, at 28 Å²/mol. However, similar to POPG, the kink associated with the start of LC phase occurred in this system at comparable surface pressures.

For the systems containing 50% 16-1PG or POPG, the observed trends were not as clear as those seen for the PCs (Figure 3.1B). Systems containing 50% POPG appeared to be more rigid compared to systems with 50% 16-1PG (with PCs, 16-1PC was more rigid). The film entered the LE phase at around 97 Å²/mol compared to 110 Å²/mol for systems contained 50% 16-1PG. The shift to lower areas for 50% POPG was seen up to a surface pressure of 25 mN/m which was around the onset of the LC phase for both fluid PGs (POPG and 16-1PG). Further
compression above this surface pressure led to overlap in the isotherms until collapse. Both systems collapsed at 48 mN/m and 30 Å²/mol (Figure 3.1B). Due to the presence of the saturated DPPG, a plateau region was seen in both systems at around 8 mN/m indicative of the formation of domains as will be discussed more below. Again, similar with DPPC, the presence of fluid lipids delays the formation of LC domains associated with saturated lipids and thus appear at a higher surface pressure (pure DPPG domains formed around 4 mN/m).

Considering that all lipid acyl chains are similar to those with the PCs, the obtained results depend on two factors: the headgroup charge and acyl chain effects. PG lipids have an overall charge of -1 from the phosphate linker in the headgroup. In terms of size, minor differences occur between the PG and PC lipid headgroups. PCs headgroup occupy an area of 0.64 nm² per lipid while PGs occupy 0.55 nm² per lipid (119). Therefore, results due to acyl chain lengths and packing are influenced by the lipid's headgroup charge.

Figure 3.2 shows more in-depth analysis of the change in the average molecular area of DPPC or DPPG as the ratio of fluid lipids varies. More systems were tested other than the 50:50 ratios and were compared at 3 distinct surface pressures of 10, 15 and 30 mN/m. These surface pressures where chosen to complement lung surfactant studies in the following chapters. 30 mN/m was chosen as the highest surface pressure to ensure analysis before system collapse. It is also biologically relevant for membranes (120-122). In figure 3.2A, the change in molecular area for PC lipids is shown. As the ratio of the saturated DPPC was increased, the shift to lower
molecular areas was seen for all systems studied. This is expected as DPPC is more rigid and
packs tighter and thus will occupy smaller areas. Other than the systems identified in table 3.1,
lung surfactant relevant combinations were also analyzed which include the following:

- 85 DPPC:15 POPC
- 85 DPPC:15 16-1PC

Both of the above systems constitute components of the lung surfactant as the ratios of POPC
and 16-1PC are equivalent (10% each of the total PCs). The trend seen above in figure 3.1A, still
holds here for the other PC systems. The molecular area of POPC containing systems appeared
to be larger than that of 16-1PC systems indicating increased fluidity (Figure 3.2A).

The trend was different for the PG systems. At low surface pressures, 16-1PG containing
DPPG systems overall occupied larger molecular areas compared to those containing POPG
(Figure 3.2B). Other than the systems mentioned in table 2, the following were studied as well
since they represent relevant lung surfactant ratios:

- 14 DPPG:86 POPG
- 33 DPPG:67 16-1PG

For more detailed comparison, the following were also tested:

- 14 DPPG:86 16-1PG
- 33 DPPG:67 POPG
Figure 3.2. Average molecular area of A) POPC or 16-1PC with varying molar ratio (%) of DPPC, B) POPG or 16-1PG with varying molar ratio (%) of DPPG analyzed at 3 surface pressures: 10, 15, and 30 mN/m.
All PG systems tested at 30 mN/m show interesting results. Systems containing 50% or more DPPG displayed no change in the average molecular area between 16-1PG and POPG systems whereas no clear trend was observed at lower surface pressures (Figure 3.2B). This can potentially be explained by lateral packing properties of the different lipids. A model by Quickenden and Tan was proposed to explain packing problems, even for homogenous population (123). Our group has adopted this model to explain packing problems in binary POPC-sphingomyelin mixtures as shown in figure 3.3 (124). The lipid headgroup is represented as a disk whereby the size differences are proportional to the respective molecular areas. Monolayers composed of mostly sphingomyelin can accommodate larger POPCs with fewer packing defects between the lipids. The opposite is not true since few smaller sphingomyelin lipids significantly disturb POPC packing resulting in larger packing defects (Figure 3.3). In our systems, the comparison is between similar glycerol based lipids (POPG vs 16-1PG) but the concept still holds. In addition to the headgroup size and architecture, differences in acyl chain and lipid charge will affect the lateral packing of monolayer. This is due to steric hindrance of unsaturated side chains or charge-charge repulsion resulting in the observed effects for systems containing less than 50% DPPG.

At 30 mN/m, 16-1PG displayed a packing density similar to that of pure DPPG. Almost no change in the average molecular area was seen as concentrations of 16-1PG were increased from that of pure DPPG. The average molecular area occupied by DPPG was 37.7 Å²/mol at 30 mN/m. This can be explained by the simple concept of packing density. At high surface
pressures, the fluid 16-1PG phase density behaves similarly to that of saturated DPPG phase density. This explains the obtained results since with further compression, the chain lengths and saturation were in an ideal mixing environment. This concept of phase density mixing at high surface pressures have been established and identified before (125-127).

Figure 3.3. Schematic of the potential lateral packing between POPC (larger grey disks) and sphingomyelin (smaller dark disks). Image adapted from (124) with permission from Elsevier through Copyright Clearance Center’s RightsLink®.
3.3 Lateral organization analysis

The lateral domain organization of DPPC during compression included the formation of LC domains in the LE-LC phase coexistence region of the pressure-area isotherm at around 7 mN/m (Figure 3.4, panel 1). Those domains had a fractal-like shape and were controlled by competing factors such as line tension and electrostatic dipole-dipole repulsion between the two different phases (103, 128). At 7 mN/m, the domains had a diameter of 15.8 ± 4.2 μm. These domains coalesced into a homogenous film upon further compression. The lateral organization of the fluid 16-1PC and POPC displayed a homogenous film throughout compression as expected for unsaturated lipids (Figure 3.4, panel 5). With increasing ratio of fluid PC lipids to DPPC, a delay in the LC domain formation was observed as also indicated from the pressure-area isotherm plateau shifts. DPPC films containing 25% POPC or 16-1PC exhibited domain formation at around 15 mN/m with similar diameters (Figure 3.4, panel 2). The respective 25% POPC system had domains with a diameter of 8.6 ± 2 μm, while domains in the 25% 16-1PC system were 8.8 ± 2 μm at 15 mN/m. With further compression at 30 mN/m, domains increased in size to 14.7 ± 3 μm for POPC and to 14.2 ± 3 μm for 16-1PC. Thus, no significant differences in terms of domain size were seen for these two systems (Figure 3.4, panel 2).

In DPPC systems with 50% fluid lipid content, the LC domains appeared much later in the compression closer to 30 mN/m and remained visible until collapse for both systems (POPC and 16-1PC) (Figure 3.4, panel 3). The appearance of those domains was consistent with the
shoulders seen in the pressure-area isotherm at around 21 and 22.5 mN/m (Figure 3.1A). The domains of DPPC with 50% POPC had a diameter of 2.3 ± 0.4 μm and those with 16:1PC had 2.6 ± 0.4 μm diameters (p-value < 0.01). Further increase of fluid lipids to 75% prevented domain formation, and the lateral organization of the monolayers resembled that of pure POPC and 16:1PC (Figure 3.4, panel 4).
**Figure 3.4.** BAM images of DPPC to POPC or 16-1PC in various ratios at surface pressures of 7, 10, 15, and 30 mN/m. For DPPC, the first image is collected at about 7 mN/m. First column is for POPC and second column for 16-1PC systems.
Pure DPPG, due to its saturated acyl chains, formed domains at low surface pressures early in the compression that coalesced into a homogenous LC phase at a surface pressure of 10 mN/m (Figure 3.5, panel 1). Bright clusters appeared in the monolayer around 30 mN/m which correspond to early collapse of pure DPPG (129, 130). Similar to PC lipid systems, the addition of fluid POPG and 16-1PG delayed the formation of the LC domains. Unlike with the PCs, a different pattern was seen between POPG and 16-1PG systems. DPPG with 25% POPG at 5 mN/m resulted in domains that were smaller than those formed with 16-1PG. These domains had a diameter of 43 ± 8 μm compared to significantly larger values of 54 ± 12 μm for 16-1PG containing systems at 5 mN/m (p-value < 0.001) (Figure 3.5, panel 2). As the monolayer was compressed further, the domains seen at 5 mN/m increased in size significantly (p-value < 0.001) and remained intact until collapse in both systems (Figure 3.5 and 3.6A). No size difference was seen between POPG and 16-1PG systems above 5 mN/m.

For DPPG systems containing 50% fluid lipids, few small domains were seen at 5 mN/m (Figure 3.5, panel 3). DPPG films including 50% POPG had domains with a diameter of 13.7 ± 2 μm while those containing 50% 16-1PG had smaller domains with a diameter of 8.3 ± 0.4 μm (p-value < 0.001). Furthermore, the frequency of domains was much smaller than that seen with 50% POPG at similar surface pressures. At 10 mN/m, domains of both systems had similar diameters with an average of 33 ± 7 μm. As compression was continued, 50% POPG system displayed 40.9 ± 9.6 μm domains which were larger than 16-1PG systems (p-value < 0.0001) (Figure 3.5, panel 3).
Further increase to the concentration of fluid PG lipids resulted in a further delay in domain formation. With 75% POPG domains appeared closer to 15 mN/m while with 75% 16-1PG they appear around 25 mN/m (Figure 3.5, panel 4). Interestingly, 75% POPG system domains at 30 mN/m were present in two populations with the larger domains having an average diameter of 24 ± 4 μm. The domains were significantly smaller at 30 mN/m in the presence of 75% 16-1PG with an average diameter of 13 ± 3 μm (p-value <0.001).

From the pressure-area isotherms, POPG containing systems overall were found to be more rigid than 16-1PG systems at surface pressures below 30 mN/m (Figure 3.2B). This increased rigidity resulted in earlier phase separation. As the ratio of fluid lipids increased, domains formed earlier in POPG systems compared to 16-1PG. As seen in Figure 3.6, domains of POPG systems were significantly larger overall due to them being formed earlier and continued to grow with monolayer compression.
Figure 3.5. BAM images of DPPG to POPG or 16-1PG in various ratios at surface pressures of 5, 10, 15, and 30 mN/m. First column is for POPG and second column for 16-1PG systems.
The results obtained from the lateral organization between the two different lipid classes suggest stronger effects within the PG lipids than PC lipids. Lateral organization was significantly different between DPPG systems containing 16-1PG and POPG lipids. This was not the case with the lateral organization of PC systems containing 16-1PC and POPC as seen in figure 3.4. In the isotherms of DPPC systems containing 16-1PC and POPC, the difference was
seen in an earlier take off by POPC containing systems (larger molecular areas). The shape and slope of the isotherm was consistent with that of 16-1PC systems. Therefore, it is not surprising that the lateral organization is consistent between the two lipids (POPC and 16-1PC). In the PG containing systems, the isotherms are very different between 16-1PG and POPG containing systems and thus the difference in lateral organization is justifiable.

3.4 Thermotropic analysis of 16-1PC/PG systems

As mentioned earlier in chapter 2, DSC is a technique that allows the measurement of the thermotropic phase behaviour of various materials including lipids. Characteristic parameters about the phase transition of lipids and their mixtures from the gel phase ($L_{\beta}$) to the liquid crystalline phase ($L_{\alpha}$) can be determined. These are similar to the LC and LE phases in monolayer studies respectively. Other parameters include the phase transition temperature, the associated change in enthalpy ($\Delta H$), and the cooperativity ($T_{1/2}$) of the transition (108).

In this chapter, the effect of fluid lipids (PCs and PGs) on DPPC or DPPG were measured. An important part of characterizing 16-1 lipids other than monolayer studies involve the identification of their phase behaviour. Again, similar to monolayer studies, no characterization on their thermotropic phase behaviour is available. Therefore, it was important to perform DSC studies on these lipids at ratios similar to that used in monolayer studies for ease of comparison. A heating scan (upscan) and a cooling scan (downscan) were performed in order to
determine melting ($T_m$) and crystallization ($T_c$) temperatures to assess the reversibility of the transition.

### 3.4.1 DPPC vs. DPPG

The thermographs of pure DPPC and DPPG are presented in figure 3.7 below with the numerical data assembled in tables 3.3 and 3.4, respectively. The DPPC heating scan showed a pre-transition peak at around 34.2 °C followed by a main transition peak at 41.5 °C (Figure 3.7A, Table 3.3). The pre-transition peak is for the transition from the gel phase to the ripple gel phase. This pre-transition is characterized by the formation of ripples or undulations in the bilayer before formation of liquid crystalline phase (131). The main transition peak from the gel (L$\beta$) to liquid crystalline (L$\alpha$) was very sharp with a $T_{1/2}$ value of 0.2 °C. The enthalpy of the endothermic main transition was calculated to be 14.3 ± 0.3 kcal/mol. These values are similar to that obtained in the literature (106, 132, 133). The reverse of this transition was only slightly different. The $T_c$ temperature associated with the cooling scan was found to be 41.1 °C with a $T_{1/2}$ value of 0.3 °C (Figure 3.7A). Since there was no significant difference between the $T_{1/2}$ values of heating and cooling scans, the transition was highly reversible.

The DPPG heating scan had a thermograph that looked almost identical to that of DPPC (Figure 3.7B). Two peaks were seen with the pre-transition peak occurring at 32.5 °C and the main transition at 40.4 °C (Table 3.4). These temperatures correspond to values obtained in the literature (133, 134). Unlike DPPC, a broader asymmetrical main transition was seen during the heating scan (Figure 3.7B). The $T_{1/2}$ of the heating scan transition was 0.8 °C with an enthalpy
value of $9.0 \pm 0.2$ kcal/mol (Table 3.4). This indicates that the gel (L$_{\beta}$) to liquid crystalline (L$_{\alpha}$) transition of DPPG was still cooperative but to a lesser degree compared to DPPC ($T_{1/2}$ of 0.2). The cooling scan’s main transition peak of DPPG was much more complex. No pre-transition was seen and the main transition appeared to be divided into 3 subpopulations. The $T_m$ of the cooling scan’s main transition was at 40°C with a $T_{1/2}$ value of 1.13 °C which is broad but still reversible since multiple cycles were performed of heating and cooling. The other two subpopulations had a $T_m$ values of 39.6°C and 39°C.

The asymmetry seen with DPPG in its complex cooling scan could be due to the presence of the charge in the PG lipids otherwise absent in PC lipids. The electrostatic repulsion between the lipids affected the packing of the system. This resulted in reduced cooperativity and complicated cooling scan with multiple peaks suggesting the formation of subpopulations (135). DPPG in HEPES buffer alone displays a very complex thermograph of multiple peaks (131, 135, 136). However, when NaCl salt is added, the main transition becomes more distinct with only one pre-transition and one main transition temperatures (131). DPPG in 100 mM NaCl solution has a thermal behaviour similar to that of DPPC. The presence of the salt changes the ionic strength resulting in a comparable thermograph. Here, a HEPES buffer that contains salt (20 mM HEPES, 100 mM NaCl, pH 7.4) was used as suggested by the manufacturer, Malvern Inc. Therefore, the presence of multiple subpopulations in the cooling scan was expected. However, the heating scan was not different from literature even after multiple cycling (133, 134).
Multiple heating and cooling scans were performed (n ≥ 4) with high reversibility and this phenomenon only appeared in the cooling scans.
Figure 3.7. Thermographs of heating scan (blue) and cooling scan (red) of A) DPPC, and B) DPPG. Absolute Cp values are used. Cp values of upscan are endothermic and exothermic for downscans. Each scan is an average of multiple scans (n ≥ 6). Scan rate used was 10 °C/hour.

3.4.2 POPC vs. 16-1PC systems

Pure POPC has a $T_m$ value of -2 °C as reported in the literature (137). Due to instrument restrictions based on condensation, low $T_m$ values below 10 °C are difficult to obtain. No $T_m$ value of 16-1PC have been reported but based on its acyl chain composition the $T_m$ can be estimated to be well below 10 °C. Therefore, various ratios in DPPC were tested in order to characterize it indirectly.

The heating and cooling scans of 75 DPPC:25 POPC are shown below in figure 3.8A with numerical analysis in table 3.3. The addition of 25% POPC resulted in a broader endothermic transition compared to pure DPPC with a $T_{1/2}$ of 5.04 °C (Figure 3.8A). This correspond to an increase of about 4.8 °C. A broadening of the peak indicates packing defects affecting transition cooperativity caused by mixing of lipids with different levels of fluidity (138) (Figure 3.3). Furthermore, the packing defects caused a decrease in the $T_m$ value from 41.5 to 37.20 °C with an associated enthalpy of 6.8 ± 0.03 kcal/mol (Table 3.3). Upon cooling of the system, minor differences were seen compared to the heating scan. The $T_c$ value was 36.6 °C with a $T_{1/2}$ of 4.1 °C. This particular system was tested before in the literature with similar $T_m$ values (139, 140).

For the 75 DPPC:25 16-1PC, the $T_m$ of the phase transition was 40.50 °C with an associated $T_{1/2}$ value of 2.1 °C and an enthalpy of 5.7 ± 0.04 kcal/mol (Figure 3.8B,Table 3.3). The cooling scan
of this system also shows a good reversibility with a $T_c$ value of 39.9 °C and a $T_{1/2}$ of 2.3 °C. Again, multiple cycles were performed and no effects on the transition temperatures were seen. The smaller $T_{1/2}$ value seen with the 16-1PC system complements the monolayer studies where it was found to be more rigid than POPC (Figure 3.1A). Therefore, the transition is expected to be more cooperative.

**Figure 3.8.** Thermographs of heating scan (blue) and cooling scan (red) of A) 75 DPPC:25 POPC, and B) 75 DPPC:25 16-1PC. Absolute Cp values are used. Cp values of upscan are endothermic and exothermic for downscans. Each scan is an average of multiple scans ($n \geq 6$). Scan rate used was 10 °C/hour.
Other systems of higher than 25% POPC (50 and 75%) were tested but the obtained thermographs were very broad, and the software did not allow proper analysis (example in figure 3.9). Nonetheless, the thermographs from systems containing 50 and 75% 16-1PC were obtained and analyzed as shown below (Figure 3.10). Again, this further indicated the higher fluidity of POPC compared to 16-1PC lipid. For the 50 DPPC:50 16-1PC system, the asymmetric thermograph showed a pre-transition peak at 34.6 °C. The main peak of this system was divided into two subpopulations at 41.2 °C and 41.7 °C with an endothermic enthalpy of 5.5 ± 0.1 kcal/mol (Figure 3.10A, Table 3.3). The cooling scan of this system still maintained the pre-transition albeit at a lower temperature of 31°C. The main transition was asymmetric and had a Tc value of 41 °C. No subpopulations were seen with the cooling scan (Figure 3.10A). The T1/2 was around 0.5 °C indicating reversibility of this system. Demixing is observed in the presence of 50% 16-1PC as opposed to 25%. The lower T1/2 values as well as the presence of both pre transition temperature and main transition is similar to that of pure DPPC. The presence of two subpopulations in the heating scan can be explained by gel phase immiscibility of DPPC into regions that are purely DPPC and regions that contain some 16-1PC and thus the slightly reduced Tm value.

Increasing the fluidity in the system by adding 75% 16-1PC resulted in a thermograph that still maintains a pre-transition temperature at 33 °C and a main transition at 41.3 °C with a T1/2 of 0.7 °C (Figure 3.10B). The associated enthalpy of main transition was 1.7 ± 0.3 kcal/mol (Table 3.3). Again, similar to the above 50:50 system, demixing between the two lipids was
present with thermograph and cooperativity similar to that of DPPC. Due to the temperature range available, only DPPC fractions are seen. The cooling scan for this system still maintained the pre-transition around 32 °C and a $T_c$ value of 40.82 °C. The $T_{1/2}$ of the cooling scan was the same as the heating scan with a value of 0.7 °C. All PC systems presented here displayed high reversibility.

With DSC, a decrease in the $T_m$ value indicates increased fluidity of the system with the $L_\alpha$ (liquid crystalline phase) being favoured over the $L_\beta$ (gel phase). Comparing the two results of DPPC systems containing POPC and 16-1PC, the $T_m$ values of POPC systems was found to be lower than 16-1PC systems. The $T_m$ value of 75 DPPC:25 POPC was 37.2 °C which is 4.3 °C lower than pure DPPC. On the other hand, the $T_m$ of 75 DPPC:25 16-1PC was a 1 °C decrease compared to DPPC. Similarly, the $T_{1/2}$ values associated with 16-1PC system were smaller than those for POPC indicating higher cooperativity of the phase transition (Table 3.3). This increased fluidity of the bilayer complements the monolayer pressure-area isotherms in which POPC was found to be more fluid at 30 mN/m and throughout the compression (Figure 3.1A).
Figure 3.9. Thermographs of heating scan of 50 DPPC:50 POPC. Absolute Cp values are used. Cp values of upscan are endothermic. Each scan is an average of multiple scans (n ≥ 6). Scan rate used was 10 °C/hour.

Figure 3.10. Thermographs of heating scan (blue) and cooling scan (red) of A) 50 DPPC:50 16-1PC, and B) 25 DPPC:75 16-1PC. Absolute Cp values are used. Cp values of upscan are endothermic and exothermic for downscans. Each scan is an average of multiple scans (n ≥ 6). Scan rate used was 10 °C/hour.
Table 3.3. Thermodynamic parameters including $T_m$, $\Delta H$ and $T_{1/2}$ of PC containing MLVs. Data are from heating scans.

<table>
<thead>
<tr>
<th>System</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T_{1/2}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>41.5 ± 0.03</td>
<td>14.3 ± 0.30</td>
<td>0.2</td>
</tr>
<tr>
<td>75DPPC:25POPC</td>
<td>37.20 ± 0.01</td>
<td>6.8 ± 0.03</td>
<td>5.04</td>
</tr>
<tr>
<td>75DPPC:2516-1PC</td>
<td>40.50 ± 0.03</td>
<td>5.7 ± 0.04</td>
<td>2.1</td>
</tr>
<tr>
<td>50DPPC:50POPC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50DPPC:5016-1PC</td>
<td>41.70 ± 0.08</td>
<td>5.5 ± 0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>25DPPC:75POPC</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25DPPC:7516-1PC</td>
<td>41.3 ± 0.6</td>
<td>1.7 ± 0.3</td>
<td>0.70</td>
</tr>
</tbody>
</table>

3.4.3 POPG vs. 16-1PG systems

Similar to analysis for POPC and 16-1PC, systems containing POPG and 16-1PG were investigated in order to characterize 16-1PG’s behaviour. Pure POPG has a $T_m$ value of close to -2 °C which is similar to that of POPC (141-143). For the 25% POPG containing DPPG system, the $T_m$ shifted from 40.4 °C for pure DPPG to 38.02 °C for the mixture (Figure 3.11A, Table 3.4). This decrease in $T_m$ correspond to increased fluidity of the bilayer where the liquid crystalline phase was stabilized over the gel phase compared to pure DPPG. The $T_{1/2}$ of the heating scan transition was 6 °C with an endothermic enthalpy of 5.25 ± 0.03 kcal/mol (Table 3.4). The cooling scan of this system indicated high reversibility since it almost overlapped with the heating scan with a $T_c$ of 37.90 and a $T_{1/2}$ of 6°C. The 25% 16-1PG system (75 DPPG:25 16-1PG) displayed a $T_m$ value slightly lower at 37.60 °C with a $T_{1/2}$ of 4.87 °C and an enthalpy of 6.10 ±
0.03 kcal/mol (Figure 3.11B, Table 3.4). The cooling scan of this system almost overlaps with the heating scan with a slight decrease in $T_m$ to 37.3 °C with a $T_{1/2}$ of 4.3 °C.

The presence of fluid lipids decreased the cooperativity of the phase transition of DPPG significantly as was seen for both 25% POPG and 16-1PG (Figure 3.11). The $T_{1/2}$ values associated with the broad peaks increased by 5.2 °C for POPG and 4.1 °C for 16-1PG compared to pure DPPG. It appears that 16-1PG slightly favoured and stabilized the liquid crystalline phase by lowering the $T_m$ more compared to systems containing POPG. This again complements results seen with monolayer studies where 16-1PG was overall more fluid compared to POPG.

The other systems including higher percentages of POPG and 16-1PG were also tested but the phase transitions were very broad making it difficult to integrate the enthalpies and identify $T_m$ values.
Figure 3.11. Thermographs of heating scan (blue) and cooling scan (red) of A) 75 DPPG:25 POPG, and B) 75 DPPG:25 16-1PG. Absolute Cp values are used. Cp values of upscan are endothermic and exothermic for downscans. Each scan is an average of multiple scans (n ≥ 6). Scan rate used was 10 °C/hour.
Table 3.4. Thermodynamic parameters including $T_m$, $\Delta H$ and $T_{1/2}$ of PG containing MLVs. Data are from heating scans.

<table>
<thead>
<tr>
<th>System</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T_{1/2}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPG</td>
<td>40.36 ± 0.05</td>
<td>8.99 ± 0.2</td>
<td>0.76</td>
</tr>
<tr>
<td>75DPPG:25POPG</td>
<td>38.02 ± 0.02</td>
<td>5.26 ± 0.03</td>
<td>6.00</td>
</tr>
<tr>
<td>75DPPG:2516-1PG</td>
<td>37.60 ± 0.01</td>
<td>6.10 ± 0.03</td>
<td>4.87</td>
</tr>
<tr>
<td>50DPPG:50POPG</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50DPPG:5016-1PG</td>
<td>-</td>
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<tr>
<td>25DPPG:75POPG</td>
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<td>-</td>
</tr>
<tr>
<td>25DPPG:7516-1PG</td>
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</tr>
</tbody>
</table>

3.5 Chapter summary and conclusions

The purpose of this chapter was to characterize the biophysical behaviour of 16-1PC and 16-1PG lipids although indirectly. Through the use of pressure-area isotherms, imaging of lateral organization of monolayers with BAM and thermotropic analysis of bilayers through DSC, first steps with the characterization were achieved. The use of both monolayer studies allowed us to characterize the stability of 16-1PC and 16-1PC lipids which were unknown before. It is important to identify their roles in the human lung surfactant. Bilayer studies through the use of DSC helped further characterize those lipids in terms of their transition temperature and thermotropic phase behaviour. With the human lung surfactant, fluidity is key in order to allow for smoother cycles of compression and expansions during breathing cycles.
Therefore, understanding the function of each lipid component and how lipids work together is important.

In the obtained results above, the contribution of 16-1PC and 16-1PG in terms of fluidity were identified and compared to the other major fluid lipids in the lung surfactant such as POPC and POPG. POPC’s induced fluidity was higher than that of 16-1PC lipid as seen with the pressure-area isotherms (Figure 3.1 and 3.2). Interestingly, the same cannot be said for PG lipids with similar acyl chains. It was found that 16-1PG will induce higher fluidity to the model lung surfactant allowing better mixing compared to POPG. This was clearly seen in the BAM images where more homogeneity was with 16-1PG systems compared to POPG systems (Figure 3.5).

From DSC data in the literature of binary systems, as the difference between the two lipids increase, the associated gel phase immiscibility increases (144-146). For instance, comparing DPPC:POPC to DPPC:SOPC, more immiscibility was seen in the latter system. This was due to the longer chains of SOPC compared to POPC. When the difference between the lipids is high, the $T_m$ is expected to shift to the direction of the fluid lipid with the lower $T_m$ and decrease cooperativity (146). This was similar to what was obtained in this chapter with the PC lipid systems. 16-1PC contains both 16 carbon long acyl chains similar to DPPC. This is not the case with POPC which has one 18 carbon long acyl chain. Therefore, with POPC, the $T_m$ is
expected to decrease and become less cooperative ($T_{1/2}$ value lower than 16-1PC systems).

Therefore, our obtained results comply well with literature data (Table 3.3).

Interestingly, with PG lipids, the systems including 16-1PG which is expected to be less different in chain length and density to DPPG had a lower $T_m$ value than POPG. However, the difference between the two systems (75 DPPG:25 POPG and 75DPPG:25 16-1PG) transition temperatures was very small of about 0.42 °C compared to a difference of 3.3 °C between the PC lipids. Nonetheless, in terms of cooperativity, the trend seen with PG lipids was similar to that of PC lipids. The transition was more cooperative with 16-1PG (shorter and more matching chain length to DPPG) than POPG with a difference of 1.13 °C (2.94 °C with PC lipids at similar ratios).
Chapter Four: Biophysical analysis on the interaction of PLGA nanoparticles with model lung surfactant system

4.1 Background and Objectives

4.1.1 In vitro model systems

As mentioned earlier in the introduction, the use of an *in-vitro* model allows for a fast, initial screening method for nanotoxicology of drugs and drug delivery systems. This is generating a lot of interest with various groups to develop simple, 2 or 3 component mimics of the human lung surfactant (88, 147-151). A simple mimic based on few components, albeit useful, does not provide a complete picture of the human lung surfactant. Therefore, outcomes about safety of certain nanoparticles generated with these models are not complete. The realization of this situation resulted in groups looking to common surfactant therapies such as BLES, Curosurf, Survanta, Alveofact, and Infasurf (152-155). These models are a good mimic of the human lung surfactant and are being used in surfactant replacement therapy for children with respiratory distress syndrome. These systems will provide researchers with a fast analysis on the toxicology of drugs and nanoparticles. Nonetheless, the underlying mechanism for observed effects cannot be identified. Thus, specific or preferential interactions of drugs and nanoparticles with individual components in the system cannot be readily assessed but would be important to understand to help develop safer drugs and carrier systems. The effect of charge, size and combination of those factors among others will be better understood by comparing data for individual lipid systems with results from the more complex models that...
mimic the human lung surfactant. This will greatly help in the development of better therapeutics rather than randomized testing that could take a lot of time and money.

4.1.2 Chapter objectives

In this chapter, the effects of PLGA nanoparticles on the surface pressure, surface potential, and elasticity on the lung surfactant biomimetic models are characterized. Furthermore, the lateral organization of the monolayer will be visualized. These techniques collectively help characterize the monolayer behaviour of the lung surfactant components. The surface pressure studies along with the compression modulus are important in identifying the stability of the lipids and the complex models. In addition, other than testing the effects of PLGA nanoparticles on the stability of the model, effects on the system’s elasticity can be identified. These two methods together can explain how the stability is affected in terms of the system’s elasticity. Additionally, surface potential measurements as well as imaging on the lateral organization provide an important tool to assess effects of nanoparticles on the

Tests will be done on all individual lipids included in the model followed by testing the effects on mixtures of the major lipid classes, PC and PG, before analyzing the complex multi-component model. In this chapter, the PLGA nanoparticles will be introduced through the subphase. This is because PLGA nanoparticles are not stable in organic solvents preventing their delivery along with the lipids. Due to the subphase being large, 120 mL, it is difficult to quantify how many nanoparticles are at the interface. Therefore, in addition to the 10:1 lipid to nanoparticle weight ratio used, a 1:1 ratio was tested as well.
The aim of this chapter is to characterize the effects of PLGA nanoparticles on the lung surfactant in terms of charge, fluidity, and overall charge density of the system. Therefore, more information about the safety of PLGA nanoparticles in the lung surfactant can be elucidated. This is obtained here in terms of how PLGA nanoparticles are interacting with the models can be characterized.

4.2 Nanoparticle Characterization

PLGA nanoparticles were synthesized through an emulsification-diffusion method using ethyl acetate as the organic solvent and Pluronic F68 as the stabilizing agent. This yielded nanoparticles that were 141 nm in diameter with a narrow polydispersity index (PDI) of 0.130. As outlined by Malvern instruments, PDI values higher than 0.7 indicate a broad size distribution and values below are mostly monodisperse (156). Therefore, our nanoparticles with PDI of 0.13 is considered fairly monodisperse in nature. In addition, the nanoparticles had a zeta potential value of -26 mV. An absolute zeta potential value between 20 and 30 mV indicates stable nanoparticle suspensions (157).

4.3 Individual Lipid Systems

4.3.1 Pressure-Area isotherms and compression modulus

The surface pressure-area isotherms of the pure PLGA nanoparticles in the subphase were recorded to assess the surface activity of the nanoparticles. Due to the instability of PLGA
nanoparticles in organic solvents, nanoparticles had to be introduced through the aqueous subphase (120 mL). The results in figure 4.1 demonstrated that the surface activity of PLGA nanoparticles was very low as indicated by the minimal increase in surface pressure upon compression; this is not unexpected for very polar materials suspended in a large bulk volume.

Figure 4.1. Pressure-Area isotherms of PLGA nanoparticles from the subphase. Water scan is with no nanoparticles present in the subphase (Blue), the 10:1 ratio correspond to the presence of PLGA nanoparticles in the subphase at 10% of the total weight of the lipids to be deposited at the interface (Red). The 1:1 system is that when nanoparticles are added in the subphase at the same weight ratio of lipids to be deposited. The obtained isotherm is that of the pure nanoparticles from the subphase (Green). All isotherms collected are average of various replicates (n ≥ 3)
Monolayers of the individual lipids found in the lung surfactant were tested in the absence and presence of PLGA nanoparticles at the two different weight ratios. The first class of lipids analyzed were the phosphatidylcholines (PCs), which constitutes the majority of the lung surfactant (71, 80). DPPC is the main component (~ 50% w/w) of the lung surfactant PCs. Pure DPPC monolayer lifted off from the gas phase at a molecular area of 110 Å²/mol and entered the LE phase (Figure 4.2A). At molecular areas between 85 and 65 Å²/mol, DPPC monolayers displayed a phase coexistence between the LE and LC phases. With further compression, the slope of the isotherm changed and the monolayer entered a LC phase until at a maximal pressure was exceeded leading to film collapse and the formation of multilamellar 3D structures. The pressure at which the 3D structures form is called the collapse pressure. For DPPC, this was around 45 Å²/mol at surface pressures of 41 mN/m. This collapse pressure was lower than that of the literature for DPPC which is expected to be around 72 mN/m (109). The measured values strongly depend on the geometry of the Langmuir trough used (in this case, 200 cm²) as well as the subphase composition. No major changes occurred to the pressure-area isotherm of DPPC upon addition of PLGA nanoparticles at a 10:1 ratio other than the shift to higher collapse surface pressures (Figure 4.2A). An increase from 41 mN/m to 43.5 mN/m was seen in the presence of 10:1 PLGA nanoparticles. When the ratio increased to 1:1, a shift to larger areas per molecule was observed in the pressure area isotherm when compared to pure DPPC. This 1:1 system entered the LE phase before the start of monolayer compression. This indicated that the PLGA nanoparticles indeed interact with the lipid monolayer inducing the increase in surface pressure at comparable molecular areas. Further compression to surface
pressures of 25 mN/m, the isotherm overlapped with the control DPPC system until a collapse pressure of 45 mN/m was reached (Figure 4.2A).

The compression modulus profile for control DPPC displayed a dip in the isotherm at surface pressures that correspond to the phase coexistence region seen in the pressure-area isotherms (Figure 4.2B, arrow). This technique allows for the measurement of the interfacial elastic packing of the monolayer and thus whether it changes in the presence of nanoparticles can be tested (158, 159). As the monolayer entered the LC phase after the coexistence region, an increase in rigidity or compression modulus is seen with a peak modulus value of 134.3 mN/m (Figure 4.2B, asterisk). Similar to the pressure-area isotherms, PLGA nanoparticles at the 10:1 ratio did not result in a significant change to the elasticity of the monolayer (Figure 4.2B). For the 1:1 system with PLGA nanoparticles, a decrease in the compression modulus was seen indicative of increased elasticity or fluidity of the system with the peak modulus reduced to 128 mN/m (Figure 4.2B). Table 4.1 below compares the collapse pressure of DPPC systems and their significance.
The second PC analyzed was POPC (Figure 4.2C). In terms of structure, this lipid has one
16 carbon acyl chain at sn-1 position and a longer 18 carbon chain with a cis double bond at sn-
2 in position 9. This double bond introduced fluidity into the system (71, 160), which was
indicated by the earlier lift off at 151 Å²/mol versus 110 Å²/mol for fully saturated DPPC. POPC
collapsed at 38.6 mN/m at a molecular area of 64 Å²/mol (Figure 4.2C). The addition of PLGA
nanoparticles caused an isotherm shift to larger molecular areas, most pronounced for the
higher 1:1 ratio tested. Unlike DPPC, a significant effect (shift to higher molecular areas) with
the 10:1 lipid to nanoparticle ratio occurred (Figure 4.2C). Since both lipids have the same
phosphocholine headgroup, the increased effect seen with POPC was presumably due to less
tighter packing of partly unsaturated and more fluid films. The pressure-area isotherms show LE
phase formation until film collapse for POPC in the absence and presence of nanoparticles,
which is confirmed by the compression moduli values below 100 mN/m. Systems that include
PLGA nanoparticles displayed an increase in monolayer elasticity up until peak modulus of

<table>
<thead>
<tr>
<th></th>
<th>DPPC + PLGA 10:1 (43.5 ± 0.06 mN/m)</th>
<th>DPPC + PLGA 1:1 (45 ± 0.1 mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC (41 ± 0.01 mN/m)</td>
<td>Significant p value ≤ 0.0001</td>
<td>Significant p value ≤ 0.0001</td>
</tr>
</tbody>
</table>

Table 4.1. Statistical analysis on the collapse pressures of DPPC systems. Two tailed student t-
test
maximum rigidity was reached at around surface pressures of 22 mN/m and compression modulus of 76 mN/m (Figure 4.2D).

To further characterize the fluidizing effect of PLGA nanoparticles the next lipid analyzed was 16-1PC. This lipid was also present in the lung surfactant at equal ratios to POPC (83). Since this lipid is not commercially available, it was acquired by custom synthesis offered by the largest lipid supplier, Avanti Polar Lipids Inc. The pressure-area isotherm of 16-1PC showed a monolayer lift off from the gas phase into the LE phase at a molecular area of 128 Å²/mol (Figure 2E), which is between lift off areas for DPPC and POPC. 16-1PC collapsed at 40.3 mN/m at a molecular area of 48 Å²/mol. Systems including PLGA nanoparticles at the 10:1 ratio entered the LE phase at a slightly larger molecular area of 140 Å²/mol and collapsed at a similar surface pressures as 16-1PC alone. Increasing nanoparticle concentration up to a 1:1 ratio increased the surface pressure of the monolayer where the system entered the LE phase much earlier before starting the compression. A slight decrease in collapse pressure occurred from 40.3 to 38.9 mN/m. The monolayer’s elasticity of 16-1PC reached a peak maximum at around 84 mN/m (Figure 4.2F). Minor changes were caused by systems that include 10:1 PLGA nanoparticles. However, systems that include higher concentration of PLGA nanoparticles (1:1) displayed increased monolayer elasticity before peak compression modulus at 80 mN/m. The modulus peak of 16-1PC falls between that of DPPC and POPC with 134.3 mN/m and 76 mN/m, respectively (Figure 2F). This indicated that 16-1PC was less fluid than POPC due to its shorter unsaturated chain (16:1 vs 18:1 carbons for POPC). This is confirmed by the observation that
there were minimal effects of nanoparticles on 16-1PC elasticity, and only the 1:1 ratio showed significant increase in molecular area and film elasticity. These results fell in between that seen for DPPC and POPC at the 10:1 ratio but were closer to the saturated DPPC system.
Figure 4.2. Pressure-Area isotherms and compression modulus of phosphatidylcholine lipids on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of DPPC systems respectively, C) the pressure-area isotherm and D) compression modulus of POPC systems, E) the pressure-area isotherm and F) compression modulus of 16-1PC systems. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3).
The next class of phospholipids analyzed were the phosphatidylglycerols (PGs), which are the second largest class of lipids found in the lung surfactant. They carry a net negative charge of \(-1\) and constitute around 10\% by weight of the total lung surfactant composition (71, 80). In terms of the headgroup size, PCs are slightly larger than the PGs with an area of 0.64 nm\(^2\) per lipid versus 0.55 nm\(^2\) respectively (119).

The pressure-area isotherm of the saturated DPPG lipid can be seen in figure 4.3A. DPPG entered the LE phase at a molecular area of 60 Å\(^2\)/mol. The control DPPG system alone quickly entered the LC phase as seen by the sharp slope of the pressure-area isotherms. DPPG collapsed into multilamellar structures at surface pressure of 40 mN/m. Systems including 10:1 PLGA nanoparticles entered the LE phase at a molecular area of 85 Å\(^2\)/mol. Due to the presence of nanoparticles, the system experienced higher surface pressures at similar molecular areas resulting in the isotherm to shift to the right. No change to collapse pressure was recorded with systems at the 10:1 ratio. Increasing the PLGA concentrations to 1:1 ratio caused the system to enter the LE phase before start of compression due to increased monolayer surface pressures. Furthermore, a shoulder was seen around surface pressure of 10 mN/m (Figure 4.3A, arrow). There was a slight stabilization to the system through the increased collapse pressure of up to 42.5 mN/m from 40 mN/m. DPPG’s elasticity showed compression modulus values of LC phase at surface pressures above 5 mN/m (Figure 4.3B). The monolayer remained in LC phase with further compression until peak modulus value of highest rigidity was reached at 118 mN/m at surface pressure of 20 mN/m. Further compression caused the system to undergo collapse as
seen by the decrease in compression modulus. PLGA nanoparticles at the 10:1 ratio caused an increase in monolayer elasticity or fluidity in which the system entered the LC phase at surface pressures of 15 mN/m as opposed to 5 mN/m. Furthermore, the peak compression modulus decreased to about 112.3 mN/m. With further increase in PLGA nanoparticle concentration, a dip in the modulus was seen around 10 mN/m corresponding to the shoulder region in the pressure-area isotherm (Figure 4.3B, arrow). A delay in which the system entered the LC phase was seen up until around 20 mN/m. This corresponds to a delay of 15 mN/m from control DPPG alone indicative of further increase to the system’s fluidity.

The lung surfactant also contains unsaturated forms of PGs such as POPG, SOPG and 16-1PG which are all present at higher ratios than the saturated DPPG. POPG has two acyl chains, one saturated containing 16 carbons and the other 18 carbons with a double bond at position 9, similar to POPC with the exception of the headgroup structure. Due to the Langmuir trough geometry (200 cm²), it was difficult to capture the lift off molecular area and collapse pressure of this lipid. Therefore, before start of compression, POPG was already in the LE phase. The full isotherm of pure POPG can be seen in chapter 5 where a different trough was used (720 cm²). POPG alone had a collapse pressure of 40.4 mN/m at 32 Å²/mol. PLGA nanoparticles at both ratios caused only a slight shift to higher molecular areas up to a surface pressure of 20 mN/m. Upon further compression a shift to smaller areas in the presence of the nanoparticles was observed (Figure 4.3C). Both systems including PLGA nanoparticles caused a decrease in collapse pressure from 40.4 to 37.4 mN/m. In terms of monolayer elasticity, POPG alone
persisted in a LE phase throughout compression (Figure 4.3D). The presence of PLGA nanoparticle at both ratios, resulted in the dip in the modulus around 20 mN/m which supported the isotherm shift seen around that surface pressure where monolayer elasticity changed. POPG in the presence nanoparticles experienced increased fluidity around surface pressures of 20 mN/m (Figure 4.3D).

SOPG has two 18 carbon acyl chains with one of them having a double bond at position 9. This system entered the LE phase at molecular areas closer to 135 Å²/mol (Figure 4.3E). The collapse pressure was reached at 36.2 mN/m at a molecular area of 43.4 Å²/mol. For systems that include 10:1 PLGA nanoparticles, the pressure-area isotherm was similar to that of SOPG alone with the exception of increased collapse pressure to 38.2 mN/m at similar molecular areas (an increase of 2 mN/m). Increasing the concentration further to a 1:1 ratio, resulted in a significant rise in the surface pressure of the monolayer, therefore a shift to larger molecular areas occurred. In addition, collapse pressure was increased to 39 mN/m (an increase of 2.8 mN/m from control). The PLGA nanoparticle interaction with SOPG overall had a stabilizing effect. The compression modulus profile of SOPG displayed a peak at around 50.7 mN/m indicative of a LE phase throughout compression (Figure 4.3F). Systems including 10:1 PLGA nanoparticles had a delay in peak rigidity of the monolayer which occurred at a surface pressure of 25.8 versus 20 mN/m. A further delay was seen in the 1:1 system with the peak modulus occurring at surface pressure of 27 mN/m.
The third fluid PG lipid found in the lung surfactant analyzed here was 16-1PG. This lipid is very similar to DPPG with the addition of the double bond at carbon number 9 in the sn-2 position. This system entered the LE phase at molecular area of 120 Å²/mol (Figure 4.3G). 16-1PG alone reached a collapse pressure of 34 mN/m at a molecular area of 38 Å²/mol. The addition of PLGA nanoparticles at the 10:1 ratio caused a slight increase in the monolayer surface pressure causing the isotherm to shift to larger molecular areas up to surface pressures of around 20 mN/m. Further compression caused this system to experience lower surface pressures than control 16-1PG reaching a lower collapse pressure of 29.3 mN/m (a decrease of 4.7 mN/m). Systems including 1:1 PLGA nanoparticles displayed further increase in the surface pressure of the monolayer. This system entered the LE phase before the start of monolayer compression. The collapse pressure of this 1:1 system reached 41.6 mN/m which was higher than the control. Thus the presence of nanoparticles were able to alter the structure and composition of the monolayer in a concentration dependent manner. The compression modulus data displayed increased elasticity with systems including nanoparticles at the 10:1 ratio compared to control. For the 1:1 ratio system, there was an increase in rigidity of the monolayer compared to pure 16-1PG (Figure 4.3H).
Figure 4.3. Pressure-Area isotherms and compression modulus of phosphatidylglycerol lipids on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of DPPG systems respectively, C) the pressure-area isotherm and D) compression modulus of POPG systems, E) the pressure-area isotherm and F) compression modulus of SOPG systems, G) the pressure-area isotherm and H) compression modulus of 16-1PG systems. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3)
The obtained results of the interactions of negatively charged PLGA nanoparticles with PC lipids presented the potential effect of electrostatic interactions. Negatively charged PLGA nanoparticles could be interacting with the positively charged amine groups of PC lipids. Similar electrostatic interactions were seen before between charged nanoparticles and the amine group of DPPC monolayers (161-163). Citrate capped gold nanoparticles with an overall negative charge were shown to interact with DPPC through an increase to the surface pressure of the monolayer more so than with positively charged gold nanoparticles (161). Overall, with results obtained with the individual lipids here, a stronger effect of the electrostatic interactions between PLGA nanoparticles with POPC > 16-1PC > DPPC were seen. This suggests less effects for lipids with increased acyl chain rigidity.

Considering the fact that PLGA nanoparticles and PG lipids are negatively charged, the obtained results are counterintuitive. Similar results were obtained in the literature where interactions of negative charged gold nanoparticles with DPPG resulted in a shift to larger areas in the pressure-area isotherm (161). The authors suggest electrostatic interactions with DPPG counterion (Na\(^+\)) would account for this. DPPG from Avanti Lipids Inc is supplied along with its counter ion Na\(^+\). As a result, we cannot ignore the contribution of this ion in the monolayer. Nonetheless, this is not the only explanation why negatively charged nanoparticles are interacting with negatively charged PG lipids. It is clear from the obtained results that there is another driving force at play other than electrostatic interactions with counterions. One potential explanation is charge repulsion between the particles and the lipids causing the lipids
to change headgroup orientation away from the charged particles (164). This can cause a fluidizing effect to the monolayer as seen with zwitterionic DPPC with positively charged nanoparticles (165). In this case, the positive amine group of DPPC and positively charged nanoparticles are repelling each other resulting in a change in headgroup orientation that causes membrane or monolayer fluidity.

Overall, for the PG systems tested here, the strongest effects seen are for DPPG and 16-1PG > SOPG and POPG. This shows that the electrostatic repulsion effect between lipids and nanoparticles resulting in monolayer fluidization was greatest for shorter acyl chain lipids.

4.3.2 Lateral organization

Other than the changes to the membrane fluidity and elasticity seen through the pressure-area isotherms and the compression modulus, BAM was also employed to image the lateral organization of monolayers to assess the impact of nanoparticles on the film structure.

The lateral organization of DPPC systems can be seen below in figure 4.4. At the phase coexistence region in the pressure-area isotherm (Figure 4.2A), domains start to appear. These domains had a fractal-like shape at around 7 mN/m (Figure 4.4, panel 1). There are many factors that control the shape of lipid domains. Factors such as monolayer compression speed, nature of subphase (water versus buffer), and incubation time before compression play a major role. Furthermore, intrinsic factors such as the line tension between the LE and LC phases, along
with electrostatic dipole-dipole repulsion have an effect as well (103, 128). DPPC domains had a diameter of 15.8 ± 4.2 μm which coalesced into a homogenous film with further compression. This film remained homogenous until monolayer collapse. Systems that include 10:1 PLGA nanoparticles had larger domains at 7 mN/m with a diameter of 23 ± 5 μm (p-value < 0.001). Furthermore, domain frequency was greatly reduced in presence of PLGA nanoparticles at the 10:1 ratio (Figure 4.4, panel 2).

The addition of higher concentration of nanoparticles with a 1:1 ratio caused the coexistence of 3 phases (Figure 4.4, panel 3). This indicates phase demixing due to the presence of nanoparticles at the 1:1 ratio. This complemented monolayer isotherm and compression modulus data suggesting the fluidizing effect of nanoparticles at the 1:1 ratio versus the 10:1 were minimal changes were observed (Figure 4.2A and B). With further compression, at surface pressures of 10 mN/m, domains for control DPPC alone had a diameter of 13 ± 4 μm. Systems including PLGA at 10:1 ratio had larger domains with diameters of 25 ± 5 μm which further increased with the 1:1 system to 39 ± 6 μm (p-value < 0.001).

Both POPC and 16-1PC are fluid lipids, thus no domains were expected for the controls. The presence of PLGA nanoparticles at both ratios did not appear to affect the lateral organization of both lipids (Figure 4.5, 4.6).
Figure 4.4. BAM images of the lateral organization of DPPC systems on an aqueous subphase.

Panel 1) control DPPC, Panel 2) DPPC + PLGA nanoparticles at 10:1 weight ratio, Panel 3) DPPC + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 4.5. BAM images of the lateral organization of POPC systems on an aqueous subphase. Panel 1) control POPC, Panel 2) POPC + PLGA nanoparticles at 10:1 weight ratio, Panel 3) POPC + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 4.6. BAM images of the lateral organization of 16-1PC systems on an aqueous subphase.

Panel 1) control 16-1PC, Panel 2) 16-1PC + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 16-1PC + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Next the PG lipid systems were investigated starting with saturated DPPG which exhibited domains at very low surface pressures that quickly coalesced into a homogenous film at surface pressures below 10 mN/m (Figure 4.7, panel 1). At surface pressures closer to 30 mN/m, bright clusters indicative of monolayer protrusions starts to appear (Figure 4.7, blue arrows). These are signs of early collapse characteristic of DPPG monolayers as it has been recorded in the literature before (129, 130). Systems including PLGA nanoparticles at the 10:1 ratio displayed phase demixing that caused a delay in the formation of the LC domains (Figure 4.7, panel 2). Similarly, systems with 1:1 PLGA nanoparticles experienced phase demixing up to surface pressures of 12 mN/m (Figure 4.7, panel 3). This stronger demixing was seen as the concentration of nanoparticles was increased. The presence of nanoparticles caused the appearance of bright clusters imaged at the low surface pressures during phase demixing regions (Figure 4.7, red arrows) which could be attributed to lipid-nanoparticle interactions since they are not seen in the control.
Figure 4.7. BAM images of the lateral organization of DPPG systems on an aqueous subphase.

Panel 1) control DPPG, Panel 2) DPPG + PLGA nanoparticles at 10:1 weight ratio, Panel 3) DPPG + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
POPG, SOPG, and 16-1PG remained in a homogeneous LE phase throughout the entire compression with no LC domains (Figure 4.8, 4.9, and 4.10 panel 1). This was due to the fluidity of the monolayer similar to that seen for the fluid PC lipids. No change to the homogenous lateral organization of POPG was seen with the 10:1 system (Figure 4.8, panel 2). Lipid nanoparticle clusters appeared around 30 mN/m until collapse with the 1:1 ratio system (Figure 4.8, panel 3). Similar to POPG, SOPG displayed a homogenous monolayer throughout compression (Figure 4.9, panel 1). No changes occurred with system of the 10:1 ratio yet demixing was seen with the 1:1 ratio system at low surface pressures which delay homogenous film formation (Figure 4.9, panel 3).

16-1PG presented a lateral organization similar to that of SOPG (Figure 4.10). Control 16-1PG displayed a homogenous film throughout compression that was not altered for systems that include 10:1 PLGA nanoparticles. Phase demixing was seen at low surface pressures below 10 mN/m for the 1:1 system. Furthermore, clusters appeared throughout compression (Figure 4.10, panel 3 arrows).
Figure 4.8. BAM images of the lateral organization of POPG systems on an aqueous subphase. Panel 1) control POPG, Panel 2) POPG + PLGA nanoparticles at 10:1 weight ratio, Panel 3) POPG + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 4.9. BAM images of the lateral organization of SOPG systems on an aqueous subphase. Panel 1) control SOPG, Panel 2) SOPG + PLGA nanoparticles at 10:1 weight ratio, Panel 3) SOPG + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 4.10. BAM images of the lateral organization of 16-1PG systems on an aqueous subphase. Panel 1) control 16-1PG, Panel 2) 16-1PG + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 16-1PG + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
4.3.3 Surface potential measurements

Surface potential measurements as a function of the molecular area were performed to better understand the lipid nanoparticle interactions. With this technique changes to the dipole orientation of the lipid monolayer during compression due to the presence of nanoparticles can be monitored. As mentioned earlier in chapter two, the surface potential is strongly affected by the dipole orientation of the terminal CH$_3$, headgroup dipoles, and the interfacial water dipole contribution (96, 166). Changes in dipole orientation are correlated to monolayer stability (95, 167). Therefore, this technique was used as an additional important method to help assemble building blocks to further characterize lipid-nanoparticle interactions for the pulmonary drug delivery using nanoparticles.

The surface potential is measured as the difference between the electrical potential of a clean subphase and that of the lipid containing interface (168). The effect of the headgroup characteristic between the PCs and PGs and their interaction with the PLGA nanoparticles was tested here. DPPC and DPPG were chosen in order to maintain the same acyl chains and their respective dipole moment contributions to the surface potential. This allows for the characterization of the dipole moment of the headgroup and surrounding water molecules only and thus differentiate nanoparticle effects between the two lipid groups.

As the monolayer was being compressed, the van der Waals forces between the lipids increased affecting the dipole orientation. It is the dipole moment that is normal to the interface that contributes to surface potential measured here (169, 170). Therefore, as the
lipids were more aligned and ordered, the normal dipole moment contribution was increased along with the lipid phases which ultimately increased the surface potential (170). This is due to the direct relationship between the surface potential and normal dipole moment according to the Helmholtz equation (169, 171). While when the lipids were in the LE phase or the gas phase, less ordering was observed and the normal contribution of the dipole moment was lower.

Results in figure 4.11A show the surface potential-area isotherms for DPPC systems. At high molecular areas up to 100 Å²/mol, while the lipids were in the gas phase, dipole reorientation can be seen occurring over a 2 step process (Figure 4.11A, box). This was indicated by the sharp increase in the surface potential and ultimately dipole moment of the lipids in the gas phase as they are being compressed. With further compression to molecular areas of 66 Å²/mol, another sharp increase to the dipole orientation occurred as seen by the change in isotherm slope. This coincided with the pressure-area isotherm where the system entered the LC phase (Figure 4.2A). A maximum surface potential value of 281.8 mV was reached at DPPC collapse molecular areas. Systems including 10:1 PLGA nanoparticles resulted in a significant increase in the detected surface potential even before the start of monolayer compression. The starting surface potential of the system was found to be around 115.2 mV as opposed to 0 mV for the control DPPC. Furthermore, the dipole reorientation region was greatly reduced at the same molecular areas. Nonetheless, the dipole reorientation associated with the LC phase was maintained and seen at molecular areas of 68 Å²/mol. The maximum
surface potential value reached at system collapse was 322 mV. For the 1:1 system, the starting surface potential was 133 mV (Figure 4.11A). Dipole reorientation at the same high molecular areas was further reduced. The LC phase dipole reorientation can be seen yet at larger molecular areas of 75.5 Å²/mol. A maximum surface potential value of 322 mV was reached at system’s collapse. This was similar to that reached with the 10:1 system. Interestingly, the second dipole reorientation occurring close to 100 Å²/mol as seen with pure DPPC was diminished, indicating that the presence of nanoparticles and their interaction with the lipid monolayer prevented the dipole reorientation of the lipid at these areas. Nonetheless, the second dipole reorientation upon entering the LC phase at above mentioned molecular areas was maintained for both PLGA nanoparticles concentrations tested. With DPPG, the starting surface potential was -49 mV and remained constant during compression up to a molecular area of 88 Å²/mol (Figure 4.11B). Compression past this molecular area resulted in an increase in the surface potential indicative of a change in the dipole moment of the monolayer not detected in the pressure-area isotherms (Figure 4.3A). This interaction was not detected in the pressure isotherms since that technique is not as sensitive as the surface potential. A second dipole reorientation was seen at molecular areas of 60 Å²/mol indicative of the phase change from LE to LC as seen in the pressure area isotherms. The maximum surface potential reached was 120.6 mV before monolayer collapse. Systems including PLGA nanoparticles at the 10:1 ratio displayed a higher starting potential of around
10.4 mV. Dipole reorientation at large molecular areas was greatly reduced yet it occurred over multiple stages (Figure 4.11B, asterisks). The maximum surface potential reached before collapse was 135 mV which was higher than control. Systems including higher PLGA concentrations of 1:1 caused a further increase in the starting surface potential to values around 55.3 mV. Dipole reorientation regions were greatly reduced. The maximum surface potential reached was 127.2 mV.

**Figure 4.11.** Surface potential-area isotherms of A) DPPC and B) DPPG systems. PLGA nanoparticles are added at both 10:1 (red) and 1:1 (green) weight ratio to the subphase. All isotherms collected are an average of various replicates (n ≥ 3).
Results show that PLGA nanoparticles had a significant effect on the dipole orientation of both DPPC and DPPG monolayers. With DPPC, an increase in the surface potential was seen at a constant molecular area. This indicated that the interaction of PLGA nanoparticles with this lipid was further enhancing the dipole moment reorientation during compression. The dipole reorientation seen in the gas phase at molecular areas of 100 Å²/mol for control DPPC was not observed in the presence of PLGA nanoparticles, yet the measured surface potential was higher. This implied that the nanoparticles could have already changed the dipole orientation before the start of compression. Similar to the pressures-area isotherms, an increase in the surface pressures was recorded before the start of compression (Figure 4.2A). The slope of the phase change from the LE phase to the LC phase was maintained in the presence of nanoparticles similar to control which occurred at a molecular area of around 60 Å²/mol. Furthermore, the jump in the surface potential for this phase was not altered with PLGA nanoparticles. Therefore it can be concluded that the nanoparticles are affecting the dipole orientation early on in the compression from the gas phase to the LE phase only. No added effect on dipole orientation was seen for the LE-LC phase transition.
4.4 Phosphatidylcholine and Phosphatidylglycerol Systems

4.4.1 Pressure-Area isotherms and compression modulus

A binary PC system of 5.6:1 molar ratio of DPPC to POPC is equivalent to that found in human lung surfactant (83, 118). The pressure-area isotherm of this system had an overall shape similar to that of pure DPPC since it is the major component in the system (Figure 4.12A). POPC at about 15% was able to change the lipid packing characteristic of DPPC at the LE-LC coexistence region. The plateau region of DPPC was reduced to a shoulder and occurred at a higher surface pressure of around 10 mN/m versus 7 mN/m for DPPC alone. Binary PC system lifted off from the gas phase into the LE phase at molecular areas of 130 Å²/mol and collapsed at 46.6 Å²/mol at surface pressure of 43 mN/m. For systems that include PLGA nanoparticles at the 10:1 ratio, a shift to larger molecular areas was seen indicative of increased surface pressure of the film. Due to interaction and the high surface pressure of the film, the system entered the LE phase before the start of compression. The shoulder of phase coexistence was maintained at 10 mN/m. The collapse occurred at surface pressure of 44.4 mN/m at similar molecular areas as the control. For systems including higher PLGA concentration of 1:1, the isotherm started of similar to the 10:1 system but with further compression shifted to smaller areas. This indicated increased rigidity of the system due to nanoparticles. The shoulder was slightly reduced and occurred at surface pressure of 9 mN/m. Monolayer collapse was achieved at 42.5 mN/m (Figure 4.12A). The differences seen between the two nanoparticle concentration systems indicated that interactions were concentration dependent. In terms of monolayer elasticity, the dip in the modulus of phase coexistence can be seen similar to that of DPPC.
Throughout compression, this system remained in a LE phase as indicated by compression modulus peak of 77.4 mN/m which is well below 100 mN/m characteristic of an overall LC phase. Systems including 10:1 PLGA nanoparticles maintained the phase coexistence dip at around 10 mN/m. Increasing PLGA concentration to 1:1 ratio increased the fluidity of the system up to the phase coexistence region around 9 mN/m. The peak modulus reached before this point was greatly reduced from 39.7 to 22.3 mN/m. Furthermore, this peak rigidity occurred earlier than that of binary PC alone.

The next system studied was the total PC lipids to better understand their role in nanoparticle interactions. In addition to the two most commonly studied lung surfactant PCs, 16-1PC was added at an equivalent molar percentage to that of POPC (83). This more complex mixture had not been studied before and best describe the PC matrix in human lung surfactant. It entered the LE phase at a molecular area of 113 Å²/mol (Figure 4.12C). The phase coexistence region seen for DPPC and the binary PC systems was even further reduced to a small shoulder at around 12 mN/m due to the increased percentage of unsaturated PC lipids in the system (POPC and 16-1PC) of up to 26%. This system collapsed at 42 mN/m at a molecular area of 43.5 Å²/mol. The presence of nanoparticles at the 10:1 ratio caused a shift to larger molecular areas due to increased surface pressure with a lift off molecular area of 132 Å²/mol. Moreover, the nanoparticles caused a further decrease in the phase coexistence shoulder which appeared at a higher surface pressure of around 14 mN/m. The collapse pressure of this system occurred at 43.3 mN/m. With systems including higher PLGA concentrations, the shoulder was almost
diminished and reduced to a slight kink (Figure 4.12C, arrow). The collapse pressure did not change from the 10:1 system. The compression modulus profile of the PC systems can be seen in figure 4.12D. The control PC system presented a peak modulus around 82 mN/m which decreased to 77.6 mN/m with the system including PLGA nanoparticles at the 10:1 ratio. Systems including PLGA nanoparticles at the 10:1 ratio displayed lower compression modulus values up until the peak modulus indicative of increased monolayer elasticity due to nanoparticles. This increased elasticity was further enhanced with systems including higher PLGA concentrations of 1:1. In addition, the peak modulus of highest rigidity for the 1:1 system occurred at 73 mN/m which was lower than control and 10:1 system.
Figure 4.12. Pressure-Area isotherms and compression modulus of phosphatidylcholine containing systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of binary PC systems respectively, C) the pressure-area isotherm and D) compression modulus of PC system. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3)
Similar to the rationale for studying the binary PC system, the binary PG model composed of the most common lung surfactant PG lipids was studied. DPPG and POPG are present in the human lung surfactant at a mol ratio of 1:6 respectively (83, 118). The pressure-area isotherms of binary PG systems can be seen in Figure 4.13A. The shape of the isotherm was dominated by the major component POPG reaching a collapse pressure of 39 mN/m. The presence of PLGA nanoparticles at the 10:1 ratio did not result in any major deviations in the pressure-area isotherm when compared to control. Only a slight increase in the collapse pressure was seen up to 40 mN/m. With systems including higher concentration of PLGA nanoparticles of 1:1, an increase in the surface pressure occurred during early compression up to surface pressures of 10 mN/m. Further compression resulted in a decrease in the surface pressure as seen by the shift to slightly lower molecular areas indicative of increased rigidity. Overall, the effects of nanoparticles at both ratios tested show minimal effects on the shape of the pressure-area isotherm.

In terms of the monolayer elasticity of binary PG, the profile was similar to control POPG (Figure 4.3D) due to it being the major component of the system (Figure 4.13B). A dip in the modulus was seen at a surface pressure of 23 mN/m indicative of a phase coexistence region (Figure 4.13B, arrow). Systems with 10 mol % PLGA nanoparticles displayed a compression modulus profile that overlaps with that of the control until the phase coexistence region. Following this region with further compression, an increase in rigidity occurred until collapse.
The 1:1 system also displayed the increased rigidity after the coexistence region. Nonetheless, before the coexistence region, the 1:1 system had higher elasticity.

Next the total PG lipids system which contains all major PGs of the lung surfactant was tested to assess the impact of nanoparticles on this class of lipids. This system contains DPPG:POPG:SOPG:16-1PG at 1:6:4.4:2 mol ratios (83) and entered the LE phase at molecular areas of 120 Å²/mol (Figure 4.13C). The collapse pressure occurred at 40 mN/m at molecular area of 42.2 Å²/mol. For the 10:1 system, the presence of PLGA nanoparticles caused an increase in the detected surface pressure of the monolayer resulting in a shift to larger molecular areas. Therefore, the system lifted off from the gas phase into the LE phase before the start of compression. At 30 mN/m and higher, the surface pressure was similar to that of the control. Further increase in nanoparticle concentrations up to 1:1, resulted in a further increase to the surface pressure up to around 30 mN/m. Collapse pressure was not affected in the presence of nanoparticles at both ratios tested. The compression modulus profile of the PG system displayed a peak modulus of 56.7 mN/m at a surface pressure of 26 mN/m after which the system started to collapse. The presence of PLGA nanoparticles caused an increase in the system’s elasticity up until a surface pressure of 30 mN/m similar to pressure-area isotherm data. This indicates that the increase in monolayer surface pressure had a fluidizing effect on the system.
Figure 4.13. Pressure-Area isotherms and compression modulus of phosphatidylglycerol containing systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of binary PG systems, C) the pressure-area isotherm and D) compression modulus of PG system. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3)
Obtained results show the effect of electrostatic interactions of PLGA nanoparticles and PC lipids. As discussed earlier with the individual lipid systems, strongest interactions were seen with POPC > 16-1PC > DPPC. This was judged based on the effects caused by adding PLGA nanoparticles at the lowest concentration of 10:1. The binary PC system here showed minor effects at the 10:1 ratio since that system contains mostly DPPC (85%) as is expected. Increasing the level of fluid lipids, resulted in detectable effects at the 10:1 ratio by increasing the surface pressure of the monolayer. The ratio of fluid lipids in the PC system was 26% (up from 15% for the binary PC system).

Similarly, with the PG lipid containing systems, the electrostatic repulsion effects were higher with short acyl chain lipids in the following order DPPG and 16-1PG > SOPG and POPG. Therefore, the binary PG lipid which contains around 15% DPPG did not cause major changes to the pressure-area isotherms. On the contrary, the PG system which contains 22.4% of DPPG and 16-1PG results in a significant increase in the surface pressure at the lowest nanoparticle concentration of 10:1.

4.4.2 Lateral organization

Compared to pure DPPC, the presence of the fluid POPC at 15% in the binary PC system delayed the formation of domains in agreement with the delay of the phase coexistence region of the pressure-area isotherm. For the control binary PC system, domain formation occurred at around 10 mN/m with a diameter of 5.5 ± 1 μm (Figure 4.14, panel 1). With further compression at 15 mN/m, these domains increased in size up to 8.5 ± 1.6 μm. At 30 mN/m
domains displayed a diameter of 10 ± 2 μm. The presence of the more fluid POPC prevented homogenous film formation seen with DPPC at high surface pressures. This is due to packing defects induced by the presence of a larger (more fluid) lipid into a rather homogenous film of pure DPPC. This phenomena has been described in details before for the binary system of sphingomyelin and fluid POPC (124).

The presence of nanoparticles at the 10:1 weight ratio resulted in smaller domains of around 3.6 ± 0.7 μm at surface pressures of around 10 mN/m (Figure 4.14, panel 2). These domains were significantly smaller than those of the control system with a p-value < 0.001. With further compression at surface pressures of 15 mN/m and higher, the size of the domains was not significantly different from nanoparticle free binary PC. With higher concentration of PLGA nanoparticles at the 1:1 ratio, larger fractal-like domains with a diameter of 12.5 ± 2.7 at 10 mN/m were seen (p-value < 0.001) (Figure 4.14, panel 3). Further compression maintained the size of the domains yet with higher frequency. At 30 mN/m the domain increased in size up to 16 ± 3.4 μm. This size was significantly larger than control and 10:1 systems at 30 mN/m (p-value < 0.001). Furthermore, with the 1:1 system, phase demixing occurred at low surface pressures up to 5 mN/m characterized by film voids (Figure 4.14, panel 3 insets).
Figure 4.14. BAM images of the lateral organization of binary PC systems on an aqueous subphase. Panel 1) control binary PC, Panel 2) binary PC + PLGA nanoparticles at 10:1 weight ratio, Panel 3) binary PC + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The lateral organization of the PC systems are represented in figure 4.15. Similar to the binary PC model, a delay in the appearance of the phase coexistence region associated with DPPC was seen. Unlike the binary PC system, no domains were visible around 10 mN/m. Only at surface pressures of 12 mN/m and higher can they be seen. At 15 mN/m domains had a diameter of 2.8 ± 0.6 μm (Figure 4.15, panel 1). Further compression caused these domains to increase in size up to 7 ± 1 μm at 30 mN/m. Systems including PLGA nanoparticles at the 10:1 ratio resulted in domains with a diameter of 3.3 ± 0.7 μm at 15 mN/m (Figure 4.15, panel 2). These domains were significantly larger than the control system (p-value < 0.001). Nonetheless, at 30 mN/m, the domains increased in size up to 7 ± 1 μm similar to control. The presence of nanoparticles at the 1:1 ratio resulted in phase demixing at low surface pressures delaying the formation of a homogenous film as seen in control. This caused an increase in monolayer fluidity (Figure 4.15, panel 3). At surface pressures of 15 mN/m, domains with a diameter of 3.7 ± 0.4 appeared which were larger than those seen with control and 10:1 system (p-value < 0.05). With further compression at 30 mN/m the domains of 9 ± 2 were larger than the pure and 10:1 PC systems (p-value < 0.001). Additionally, bright lipid-nanoparticle clusters were seen at 30 mN/m in the 1:1 system. Figure 4.16 below shows the 3D image of the system.
Figure 4.15. BAM images of the lateral organization of PC systems on an aqueous subphase.

Panel 1) control PC system, Panel 2) PC system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) PC system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The lateral organization of the binary PG system only resulted in domains at high surface pressures close to 30 mN/m (Figure 4.17, panel 1). This was expected as it is mostly a fluid system (86%). The effect of the saturated lipid component DPPG only appeared upon compression to smaller molecular areas. This was illustrated by the fact that domains were formed at high pressures which were not seen in pure POPG. The average size of those domains was $3.1 \pm 0.5 \mu m$. There was no difference in the size of the domains or frequency upon nanoparticle addition in the 10:1 and 1:1 systems (Figure 4.17, panel 2 and 3). Consistent with the compression modulus data of high fluidity (Figure 4.13B), a delay in the formation of a homogenous LE phase at low surface pressures occurred with the 1:1 system. This is due to nanoparticle causing phase demixing in the system.
Figure 4.17. BAM images of the lateral organization of binary PG systems on an aqueous subphase. Panel 1) control binary PG, Panel 2) binary PG + PLGA nanoparticles at 10:1 weight ratio, Panel 3) binary PG + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The majority of the PG system consists of fluid lipids (92.5%) and thus domain formation was not expected to be seen during compression (Figure 4.18). The control PG system displayed a homogenous LE film that persisted until monolayer collapse. The addition of nanoparticles at both ratios tested, caused phase demixing at low surface pressures (Figure 4.18, panel 2 and 3). In the 1:1 system, bright lipid nanoparticle clusters can be seen in the BAM images and appeared throughout compression.
Figure 4.18. BAM images of the lateral organization of PG systems on an aqueous subphase.

Panel 1) control PG system, Panel 2) PG system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) PG system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
4.5 Lung Surfactant Model systems

4.5.1 Pressure-Area isotherms and compression modulus

The first complex model system tested was the 4 lipid system composed of the major and most commonly studied PC and PG lipids human lung surfactant biomimetic. It contains DPPC, POPC, DPPG, and POPG at 45:8:1:6 representing the molar lipid ratio found in human lung surfactant (83). The pressure-area isotherm of this system displayed a lift off from the gas phase at a molecular area of around 116 Å²/mol. This system reached collapse at 40 mN/m at a molecular area of 43 Å²/mol. A small shoulder was seen at 10 mN/m indicative of the LE-LC phase coexistence (Figure 4.19A, arrow). Systems including PLGA nanoparticles at the 10:1 ratio presented a pressure-area isotherm that overlapped with the control until collapse. The 1:1 system resulted in increased surface pressure of the monolayer as indicated by the isotherm shift to larger molecular areas. Furthermore, the collapse pressure was increased to 44 mN/m, an increase of 4 mN/m.

The compression modulus profile of the 4 lipid system exhibited a peak modulus of 78 mN/m at surface pressures of 27.2 mN/m (Figure 4.19B, asterisk). The presence of PLGA nanoparticles at the 10:1 ratio increased the peak rigidity of the monolayer of up to 85.8 mN/m at similar surface pressures. This system remained more rigid than control until collapse. The 1:1 system displayed increased elasticity or fluidity of the system compared to control up to a surface pressure of around 20 mN/m. The peak modulus reached was similar to that of the 10:1 system and remained rigid until collapse. A dip in the modulus profile can be seen around
surface pressures of 10 mN/m in all three 4 lipid systems which was attributed mostly to DPPC for the phase coexistence region.

As mentioned earlier in the introduction, the human lung surfactant contains around 2-8% neutral lipids such as cholesterol (80, 172, 173). Therefore, it is important to study the effects of this lipid on the system to have a better biomimetic of the human lung surfactant. The addition of cholesterol at 2% of the total weight to the 4 lipid system constitutes the 5 lipid model tested here. The pressure-area isotherm of the 5 lipid model can be seen in figure 4.19C. The sterol addition caused the system to enter the LE phase at a molecular area of 108 Å²/mol. This was followed by a sharp slope increase up to collapse. Gradual collapse was seen with this system which started from 38 to 47 mN/m over molecular areas of 60 to 52 Å²/mol (Figure 4.19C, square inset). The presence of PLGA nanoparticles in 10:1 systems caused the monolayer to lift off from the gas phase at a larger molecular area of 130 Å²/mol compared to the control (108 Å²/mol). Higher surface pressures were seen up to a molecular area of 75 Å²/mol and surface pressure of 10 mN/m. Further compression resulted in a shift to lower molecular areas indicative of lower surface pressures compared to control. This shift indicated potential loss of lipids into the subphase or increased rigidity in the monolayer. Similar trend was seen with the 1:1 system. Both systems displayed small kinks in the isotherm around surface pressures of 10 mN/m not seen in control (Figure 4.19C).
The 5 lipid film presented a peak modulus of around 141.4 mN/m indicative of a LC monolayer (Figure 4.19D). PLGA nanoparticles at both ratios tested caused a significant increase in monolayer elasticity as was seen in the decrease of compression modulus peak to 92 and 86 mN/m for 10:1 and 1:1 systems respectively. Therefore, the shift in the isotherm to lower molecular areas in the presence of nanoparticles can be attributed to loss of lipids as opposed to increased rigidity since the opposite was seen in the compression modulus. Furthermore, the kinks in the isotherm seen around 10 mN/m for phase coexistence display a minor dip in the modulus around similar surface pressures yet not as distinct as those seen with other PC containing systems. Interestingly, the isotherm kink and modulus dip were not seen in the control 5 lipid system. It appears that the rigidifying effect of the sterol was in competition with PLGA nanoparticles. It is known that cholesterol can induce a rigidifying effect to an otherwise fluid system (174-176). It does this by forcing the fluid hydrocarbon acyl chains to stack more closely together in an orderly fashion.
Figure 4.19. Pressure-Area isotherms and compression modulus of 4 and 5 lipid systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of 4 lipid systems respectively, C) the pressure-area isotherm and D) compression modulus of 5 lipid systems. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3)
Next, the complete phospholipid model (7 lipid system) was analyzed first followed by the 8 lipid system including which included cholesterol. The control 7 lipid model entered the LE phase at a molecular area of 115 Å²/mol (Figure 4.20A). A kink can be seen at a surface pressure of 12 mN/m indicative of phase coexistence. This system collapsed at 40.8 mN/m at a molecular area of 42.8 Å²/mol. The presence of PLGA nanoparticles at the 10:1 ratio caused an earlier lift off at a molecular area of 130 Å²/mol. A higher surface pressure was experienced until the kink of phase coexistence region at 12 mN/m. It is where the isotherm overlapped with the control until system collapse. Increasing PLGA concentration resulted in a further increase in the surface pressure experienced in the monolayer all the way until collapse which occurred slightly higher than control at 42.4 mN/m (compared to 40.8 mN/m). The compression modulus profile of the 7 lipid system displayed the dip for the phase coexistence region at around 12 mN/m with a peak modulus of around 75 mN/m indicative of a LE phase (Figure 4.20B). The presence of PLGA nanoparticles increased the elasticity of the system up to the peak modulus point which was similar to that of control.

The addition of 2% by weight cholesterol did not affect the shape of the isotherm significantly when compared with the 7 lipid system (Figure 4.20C). The kink seen with the 7 lipid system around 12 mN/m was diminished in the presence of 2% by weight cholesterol. The compression modulus data showed that the 8 lipid system was slightly more rigid with a peak modulus value of around 90 mN/m versus 75 mN/m for the 7 lipid system. This was expected
due to the presence of cholesterol disrupting the monolayer packing (Figure 4.20D). Table 4.2 below compares the collapse pressure of the 8 lipid systems and their significance.

**Table 4.2.** Statistical analysis on the collapse pressures of the 8 lipid systems. Two tailed student t-test

<table>
<thead>
<tr>
<th></th>
<th>8 lipid + PLGA 10:1 (40.8 ± 0.1 mN/m)</th>
<th>8 lipid + PLGA 1:1 (42.4 ± 0.5 mN/m)</th>
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<tr>
<td><strong>8 lipid</strong></td>
<td>Non-significant</td>
<td>Significant</td>
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<tr>
<td>(40.8 ± 0.1 mN/m)</td>
<td></td>
<td>p value ≤ 0.05</td>
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Figure 4.20. Pressure-Area isotherms and compression modulus of 7 and 8 lipid systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of 7 lipid systems respectively, C) the pressure-area isotherm and D) compression modulus of 8 lipid systems. All systems show results for lipid controls (Blue) and in the presence of PLGA nanoparticles at the 10:1 (Red) and 1:1 ratio (Green). All isotherms collected are average of various replicates (n ≥ 3)
4.5.2 Lateral organization

The lateral organization of the 4 lipid system exhibited the formation of domains at surface pressures of around 10 mN/m, which was consistent with the shoulder seen in the pressure-area isotherm (Figure 4.19A). These domains had a diameter of 4.2 ± 1 μm (Figure 4.21, panel 1). Upon further compression, at surface pressure of 15 mN/m, 2 domain populations appeared. One population with a diameter of 2.8 ± 0.5 μm and another with 9.2 ± 1.2 μm. At 30 mN/m with smaller molecular areas, domains had a diameter of 8.7 ± 1.3 μm with higher frequency. These domains remain until collapse.

Systems including PLGA nanoparticles at the 10:1 ratio induced domains that were slightly bigger at 10 mN/m with a diameter of 6 ± 1.3 μm (p-value < 0.001) (Figure 4.21, panel 2). At 15 mN/m, these domains increased in size to a diameter of 8 ± 1.5 μm. Unlike the control system, no distinct two populations were seen in this system. Interestingly, at 30 mN/m, a high frequency of the protruding lipid nanoparticle clusters were seen as indicated by the increased brightness. For the 1:1 system, at low surface pressures phase demixing can be seen as film voids that delayed the formation of a more homogenous film compared to the control system (Figure 4.21, panel 3). At 10 mN/m, domains started to appear with a diameter of 12.6 ± 3.6 μm which were larger than control of 4.2 ± 1 μm (p-value < 0.001). Two populations of domain sizes appeared at 15 mN/m; one with a diameter of 4.11 ± 1 μm and another with a diameter of 18 ± 2.4 μm. At 30 mN/m and similar to the 10:1 system high ratio of lipid nanoparticle clusters can be seen indicative of stronger effects of the nanoparticles with this system.
Figure 4.21. BAM images of the lateral organization of 4 lipid systems on an aqueous subphase.

Panel 1) control 4 lipid system, Panel 2) 4 lipid system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 4 lipid system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The addition of cholesterol to the 4 lipid system resulted in the formation of very small domains at 12 mN/m that persisted until monolayer collapse with a diameter of 6.3 ± 1.2 μm (Figure 4.22, panel 1). The addition of PLGA nanoparticles at the 10% weight ratio maintained the small domains seen in the control system in addition to few larger domains with a diameter of 26.4 ± 6 μm. At 30 mN/m the larger domains disappeared and smaller domains similar to control were seen with diameters of 6.3 ± 1.2 μm (Figure 4.22, panel 2). When nanoparticles were added at the 1:1 ratio, the fluidizing effect of the nanoparticles can be seen in the monolayer by the delay in the formation of the more homogenous LE phase. Phase demixing as film voids occurred at low surface pressures (Figure 4.22, panel 3). At 30 mN/m, strong lipid nanoparticle clusters can be seen resulting in monolayer protrusions (Figure 4.23).
Figure 4.22. BAM images of the lateral organization of 5 lipid systems on an aqueous subphase.

Panel 1) control 5 lipid system, Panel 2) 5 lipid system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 5 lipid system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
In the more complex 7 lipid system, the domains formed at 15 mN/m had a diameter of 3.61 ± 0.6 μm (Figure 4.24, panel 1). Systems including PLGA nanoparticles at similar surface pressures did not result in significant changes to the lateral organization. Further compression to 30 mN/m resulted in larger domains with a diameter of 7 ± 1.2 μm that did not change in the presence of nanoparticles. Phase demixing and film voids were seen with the 1:1 system at low surface pressures below 10 mN/m, consistent with most previous results seen at this nanoparticle ratio (Figure 4.24, panel 3).
Figure 4.24. BAM images of the lateral organization of 7 lipid systems on an aqueous subphase.

Panel 1) control 7 lipid system, Panel 2) 7 lipid system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 7 lipid system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The addition of cholesterol to the 7 lipid system constitutes the final and most complex 8 lipid model system. The presence of cholesterol resulted in a more homogenous LE film throughout compression (Figure 4.25). Nanoparticles delayed the formation of the LE phase at both ratios tested (10:1 and 1:1) through phase demixing. Regions of LC domains as well as lipid nanoparticle clusters can be seen with the 10:1 system up to 10 mN/m (Figure 4.25, panel 2). Demixing and film voids occurred with the 1:1 system before 10 mN/m.
Figure 4.25. BAM images of the lateral organization of 8 lipid systems on an aqueous subphase.

Panel 1) control 8 lipid system, Panel 2) 8 lipid system + PLGA nanoparticles at 10:1 weight ratio, Panel 3) 8 lipid system + PLGA nanoparticles at the 1:1 ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
**4.5.3 Surface potential measurements**

Surface potential-area isotherms of the final 8 lipid system was analyzed as seen in Figure 4.26. This system contained about 82% PC lipids and thus one could assume that results would be similar to that of pure DPPC system measured above figure 4.11A. The control 8 lipid system showed a sharp increase in the surface potential upon compression up to molecular areas of around 125 Å²/mol. This increase due to dipole reorientation of the monolayer occurred early in compression before any detectable changes were registered in the pressure-area isotherms due to the high sensitivity of the technique (Figure 4.20C). Further compression to this system resulted in minor changes to the dipole orientation up until a maximum surface potential value of around 160 mV at monolayer collapse.

Systems including PLGA nanoparticles at the 10:1 ratio caused the starting surface potential of the monolayer to increase from 0 mV to 68.2 mV. Dipole reorientation potentially occurred before the start of monolayer compression as was seen by the increased starting surface potential at comparable molecular areas. In addition, the maximum surface potential reached at collapse was higher than control reaching 188 mV. For the 1:1 system, the starting surface potential was similar to that of the 10:1 system reaching a maximum surface potential slightly lower at 171 mV.

The maximum surface potential reached with the 8 lipid system was much lower than that of control DPPC (160 vs. 281.1 mV). This can be explained by the presence of PG lipids in the system. Pure DPPG had a surface potential value of -49 mV before the start of compression.
and reached a maximum potential of 120.6 mV (Figure 4.11B). DPPG at high molecular areas when lipids are far apart from each other in the gas phase, experience a negative dipole moment pointing from the monolayer (-) into the water (+) and thus display a negative surface potential (177, 178). The dipole moment orientation was altered with further compression since positive surface potential values were obtained which correspond to dipole values pointing from the monolayer (-) into the air (+) (169, 171) (Figure 4.11B). Nonetheless, the maximum surface potentials reached with DPPG are lower than that of DPPC which has a dipole moment directed from the monolayer (-) to the air (+) throughout compression.

Therefore, since the 8 lipid system contain a fair amount of negatively charged lipids, the reduction in the maximum surface potential from DPPC was expected. Overall, the surface potential of the lung surfactant controls the stability of the system (167). It controls whether airborne particles and ultimately drug delivery particles can pass through into the blood stream and which cannot (179). Furthermore, the lateral organization depends on the surface potential. The dipole orientation in the monolayer can dictate formation and shape of LC domains (128, 180). Therefore, the surface potential measurements in addition to the surface pressure isotherms are important for analyzing the interaction of nanoparticle with the lung surfactant.
Figure 4.26. Surface potential-area isotherms of 8 lipid systems. PLGA nanoparticles are added at both 10:1 (red) and 1:1 (green) weight ratio to the subphase. All isotherms collected are an average of various replicates (n ≥ 3).
4.6 Chapter summary and conclusions

Overall, PLGA nanoparticles had a fluidizing effect to all 3 individual PC lipids in the following order; POPC > 16-1PC > DPPC. This indicated that PLGA nanoparticles had a stronger effect on fluid PC lipids than saturated lipids. With individual PG lipids, rigidifying effects occurred upon interaction with nanoparticles in DPPG and 16-1PG more so than POPG. The trend seen with individual PGs was as follows; DPPG and 16-1PG > SOPG and POPG. In this case, due to the added charge repulsion between the negatively charged nanoparticles and PG lipids, stronger effects were seen with shorter acyl chain lipids than longer ones.

The complex 4 lipid model system showed a rigidifying effect due to the presence of cholesterol. However, when nanoparticles were present, the system displayed characteristics similar to that without cholesterol. This is an interesting finding that may suggest the opposite effects of both components (cholesterol and PLGA nanoparticles).

For purposes of drug delivery, it is crucial to study the effects of nanoparticles on the surface potential of the lung surfactant and cell membranes and how it can affect nanoparticle drug deposition. Surface potential of the lung surfactant directly controls the lateral organization and domain formation. Any disturbance to the lateral organization can affect the stability of the system as seen in the above results. Therefore, understanding how the nanoparticles impact the surface potential of monolayers can be useful for designing optimal drug delivery systems that depend greatly on the barrier system in study (124, 148, 167).
Results show that in DPPC, DPPG, as well as the complex 8 model system, the presence of PLGA nanoparticles significantly increased the surface potential of the monolayers altering the lateral organization as also evident from the BAM images. This indicates the potential negative effects of PLGA nanoparticles stabilized with Pluronic acid for the pulmonary drug delivery. Whether the negative effects are solely due to PLGA or the stabilizer needs to be further studied.

Another interesting outcome displayed through the BAM images is the effect of PLGA nanoparticles at high surface pressures. Both 4 and 5 lipid systems displayed bright lipid-nanoparticle clusters that were not seen in the other two more complex systems (7 and 8). From both the compressibility isotherms and lateral organization, nanoparticles caused significant changes to 4 and 5 lipid systems. A significant rigidifying effect due to nanoparticles was seen with the 4 lipid systems. With the 5 lipid system, a strong fluidizing effect was seen in addition to the competitive effect between cholesterol and PLGA as mentioned above. However, with the 7 and 8 systems, no clusters were seen at high surface pressures and no significant changes occurred to the monolayer’s elasticity. Therefore, these results could suggest the importance of having a more representative model (better mimic) to study effects of nanoparticles. A good mimic system can be more informative on lipid-nanoparticle interaction than a simpler model which can not be considered a true model. However, the changes in the lateral organization seen at low surface pressures with 7 and 8 lipid systems due to nanoparticles indicate the potential toxic effects of PLGA nanoparticles. This indicates the
importance of having a good mimic system and that the mode of interaction can be different between a simple mimic and a more representative system.
Chapter Five: **Biophysical analysis on the interaction of gelatin nanoparticles with model lung surfactant system**

### 5.1 Background and Objectives

In this chapter, the effects of gelatin nanoparticles on the stability of biomimetic lung surfactant models were investigated similar to previous chapter on PLGA nanoparticles. Gelatin nanoparticles were chosen as they are biodegradable and biocompatible, which are two important characteristics for drug delivery vehicles as outlined in the introduction. Furthermore, they are natural polymers obtained from collagen and thus possess low antigenicity (32).

Stability measurements included pressure-area isotherms, surface potential-area isotherms and visualization of the monolayer’s lateral organization. Similar to the previous PLGA chapter, measurements were first conducted on individual lipid systems before analyzing complex models. This approach allows identifying both individual and cumulative contributions of the different lipid headgroups (PC vs PG) and acyl chain structure (saturated vs. monounsaturated) on the interactions with gelatin nanoparticles. In this chapter, the interaction of gelatin nanoparticles with a biomimetic model of the human lung surfactant and its components are investigated. Factors dictating the interactions are identified.
5.2 Nanoparticle Characterization

Gelatin nanoparticles were synthesized through a double desolvation method which was further optimized by previous fellow graduate student (Dr. Patrick Lai). This method resulted in nanoparticles with a diameter of 110 nm with a polydispersity index (PDI) of 0.2. This PDI value indicates that the particles are mostly monodisperse in solution (156). The nanoparticles had a zeta potential value of +17 mV at pH 7.0. The slight positive charge of the particles introduces stability in an aqueous suspension.

5.3 Individual Lipid Systems

5.3.1 Pressure-Area isotherms and Compression modulus

The effect of gelatin nanoparticles on the stability of the lung surfactant mimic was first tested in individual lipid systems that constitute the majority of the human lung surfactant. The first class were the PCs which correspond to about 70% of the total lipid of the human lung surfactant by weight (71, 80). The pressure-area isotherm of DPPC in the absence and presence of gelatin nanoparticles can be seen in figure 5.1A. Gelatin nanoparticles were co-deposited at the interface along with the lipid sample at a 10:1 weight ratio of lipid to nanoparticle. Control DPPC lifted off from the gas phase at a molecular area of 98 Å²/mol and entered the LE phase. This take off area was different than that seen for PLGA nanoparticle studies due to the different size of the Langmuir trough used (720 vs. 200 cm²). Phase coexistence between the LE and LC phases appeared through the plateau region at a surface pressure of around 7 mN/m.
This occurred over the range of molecular areas between 77 and 57 Å²/mol. With further compression, DPPC monolayers collapsed into multilayer structures at a molecular area of 37 Å²/mol (181, 182) and a surface pressure of around 58 mN/m. Systems including gelatin nanoparticles at the 10% weight ratio resulted in a slightly earlier lift off at around 105 Å²/mol (Figure 5.1A). This greater molecular area indicated interactions of gelatine nanoparticles with lipids resulting in larger areas and slight monolayer fluidization. Upon further compression of DPPC + gelatin nanoparticle system, a shoulder was observed between 8 and 16 mN/m which exhibited a steadily increasing slope in contrast to the flat plateau seen in DPPC alone. This drastic change in the slope of the isotherm corresponded to phase coexistence between LE and LC phases. The system collapsed at a surface pressure of 56 mN/m and at a molecular area of 30 Å²/mol (Figure 5.1A). The limited pressure reduction by just 2 mN/m compared to pure DPPC suggested a minor effect of the nanoparticles on film stability. In addition, smaller overall areas were observed in the presence of the nanoparticles at surface pressures above 20 mN/m due to particle induced packing or potential clustering of lipids. At a surface pressure of 30 mN/m, the shift was from a molecular area of 45.8 to 42.6 Å²/mol (Figure 5.1A). The compression modulus of DPPC presented a modulus dip at surface pressures of 7 mN/m indicative of phase coexistence region (Figure 5.1B, purple arrow). With further compression, at surface pressures of 20 mN/m, a plateau region was seen in the modulus profile (Figure 5.1B, purple asterisk). This correlated with the formation of a homogenous LC phase as was indicated by the modulus values above 100 mN/m (97-99). The peak modulus of highest rigidity was at a surface pressure of 30 mN/m with a value of 240 mN/m. In the presence of gelatin nanoparticles, the dip for
phase coexistence was shifted to larger surface pressures at around 15 mN/m (Figure 5.1B, orange arrow). Additionally, the monolayer fluidity was much higher compared to control DPPC as indicated by the compression modulus data (Figure 5.1B) since lower compression moduli correspond to a more fluid monolayer.

The effect of gelatin nanoparticles on the second class of PCs can be seen in Figure 5.1C. As mentioned earlier, POPC has one 18 carbon acyl chain with a cis double bond in position 9. The other acyl chain has 16 carbons and is fully saturated. The unsaturated acyl chain resulted in increased fluidity to the system compared to DPPC. With compression, POPC existed in the gas phase until a molecular area of around 115 Å²/mol. This area was larger than that of DPPC due reduced packing in the more fluid films. Upon further compression, POPC entered the LE phase and remained there until collapse as confirmed by modulus values below 100 mN/m throughout compression (Figure 5.1D). POPC collapsed at a surface pressure of around 48 mN/m and at a molecular area of 41 Å²/mol. When gelatin nanoparticles were co-deposited, no change to the shape, slope or final collapse pressure was seen. Nonetheless, collapse started earlier in compression starting at 40 mN/m until a final collapse pressure similar to control at 48 mN/m at 41 Å²/mol was reached. The compression modulus profile of the POPC + gelatin nanoparticle system maintained the same peak modulus as the control at around 96 mN/m at surface pressures of 30 mN/m. However, the slope of collapse after peak compression was sharper with gelatin nanoparticles indicating faster collapse.
Figure 5.1. Pressure-Area isotherms and compression modulus of phosphatidylcholine lipids on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of DPPC systems respectively. C) the pressure-area isotherm and D) compression modulus of POPC systems. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
Next PGs were analyzed, constituting the second largest class of lipids in human lung surfactant \((71, 80)\). DPPG entered the LE phase at a molecular area of around 55 \(\text{Å}^2/\text{mol}\) (Figure 5.2A). After Compression to an area of 45 \(\text{Å}^2/\text{mol}\) and a surface pressure of 4 mN/m, the DPPG monolayer entered into the LC phase until monolayer collapse at 57 mN/m. In the presence of nanoparticles, DPPG entered the LE phase at a slightly earlier lift off area of 59 \(\text{Å}^2/\text{mol}\) (earlier than control by 4 \(\text{Å}^2/\text{mol}\)). The nanoparticles also caused a decrease in the stability of DPPG films indicated by a collapse pressure reduction of 7 mN/m. The control DPPG displayed a compression modulus profile with a plateau around surface pressures of 35 mN/m followed by a sharp increase in modulus slope (Figure 5.2B, purple arrow). The peak modulus value reached 240 mN/m at surface pressure of 30 mN/m. In the presence of gelatin nanoparticles, the compression profile shifted to lower values indicating increased fluidity. Furthermore, in addition to the plateau seen with control at 25 mN/m indicative of collapse, another plateau occurred earlier at surface pressure of 10 mN/m (Figure 5.2B, orange arrow). This potentially could be due to lipid nanoparticle interactions.

The shape and slope of POPG isotherm was similar to that of POPC, as to be expected for a partially unsaturated and thus fluid lipid. POPG entered the LE phase at 115 \(\text{Å}^2/\text{mol}\) and the isotherm displayed a constant slope up to surface pressure of 25 mN/m (Figure 5.2C). Following this, the isotherm slope increased until collapse pressure was reached at 48 mN/m at a molecular area of 25.4 \(\text{Å}^2/\text{mol}\). Gelatin nanoparticles did not change the lift-off area but with further compression caused the isotherm to shift to smaller areas above surface pressures of 20
mN/m. Positively charged gelatin nanoparticles are presumably interacting with the negatively charged headgroup of POPG and may remove a limited number of lipids resulting in the moderate shift in area (44.3 to 41 Å²/mol at surface pressure of 30 mN/m). Nevertheless, the particle impact on POPG was limited as both systems collapsed at 47 mN/m (Figure 5.2C). The compression modulus profile of control POPG displayed a dip where the slope of the moduli changed with further compression (Figure 5.2D, purple arrow). This occurred around a surface pressure of 23 mN/m complementing the change in isotherm slope. Following this, the highest rigidity of the system was reached at surface pressures of 35 mN/m with a maximum modulus of 61.6 mN/m. Similar to previous systems, the gelatin nanoparticles caused an increase in system’s elasticity by the decreased modulus profile values. The peak rigidity was also reduced to 44.8 mN/m (a reduction of 16.8 mN/m) and occurred at similar surface pressures of 35 mN/m.
Figure 5.2. Pressure-Area isotherms and compression modulus of phosphatidylglycerol lipids on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of DPPG systems respectively. C) the pressure-area isotherm and D) compression modulus of POPG systems. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
5.3.2 Lateral organization

The effect of gelatin nanoparticles on the lateral organization of DPPC monolayers is shown in figure 5.3. The observed fractal-like domains of DPPC in the plateau region (~7 mN/m) characteristic of the LE-LC phase co-existence started to coalesce at 15 mN/m resulting in homogenous film at higher surface pressures (Figure 5.3, panel 1). When gelatin nanoparticles were co-deposited, smaller fractal-like domains started to form at the shoulder region of the isotherm (Figure 5.1A) at surface pressures around 10 mN/m (Figure 5.3, panel 2). The gelatin nanoparticles significantly delayed the formation of domains. Control DPPC resulted in domains with an average diameter of 15 ± 2.5 μm in diameter. With the gelatin containing systems, the domains significantly reduced to half the size to 7.6 ± 1.4 μm (p-value < 0.0001). The interaction of nanoparticles with DPPC lipids appeared to increase the line tension between the LE and LC phases favouring smaller and less fractal-like domains (103, 128). Furthermore, the nanoparticles delayed the coalescence of the monolayer above surface pressures of 15 mN/m and were still visible at 30 mN/m (Figure 5.3, panel 2). Further interactions of the nanoparticles with DPPC were also indicated by the presence of bright spots or lipid-nanoparticle clusters that are seen either in the center or along the edges of the LC domains (Figure 5.3, panel 2 red arrows). These clusters started at low surface pressures and continued until collapse with increasing frequency. These bright clusters are protrusions from the monolayer as evidenced by the increased grayscale intensity in figure 5.4 which provide a qualitative analysis of the monolayer thickness.
Figure 5.3. BAM images of the lateral organization of DPPC systems on an aqueous subphase. Panel 1) control DPPC, Panel 2) DPPC + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 5.4. BAM images and 3D analysis of DPPC systems. A) pure DPPC at 7 mN/m, B) DPPC with gelatin nanoparticles at 10% weight ratio at 10 mN/m.
The more fluid unsaturated POPC did not exhibit LC domain formation as was expected thus the monolayer appeared as a homogenous LE film throughout compression (Figure 5.5, panel 1). However, the gelatin nanoparticles resulted in the formation of bright clusters that appeared at low surface pressures of 5 mN/m and were observed until collapse with increasing frequency (Figure 5.5, panel 2 red arrows and inset). Furthermore, the nanoparticles disturbed the lateral homogeneity of the film. Three distinct features can be seen at 30 mN/m: 1) a fluid LE phase (dark grey area), 2) rigid LC phase (lighter grey), and 3) lipid-nanoparticle cluster protrusions (Figure 5.5, panel 2). These clusters localized within the LC region similar to what was seen with DPPC systems in presence of gelatin nanoparticles (Figure 5.3, panel 2).
Figure 5.5. BAM images of the lateral organization of POPC systems on an aqueous subphase. Panel 1) control POPC, Panel 2) POPC + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Similarly, the effect of gelatin nanoparticles on the lateral organization of individual PGs was investigated. BAM images of the pure saturated DPPG showed the formation of LC domains at low surface pressures that quickly coalesced into a homogenous film at around 10 mN/m (Figure 5.6, panel 1). At high surface pressures of around 30 mN/m, bright clusters and monolayer defects in the form of film voids started to appear. These multilayered formations are an indication of an early ‘start of collapse’ and have been previously reported in the literature for DPPG (129, 130). The presence of gelatin nanoparticles expedited the development of these bright clusters starting at very low surface pressures of around 10 mN/m (Figure 5.6, panel 2 arrows and inset). Gelatin nanoparticles increased the fluidity of the monolayer as seen in the compression modulus profile (Figure 5.2C) and thus those rigid lipid nanoparticle clusters increase the fluidity of the system overall. Opposite to PC systems, these clusters appeared to form at the edges of the PG domains, more so with the LE phase.
Figure 5.6. BAM images of the lateral organization of DPPG systems on an aqueous subphase. Panel 1) control DPPG, Panel 2) DPPG + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Similar to POPC, POPG is a fluid system with mono-unsaturation that does not result in formation of LC domains (Figure 5.7, panel 1). The film’s lateral organization remained homogenous throughout compression. When nanoparticles were present, lipid-nanoparticle clusters started to appear at around 5 mN/m and increased in frequency with further compression. Darker structures appeared that correspond to voids in the monolayer that indicate packing problems that could lead to early collapse (Figure 5.7, panel 2 insets and arrows). Similar to DPPG, while the voids in the film were an indication of early onset of collapse however, unlike DPPG the actual collapse pressure of the compression isotherm appeared to be unaffected (Figure 5.2).
Figure 5.7. BAM images of the lateral organization of POPG systems on an aqueous subphase. Panel 1) control POPG, Panel 2) POPG + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The effect of gelatin nanoparticles was more drastic with individual PG lipids versus PC lipids. From the pressure-area isotherms, a significant decrease in the collapse pressure was seen for DPPG of 7 mN/m. With individual DPPC, collapse occurred gradually yet ultimately reaching the same final pressure. The lateral organization show the formation of bright clusters that were more significant with DPPG than DPPC. With unsaturated lipids, these clusters appeared early in the compression and continued until collapse with POPG. In POPC systems, these clusters only appeared at high surface pressures. This indicated the significant role of electrostatic attraction between the positively charged gelatin nanoparticles and negatively charged lipids. Furthermore, the preferential interaction of the nanoparticle with lipids was found to be phase dependent. Lipid nanoparticle clusters with both individual PCs was found within LC domains. The opposite was seen with PG lipids where the clusters formed at the LE phase in both DPPG and POPG.

The role of lipid saturation dictating interactions can be seen as well. The elasticity of saturated DPPC and DPPG was significantly enhanced as seen in the compression modulus profiles more so than with the unsaturated POPC and POPG. This helps further characterize the mode of interaction of nanoparticles with lipid systems. Increased elasticity was also seen with unsaturated lipids yet to a lesser extent. Therefore, the interaction of gelatin with at least these 4 lipids can be attributed to a fluidizing effect. This explains the large fluidization effect seen in rigid lipids than already fluid lipids such as POPC and POPG.
5.4 Phosphatidylcholine and Phosphatidylglycerol Systems

5.4.1 Pressure-Area isotherms and Compression modulus

Similar to the study with PLGA nanoparticles in chapter 4, the effects of gelatin nanoparticles on the binary PC system was also tested. The addition of 15% mol ratio of POPC had a strong impact on the behaviour of DPPC monolayer and the characteristic plateau of DPPC was only maintained as a shoulder at higher surface pressures (Figure 5.8A, purple arrow). Control binary PC started to collapse at a surface pressure of 48.4 mN/m to a final collapse pressure of 50.7 mN/m. The presence of gelatin nanoparticles at a 10:1 lipid to nanoparticle weight ratio resulted in only minor deviations of the isotherm suggesting limited destabilization. Both systems lifted off from the gas phase at a molecular area of 110 Å²/mol. This falls between the lift off areas of DPPC and POPC (98 and 115 Å²/mol respectively) which was expected due to the mixture. The collapse pressure of gelatin nanoparticle containing system was similar to that of control occurring between surface pressures of 48.4 to 50.7 mN/m. The compression modulus profile of control binary PC showed a dip at surface pressure of 10 mN/m corresponding to the phase coexistence region (Figure 5.8B, purple arrow). This system reached a peak compression modulus of 136.2 mN/m at a surface pressure of 30 mN/m. Systems including gelatin nanoparticles decreased the compression modulus peak from 136.2 to 124.6 mN/m. Between surface pressures of 10 mN/m and 35 mN/m, gelatin containing systems were more elastic than control.
The next system tested were the total PC lipids. In addition to DPPC and POPC, 16-1PC was included to test the effect of gelatin on the major PC lipids. This system was composed of 5.6:1:1 for DPPC:POPC:16-1PC molar ratio (83). The phase coexistence plateau region seen with DPPC and binary PC system was further reduced and shifted to a higher surface pressure of around 13 mN/m due to the increased percentage of fluid lipids in the system (Figure 5.8C, purple arrow). The lift off area of the PC system was similar to the binary PC system and occurred at 110 Å²/mol. Control PC system collapsed at 42 mN/m at a molecular area of 42.8 Å²/mol. The presence of gelatin nanoparticles along with the PC system resulted in a shift to larger molecular areas suggesting particle interactions with the monolayer. The lift off area occurred at 124 Å²/mol (Figure 5.8C). However, collapse pressure was not affected and occurred at a similar surface pressure as control at 42 mN/m. In terms of monolayer elasticity, a dip in the modulus for phase coexistence can be seen for the control system at surface pressures of 13 mN/m (Figure 5.8D, purple arrow). The peak modulus of this system occurred at 82 mN/m at a surface pressure of 26.7 mN/m. This peak rigidity was much lower compared to binary PC (82 vs. 136.2) as was expected due to increased ratio of fluid lipids (16-1PC). The presence of gelatin nanoparticles caused the monolayer to reach the peak rigidity early at a surface pressure of 22 mN/m compared to 26.7 mN/m of control although the collapse pressure of 42 mN/m was not affected. This showed gradual collapse as was also seen in the pressure area isotherm (Figure 5.8C) where the final collapse was gradual indicative of a stabilizing effect.
Figure 5.8. Pressure-Area isotherms and compression modulus of phosphatidylcholine containing systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of binary PC systems respectively. C) the pressure-area isotherm and D) compression modulus of PC systems. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
Similarly, the effect of gelatin on the binary PG and the overall lung surfactant PG system were analyzed. The pressure-area isotherms of the binary PG system composed of 1:6 mol ratio of DPPG to POPG with and without gelatin nanoparticles are shown in figure 5.9A. The control binary PG system entered the LE phase at a molecular area of 110 Å²/mol. A shoulder-like inflection or a kink in the isotherm was seen at around 20 mN/m corresponding to the LE-LC phase coexistence. The system collapsed at a surface pressure of 48 mN/m at a molecular area of 20 Å²/mol. When the nanoparticles were present, the effects on the shape of the isotherm were minor. The inflection point kink of phase coexistence occurred later in the compression at surface pressures of 23 mN/m (Figure 5.9A, purple arrow) versus 20 mN/m of the control system. The elasticity of both systems was similar up to surface pressures of 20 mN/m (Figure 5.9B). Following this dip, the system with gelatin nanoparticles displayed more elastic properties than the control.

Next the overall PG model was analyzed which contains DPPG:POPG:SOPG:16-1PG at 1:6:4.4:2 mol ratio corresponding to the major PG lipids of the lung surfactant. The addition of SOPG and 16-1PG at ~33% and 15% mol ratios respectively introduced fluidity to the system. The control PG system entered the LE phase at a molecular area of 120 Å²/mol and collapsed at 42 Å²/mol and 39.3 mN/m (Figure 5.9C). In the presence of gelatin nanoparticles, the system entered the LE phase at 135 Å²/mol and collapsed at 30 mN/m and 43 Å²/mol. This decrease in collapse pressure was the most significant effect of gelatin nanoparticles seen. The reduction in the collapse pressure from 42 mN/m for controls to 30 mN/m and increased fluidity (Figure
5.9D) were an indication of the destabilization effect of the nanoparticles to the system. Packing changes were introduced by the nanoparticles to PG lipids consisting mostly of unsaturated lipids of different acyl chain lengths and saturated DPPG.

**Figure 5.9.** Pressure-Area isotherms and compression modulus of phosphatidylglycerol containing systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of binary PG systems respectively. C) the pressure-area isotherm and D) compression modulus of PG systems. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
5.4.2 Lateral organization

Figure 5.10 shows the lateral organization of the binary PC systems with and without gelatin nanoparticles at 10% weight ratio. Since this system contained 15% of the fluid POPC, it was expected that there will be a delay in domain formation characteristic of saturated lipids. Indeed, domain formation started at a surface pressure close to 10 mN/m versus 7 mN/m for pure DPPC (Figure 5.10, panel 1). At 15 mN/m, these domains had a diameter of 14 ± 3 μm. The domains remained intact until monolayer collapse at 50.7 mN/m. No mixing of the phases was seen during compression indicating the high line tension between the two different phases. The gelatin nanoparticles decreased the domain diameter to 8 ± 1.4 μm at 15 mN/m with a p-value < 0.0001 (Figure 5.10, panel 2). With further compression of gelatin containing system, the domains coalesced resulting in a homogenous film at 30 mN/m. Furthermore, lipid-nanoparticle cluster were seen around 10 mN/m that continued until monolayer collapse at 50.7 mN/m. These clusters appeared within or at the edges of LC domains (Figure 5.10, panel 2 red arrows and inset). In addition, systems including gelatin nanoparticles displayed film void defects at high surface pressures leading to collapse (Figure 5.10, panel 2 blue inset at 30 mN/m).
Figure 5.10. BAM images of the lateral organization of binary PC systems on an aqueous subphase. Panel 1) control binary PC, Panel 2) binary PC + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The lateral organization of the PC system with and without gelatin nanoparticles is shown in Figure 5.11. Due to the increased molar percentage of fluid lipids (26% versus the 15% for the binary PC system), an even further delay in domain formation was seen. These domains started to appear at surface pressures close to 15 mN/m (Figure 5.11, panel 1). Moreover, domains had a diameter of $2.8 \pm 0.6 \, \mu m$. As the molecular area was decreased with further compression, domain size increased up to $7 \pm 1 \, \mu m$ at 30 mN/m and remained intact until monolayer collapse at 42 mN/m. PC system including gelatin nanoparticle displayed lipid nanoparticle clusters earlier in compression at surface pressures below 5 mN/m that continued until collapse (Figure 5.11, panel 2 red arrows and inset). Similar to all PC systems analyzed thus far, these gelatin nanoparticle-induced clusters were either on the edge or within the LC domains. Furthermore, the nanoparticles caused some defects in the monolayer in the form of black void-like regions (Figure 5.11, panel 2 blue arrow and inset at 30 mN/m). These areas are an indication of packing defects caused by the nanoparticles. Domain size at 15 mN/m was significantly larger than control with a diameter of $3.7 \pm 0.6 \, \mu m$ at 15 mN/m versus $2.8 \pm 0.6 \, \mu m$ (p-value < 0.0001). Similarly, at 30 mN/m the domain diameter increased to $9.5 \pm 2.4 \, \mu m$ at 30 mN/m in the presence of gelatin nanoparticles compared to $7 \pm 1 \, \mu m$ of control (p-value < 0.0001).
Figure 5.11. BAM images of the lateral organization of PC systems on an aqueous subphase. Panel 1) control PC system, Panel 2) PC system + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The PG analysis was started with the binary PG system containing about 85% unsaturated POPG and thus no domains were seen at low surface pressures (Figure 5.12, panel 1). Domains mostly enriched in DPPG only appeared at smaller molecular areas upon further compression closer to 30 mN/m. These domains were smaller and more branched in shape with a diameter of 9 ± 1.4 μm compared to circular domains of DPPG (Figure 5.12, panel 1). These domains remained intact until monolayer collapse at 48 mN/m. In the presence of gelatin nanoparticles, the domain size decreased to 8 ± 1.6 μm (p-value < 0.05) (Figure 5.12, panel 2). Lipid nanoparticle clusters also appeared early in compression and persisted until collapse. However, unlike PC systems, these clusters localized within the LE phase outside the LC domains (Figure 5.12, panel 2 red arrows and inset). Furthermore, void-like structures were observed in the film indicating significant packing defects.
Figure 5.12. BAM images of the lateral organization of binary PG systems on an aqueous subphase. Panel 1) control binary PG, Panel 2) binary PG + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The overall PG system contains 92.5% unsaturated lipids and these lipids will dominate the lateral organization (Figure 5.13, panel 1). A homogenous LE phase without any domain formation was maintained until collapse. The PG system containing nanoparticles led to lipid nanoparticle clusters that resulted in a significant decrease in collapse pressure from 42 mN/m to 30 mN/m (Figure 5.13, panel 2 red arrows).
Figure 5.13. BAM images of the lateral organization of PG systems on an aqueous subphase. Panel 1) control PG system, Panel 2) PG system + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Similar to the individual lipid results, a significant destabilization effect was seen with PG system while minimum changes were seen with PC systems. The collapse pressure of PG system in the presence of gelatin nanoparticles had a decrease of 12 mN/m (Figure 5.9C). The lateral organization was affected to a similar degree in the presence of gelatin in both PC and PG containing systems in the form of clusters. Remarkably, the effect of phase preference seen above with individual lipids was still seen here. In both PC containing systems (binary PC and PC system), clusters appeared to localize in LC domains. With PG lipids, these clusters were present in the LE phase. This indicates that gelatin has a preferential interaction that is phase dependent between the different lipid classes. In PG containing systems, nanoparticle induced clusters are only seen in fluid lipids or lipid in the LE phase. In PC containing systems, nanoparticle induced clusters are only seen in saturated or highly compressed LC lipid phases. Due to the increased fluidity of PG system (92.5%) versus PC system (26%), no domains were seen yet clusters appeared throughout the LE film due to their preferential interaction with the fluid lipid phase.

5.5 Lung Surfactant Model Systems

5.5.1 Pressure-Area isotherms and Compression modulus

The first complex system analyzed with the gelatin nanoparticles was the 4 lipid system. This system is composed of 45:8:1:6 molar ratio of DPPC:POPC:DPPG:POPG. The model contains the major 4 physiologically relevant PC and PG lipid classes found in the lung surfactant. This system entered the LE phase at a molecular area of 110 Å²/mol (Figure 5.14A). The pressure-
area isotherm of this system displayed a steady slope with a kink at 10 mN/m indicative of phase coexistence (Figure 5.14A, purple arrow). This system displayed gradual collapse which started at 48.3 to 53.8 mN/m (Figure 5.14, green box). Systems including gelatin nanoparticles lifted off from the gas phase at the same molecular area as the control of 110 Å²/mol. Similarly, this system exhibited gradual collapse albeit starting earlier at surface pressure of 45 to 59 mN/m. This gradual collapse is indicative of the ‘squeezing out’ of the unsaturated more fluid lipids since it occurred over the same pressure range corresponding to the collapse pressures of those lipids. The remaining saturated lipids are responsible for maintaining the high surface pressure corresponding to the low surface tension and stability of the film. This phenomena is well documented in the literature (70, 183-186). The unsaturated lipids along with the surfactant proteins such as SP-B and SP-C are responsible for maintaining the integrity of the monolayer during breathing cycles corresponding to compression and expansion. Specifically, these proteins along with unsaturated lipids help in the folding of the monolayer to prevent squeezing out and loss of lung surfactant materials (89, 185, 186). They are additionally important for film spreading and film adsorption upon inhalation (187). The compression modulus of the 4 lipid system showed a dip in the modulus profile complementing the kink in the pressure area isotherm (Figure 5.14B, purple arrow). The control 4 lipid system reached peak rigidity at surface pressure of 30 mN/m reaching a modulus value of 104 mN/m. Systems including gelatin nanoparticles decreased the monolayer’s rigidity to a peak modulus value of 89 mN/m at similar surface pressures.
The effect of introducing cholesterol at 2% by weight (80, 172, 173) to the 4 lipid systems was analyzed next. This 5 lipid system lifted off from the gas phase at 105 Å²/mol and displayed a phase coexistence kink at around 10 mN/m (Figure 5.14C, purple arrow and inset). This system also displayed a gradual collapse from 48 to 53.8 mN/m (Figure 5.14C, green box). 5 lipid system including gelatin nanoparticles entered the LE phase at a similar molecular area as control (105 Å²/mol). Collapse started earlier than control at around 45 mN/m and reached 60 mN/m (Figure 5.14C, green box). The kink of phase coexistence was diminished and cannot be identified from the pressure area isotherm. The compression modulus of the control 5 lipid system reached peak rigidity of 104 mN/m at surface pressure of 30 mN/m (Figure 5.14D). Similar to the 4 lipid system, the gelatin nanoparticles caused a decrease in the peak rigidity to 95 mN/m indicative of increased elasticity.
Figure 5.14. Pressure-Area isotherms and compression modulus of 4 and 5 lipid systems on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of 4 lipid systems respectively. C) the pressure-area isotherm and D) compression modulus of 5 lipid systems. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
The final complex lung surfactant model analyzed with gelatin nanoparticles was the 8 lipid system. This system contains all of the major lipids found in the PC and PG lipid classes with 2% w/w ratio of cholesterol. This system left the gas phase at a molecular area of 113 Å²/mol (Figure 5.15A). Collapse was reached at 43 mN/m at a molecular area of 42.8 Å²/mol. The lift off area was not affected by the presence of gelatin nanoparticles. However, collapse was reduced occurring between 39 and 41 mN/m. The compression modulus profile of the control system reached maximum rigidity at 89 mN/m at a surface pressure of 32 mN/m (Figure 5.15B). While in the presence of gelatin nanoparticles, peak rigidity of 79.5 mN/m was reached at surface pressure of 24 mN/m. Furthermore, the nanoparticles increased the elasticity of the system as the peak rigidity achieved was lower than control.
Figure 5.15. Pressure-Area isotherms and compression modulus of complex system on an aqueous subphase. A) the pressure-area isotherm and B) compression modulus of 8 lipid systems respectively. All systems show results for lipid controls (Blue) and in the presence of gelatin nanoparticles (Red) which are added at a 10% weight ratio. All isotherms collected are an average of various replicates (n ≥ 3)
5.5.2 Lateral organization

The lateral domain organization during monolayer compression of the 4 lipid system can be seen in figure 5.16. LC domains appeared close to 10 mN/m and increased in size with further compression reaching a diameter of 4.4 ± 0.5 μm at 15 mN/m. Further compression led to coalescence of these domains at 25 mN/m that resulted in a homogenous film that remained intact until collapse (Figure 5.16, panel 1). With systems that contain gelatin nanoparticles, the size of the domains formed were significantly larger at similar surface pressures compared to controls. At 15 mN/m, the domains had a diameter of 6 ± 1 μm (p-value < 0.0001) (Figure 5.16, panel 2). In addition, these domains did not appear to coalesce as homogenously as the control. Few domains can be seen at 30 mN/m (Figure 5.16, panel 2 blue box).

The addition of 2% w/w ratio of cholesterol resulted in significant changes to the lateral organization when compared to the 4 lipid system (Figure 5.17). Domains formed closer to 10 mN/m and started coalescing into a homogenous film before 30 mN/m (Figure 5.17, panel 1). At 15 mN/m, the domains were hard to distinguish unlike the 4 lipid system. At 10 mN/m the domains had a diameter of 2.5 ± 0.2 μm. When nanoparticles were present, these domains were significantly larger with a diameter of 3 ± 0.5 μm (p-value < 0.0001) (Figure 5.17). In addition, the nanoparticles resulted in the formation of void-like defects in the system that started early in compression and affected the overall film lateral stability (Figure 5.17, panel 2 red box).
Figure 5.16. BAM images of the lateral organization of 4 lipid systems on an aqueous subphase. Panel 1) control 4 lipid system, Panel 2) 4 lipid system + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm.

Each image is a representation of at least 3 images
Figure 5.17. BAM images of the lateral organization of 5 lipid systems on an aqueous subphase. Panel 1) control 5 lipid system, Panel 2) 5 lipid system + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The compression of the 8 lipid system did not result in detectable domain formation (Figure 5.18, panel 1). The 8 lipid system that contained nanoparticles showed the formation of lipid-nanoparticle clusters that started before monolayer compression and continued until collapse (Figure 5.18, panel 2). These clusters are protruding from the monolayer as indicated by the increased grayscale of the system (Figure 5.19, colourized 3D).
Figure 5.18. BAM images of the lateral organization of 8 lipid systems on an aqueous subphase. Panel 1) control 8 lipid system, Panel 2) 8 lipid system + gelatin nanoparticles at 10:1 weight ratio. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Results of the lateral organization of the complex systems showed better lipid mixing when compared to the PC and PG systems explored previously (Figure 5.10-13). The appearance of a homogenous film at higher surface pressures after domain formation has been reported before (125). A more homogenous film at high surface pressures was explained by the density similarity between the 2 lipid phases (LE and LC). This reappearance depends greatly on the molecular area available at high surface pressures. The LC domains in a monolayer reach a point where they cannot be further compressed, or the change in the molecular area is not significant. Therefore, to explain the reduction in molecular area with further compression...
must be due to the LE phase being further compressed to a density that is almost similar to that of the LC domains that result in better phase mixing and a more homogenous film (125-127).

As the ratio of fluid lipids were increased in the more complex systems, the elasticity or fluidity was increased when compared to that of saturated DPPC or DPPG (decreased compression modulus (Figure 5.1B and 5.2B). The three complex models had a maximum rigidity peak that was lower than that of DPPC or DPPG and displayed better mixing in the lateral organization as seen with BAM.

The lateral organization after the addition of cholesterol showed a disruption to the domains formed with the 4 lipid system. Cholesterol appeared to result in a homogenizing effect between the two lipid phases. Similar effects were previously seen in which the lateral organization of BLES, a commercially available lung surfactant replacement therapy, was significantly altered in the presence of cholesterol as low as 5% (188). Another group also characterized the interaction of cholesterol in lung surfactant model systems (189). It was suggested that cholesterol interacts with LC phase lipids and thus altering the phase separation and density between the two phases. These studies agree with our results, where the domains formed at around 10 mN/m quickly ‘broke’ into a homogenous film earlier when cholesterol was present (4 versus 5 lipid systems) (118).
5.5.3 Surface potential measurements

The effect of gelatin nanoparticles on the orientation of the dipoles in the 8 lipid system during compression can be seen in figure 5.20. The pure lipid system exhibited no detectable surface potential at large molecular areas before compression. As the monolayer was compressed, a sharp increase in the slope from around 133 to 124 Å²/mol suggested dipole reorientation corresponding to a potential increase from 0 to 44 mV (Figure 5.20). These areas are within the gas phase of the monolayer before any increase to surface pressure was detected (Figure 5.15A). This indicates the sensitivity of this technique when compared to pressure-area isotherms. Further compression to areas smaller than 124 Å²/mol resulted in smaller changes to the surface potential measured with only a progressive increase until the maximum surface potential was reached at 160 mV (Figure 5.20). Systems containing gelatin nanoparticles experienced an increase from 0 mV to 86 mV during dipole reorientation. The starting surface potential was not affected unlike results with PLGA nanoparticles in chapter 4 (starting surface potential values were 68.2 mV for the 10:1 system).

Gelatin nanoparticles delayed the initial dipole reorientation to occur between 133 and 109.3 Å²/mol. The dipole reorientation was not as fast as the control system since the slope of the transition was not as sharp (Figure 5.20). It appears that the nanoparticles delay the dipole reorientation of the monolayer during compression. Furthermore, with continued compression there was a significant increase in the surface potential of around 37 mV until monolayer collapse which occurred at 197 mV (Figure 5.20).
Even though the pressure-area isotherm of 8 lipid system showed no destabilizing effect (collapse pressure was not affected) (Figure 5.15A), the changes in the lateral organization (Figure 5.18) as well as the surface potential showed significant changes to the system. This indicates the direct effect on the stability of the monolayer (167). Therefore changes to the lateral organization due to nanoparticles could potentially be detrimental to the functionality of the system.

**Figure 5.20.** Surface potential-area isotherms of 8 lipid systems. Gelatin nanoparticles at 10:1 lipid to nanoparticle weight ratio are deposited along with the lipids at the interface. All isotherms collected are an average of various replicates (n ≥ 3).
5.6 Chapter summary and conclusions

To summarize, results from this chapter showed that lipid-gelatin nanoparticle interactions were specific in terms of headgroup charge and acyl chain saturation. From the pressure-area isotherms, PG containing systems resulted in a significant decrease in the collapse pressure in the presence of gelatin nanoparticles, which was not seen for PC containing systems. No significant decrease of the collapse pressure of PC systems were seen. For DPPG alone, the collapse pressure dropped from 57 to 50 mN/m (Figure 5.2A). Similarly, the PG system containing DPPG:POPG:SOPG:16-1PG at 1:6:4.4:2 ratio displayed a major decrease in the collapse pressure from 42 to 30 mN/m (Figure 5.9C). This indicates that gelatin nanoparticles are affecting PG lipids more so than PC lipids. Moreover, results show that there is a strong preferential interaction between the PG lipids with stronger destabilization with fluid POPG compared to saturated DPPG.

Furthermore, the effects on the lateral domain organization were more pronounced for the PG containing systems. BAM images displayed higher frequency of the lipid nanoparticle clusters appearing in PG systems compared to their PC counterparts. These alterations can be explained by the slightly positive charge of gelatin nanoparticles and their electrostatic interactions with the negatively charged PG lipids. Nonetheless, these interactions include other factors such as particle effects on lipid packing as well since changes in lateral domain organization are also seen for the zwitterionic PCs. However, the changes with PCs were not as drastic as PGs. For example, LC domains of DPPC were significantly reduced in size in the
presence of nanoparticles (Figure 5.3 panel 2, and figure 5.4). Furthermore, a strong particle impact on lateral organization was also seen for POPC as indicated by the formation of 3 distinct features; 1) regions of dark grey (LE), 2) regions of light grey (LC), and 3) bright lipid nanoparticle clusters occurring in the LC phase (Figure 5.5, panel 2).

Another interesting outcome from this study was the preferential interaction of nanoparticles within PG and PC systems. With the PC systems, the lipid nanoparticle clusters appeared to form within or at the edges of LC domains. Gelatin nanoparticles had a preferential interaction with the saturated PC species as opposed to the more fluid ones (DPPC vs. POPC). The opposite was seen with PG lipids, as clusters appeared to localize within the LE phase surrounding the LC domains. Unlike with the PCs, no preferential interactions were seen between the saturated and unsaturated PGs as both systems in the presence of gelatin nanoparticles had a significant effect. With DPPG, a reduction in the collapse pressure of 7 mN/m (Figure 5.2A) was seen along with formation of bright clusters in BAM images (Figure 5.6, panel 2). With unsaturated POPG, bright clusters were visible throughout compression (Figure 5.7, panel 2).

Therefore, factors determining these interactions include headgroup structure, acyl chain architecture (saturation), charge, and charge density of the monolayer. The molar ratio of the mixtures plays a significant role as well since it strongly affects lipid packing. Lipid components present only at low concentrations have a great impact on monolayer’s behaviour. Addition of
only 15% of one component (POPC or DPPG) caused significant changes to the lateral domain organization. A similar effect with lipid mixtures was seen in which POPC mixed with sphingomyelin altered the lateral packing of the monolayer when different ratios are used (124). Systems including 8:2 sphingomyelin:POPC displayed better packing than 2:8 sphingomyelin:POPC system. This sheds the light into the importance of the lateral packing and how the addition of a different lipid can disturb the optimal packing of a system.

To summarize, eventhough gelatin is a natural polymer that is both biodegradable and biocompatible, results here show that it is causing significant negative effects to the stability of the human lung surfactant components and complex mimics. This sheds the light into the importance that not all promising biodegradable nanoparticles are safe for all drug delivery routes.
Chapter Six: **Biophysical analysis of SP-B_{1-25} containing biomimetic lung surfactant models with polymeric nanoparticles**

### 6.1 Background and Objectives

The human lung surfactant contains 10% by weight surfactant proteins (69). Therefore, it is important to also include surfactant proteins to the model and identify whether results seen in previous chapters still hold when proteins are present. Furthermore, having the proteins in the model is a better mimic of the human lung surfactant than the lipids alone.

This chapter investigated the interaction of the NH$_2$-terminal peptide 1-25 (SP-B$_{1-25}$) of the lung surfactant protein SP-B with the complex 8 lipid biomimetic lung surfactant model. Its behaviour in the presence of different lipid classes was assessed. Both SP-B and SP-C are hydrophobic proteins that aid in the adsorption and respreading of the lung surfactant during breathing cycles. The absence of SP-B was found to be lethal indicating its important role in the human lung surfactant among all surfactant proteins including SP-C, SP-A, and SP-D (190, 191). Therefore, due to the high importance of SP-B, it was chosen as the first protein to add to the model.

The SP-B$_{1-25}$ segment has been shown to maintain the surface activities of the native SP-B (84, 85) thus potentially providing a more synthetic alternative to the full 79 residue SP-B protein. Molecular dynamic simulation and FTIR studies have shown that SP-B$_{1-25}$ is aligned
parallel to the interface with the first 7 amino acid residues anchor and insert into the monolayer. The next 8-22 residues adopt an alpha helix parallel to the interface (192-194).

The mode of interaction of SP-B\textsubscript{1-25} with the different individual lipids in the system was assessed in order to better characterize lipid-protein interactions. Moreover, the effects of polymeric nanoparticles such as PLGA and gelatin on the model were investigated by using pressure-area isotherms, compression moduli, surface potential measurements and Brewster angle microscopy. Similar techniques and model systems were employed here in order to characterize the interaction of the SP-B\textsubscript{1-25} with the various model systems.

6.2 Individual Lipid Systems

6.2.1 Pressure-Area isotherms and compression modulus

The effects on the pressure-area isotherms of DPPC after the addition of 10\% by weight SP-B\textsubscript{1-25} are shown in Figure 6.1A. This system entered the LE phase at a molecular area of 115 Å\textsuperscript{2}/mol and plateaued between 89 to 69 Å\textsuperscript{2}/mol and surface pressures of 6 and 8 mN/m. This is the LE-LC phase coexistence region. Due to the earlier take off from the gas phase compared to DPPC alone (115 vs. 110 Å\textsuperscript{2}/mol), the plateau was slightly shifted to larger areas. For DPPC only system, the plateau region was between 77 to 57 Å\textsuperscript{2}/mol. Further compression increased the DPPC + SP-B\textsubscript{1-25} isotherm’s slope up to 20 mN/m. The presence of 10\% SP-B\textsubscript{1-25} resulted in the appearance of a shoulder at 20 mN/m (Figure 6.1A, purple arrow and inset). Another shoulder was seen at 42 mN/m indicative of gradual collapse similar to complex systems seen in chapter
5 (Figure 6.1A, square box). In this case, the presence of the peptide stabilized the collapse of DPPC allowing it to undergo collapse gradually. Complete collapse occurred around 48 mN/m at a molecular area of 39.5 Å²/mol. Compared to DPPC alone, this increase of about 8 mN/m indicates that SP-B₁₋₂₅ increased the stability of DPPC monolayers. The addition of 10% PLGA nanoparticles (wt ratio) resulted in an earlier take off and the LE phase formation was already observed at the start of compression at molecular areas above 118 Å²/mol. The larger molecular areas per molecule or increased surface pressures were only observed until the shoulder that ended at 20 mN/m. After this surface pressure, both isotherms almost overlapped until start of gradual collapse at 42 mN/m. Both the shoulder seen around 20 mN/m and gradual collapse were maintained in both systems.

Increasing the concentration of PLGA nanoparticles to a 1:1 ratio caused the system to enter the LE phase at an even larger molecular areas (Figure 6.1A). This indicates a larger surface pressure experienced in the monolayer at certain molecular areas. Similar to the 10:1 system, the isotherm of the 1:1 system above 20 mN/m appeared to almost overlap with the DPPC + SP-B₁₋₂₅ control system up until collapse. The shoulder and gradual collapse were also maintained in this system similar to the control. With gelatin nanoparticles, results followed a different trend. The isotherm took off from the gas phase at a similar area to DPPC + SP-B₁₋₂₅ control but with further compression it shifted to lower molecular areas. The shoulder at 20 mN/m and the gradual collapse in this system were less pronounced. In addition, gelatin nanoparticles caused a significant decrease in the final collapse pressure from 48 mN/m to 44
mN/m. However, due to the presence of SP-B\textsubscript{1-25}, the collapse pressure of this system was higher than that of DPPC with no SP-B\textsubscript{1-25} which occurred at 40 mN/m at a molecular area of 43 Å\textsuperscript{2}/mol. Table 6.1 below compares the collapse pressure of DPPC systems and their significance.

**Table 6.1.** Statistical analysis on the collapse pressures of DPPC systems with SP-B\textsubscript{1-25}. Systems are compared to DPPC + SP-B. Two tailed student t-test was used.

<table>
<thead>
<tr>
<th>System</th>
<th>Collapse Pressure (mN/m)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC + SP-B (48 ± 0.2 mN/m)</td>
<td>Significant p value ≤ 0.0001</td>
<td></td>
</tr>
<tr>
<td>DPPC + SP-B + PLGA 10:1</td>
<td>Non-significant</td>
<td></td>
</tr>
<tr>
<td>DPPC + SP-B + PLGA 1:1</td>
<td>Non-significant</td>
<td></td>
</tr>
<tr>
<td>DPPC + SP-B + Gelatin</td>
<td>Significant p value ≤ 0.05</td>
<td></td>
</tr>
</tbody>
</table>

In terms of monolayer elasticity, SP-B\textsubscript{1-25} caused a significant decrease in the compression modulus peak of DPPC. The reduction was seen from 134.4 mN/m to 115 mN/m. A peak in the compression modulus indicate locations of maximum rigidity. This reduction therefore specified increased elasticity or fluidity of the system due to the presence of SP-B\textsubscript{1-25} (Figure 6.1B). The shoulder seen in the pressure-area isotherm at around 20 mN/m resulted in a peak followed by a dip or decrease in the modulus (Figure 1B, arrow). The presence of SP-B\textsubscript{1-25} exhibited two peaks in contrast to DPPC system only, one before the dip with a modulus of 60 mN/m and another at 115 mN/m. The presence of PLGA and gelatin nanoparticles maintained the overall increased elasticity and dip seen in the modulus. As the ratio of PLGA nanoparticles increased, the dip occurred at a smaller compression modulus value indicating increased
elasticity. With gelatin nanoparticles, the dip was slightly shifted and occurred later in compression at a surface pressure of 22 mN/m. Furthermore, with gelatin nanoparticles the peak compression modulus was significantly reduced from 115 mN/m of control system to 92 mN/m.

The POPC + SP-B1-25 system entered the LE phase at 141 Å²/mol and maintained a constant slope upon compression up to the collapse pressure of 41 mN/m at 54 Å²/mol (Figure 6.1C). SP-B1-25 caused a delay of 10 Å²/mol in the lift off area into the LE phase. Similar to DPPC systems above, an increase in the collapse surface pressure of about 3 mN/m was seen with SP-B1-25. The addition of 10% PLGA resulted in a slight decrease of collapse pressure of about 2 mN/m. Furthermore, a kink appeared in the isotherm around 10 mN/m (Figure 6.1C, arrow and inset). With the 1:1 system with PLGA nanoparticles, it entered the LE phase before start of compression. Similar to the 10:1 system with PLGA nanoparticles, the kink at 10 mN/m was present yet more pronounced (Figure 6.1C, inset).

The peak of the compression modulus for POPC at 76 mN/m was flattened out and decreased with the presence of SP-B1-25 indicating increased elasticity (Figure 6.1D, asterisk). The presence of PLGA nanoparticles further increased the elasticity of the monolayer up to surface pressures of 20 mN/m above which no differences from control were seen. The system with 1:1 PLGA nanoparticles increased the elasticity of the monolayer more than the 10:1
system. A dip in the modulus occurred with both PLGA systems around 10 mN/m consistent with the shoulder seen in the pressure-area isotherm (Figure 6.1D, arrow).

Likewise, the other fluid PC lipid system, 16-1PC + SP-B_{1-25} was analyzed with and without PLGA nanoparticles (Figure 6.1E). Due to the presence of SP-B_{1-25}, this system entered the LE phase before the start of compression. While with the 16-1PC alone, the system entered the LE phase at a molecular area of 128 Å^{2}/mol. Unlike the previous PC systems, the collapse pressure was not affected by the presence of SP-B_{1-25} and was maintained at 40 mN/m. The presence of PLGA nanoparticles caused a slight shift to larger areas during compression up to 30 mN/m after which the isotherm overlapped with the control. These shifts to larger areas correspond to increased fluidity as seen in compression modulus data (Figure 6.1F). Similar to POPC system above, SP-B_{1-25} reduced and flattened out the peak modulus (from 85 to 67 mN/m) representing increased elasticity of the system. Moreover, PLGA nanoparticles resulted in a dip in the compression modulus at 10 mN/m similar to the POPC system. This was present in the pressure-area isotherm yet reduced to a kink in the isotherm and a change in the slope (Figure 6.1E, arrow and inset).
Figure 6.1. Pressure-Area isotherms and compression modulus of phosphatidylcholine lipids on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of DPPC respectively, C) the pressure-area isotherm and D) compression modulus of POPC systems, E) the pressure-area isotherm and F) compression modulus of 16-1PC systems. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with DPPC at the interface. All isotherms collected are average of various replicates (n ≥ 3)
The first lipid analyzed from the PG systems was DPPG. The presence of 10% by weight SP-B$_{1-25}$ changed the shape of DPPG isotherm significantly (Figure 6.2A). The isotherm lifted off from the gas phase earlier in compression at around 89 Å$^2$/mol versus 60 Å$^2$/mol for DPPG alone. A plateau region occurred around 23 mN/m at 50.4 Å$^2$/mol, which was not seen with DPPG alone. This system collapsed at 42 mN/m at a molecular area of 36 Å$^2$/mol. SP-B$_{1-25}$ stabilized DPPG as indicated by the increased collapse pressure (39 vs. 42 mN/m). The presence of PLGA nanoparticles in the subphase at 10:1 lipid to nanoparticle ratio caused the system to enter the LE phase at 96 Å$^2$/mol (about 7 Å$^2$/mol earlier than control). Both isotherms overlap close to the plateau region at 25 mN/m and remain so until collapse at 42 mN/m. Increasing the PLGA concentration caused the isotherm to enter the LE phase before the start of compression with a shoulder that appeared at 10 mN/m (Figure 6.2A, arrow). In addition, the plateau region started earlier around a surface pressure of 21 mN/m as opposed to 23 mN/m. Collapse pressure of PLGA containing systems did not deviate from the SP-B$_{1-25}$ containing control system. Similar to the DPPC + SP-B$_{1-25}$ system, the addition of gelatin nanoparticles resulted in an opposite effect of PLGA nanoparticles. The molecular area for entry into the LE phase was maintained similar to controls but the system remained in the LE phase longer up to a molecular area of 60 Å$^2$/mol (Figure 6.2A).

DPPG + SP-B$_{1-25}$ displayed a different compression modulus profile than that of DPPG alone. The peak compression modulus was significantly reduced from 117 mN/m to 76 mN/m in the presence of the peptide (Figure 6.1B). Furthermore, this peak rigidity occurred later in
compression compared to DPPG alone. It happened at a surface pressure close to 38 mN/m.

The plateau region in the isotherm around 23 mN/m, caused a peak followed by a dip in the modulus curve (Figure 6.2B, arrow). PLGA and gelatin nanoparticles increased the rigidity of DPPG + SP-B1-25 system significantly as indicated by the higher compression modulus values. The shoulder that appeared in the isotherm for DPPG + SP-B1-25 at the 1:1 ratio with PLGA nanoparticles can be seen as a dip in the modulus as well (Figure 6.2B, asterisk). A similar dip was seen with the 10:1 PLGA system but to a smaller extent.

SP-B1-25 in POPG caused the system to enter the LE phase much earlier than POPG alone. This indicated interactions of SP-B1-25 with the lipid that resulted in this shift to larger areas (Figure 6.2C). The collapse pressure was not altered by the presence of SP-B1-25. PLGA nanoparticles at both ratios did not seem to affect the pressure-area isotherms significantly during compression. A minor decrease to the isotherm slope was seen in the presence of PLGA nanoparticles. Opposite to the trend seen so far, SP-B1-25 increased the compression modulus and thus the rigidity of POPG (Figure 6.2D). It increased the peak modulus value to 77.8 mN/m from 42.5 mN/m for POPG alone. Albeit being a significant increase, the monolayer overall remained in the LE phase since the peak was below 100 mN/m modulus value. A slight increase in elasticity of the monolayer can be seen due to PLGA nanoparticles below a surface pressure of 30 mN/m. Above this surface pressure the elasticity was the same between all systems until collapse.
The pressure-area isotherm of SOPG + SP-B_{1-25} was analyzed next (Figure 6.2E). SP-B_{1-25} caused a stabilization effect to the SOPG system resulting in an increase in the collapse pressure from 36.3 to 40.3 mN/m. The presence of the peptide shifted the entry into the LE phase to 130 Å²/mol versus 135 for SOPG alone. Systems with PLGA nanoparticles at the 10:1 ratio did not alter the pressure-area isotherm. However, in the presence of nanoparticles at the 1:1 ratio with SOPG + SP-B_{1-25}, the system entered the LE phase before the start of compression. Similar to POPG, the presence of SP-B_{1-25} caused an increase in the modulus peak value from 53 to 71 mN/m (Figure 6.2F). Moreover, this peak of highest rigidity was delayed and occurred further in compression (25 vs. 17 mN/m).

The last single PG system was the 16-1PG + SP-B_{1-25} (Figure 2G, H). The isotherm took off from the gas phase at a molecular area similar to that of 16-1PG alone at 120 Å²/mol. However, the slope of the SP-B_{1-25} containing system was sharper indicating increased interactions. This resulted in systems experiencing increased van der Waals interactions and thus causing an earlier increase in surface pressures. In addition, the collapse pressure was increased from 35 to about 42 mN/m signifying a stabilization effect. The presence of PLGA nanoparticles caused a shift to even larger areas throughout compression that increased with the particle concentration. The collapse pressure was not affected in the presence of PLGA nanoparticles at the 10:1 ratio. However, the 1:1 system caused a decrease in the collapse pressure which occurred at 40 mN/m. Similar to the other unsaturated PG lipids, interactions with SP-B_{1-25} appeared to increase the rigidity of the monolayer. The peak of the compression modulus of
SP-B_{1-25} containing system had a maximum value of 62 mN/m compared to 37 mN/m for 16-1PG alone (Figure 6.2H). In the presence of PLGA nanoparticles, a slight increase in the monolayer’s elasticity was seen up to a surface pressure of 20 mN/m where with further compression, the elasticity was similar to that of control.
**Figure 6.2.** Pressure-Area isotherms and compression modulus of phosphatidylglycerol lipids on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of DPPG respectively, C) the pressure-area isotherm and D) compression modulus of POPG systems, E) the pressure-area isotherm and F) compression modulus of SOPG systems, G) the pressure-area isotherm and H) compression modulus of 16-1PG. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with DPPG at the interface. All isotherms collected are average of various replicates (n ≥ 3)
Due to the nature of SP-B, it is known to interact with lung surfactant through; 1) electrostatic interactions with anionic lipids such as PGs and 2) hydrophobic interactions with lipid acyl chains near the surface (76, 82, 195, 196). SP-B along with SP-C facilitate adsorption and spreading of the monolayer during breathing cycles. This is achieved through the formation of “surface associated reservoirs” that remain attached to the monolayer (Figure 6.3). During inhalation, they are reintroduced back into the lung surfactant (183, 197). The interaction of SP-B with associated lipids and potential formation of the reservoirs can be seen in the obtained pressure-area isotherms.
With DPPC and DPPG, the shoulders seen at 20 and 23 mN/m respectively in the pressure-area isotherms (Figure 6.1A and 6.2A) are associated with the interaction of the peptide or peptide-lipids complexes. It is estimated that the peptide will interact with the monolayer at surface pressures of 15 mN/m (199). With further compression, the peptide is squeezed out into reservoirs and therefore less lipids are directly in the monolayer interface. This explains the shift to smaller areas seen after the shoulder formation. The observation seen
here is similar to literature reports for these lipids, individually or in mixtures (200, 201).

Furthermore, the shifts to larger areas or increase in area per molecule seen for DPPC and
DPPG due to the presence of the peptide were also reported (84, 201, 202).

Due to the net positive charge of the peptide, it is estimated to interact with anionic
lipids such as the PGs, yet literature does exist for preferential interactions with zwitterionic PC
lipids (203, 204). It was suggested that even a stronger interaction is seen with PC lipids versus
PG lipids. Fluorescence microscopy as well as mass spectrometry were performed and it was
evident that SP-B interacted with DPPC lipid (203, 204). This is explained through the solubility
of the protein. It can partition into DPPC films more easily than DPPG due to the ability of DPPG
to form hydrogen bonds between the lipids resulting in a rigid system that prevents insertion of
the protein. However, to my knowledge, support does exist for interaction of SP-B with anionic
and zwitterionic lipids equally which also complements results seen here. Due to the extensive
analysis and comparison done here, the interaction of SP-B with the two lipid classes is further
analyzed in terms of fluidity and acyl chain length. All PCs showed a fluidization effect in the
presence of the peptide while with the PGs, a fluidization was only seen with DPPG and all
other PGs tested a rigidification effect was seen.

After the interaction and potential expulsion of the SP-B_{1-25} from the monolayer surface
into the reservoirs, the isotherm displayed a shift back to lower areas almost overlapping with
the control (Figure 6.1A and 6.2A). In addition, the compression modulus of DPPC and DPPG
systems show that after the isolation of SP-B$_{1-25}$ (dip in the modulus), the monolayer increased rigidity yet not to the same extent as the peptide free systems (Figure 6.1B and 6.2B).

The unsaturated PC lipids (POPC and 16-1PC) both displayed the shift to larger areas per molecule in the pressure-area isotherms similar to DPPC indicating a fluidizing effect (Figure 1C,E). The peak compression modulus of both systems was reduced and flattened suggesting increased elasticity of the monolayer due to the presence of SP-B$_{1-25}$ (Figure 6.1D and F, asterisks). This was not the case for unsaturated PG lipids (POPG, SOPG, and 16-1PG) that are able to undergo electrostatic interactions with SP-B$_{1-25}$. The shift to larger areas per molecule as well as the increased compression modulus indicated that the interaction of SP-B$_{1-25}$ with the unsaturated PGs was different than the PCs, presumably due to their negative charge. In addition, obtained results showed that SP-B$_{1-25}$ increased the collapse pressure of individual lipid systems analyzed, increasing their stability (85, 91).

The presence of nanoparticles further increased the elasticity of all individual PC systems with the exception of DPPC where a slight increase in rigidity was seen above a surface pressure of 25 mN/m as seen in the compression modulus profiles (Figure 6.1B). This could be due to packing defects caused by the nanoparticle at that concentration when interacting with DPPC as explained in previous chapters (124). With PG lipids, all nanoparticles caused an increase in rigidity. These results indicate interactions of the peptide and nanoparticles with lipids systems analyzed.
6.2.2 Lateral organization

The lateral organization of DPPC + SP-B\textsubscript{1-25} was characterized below in figure 6.4. The fractal like shape of pure DPPC domains was maintained yet increased in size to a diameter of 77.3 ± 18 μm at 7 mN/m (Figure 6.4, panel 1). Pure DPPC had 15.8 ± 4.2 μm domains at the same surface pressure (Figure S6.1, panel 1).

PLGA nanoparticles caused DPPC + SP-B\textsubscript{1-25} domains to significantly decrease in size. With the 10:1 PLGA ratio, the domains had a diameter of 22.5 ± 6.5 μm (Figure 6.4, panel 2). At a surface pressure of 10 mN/m, the domains started to coalesce into a homogenous film. Increasing PLGA concentration (1:1 ratio) resulted in DPPC + SP-B\textsubscript{1-25} domains with a diameter of 34.5 ± 9.4 μm (Figure 6.4, panel 3). Further compression of the isotherms caused these domains to coalesce into a homogenous film until monolayer collapse. Film packing defects were seen as black voids with the 1:1 lipid to PLGA nanoparticle system up to 10 mN/m (Figure 6.4, panel 3 insets). Gelatin nanoparticles at the 10:1 ratio abolished the formation of domains at 7 mN/m (Figure 6.4, panel 4). Bright clusters appeared early in the compression and remained throughout the entire compression range. In addition to the bright clusters, film void were also seen up until collapse (Figure 6.4, panel 4 inset). These film defects were seen with DPPC + gelatin nanoparticles (Chapter 5), therefore, the defects were attributed mostly to gelatin nanoparticles. However, in the DPPC + gelatin nanoparticles system, domains were still visible, therefore the disappearance of domains can be attributed to the presence of SP-B\textsubscript{1-25}. 
Figure S6.1. The lateral organization of PC systems in the absence of SP-B$_{1-25}$. 
**Figure 6.4.** BAM images of the lateral organization of Panel 1) DPPC + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Panel 4) gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The next system analyzed was POPC + SP-B$_{1-25}$ (Figure 6.5). SP-B$_{1-25}$ did not affect the homogenous lateral organization of POPC (Figure S6.1, panel 2). The presence of PLGA nanoparticles also did not disturb the homogenous film at both concentrations tested (Figure 6.5, panel 2 and 3). The second fluid PC lipid system, 16-1PC + SP-B$_{1-25}$, remained homogenous throughout compression (Figure 6.6, panel 1). This was similar to what was seen for the 16-1PC without the peptide (Figure S6.1, panel 3). PLGA nanoparticles at the 10:1 ratio caused phase demixing between gas and LE phases at 5 mN/m (Figure 6.6, panel 2). Nonetheless, this effect was not observed at the 1:1 lipid to PLGA nanoparticle ratio (Figure 6.6, panel 3).
Figure 6.5. BAM images of the lateral organization of Panel 1) POPC + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 6.6. BAM images of the lateral organization of Panel 1) 16-1PC + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The presence of SP-B_{1-25} with DPPG caused phase demixing that delayed the formation of LC domains seen at 5 mN/m with DPPG only systems (Figure S6.2, panel 1). Lobed Domains started to appear around 10 mN/m with a diameter of 17.4 ± 4.8 μm (Figure 6.7, panel 1). The DPPG system with no peptide, had coalesced domains at similar surface pressures. Those domains became more circular with further compression and decreased in size to 8.4 ± 1.6 μm at 15 mN/m. Unlike DPPG alone, no domain coalescence was seen up to 15 mN/m. In the presence of PLGA nanoparticles at the 10:1 ratio, domain formation started at 5 mN/m with a diameter of 9 ± 1.3 μm (Figure 6.7, panel 2). With further compression, these domains decreased in size to 7 ± 1.1 μm and remained intact up to 15 mN/m. At 30 mN/m bright clusters were recorded with a high concentration indicating packing defects due to nanoparticles.

Increasing the concentration of PLGA nanoparticles to the 1:1 ratio resulted in domains with a diameter of 8.3 ± 1.3 μm at 5 mN/m which was similar to the 10:1 system (Figure 6.7, panel 3). At 10 mN/m domains formed with a diameter of 9.3 ± 1.3 μm which was significantly larger than those seen with the 10:1 system (p-value < 0.001). These domains maintained their size until a surface pressure of 15 mN/m. With gelatin nanoparticles at 10:1 ratio, domains started to form early in compression with a diameter of 14 ± 1.3 μm at 5 mN/m (Figure 6.7, panel 4). These domains coalesced after surface pressures of 15 mN/m. Furthermore, similar to DPPC + SP-B_{1-25} + gelatin system, bright clusters appeared early in compression that persisted until collapse which could be mostly attributed to gelatin nanoparticles since these clusters appeared in gelatin containing DPPG with no peptide as well.
Figure S6.2. The lateral organization of PG systems in the absence of SP-B$_{1-25}$. 
Figure 6.7. BAM images of the lateral organization of Panel 1) DPPG + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Panel 4) gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
POPG + SP-B_{1-25} formed clusters at 20 mN/m that disappeared with further compression (Figure 6.8, panel 1). Furthermore, these clusters were not seen in POPG systems without SP-B_{1-25} (Figure S6.2, panel 2). Since these clusters only appeared with SP-B systems, they could be attributed to POPG- SP-B_{1-25} reservoir formation resulting in increased clustering associated with proper functioning of the lung surfactant. Images at 5 mN/m were not captured since due to the presence of the peptide, an increase in the surface pressure was seen in the pressure-area isotherm before the start of compression (Figure 6.2C). Systems that include 10:1 PLGA nanoparticles resulted in cluster formation at a similar surface pressure of 20 mN/m that disappeared with further compression (Figure 6.8, panel 2). Images were collected at 20 mN/m as opposed to 15 mN/m since clusters only appeared at that surface pressure. Similarly, with the 1:1 ratio of PLGA, SP-B_{1-25} resulted in clusters that started at 15 mN/m and continued until 20 mN/m (Figure 6.8, panel 3). The intensity and frequency of these clusters was highest in the POPG + SP-B_{1-25} system including PLGA at the 1:1 ratio. The second fluid PG lipid SOPG, showed clusters at low surface pressures up to 10 mN/m (Figure 6.9, panel 1). These clusters were not seen in the SOPG only system (Figure S6.2, panel 3). Systems that include PLGA nanoparticles resulted in phase demixing at low surface pressures delaying the formation of the homogenous film seen in controls (Figure 6.9, panel 2 and 3). The different ratio of PLGA nanoparticles delayed film formation differently. In the 10:1 system, domains were seen with various sizes which did not fully coalesce at 10 mN/m (Figure 6.9, panel 2). Voids were also seen throughout the film. On the other hand, in the 1:1 system, the domains were smaller at 5 mN/m. At 10 mN/m the film was coalesced yet not homogenously. Regions of higher brightness were
scattered across indicating regions of increased van der Waals interactions (Figure 6.9, panel 3).

Clusters still appeared similar to control with both PLGA systems until 10 mN/m.

Figure 6.8. BAM images of the lateral organization of Panel 1) POPG + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Figure 6.9. BAM images of the lateral organization of Panel 1) SOPG + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
16-1PG was the third fluid PG lipid found in the model and its lateral organization in the presence of the peptide was characterized. The control system (Figure 6.10, panel 1) showed phase demixing and cluster formation due to the presence of SP-B\textsubscript{1-25} as this was not seen in 16-1PG alone (Figure S6.2, panel 4). This phase demixing was also present in systems including PLGA nanoparticles at the 10:1 ratio (Figure 6.10, panel 2). Bright clusters appeared with these two systems up to around 15 mN/m but disappeared with further compression. Increasing PLGA concentrations to 1:1 ratio seemed to counteract the effects of SP-B\textsubscript{1-25} since a homogenous film was seen throughout compression (Figure 6.10, panel 3). The clusters still appeared at 10 mN/m yet to a much lower frequency than the 10:1 system (Figure 6.10, panel 3 arrows and inset). No clusters were visible above 10 mN/m.
Figure 6.10. BAM images of the lateral organization of Panel 1) 16-1PG + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The lateral organization of individual PC and PG lipids in the presence of SP-B_{1-25} showed preferential interaction of the peptide with PG lipids. This was seen through the cluster formations that remained up to surface pressures corresponding to the interaction and potential exclusion of the peptide (205, 206). In addition, the increased phase demixing and delay in homogenous condensed phase formation indicated preference of LE phase. These results agree with the literature data that indicate SP-B_{1-25} interaction with LE phase and thus stabilizing it and preventing condensed phase formation (84, 85, 205). This is important in order to facilitate respreadability of the lung surfactant during breathing cycles. A fully rigid surfactant system will achieve low surface tensions but will not be able to accommodate breathing cycles. Within the PC lipids, only DPPC presented a change in the lateral domain organization upon presence of SP-B_{1-25}. This was seen through an increase in the size of LC domains at the phase coexistence region.

Film defects were seen in the presence of nanoparticles in the form of film voids or delay in homogenous film formation. These effects can mostly be attributed to the nanoparticles due to the fact that in chapter 4 and 5, defects were seen in the presence of nanoparticles in systems with no SP-B_{1-25}.
6.3 Phosphatidylcholine and Phosphatidylglycerol Systems

6.3.1 Pressure-Area isotherms and compression modulus

The binary PC + SP-B$_{1-25}$ lifted off from the gas phase at a similar molecular area as the binary PC alone at 127 Å$^2$/mol (Figure 6.11A). A shoulder was seen at 10 mN/m at a molecular area of 85 Å$^2$/mol identifying a region of LE-LC phase coexistence. Binary PC + SP-B$_{1-25}$ collapsed at a molecular area of 41.8 Å$^2$/mol with a surface pressure of 46 mN/m. Again, a stabilization effect was observed due to the peptide with an increase of 3 mN/m in the collapse pressure. The addition of PLGA nanoparticles at 10:1 lipid to nanoparticle ratio did not cause a change to the pressure-area isotherm of the control binary PC + SP-B$_{1-25}$. The 1:1 lipid to PLGA nanoparticle ratio caused an increase in the areas per molecule of 12 Å$^2$/mol up to a surface pressure of 10 mN/m after which the isotherms overlapped with the control. The presence of PLGA nanoparticles at the 1:1 ratio caused the system to enter the LE phase earlier than control. Nonetheless, no change to the collapse pressure was observed. The compression modulus peak of the binary PC system without SP-B reached a peak maximum of 77.4 mN/m at a surface pressure of 26 mN/m (Figure 6.11B). The presence of SP-B$_{1-25}$ shifted this peak to later in compression occurring at 30 mN/m. All systems displayed a dip around 10 mN/m which signified phase coexistence. Due to the earlier lift off of the 1:1 PLGA system, an increase in the elasticity was seen by the decreased compression values below 10 mN/m.

The pressure-area isotherm of PC system + SP-B$_{1-25}$ is shown in figure 6.11C. This system includes DPPC:POPC:16-1PC at 5.6:1:1 molar ratio. The PC system alone lifted off from the gas
phase at a molecular area of 110 Å²/mol. A phase coexistence shoulder at 13 mN/m occurred followed by an increase in the isotherm slope until collapse at 42 mN/m. The presence of SP-B₁₂₅ caused the isotherm to lift off at a larger molecular area of 120 Å²/mol. The phase coexistence shoulder was still maintained at 13 mN/m. This system collapsed at 44 mN/m at a molecular area of 40 Å²/mol. Systems including PLGA nanoparticles at the 10:1 ratio caused an earlier lift off of about 8 Å²/mol. The slope was maintained up to the phase coexistence region, which was significantly reduced to a slight kink around 13 mN/m. Increasing nanoparticle concentration to a 1:1 ratio, caused a significant increase to the molecular area by around 24 Å²/mol up to the phase coexistence region. Following this phase coexistence region, PLGA nanoparticles both resulted in a shift to larger areas yet a larger shift was seen with the 1:1 system. Collapse pressure was not affected with the 10:1 system and it occurred gradually from around 38 Å²/mol to 44 Å²/mol with the 1:1 system (Figure 6.11C, square box). In the presence of gelatin nanoparticles, the isotherm lifted off at a similar molecular area to control of 120 Å²/mol. The isotherm started off occupying molecular areas similar to the SP-B₁₂₅ control but slightly shifted to smaller areas at higher surface pressures. The phase coexistence shoulder was maintained similar to control around 13 mN/m, whereas the collapse pressure was reduced by 4 mN/m to about 40 mN/m indicative of a destabilizing effect.

The compression modulus of PC system displayed a dip at around 13 mN/m consistent with the shoulder seen in the pressure area isotherm for phase coexistence (Figure 6.11D). The peak modulus value reached was 82.3 mN/m at a surface pressure of 23 mN/m. PC system +
SP-B_{1-25} also showed a dip around 13 mN/m consistent with the isotherm shoulder (Figure 6.11D, arrow). Compared to PC system alone, the presence of SP-B_{1-25} increased the elasticity of the system by reducing the peak modulus of highest rigidity from 82.3 to 56 mN/m. This was similar to what was seen for all individual PC systems tested above. PLGA nanoparticles caused a slight increase in elasticity of the system before the phase coexistence region. The compression modulus values were decreased from the control before surface pressure of 10 mN/m (Figure 11D, purple oval).
Figure 6.11. Pressure-Area isotherms and compression modulus of phosphatidylcholine containing systems on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of binary PC systems respectively, C) the pressure-area isotherm and D) compression modulus of PC systems. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the PC system at the interface. All isotherms collected are average of various replicates (n ≥ 3)
The binary PG + SP-B\textsubscript{1-25} system had a pressure-area isotherm that occupied larger molecular areas when compared to binary PG alone (Figure 6.12A). A kink or a change in the isotherm slope was seen around 27 mN/m indicating phase coexistence. Collapse occurred at 43 mN/m at a molecular area of 44 Å\textsuperscript{2}/mol, which was higher by about 5 mN/m compared to the binary PG system without the peptide. Addition of PLGA nanoparticles at both ratios caused the monolayer to occupy smaller molecular areas above surface pressure of 20 mN/m until system collapse. A slight increase in the compression modulus was seen after addition of SP-B\textsubscript{1-25} (Figure 6.12B). The peak modulus of the binary PG was increased from 40.3 to 50.6 mN/m in the presence of SP-B\textsubscript{1-25}. Furthermore, with SP-B\textsubscript{1-25}, the peak followed by the dip of phase coexistence was more pronounced than binary PG alone as was seen in the compression isotherms (Figure 6.12B, arrow).

Next, the PG system in the presence of 10% by weight SP-B\textsubscript{1-25} was analyzed. This system contains DPPG:POPG:SOPG:16-1PG at 1:6:4:4:2. The pressure-area isotherm of the PG system + SP-B\textsubscript{1-25} entered the gas phase at 132 Å\textsuperscript{2}/mol, which was about 12 Å\textsuperscript{2}/mol earlier in compression than PG system alone. Collapse occurred at 40 mN/m at a molecular area of 40 Å\textsuperscript{2}/mol (Figure 6.12C). Addition of PLGA nanoparticles caused the system to enter the LE phase before the start of compression. In the presence of gelatin nanoparticles, the system left the gas phase at a similar molecular area to the control at 132 Å\textsuperscript{2}/mol. With further compression, the monolayer occupied smaller molecular areas compared to the control at similar surface pressures. Furthermore, the gelatin nanoparticles caused a significant decrease of 7 mN/m in
collapse pressure to around 33 mN/m. The presence of SP-B peptide resulted in an increase in the elasticity of the system since the compression modulus peak decreased from 56.6 to 45.6 mN/m (Figure 6.12D). The compression modulus profile of this system showed a peak followed by a dip at surface pressures of around 20 mN/m indicative of SP-B\textsubscript{1-25} interaction since it was not seen in the PG system alone (Figure 6.12D, arrow).
Figure 6.12. Pressure-Area isotherms and compression modulus of phosphatidylglycerol containing systems on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of binary PG systems respectively, C) the pressure-area isotherm and D) compression modulus of PG systems. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the PG system at the interface. All isotherms collected are average of various replicates (n ≥ 3)
6.3.2 Lateral organization

The binary PC + SP-B_{1-25} system resulted in domain formation early in the compression. At 5 mN/m, domains had a diameter of 3.8 ± 0.5 μm (Figure 6.13, panel 1). No domains were seen at this surface pressure in the absence of peptide (Figure S6.1, panel 4). Further compression caused these domains to increase in size up to 4.7 ± 0.7 μm at 15 mN/m. The domains had a diameter that was significantly smaller than the binary PC alone at similar surface pressures (p-value < 0.001). Bright clusters due to SP-B_{1-25} were seen up to surface pressures of 30 mN/m. Addition of PLGA nanoparticles delayed domain formation until a surface pressure of 15 mN/m (Figure 6.13, panel 2 and 3). The domains had a diameter of 9.5 ± 2.3 μm which was significantly larger than peptide containing control (p-value < 0.001). No change to domain size was seen with varying PLGA concentrations. SP-B_{1-25} clusters also appeared in the presence of both PLGA nanoparticle concentrations although to a smaller extent than control. Furthermore, in the 1:1 system, film defects were seen early in compression at 5 mN/m (Figure 6.13, panel 3 inset). These film defects were present in binary PC systems including PLGA at the 1:1 ratio in the absence of SP-B_{1-25}. Therefore they could be attributed mostly due to the nanoparticles than the peptide.
Figure 6.13. BAM images of the lateral organization of Panel 1) binary PC + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The model’s complete PC lipids system + SP-B₁₋₂₅ lateral organization was analyzed next. The phase coexistence region at a surface pressure of 13 mN/m resulted in domain formation. At 15 mN/m, the domains had a diameter of 3 ± 0.6 and at 30 mN/m they increased to 5.4 ± 1 μm (Figure 6.14, panel 1) which was significantly smaller than PC system alone at similar pressures with diameters of 7 ± 1 μm (p-value < 0.001) (Figure S6.1, panel 5). PC system + SP-B₁₋₂₅ that include PLGA nanoparticles at 15 mN/m had domains with a diameter of 3 ± 0.5 μm at both ratios tested (Figure 6.14, panel 2 and 3). PLGA nanoparticles did not affect the size of domains at 15 mN/m when compared to the control. However, there was a difference in domain frequency. The 1:1 system with PLGA nanoparticles formed domains of similar size yet at a smaller frequency when compared to control and 10:1 system. At 30 mN/m, a slight increase was seen in domain diameter with PLGA nanoparticles at the 1:1 ratio. The diameter increased to 6.8 ± 1.2 μm which was statistically significant compared to control and 10:1 systems at similar surface pressures (p-value < 0.001) (Figure 6.14, panel 3). Phase demixing was seen with systems that include PLGA nanoparticles at low surface pressures. The 10:1 system resulted in domains at 5 mN/m while the 1:1 system had void like defects. These effects caused a delay in homogenous LE film formation seen in control at similar surface pressures.
Figure 6.14. BAM images of the lateral organization of Panel 1) PC system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Panel 4) gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Likewise, for systems including 10:1 gelatin nanoparticles, domains appeared around 13 mN/m and at 15 mN/m had a diameter of 3 ± 0.5 μm, which was similar to lipid controls and PLGA containing systems (Figure 6.14, panel 4). However, the frequency of the domains was similar to that of the 10:1 PLGA containing system. At 30 mN/m domains grew to a diameter of 7.4 ± 1.2 μm, which was larger than the control system of 5.4 ± 1 μm (p-value < 0.001). Due to the presence of gelatin nanoparticles, clusters and film defects appeared throughout the compression similar to all other gelatin systems (Figure 6.14, panel 4 insets).

The lateral organization of the binary PG + SP-B$_{1-25}$ system was the first to be analyzed from the more complex PG mixtures with SP-B$_{1-25}$ (Figure 6.15). Phase demixing and bright clusters appeared up to 10 mN/m (Figure 6.15, panel 1). Unlike binary PG system without SP-B$_{1-25}$, no domains were seen at 30 mN/m in the presence of SP-B$_{1-25}$ (Figure S6.2, panel 5). PLGA containing systems caused packing defects in the monolayer at both ratios tested. The 10:1 system resulted in phase separation and cluster formation below 10 mN/m (Figure 6.15, panel 2). The 1:1 system reached high surface pressures above 5 mN/m even before the start of compression. At 10 mN/m, film voids were seen along with clusters (Figure 6.15, panel 3). However, the presence of both peptide and PLGA nanoparticles were able to form the domains seen in the binary PG system alone at 30 mN/m. The domains can be seen albeit vaguely at the 10:1 system but they are distinct with higher PLGA concentration of 1:1. They possess a diameter of 3.3 ± 0.5 μm.
Figure 6.15. BAM images of the lateral organization of Panel 1) binary PG + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The PG system + SP-B₁₂₅ mostly consists of fluid lipids (92.5%) and thus domain formation was not expected (Figure 6.16, panel 1). The addition of 32% SOPG and 15% 16-1PG fluid lipids to the binary PG + SP-B₁₂₅ removed the phase demixing seen at low surface pressures (Figure 6.15, panel 1) and maintained a homogenous film until collapse (Figure 6.16, panel 1). PLGA nanoparticles at 10:1 ratio did not affect the system's lateral organization (Figure 6.16, panel 2). However, increasing the concentration of nanoparticles caused film defects at high surface pressures (Figure 6.16, panel 3 inset). Gelatin nanoparticles resulted in cluster formation throughout the compression (Figure 6.16, panel 4).
Figure 6.16. BAM images of the lateral organization of Panel 1) PG system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Panel 4) gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
For both PC and PG containing systems, a significant decrease in domain diameter or a complete disappearance of domains was observed in the presence of SP-B_{1-25}. This is an expected effect of the peptide since it favours interactions with fluid and anionic lipids that mostly localize and stabilize the LE phase. Due to this localization, the line tension between the two phases increases which is energetically unfavourable leading to smaller domains. Similar results were seen in systems containing more than one lipid. For example, Survanta, a clinical lung surfactant film derived from bovine lungs was found to decrease domain size in the presence of SP-B as low as 5% by weight (206). The interaction of SP-B with the LE phase resulted in higher line tension and dipole repulsion between the two phases (LE and LC) resulting in smaller domains. Furthermore, in binary PC and PG systems, the peptide associated clusters were seen at low surface pressures which disappeared with further compression. Again this confirms the preferential interaction of the peptide with the LE phase before monolayer condensation (198).

Another result of SP-B_{1-25} peptide interaction with PC and PG systems was the increased surface pressure. This indicated that the interaction was increasing the stability of the system allowing it to reach lower surface tension values. This further supports the importance of the peptide in the human lung surfactant and ultimately its presence in biomimetic models. The presence of PLGA nanoparticles did not disturb this stability, yet gelatin nanoparticles caused a significant decrease in the collapse pressure. Furthermore, from the lateral organization images, gelatin appeared to affect the organization more so than PLGA. From the gelatin
studies chapter (Chapter 5), a decrease in collapse pressure for these similar systems was not observed. Ultimately, the decrease seen here could be due to peptide nanoparticle interactions.

6.4 Lung Surfactant Model Systems

6.4.1 Pressure-Area isotherms and compression modulus

The 4 lipid system + SP-B$_{1-25}$ entered the LE phase at 125 Å$^2$/mol which was 9 Å$^2$/mol earlier than the protein free system (Figure 6.17A). Two shoulders were seen one around 10 mN/m and another at 25 mN/m. Furthermore, gradual collapse was observed between 34 and 40 mN/m (Figure 6.17A, purple box). This gradual collapse was not seen for the 4 lipid system alone. The presence of SP-B$_{1-25}$ helped refine the collapse process by squeezing out the unsaturated lipids from the monolayer. This resulted in the saturated lipids remaining in the system which were able to reach higher collapse pressures. This helped lower the surface tension of the monolayer (183, 198, 207-209).

PLGA nanoparticles caused this system to lift off into the LE phase earlier than the control and before the start of compression. The shoulders seen at 10 and 25 mN/m as well as the gradual collapse were not affected. PLGA nanoparticle containing system at the 1:1 ratio caused a slight stabilization to the monolayer by increasing collapse pressure by 3 mN/m. The compression modulus profile displayed a dip slightly after 10 mN/m for phase coexistence as well as a peak followed by a dip around surface pressures of 20 mN/m indicative of the SP-B$_{1-25}$
interaction and squeeze out into reservoirs (Figure 6.17B, arrows). A decrease in the compression modulus was seen following the squeeze out. Minor deviations (increased elasticity) in the compression modulus were seen after addition of PLGA nanoparticles.

The addition of 2% by weight cholesterol to the 4 lipid + SP-B1-25 did not cause a significant deviation in the pressure-area isotherm nor the compression modulus profile from the 4 lipid system + SP-B1-25 (Figure 6.17C,D). Interestingly, the addition of cholesterol to the 4 lipid system without SP-B1-25 caused a significant rigidifying effect seen in the pressure-area isotherms as well as the compression modulus data. The 5 lipid system alone had a modulus peak value of 141 mN/m which corresponds to the LC phase. This increased rigidity by cholesterol appeared to be eliminated by SP-B1-25 resulting in comparable readings for both 4 and 5 lipid systems. The 4 lipid + SP-B1-25 had a peak modulus value of 58 mN/m while the 5 lipid + SP-B1-25 had a peak of 59 mN/m. A similar effect was seen with DPPC:POPG (7:3) systems containing a 34 residue portion of SP-B called Mini-B and cholesterol (210).
Figure 6.17. Pressure-Area isotherms and compression modulus of 4 and 5 lipid systems on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of 4 lipid systems respectively, C) the pressure-area isotherm and D) compression modulus of 5 lipid systems. All isotherms collected are average of various replicates (n ≥ 3)
The next system analyzed was the complete phospholipid model with SP-B<sub>1-25</sub> (7 lipid system + SP-B<sub>1-25</sub>) (Figure 6.18A). The 7 lipid system with no peptide took off from the gas phase at 115 Å²/mol and maintained the kink at around 12 mN/m. The system including peptide entered the LE phase around 130 Å²/mol and displayed two shoulders at 12 and 25 mN/m. It collapsed at 40.8 mN/m and 42.8 Å²/mol. Again, the presence of the peptide facilitated gradual collapse of the system between 38 to 43 mN/m (Figure 6.18A, square box). The first shoulder was for the characteristic phase coexistence region seen with the 7 lipids alone. The second shoulder was for the interaction and folding of the monolayer into reservoirs due to SP-B<sub>1-25</sub>. The addition of PLGA nanoparticles caused the system to enter the LE phase earlier in compression which was directly proportional to nanoparticle concentration (Figure 6.18A). A slight increase in the system’s elasticity was seen after the reservoir formation (Figure 6.18B, arrow). The system’s elasticity was not affected by the presence of PLGA nanoparticles at the 10:1 ratio. Increased elasticity was present only with the 1:1 ratio up to a surface pressure of 25 mN/m (position of the second shoulder) (Figure 6.18B).

The addition of cholesterol to the 7 lipid system + SP-B<sub>1-25</sub> did not affect the molecular area where it entered the LE phase (130 Å²/mol) (Figure 6.18C). Nonetheless, the shoulder at 12 mN/m was greatly reduced and the shoulder at 25 mN/m was diminished. No visible change occurred to the gradual collapse due to addition of cholesterol (Figure 6.18C, square box). Addition of PLGA nanoparticles at the 10:1 ratio did not cause a change to the overall shape of the isotherm with only a minor increase in collapse pressure of about 2 mN/m (from 43 to 45
mN/m). Increasing concentration of PLGA nanoparticle up to the 1:1 ratio caused the system to enter the LE phase before the start of compression due to nanoparticle interaction. In the presence of gelatin nanoparticles, the monolayer occupied smaller molecular areas indicating that the system entered the LE phase much later in compression at 120 Å²/mol which is 10 Å²/mol smaller than the control. Furthermore, a decrease in collapse pressure has occurred of about 3 mN/m indicative of destabilization by gelatin nanoparticles. The compression modulus profile of the 8 lipid + SP-B₁₋₂₅ was similar to the 8 lipid system without the peptide up to surface pressures close to 20 mN/m (Figure 6.18D). With further compression, the system including SP-B₁₋₂₅ experienced increased elasticity with a peak modulus reduction from 86.4 to 66.5 mN/m. Systems including PLGA nanoparticles at the 10:1 ratio further experienced increased rigidity yet not as rigid as the 8 lipid system without the peptide. The peak modulus of 10:1 system reached 76 mN/m. However, as the ratio of PLGA nanoparticles increased, an increase in the system’s elasticity was seen from the start of compression until collapse with a peak modulus of 57 mN/m. Table 6.2 below compares the collapse pressures of the 8 lipid systems and their significance.

**Table 6.2.** Statistical analysis on the collapse pressures of 8 lipid systems with SP-B₁₋₂₅. Systems are compared to 8 lipid + SP-B. Two tailed student t-test was used.

<table>
<thead>
<tr>
<th>Systems</th>
<th>8 lipid (40.8 ± 0.1 mN/m)</th>
<th>8 lipid + SP-B + PLGA 10:1 (45 ± 0.06 mN/m)</th>
<th>8 lipid + SP-B + PLGA 1:1 (44 ± 0.02 mN/m)</th>
<th>8 lipid + SP-B + Gelatin (40 ± 0.05 mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 lipid + SP-B</td>
<td>Significant p value ≤ 0.05</td>
<td>Significant p value ≤ 0.0001</td>
<td>Significant p value ≤ 0.01</td>
<td>Significant p value ≤ 0.01</td>
</tr>
</tbody>
</table>
Figure 6.18. Pressure-Area isotherms and compression modulus of 7 and 8 lipid systems on an aqueous subphase with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 and 1:1 weight ratio through the subphase. A) the pressure-area isotherm and B) compression modulus of 8 lipid systems respectively, C) the pressure-area isotherm and D) compression modulus of 8 lipid systems. Gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the 8 lipid system at the interface. All isotherms collected are average of various replicates (n ≥ 3)
6.4.2 Lateral organization

The first complex lung surfactant model system consisted of the 4 lipids (DPPC, POPC, DPPG, and POPG) and SP-B_{1-25}. Domains formed closer to 15 mN/m with a diameter of 3.4 ± 0.6 μm (Figure 6.19, panel 1). Unlike the peptide free system, no domains were seen at 10 mN/m and only one population of domains formed at 15 mN/m (Figure S6.3, panel 1). These domains fall in between the two population sizes of the 4 lipid system at 15 mN/m of 2.8 ± 0.5 μm and 9.2 ± 1.2 μm (p-value < 0.05). At 30 mN/m, bright clusters dominate due to the presence of SP-B_{1-25} since it was not seen in 4 lipid system (Figure S6.3, panel 1).

PLGA nanoparticles in the presence of SP-B_{1-25} caused domains at 15 mN/m to decrease in size to 2.7 ± 0.4 μm (p-value < 0.001) (Figure 6.19, panel 2 and 3). At the 10:1 ratio, clusters formed at low surface pressures up to 10 mN/m (Figure 6.19, panel 2). On the other hand, with the 1:1 system phase demixing in the form of film voids was seen up to 5 mN/m delaying the formation of the homogenous LE film seen in the control (Figure 6.19, panel 3). A similar effect of PLGA at the 1:1 ratio occurred in the 4 lipid system without peptide. Therefore, these defects could be mostly attributed to the nanoparticles. The bright clusters seen at 30 mN/m in the control system were maintained with systems including both concentrations of PLGA nanoparticles. The size of clusters were large and had a higher intensity at the 1:1 PLGA containing system compared to control and 10:1 system.
Figure S6.3. The lateral organization of complex systems in the absence of SP-B$_{1-25}$. 
Figure 6.19. BAM images of the lateral organization of Panel 1) 4 lipid system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The addition of cholesterol to the 4 lipid system + SP-B_{1-25} prevented domain formation. The 5 lipid system without SP-B_{1-25} had domains forming around 12 mN/m until collapse (Figure S6.3, panel 2). The increased fluidity in the system was due to the presence of SP-B_{1-25}. This agrees well with the decreased compression modulus profile after peptide addition (Figure 6.17D). Therefore, S-B_{1-25} resulted in a homogenous film similar to what is expected of fluid monolayers. At 30 mN/m bright clusters and film void defects can be seen (Figure 6.20, panel 1 insets). The bright clusters at high surface pressures were maintained in the presence of PLGA nanoparticles yet to a higher intensity (Figure 6.20, panel 2 and 3). The size of the clusters in the 10:1 system are larger than the 1:1 system. Phase demixing occurred at low surface pressures with PLGA at the 1:1 system in the form of film voids.
Figure 6.20. BAM images of the lateral organization of Panel 1) 5 lipid system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The next lung surfactant model is composed of 7 lipids + SP-B$_{1-25}$. This system includes 16-1PC, SOPG, and 16-1PG in addition to the 4 lipid system. At 15 mN/m, domains started to appear with a diameter of $2.8 \pm 0.4 \ \mu m$ (Figure 6.21, panel 1). These domains were significantly smaller ($p$-value $< 0.001$) than those seen at similar surface pressures of the 7 lipid system with no peptide with a diameter of $3.6 \pm 0.6 \ \mu m$ (Figure S6.3, panel 3). Compressing the monolayer to a surface pressure of 30 mN/m resulted in domains with a diameter of $3.6 \pm 0.7 \ \mu m$. These domains remained intact until collapse. Again, these domains are smaller than the peptide free 7 lipid system which had a diameter of $7 \pm 1.2 \ \mu m$ ($p$-value $< 0.001$).

The addition of PLGA nanoparticles caused the domains at 15 mN/m to increase in size and frequency to a diameter of $3.6 \pm 0.6 \ \mu m$ ($p$-value $< 0.001$) (Figure 6.21, panel 2). Increasing the concentration of PLGA nanoparticles resulted in a further increase in domain frequency at 15 mN/m (Figure 6.21, panel 3) with a diameter similar to that of control SP-B$_{1-25}$ containing system. At 30 mN/m, systems that include PLGA nanoparticles caused the domains to decrease in size to $2.5 \pm 0.3 \ \mu m$ and increase in frequency at both ratios ($p$-value $< 0.001$) (Figure 6.21, panel 2 and 3 insets). Phase demixing at low surface pressures was only seen with systems that include PLGA nanoparticles at the 1:1 ratio (Figure 6.21, panel 3). This phase demixing was also seen in the 7 lipid system with no peptide in the presence of PLGA at the 1:1 ratio.
Figure 6.21. BAM images of the lateral organization of Panel 1) 7 lipid system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
The lateral domain organization of the most complex 8 component lung surfactant model system is shown in figure 6.22. This system is composed of the 7 lipid system + SP-B_{1-25} with the addition of 2\% (wt ratio) cholesterol. Very small domains were visible and persisted until 20 mN/m but could not be properly sized (Figure 6.22, panel 1). After 20 mN/m, a homogenous film was seen until monolayer collapse. Systems that include PLGA nanoparticles displayed bright clusters at high surface pressures (Figure 6.22, panel 2). Increasing PLGA concentrations resulted in phase demixing at low surface pressures in addition to clusters at 30 mN/m (Figure 6.22, panel 3). The clusters in the 1:1 system were larger with a higher frequency than the 10:1 system. Interestingly, no clusters were seen in 8 lipid systems with PLGA at high surface pressures when no SP-B_{1-25} was present. Since these clusters are not seen in the SP-B_{1-25} control in panel 1, they can be attributed to the contribution of both PLGA nanoparticles and peptide. This indicate a potential direct interaction between the two components.

With gelatin nanoparticles, the lateral organization was similar to control system up to a surface pressure of 30 mN/m. Unlike other SP-B_{1-25} systems that include gelatin nanoparticles, clustering was only seen at high surface pressure of around 30 mN/m (Figure 6.22, panel 4). However, those clusters were larger than those with the PLGA nanoparticles. They varied in size from 3 to 38 μm in diameter. This was one of the most significant effects seen in the lateral organization of all systems analyzed.
Figure 6.22. BAM images of the lateral organization of Panel 1) 8 lipid system + SP-B at 10% weight ratio systems on an aqueous subphase. PLGA nanoparticles are added at both Panel 2) 10:1 and Panel 3) 1:1 weight ratios to the subphase. Panel 4) gelatin nanoparticles at 10:1 lipid to nanoparticle ratio are deposited along with the lipids at the interface. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.
Previously the effects of gelatin nanoparticles on BLES were assessed in our lab (Dr. Lai, PhD thesis 2017). Spikes formed in BLES films in the presence of gelatin were seen by BAM images. BLES consist of a complex mixture of PC and PG lipids along with SP-B and SP-C \((211)\). Due to the similarity in complexity between the 8 lipid + SP-B\(_{1.25}\) system developed here and commercially available BLES, interactions of PLGA nanoparticles on BLES were tested in this thesis at similar concentrations used for gelatin studies done before to allow for direct comparison (Figure 6.23). The lateral organization was found to be similar to that obtained with our 8 lipid + SP-B\(_{1.25}\) with clusters forming at surface pressures around 30 mN/m in the presence of nanoparticle which is well below the collapse pressure of the system. This indicates the toxic effects of the nanoparticles and the similarity between both systems.
Figure 6.23. BAM images of the lateral organization of Panel 1) BLES systems on an aqueous subphase. PLGA nanoparticles are added at Panel 2) 10:1 weight ratio to the subphase. Scale bar corresponds to 50 μm. Each image is a representation of at least 3 images.

From the pressure-area isotherms of the 4 complex model systems, it can be clearly seen that the presence of SP-B$_{1-25}$ increased the stability of the collapse mechanism of the systems (Figure 6.17 and 6.18). The gradual collapse otherwise not seen in the absence of peptide indicated the ability of the peptide to facilitate the squeezing out of unsaturated lipids. This results in saturated lipids occupying the majority of the monolayer. This is a stabilizing effect since saturated lipids can achieve low surface tension values needed for a proper functioning lung surfactant (183, 198, 207-209).
Another outcome of the complex lipid system studies identifies the important interaction between SP-B_{1-25} and cholesterol. Comparing the compression modulus results of the 4 and 5 lipid systems in the absence of the peptide, the rigidifying effect due to cholesterol can be seen in the 5 lipid system. Similar effects were seen in the 7 lipid system after addition of cholesterol (8 lipid system). This is one of the roles of sterols in a mostly fluid system (212, 213). However, in the presence of SP-B_{1-25}, no effect on the monolayer’s rigidity due to cholesterol can be seen. The interaction between SP-B and cholesterol were identified before and results show that when the two are present in a system, the lateral behaviour of the system was greatly affected (214-216).

The 4 and 5 lipid systems in the presence of the peptide resulted in increased clustering at high surface pressures in the absence of both nanoparticles. This effect on the lateral organization could indicate that more components are needed in the system in order to have a better mimic of the lung surfactant that are homogenous throughout compression. This was achieved as seen with the 7 and 8 lipid system + SP-B_{3-25}. No clusters were seen at high surface pressures indicating better mixing between the lipids. Clusters were only seen in these systems in the presence of nanoparticles indicating their potential toxic effects on the system’s stability.
6.4.3 Surface potential measurements

The effect of the presence of SP-B_{1-25} on the surface potential measurements of the 8 lipid system can be seen below in figure 6.24. The changes to the molecular dipoles due to the presence of SP-B_{1-25} and any changes upon nanoparticle addition can be assessed. At high molecular areas when lipids are in the gas phase, the pressure-area isotherm did not detect any lipid-lipid interactions until a molecular area of 115 Å²/mol where lift off occurred for the 8 lipid system (Figure 6.18C). Due to the high sensitivity of surface potential measurements, a sharp increase in the surface potential was seen indicative of dipole reorientation of the monolayer up to a molecular area of 125 Å²/mol to a value of 44 mV. No effects were detected in the pressure-area isotherms at similar molecular areas. Further compression increased the surface potential although to a lesser extent (reduced slope) until collapse with a maximum value of 160 mV (Figure 6.24). The surface potential isotherm for the SP-B_{1-25} containing 8 lipid system started off at a higher surface potential value of 88 mV (8 lipid system alone = 0 mV). This is due to the presence of SP-B_{1-25} which caused an increase in monolayer’s dipole moment. Due to the parallel alignment of the peptide to the monolayer’s interface, minimal effects on the total registered dipole was from the peptide itself (192-194). Therefore, the presence of the peptide facilitated the ordering of the lipid’s headgroup dipole moment and the surrounding water molecules in the vicinity. Since the system already experienced dipole reorientation before compression, only a small increase in the slope is registered during compression of about 10 mV by 130 Å²/mol. The maximum surface potential reached at collapse was 205 mV which was higher than no peptide system (160 mV). Again, pure DPPC from chapter 4 reached a maximum surface potential value of 281.6 mV, DPPG reached 120 mV, and POPC can reach 260 mV (124).
Therefore, the presence of the peptide is increasing the overall surface potential of the 8 lipid system counteracting the reduced potential associated with PG lipids due to opposite dipoles. Since the presence of SP-B$_{1-25}$ allows for a better model or representation of the lung surfactant, the effects of nanoparticles to this model were considered.

PLGA nanoparticles at the 10:1 ratio caused only minor changes to the surface potential of the SP-B$_{1-25}$ control system. A slight deviation was seen at smaller molecular areas where a sharp increase in the potential isotherm was recorded (Figure 6.24, asterisk). This potentially indicate changes to dipole orientation at the low molecular areas. However, the final surface potential reached was not affected and was similar to the control SP-B$_{1-25}$ containing system with a value of 205 mV. With the 1:1 PLGA ratio system, the presence of nanoparticles caused a further increase in the initial surface potential with a starting value of 134 mV. Unlike other 8 lipid systems, the sharp increase in slope of surface potential is reduced, indicating dipole reorientation was occurring gradually with compression. No sharp change in dipole orientation was detected unlike the control system at the initial stages of compression. The maximum surface potential of 221 mV was reached before monolayer collapse.

With 8 lipid + SP-B$_{1-25}$ + gelatin system, the starting surface potential was reduced compared to the 8 lipid system + SP-B$_{1-25}$ alone with a starting potential value of 35 mV. This indicates that gelatin is disturbing the dipole orientation of the monolayer in a negative manner. It is delaying the dipole orientation of the system at large molecular areas. However,
with further compression, a sharp increase in the surface potential occurred from 35 to 98.2 mV between molecular areas of 131 to 117 Å²/mol (Figure 6.24). The maximum surface potential was similar to that of the control system of 205 mV. Interestingly, the dipole reorientation at this stage occurred over a two-step process similar to what is seen for DPPC in chapter 4 and (169) (Figure 6.24, arrow). From the pressure-area isotherm, this two-step dipole reorientation coincides with the LE phase transition of the system (Figure 6.18C).

**Figure 6.24.** Surface potential-area isotherms of 8 lipid systems with and without 10% by weight SP-B. PLGA nanoparticles are added at both 10:1 (red) and 1:1 (green) weight ratio to the subphase. Gelatin nanoparticles at 10:1 (yellow) lipid to nanoparticle weight ratio are deposited along with the lipids at the interface. All isotherms collected are an average of various replicates (n ≥ 3).
As mentioned earlier, the peptide does not contribute directly to the surface potential measured in these experiments. This is because only the normal dipole moment that is perpendicular to the interface is contributing to the measured surface potential (167, 171). Similarly, the spherical shape of the nanoparticles will ultimately result in dipoles that cancel each other out. Therefore, the changes seen in the above systems in terms of surface potential could be due to the nanoparticle and peptide altering the dipole moment of the monolayer due to interaction with the surface. With PLGA nanoparticles, their presence resulted in an increase in the dipole moment that ultimately increased the surface potential. This trend was seen with and without the presence of SP-B\textsubscript{1-25}. However, no such trend was seen in the presence of gelatin nanoparticles. With the 8 lipid system that is missing the surfactant peptide, the gelatin nanoparticles caused an increase in the overall surface potential values. In systems including SP-B\textsubscript{1-25}, that was not the case. As figure 6.24 shows, the gelatin nanoparticles decreased the surface potential values earlier in compression up to a molecular area of 60 Å\textsuperscript{2}/mol. This is the molecular area where the system enters the solid phase at maximum rigidity (Figure 6.18D).

### 6.5 Chapter summary and conclusions

Overall, 10% by weight SP-B\textsubscript{1-25} stabilized the collapse mechanism of the complex lipid systems by increasing the collapse surface pressure (Figure 6.18). In addition, a gradual collapse was seen which was otherwise absent in peptide free systems. This functionality of SP-B was seen before with lung surfactant lipids and mixtures at different ratios (85, 91, 195, 210). Microscopy techniques showed that SP-B with concentrations ranging from 5 to 20 weight %
were able to stabilize collapse of model systems of lung surfactant. This was achieved through homogenization of collapse mechanism by the formation of buckles or reservoirs with anionic lipids at collapse (91, 217). This helps the lung surfactant maintain efficient respreading and attain low surface tension values needed during breathing cycles (91, 217). However, our results and others (200, 202) show that SP-B\textsubscript{1-25} can also stabilize zwitterionic PC lipids (saturated and unsaturated) as well by increasing the collapse pressure (Figure 6.1A). The lung surfactant contains more than just DPPC and therefore with the previously uncharacterized 16-1 lipids and the complex models developed here, more details of the SP-B\textsubscript{1-25} effects are identified. Our results show that SP-B\textsubscript{1-25} also stabilized SOPG and 16-1PG through a significant increase in the collapse pressure not seen with the commonly studied POPG alone.

The SP-B\textsubscript{1-25} peptide segment interacts with both PC and PG lipids albeit to a different extent. With PC lipids and mostly with saturated DPPC, the interactions promote increased elasticity or fluidity to the system that maintains a stabilized collapse mechanism through changes to the lateral packing of the monolayer. With PG lipids, the level of acyl chain saturation dictates a different mode of action. For saturated DPPG, the interaction resulted in an increase in the monolayer’s fluidity packing while with unsaturated PGs such as POPG, SOPG, and 16-1PG an increase in rigidity was seen. This is similar to the effects of cholesterol to a rather fluid system (212, 213). With the most complex models (7 and 8 lipid + SP-B\textsubscript{1-25}) increased fluidity was seen after the formation of SP-B\textsubscript{1-25} associated reservoirs that results in a
gradual collapse. This can be attributed to added fluidizing effect of remaining PC and DPPG lipids since PC lipids constitute the majority of the system (82%).

In terms of PLGA and gelatin nanoparticle effects, the 4 complex models that mimic the human lung surfactant show high clusters that occur around surface pressures of 30 mN/m. This correspond to surface tension of 42 mN/m which is well above the low 0-5 mN/m surface tension values needed to prevent lung collapse. The clusters caused by the nanoparticles are affecting the homogeneity and stability of the model and this clearly indicates the negative effects of those nanoparticles on the human lung surfactant. Furthermore, stronger destabilizing effects were seen with gelatin nanoparticles as is evidenced by the decreased collapse pressure from the pressure-area isotherms of almost all systems tested in this chapter. The altered surface potential and dipole density of the monolayer due to the presence of both nanoparticle is also a concerning effect since it is directly correlated to the stability of the lung surfactant (167).
7.1 Summary and conclusions

Nanomedicine is relatively a new field that emerged in the late 1900s. The ability to generate a drug delivery vehicle with controlled drug release provides many benefits over other common transport methods. Up until 2017, there were at least 50 FDA approved nanomedicines with a minimum of one product approved per year (218, 219). From these approved nanomedicines, less than 12% are for the pulmonary drug delivery. This route provides many advantages such as it being non-invasive, contain large surface area (high vascularization) and provide close proximity to the heart and blood circulation (33-35, 37). Therefore, due to these advantages, more needs to be done in order to further advance this route and FDA approved nanomedicines.

A biomimetic model was further developed in this thesis, and its monolayer biophysical characteristics were analyzed in the presence of both gelatin and PLGA nanoparticles. This model was derived from published mass spec data on the major PC and PG lipids found in the human lung surfactant (83) that was further developed to accommodate for complex combinations of the different lipid classes. Furthermore, the model contained the N-terminal end of SP-B which has a vital role in lung function (190, 191). Techniques used include surface pressure/potential area isotherms as well as interfacial elastic properties through Langmuir trough monolayers. Furthermore, the lateral organization of systems were characterized with
BAM imaging which does not involve the use of exogenous dyes therefore direct analysis on the monolayer’s film can be assessed (100). The model contained 16-1PC and 16-1PG lipids which constitute a major component of the lung surfactant. These lipids are not commercially available yet and were custom synthesized for the purposes of this thesis through Avanti Polar Lipids. Therefore, biophysical analysis on these two lipids was essential and thus were performed before incorporation into the lung surfactant model. This ultimately provided essential information about the contribution of these lipids to the human lung surfactant. 16-1PG introduced higher fluidity to the lung surfactant than 16-1PC. Furthermore, 16-1PG was found to be more fluid compared to POPG while the opposite was true between 16-1PC and POPC. Interactions of these lipids with negatively charged PLGA nanoparticles indicate greater destabilization with 16-1PG as opposed to 16-1PC.

After lung surfactant model development and detailed analysis, the effects of two types of polymeric biodegradable nanoparticles were tested on their interactions with the model. Gelatin nanoparticles were previously studied in our lab but further expanded here with the improved model along with uncharacterized PLGA nanoparticles. The nanoparticles were synthesized to be within the size limit (< 260 nm) that can avoid recognition by alveolar macrophages as a foreign material and pass through to the blood stream (43, 51-53). Gelatin nanoparticles were positively charged while PLGA nanoparticles were negatively charged. The impact of nanoparticle type and charge difference were assessed with the biomimetic model starting with its individual components to the final system. This helps identify how
nanoparticles are interacting with the model and whether it is headgroup driven, acyl chain length and saturation, or a combination of those factors.

Results from this thesis show that negatively charged PLGA nanoparticles with a diameter of 141 nm had a different mode of interaction with the lipids analyzed depending on the level of saturation and chain length. A strong fluidization effect was seen with POPC followed by 16-1PC more so than with DPPC. This indicated stronger effects on unsaturated versus saturated PC lipids. The second class assessed were the PGs and the fluidization trend were as follows: DPPG and 16-1PG > POPG and SOPG. Shorter chained PG lipids had a stronger effect. In addition, comparing the results from the two lipid classes, the electrostatic repulsion between the PLGA nanoparticles and PG lipids had a stronger effect than the electrostatic attraction of the particles with amine groups of the choline headgroup of PC lipids. In addition, comparing saturated DPPC with DPPG, stronger fluidizing effect was seen with DPPG. Therefore, it can be concluded that the electrostatic repulsion was a strong force controlling the mode of interaction of PLGA nanoparticles. The lateral organization of PG lipids were negatively affected by PLGA nanoparticles. Phase demixing at low surface pressures and cluster formation were seen throughout the BAM images.

Gelatin nanoparticles are positively charged and have a diameter of 110 nm. The elasticity of both lipid classes upon exposure to gelatin nanoparticles were altered through an increase in fluidity. Lipid nanoparticle clusters appeared throughout the monolayer from early compression
until collapse. Interestingly, there was a preferential localization of the lipid nanoparticle clusters between the two classes. With PC lipids, the nanoparticles were localized within or at the edge of LC domains while with PG lipids interaction were with the LE phase. This indicated that positively charged gelatin nanoparticles have a strong interaction with saturated zwitterionic PC lipids and unsaturated negatively charged PG lipids. The effect of charge interactions such as electrostatic attraction between positively charged gelatin nanoparticles and negatively charged PG lipids caused a significant destabilization effect. This was seen through the reduction of the collapse surface pressure and changes to the lateral organization more so with PG versus PC systems. Significant reductions in collapse pressure were seen with 16-1PG and SOPG. However, the effects on the lateral packing of zwitterionic PC lipids were significant as well yet to a lesser degree. The lateral organization of PC containing systems were strongly affected by the presence of gelatin nanoparticles where lipid nanoparticle clusters were formed affecting the overall stability of the systems.

The presence of SP-B1-25 in the biomimetic model resulted in a fluidizing effect of individual PC lipids included in the model. The opposite was true with unsaturated PG lipids were a rigidifying effect was seen (saturated DPPG was the exception). This was expected since SP-B and its NH2-terminal peptide (SP-B1-25) are known to interact with unsaturated PG lipids to facilitate breathing cycles (84, 85). The interaction of SP-B1-25 with 16-1PC, 16-1PG, SOPG, and their complex mixtures have not been studied before. The fluidizing effect seen with 16-1PC was similar to that of POPC. 16-1PG and SOPG had a different effect when compared to POPG.
The presence of SP-B_{1-25} caused 16-1PG and SOPG to increase in rigidity and display a stabilization effect not seen with commonly studied POPG where a fluidizing effect was seen. This increased stability indicates the potential importance of these unsaturated PGs in terms of facilitating normal breathing cycles. In addition, this highlights the importance of the distinction between the unsaturated PGs in terms of their function in the lung surfactant.

One interesting outcome from this thesis is the importance of the interaction between SP-B and cholesterol. The addition of cholesterol to the 4 lipid system induced rigidity through a reduction in the elastic properties of the monolayer as seen in the compression modulus data. Similarly, the 7 lipid system in the presence of cholesterol resulted in increased rigidity. This is an expected function of cholesterol in rather fluid lipid systems (212, 213). This was not the case in systems including SP-B_{1-25}. Results show that the effects of cholesterol are diminished when SP-B_{1-25} is present. No difference to the lateral elasticity of the system was seen before and after the addition of cholesterol to SP-B_{1-25} containing 4 and 7 lipid systems. Pérez-Gil and colleagues were able to identify some negative effect of cholesterol on PC-PG systems containing SP-B (214-216). In the presence of cholesterol, the system was not able to mimic the elastic properties of the native surfactant (214). Interestingly, function of PC-PG + SP-B system was restored in the presence of lipophilic SP-C (214-216). Therefore, in the presence of both cholesterol and SP-B_{1-25}, in our model, the increase in rigidity expected due to cholesterol is diminished due to their potential interaction. However, the lung surfactant requires a certain
degree of rigidity in order to maintain the low surface tension needed for proper breathing cycles.

The presence of both PLGA and gelatin nanoparticles altered the lateral organization of the biomimetic 8 lipid + SP-B_{1-25} model system. Lipid nanoparticle clusters were seen in the BAM images at surface pressures of 30 mN/m and higher until collapse. The onset of those clusters occurred below the equilibrium of a proper functioning lung surfactant which occurs at a surface pressure of 49 mN/m (equivalent to surface tension of 23 mN/m) at the beginning of exhalation (172, 220, 221). This can potentially affect the lateral stability needed for proper functioning of the lung surfactant. This indicates that more factors should be considered when designing a drug delivery system in addition to the biodegradability of the material type used to make nanoparticles.

The benefit of using a biomimetic model that is cheap and easily assembled is highly beneficial. It functions as a tool for *in vitro* analysis of nanomaterial toxicity. Previous work was able to show a link between *in vitro* and *in vivo* methods (149). Cyanoacrylate nanoparticles coated with polysorbate 80 surfactant decreased the collapse pressure of DPPC system which ultimately was associated with acute pulmonary toxicity *in vivo* with mice (149). The focus of this thesis was to develop a fully synthetic biomimetic model for nanotoxicology rather than a replacement therapy. Therefore, in order to have a better representation of the human lung surfactant, other lipids which are present in high concentrations yet not well studied in the
literature were added. The results of the mass spec studies from Postle and colleagues were used as an initial guide for the lipid composition from which our model was developed (83). PLGA and gelatin nanoparticles show a destabilizing effect on the stability and lateral organization of 16-1PG and SOPG lipids. Therefore, for purposes of nanoparticle drug delivery, the 8 lipid system + SP-B\textsubscript{1-25} is a good \textit{in vitro} model.

### 7.2 Future directions

It is beneficial to test the effects of SP-C on the behaviour of cholesterol containing SP-B systems. There is a vast amount of literature outlining the importance of both proteins for proper functioning of the lung surfactant (214-216, 222, 223). SP-B is required to maintain interfacial stability at exhalation while both SP-B and SP-C are essential for respreading during inhalation. Even though SP-B is essential for life, absence of SP-C can result in pneumonitis or lung inflammation (224). Nonetheless, for \textit{in vitro} purposes, the model used here with SP-B\textsubscript{1-25} is a good biomimetic model since a similar yet simpler system containing DPPC:POPC:POPG + 1\% SP-B was found to mimic the interfacial behaviour of native surfactant (214). However, as mentioned earlier, in order to assess the safety of nanoparticles for pulmonary drug delivery, the model needs to be expanded to include more lipids that better mimic the human lung surfactant. Both nanoparticles tested show a specific destabilizing effect with these lipids (16-1PC, 16-1PC, and SOPG) otherwise not expected.
In addition, to better mimic the lung surfactant, multiple cycles of compression and expansion would be valuable to better assess film stability. The effects of nanoparticles on multiple isocycles could be more conclusive. Optimal isocycle experiments would be those surrounding the equilibrium surface tension of the lung surfactant which is difficult with the shape of Langmuir troughs available. The maximum surface pressure that can be reached for DPPC system with our rectangular troughs was around 60 mN/m which is lower than literature values of 72 mN/m (109). Therefore, isocycles need to be performed around a surface pressure of 49 mN/m which is difficult given that some of our systems collapsed around that surface pressure. If this is possible, stability measurements on lung surfactant mimics with nanoparticles would be valuable and a better representation. Further optimization would be needed to perform proper lung surfactant isocycle experiments either through subphase or trough dimension alterations. However, isocycles between 0 mN/m and just below system collapse would also be beneficial in determining the driving force behind the interaction between nanoparticles and individual lipids. In addition, the effects on lipid’s stability can be tested this way.

Another future direction is to test other biodegradable nanoparticles commonly used in research for drug delivery. Optimization protocols are currently underway in our group for chitosan and cellulose nanoparticle synthesis. Chitosan is a natural polysaccharide derived from chitin that can display antibacterial properties (225). Cellulose nanoparticles are another biodegradable polysaccharide derived from plants. Chitosan nanoparticles are currently being
tested in the literature for pulmonary drug delivery (225, 226) but based on a literature search, not much is out there for cellulose nanoparticles. Therefore, testing the interaction of these nanoparticles with our biomimetic model is of great interest and a potential future experiment.

The biomimetic model could be beneficial for purposes other than drug delivery. Metal induced toxicity due to industrialization is not a minor concern and carries adverse health problems. For example, metals such as $\text{Co}^{2+}$ and $\text{Ni}^{2+}$ (227, 228), $\text{Pb}^{2}$ (229-231) and $\text{Mn}^{2+}$ (232, 233) have been found to affect the lung’s stability and proper breathing. Nanoparticle format of these metals need to be formulated and then tested since exposure is usually in a particulate rather than in an elemental form. This will help further analyze the mode of toxicity and potentially aid in development of therapies that circumvent the negative effects.
References


Appendix

Published papers


List of talks and conferences

Invited Talks:
- "Effect of polymeric nanoparticles on the stability of biomimetic models of the lung surfactant" April 10th 2015, Institute of Molecular Biosciences, University of Graz, Graz, Austria.

- "Effect of polymeric nanoparticles on the stability of biomimetic models of the lung surfactant" May 6th 2016, Institute of Environmental Sciences and Research (ESR), New Zealand.

Oral conference presentation:


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