A materials science approach to treating respiratory distress syndromes and advanced COVID-19 infections

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Dedication

I am immensely thankful for people who have come before me and opened the door. A heroine in my field, Agnes Pockels (1862-1935), was denied education and opportunities because of the customs of her times, yet found courage to communicate her curiosity-driven scientific findings from her homemade experimental setup. Because of her courage and intellect, the surface science community and women everywhere have benefited tremendously. My own PhD dissertation employs Langmuir trough, a tool that she conceived. This dissertation is dedicated to her and many other women in STEM who have opened the door for me. I hope I can continue doing the same for others.
Abstract

Lung Surfactant (LS) is a mixture of lipids and proteins lining the air/water interface in the alveoli. LS facilitates breathing, mainly by reducing the air/water interfacial tension, and thus the energy required to breathe. The lack of functional LS is associated with two pathological conditions: Neonatal Respiratory Distress Syndrome (NRDS) and Acute Respiratory Distress Syndrome (ARDS). NRDS occurs in premature infants who have not developed LS secretion system, while ARDS happens when the lung is injured and the inflammatory response leads to LS inactivation. To date, the state-of-art replacement surfactants are extracted from animals, of which the exact composition is not known and could vary from animal to animal. This raises concerns regarding contamination and quality-control. Additionally, the animal-derived LS has not been effective in treating ARDS as the body’s innate immune system inactivates LS, both endogenous and exogenous. This lack of quality-controlled, effective LS thus calls for a systematic understanding of clinical lung surfactant formulation, as well as an investigative study into LS inactivation mechanism during ARDS progression and its treatment.

The contribution of my PhD work can be divided into three major themes. First, I have developed an understanding of how formulation affects clinical lung surfactant viscoelasticity and subsequent intratracheal delivery to treat neonatal respiratory distress syndrome. Second, I demonstrated that Langmuir trough, a tool classically used to study lipid monolayer, can be used for measurements of dilatational modulus (resistance to area change) of the lung surfactant system and inflammation products. Third, I investigated dynamics of lung surfactant in the presence of inflammation products, and discovered a mechanism by which a potentially fatal lung collapse can take place.

Ultimately, the unified understanding of these phenomena will serve as a powerful weapon against the respiratory distress syndromes. This overarching understanding of the LS system can further be extended to other membrane and interfacial phenomena.
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6.12 Compressing the protein film from the initial 13 mN/m to 23 mN/m increases both $E^*$ and $G_s^*$ similar to insoluble DPPC. $\beta$-lactoglobulin has a dilatational modulus similar to soluble LysoPC but has a shear modulus orders of magnitude greater than LysoPC or DPPC. B) Following an initial compression of $\beta$-lactoglobulin in the trough to a surface pressure of 23 mN/m, both $\pi_{par}$ and $\pi_{perp}$ decay with time. $\frac{\Delta A_0\pi_{par}}{\Delta A} < \frac{\Delta A_0\pi_{perp}}{\Delta A}$ due to the rearrangement of $\beta$-lactoglobulin at the air-water interface inconsistent with Eqns. 6.1 and 6.2. Proteins unfold due to the change in the hydrophobic environment at the interface.

7.1 Experimental setup for bulk rheometry using cone and plate.
7.2 Steady shear viscosity vs. shear rate for the clinical surfactants Survanta, Curosurf and Infasurf at 37°C. The three surfactants follow a power-law relationship \( \eta = \sigma \dot{\epsilon}^m \) with \( m \approx -1 \) over shear rates from \( 10^{-2} \) to \( 10^2 \)/s. The viscosity of Survanta is two orders of magnitude greater than Curosurf or Infasurf although the lipid mass loading of Survanta is 25 mg/mL, while 35 mg/mL for Infasurf and 80 mg/mL for Curosurf. At the lowest shear rates, a Newtonian plateau (arrows) indicates slip of the concentrated suspension at the walls of the cone and plate. Except where noted, the error was smaller than the symbols.

7.3 Viscosity vs. shear stress. All three surfactants exhibit the features of yield stress fluids. The Newtonian plateau at shear stresses below the yield stress (arrows) is indicative of wall slip, which is common to concentrated suspensions. The viscosity drops by more than an order of magnitude at the yield stress, and the fluid is weakly shear thinning at higher stresses. The error in measurements is within the size of the symbols used unless noted.

7.4 Optical micrographs (top row (A–C)) and freeze-fracture transmission electron micrographs (bottom row (D–F)) of Survanta, Curosurf and Infasurf showing the differences in bilayer aggregate microstructures. (A) Survanta consists of 5–20 \( \mu \)m jagged, prolate bilayer aggregates (arrows) with length to width aspect ratios, \( p \sim 3–6 \). (D) The corresponding FFTEM image shows one such flat aggregate surface with multiple bilayer steps. (B) Curosurf and (C) Infasurf form spherical multilayered structures of 1–10 \( \mu \)m in diameter. However, FFTEM images show (E) Curosurf has close packed concentric lamellae that are organized like an onion. (F) Infasurf consists of aggregated unilamellar vesicles and water-filled void spaces. Hence, Curosurf packs more lipid mass within each aggregate than Infasurf so that the volume fractions are similar for these two surfactants.
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7.6 Linear shear rheology of Survanta, Infasurf and Curosurf at oscillatory strains determined to be in the linear regime for each material. All three materials had $G' > G''$ consistent with significant microstructure in the suspension that could store elastic energy over this frequency range. $|\eta^*| = \sqrt{G'^2 + G''^2}/\omega$ for Survanta is also around two orders of magnitude larger than that of Curosurf or Infasurf, similar to the trend in steady shear viscosity (Figure 7.2). Standard deviation of multiple measurements is smaller than the symbols used.

7.7 Strain amplitude sweep for linear oscillatory shear experiments. The critical strain is determined as being the strain amplitude that causes the maximum $G'$ to decrease 10%.

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(B) Optical microscopy shows that as Survanta is heated above 60 °C, the prolate aggregates (black arrow) melt into spherical shapes (white arrow), similar to those of Curosurf and Infasurf in Figure 7.4B and C.

(C) On gentle shear, the Survanta aggregates stuck to the cover slip distort and form extended tubular structures (arrow), similar to bilayer liposomes. This temperature induced change in morphology is responsible for the drop in viscosity of Survanta on heating.
7.9  Differential Scanning Calorimetry of (i) Survanta, (ii) Infasurf, (iii) Curosurf. Survanta shows a broad endothermic peak from 50-55°C due to the stabilizing effect of palmitic acid on the dipalmitoylphosphatidylcholine gel phase. Infasurf has a very broad, small endotherm from about 28-40°C. Curosurf has a broad peak similar to Survanta but from 25-30°C.

7.10  Surface active materials can adsorb to air-water interface in cone and plate geometry.

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8.1  ARDS feedback loop. Initial trauma or injury leads to inflammation whereby innate immune response leads to permeabilization of alveoli, leading to flooding of alveoli with fluids and contaminants that can alter the dynamics of lung surfactant at the interface and create lung heterogeneity in forms of dead spaces where lung cannot inflate. This leads to decrease in lung dilatational modulus, increase in surface tension, and LS inactivation. Eventually, Laplace instability happens and the alveoli loses compliance, exacerbating the initial injury and one’s ability to breathe.
8.2 Origin of depletion forces in a binary mixture of colloid and non-adsorbing polymer. (Left) The centers of the small spheres are excluded from the hatched regions within one small sphere radius ($R_g$) of the larger spheres (radius $R$) or the interface. (Right) When the larger spheres move to the interface or toward each other, the hatched regions overlap, and the total volume accessible to the small spheres increases by this amount times the number of large spheres (total increase in volume in the bottom right-hand corner). The increase in the volume accessible to the polymer increases the entropy of the system, resulting in a net “depletion” force pushing the large spheres toward the interface or each other. Figure is adapted from [27].

8.3 Structure of polyethylene glycol (PEG).

8.4 Measured viscosity of saline and saline with 5% PEG.

8.5 Reduced viscosity, $\eta/\eta_m$ as a function of shear rate on addition of polyethylene glycol (PEG) of 20 kDa. The reduced viscosity decreases by an order of magnitude or more on addition of PEG, consistent with a decrease in the particle volume fraction in the suspension caused by the combined effects of osmotic pressure induced dehydration of the surfactant aggregates and a loss of excluded volume due to the depletion attraction.

8.6 (A) Small angle X-ray diffraction patterns of Infasurf and (B) Survanta with and without added polyethylene glycol polymer (PEG). Without PEG, both Infasurf and Survanta had broad, weak reflections. The d-spacing for Infasurf varied from 7.6-7.8 nm and Survanta had 3 repeat spacings of 8.8, 8.3 and 7.8 nm, indicative of lateral phase separation within the bilayers. Adding PEG greatly increased the peak heights for both Infasurf and Survanta, indicating better correlations between the bilayers. The d-spacing for Infasurf decreased to 7.1 nm and 7.6 nm for Survanta. The freeze-fracture inset in A shows that the internal organization of Infasurf changed to concentric, tightly packed bilayers with no internal water-filled voids as in Figure 7.4F.
8.7 Centrifuged suspensions of Survanta, Infasurf, and Curosurf, with and without polyethylene glycol (PEG). Before PEG, all three suspensions had similar volume fractions of 40-50% of the yellow lipid aggregates. Following PEG additions, the volume fraction decreased significantly due to the combination of dehydration of the lipid bilayers and the depletion attraction induced flocculation.

9.1 Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of double-chain phospholipids such as DPPC to form single chain lysopalmitoylphosphatidylcholine (LysoPC) and palmitic acid (PA).

9.2 (a) Surfactant coated interface with surface concentration $\Gamma_0$ undergoing oscillations in area, $\delta A$, at frequency $\omega$ in a liquid containing soluble surfactant. (b) for $\omega \tau_s \gg 1$, surfactant exchange is slow, $\Gamma_0 \rightarrow \Gamma_0 - \delta \Gamma_0$, and $\gamma \rightarrow \gamma + \delta \gamma$ resulting in a large $E^* = A \delta \gamma / \delta A$. (c) for $\omega \tau_s \ll 1$, surfactant exchange is fast, leaving $\Gamma_0$ and $\gamma$ unchanged, resulting in $E^* \rightarrow 0$. Figure adapted from Manikantan and Squires [28].

9.3 Equilibrium surface tension vs concentration of LysoPC fit to Equation 9.8 to extract $\Gamma_\infty$, $K$. Inset: Plot of surface tension vs ln (LysoPC) can provide $\Gamma_\infty$ from the slope of the curve near the CMC concentration of 7 µM. Best fit values were $\Gamma_\infty = 1.7 \mu\text{mol/m}^2$ and $K = 330 \mu\text{M}^{-1}$.

9.4 A. A generic schematic of adsorption to a curved surface being faster than to a flat surface. B. Curvature effect on the adsorption of 1 µM LysoPC.

9.5 Adsorption of Lysolipid from bulk lysolipid concentrations ranging from 0.01 – 10000 µM. The dynamic surface tension vs time is plotted for bubble radii of 40 µm (A) and 135 µm (B). The long-time minimum values of surface tension plateau at 38 mN/m for concentrations at or above the lysolipid CMC of 7 µM. For a given concentration, the surface tension decreases more slowly for the larger bubble radius. 175 sec were required to reduce the surface tension to 60 mN/m for a 10 µM solution on the 40 µm bubble, while the same reduction required 350 s on the 135 µm bubble.
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9.9 Dilatational modulus vs. concentration of LysoPC across different frequencies measured on Langmuir trough. .......................... 151

9.10 Dilatational modulus data for LysoPC obtained from Langmuir trough with fit from Lucassen equation (Equation 6.9). The fit does not work well at higher concentration. .......................... 152

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9.13 Dilatational modulus of LysoPC 1000 $\mu$M as a function of frequency, with different colored points indicate different slopes regions.

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10.3 Dilatational modulus of DPPC interface after LysoPC is introduced and the system equilibrates. Below CMC of LysoPC (6-7 $\mu$M), the dilatational modulus of the system is relatively high and close to the value of $E^*$ of DPPC. Above the CMC, the dilatational modulus drops dramatically close to $E^*$ of LysoPC of introduced concentration.
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10.5 Compression/Expansion cycles of the interface of DPPC+LysoPC of (A) 40 µM and (B) 3 µM concentration once surface tension reaches a relatively stable plateau after oscillations as a function of time. The alphabet labels in (A) and (B) are guides for morphological evolution in figures 10.7 and 10.8, respectively.

10.6 Evolution of interfacial morphology following the introduction of 40 µM of LysoPC. Brightness and contrast were enhanced to demonstrate the evolution. Each figure is labeled based on the corresponding points in Figure 10.2. The scale bar in figures A-H indicates 20 µm, while the scale bar in the lower row for figures D*-H* is 100 µm. While not depicted in this chapter, 3 µM LysoPC creates similar changes to the interface following its adsorption and oscillation, resulting in a uniform interface.

10.7 The interface of DPPC+LysoPC 40 µM during compression/expansion cycles. Labels are according to figure 10.5A. Scale bar is 100 µm.

10.8 The interface of DPPC+LysoPC 3 µM during compression/expansion cycles. Labels are according to figure 10.5B. Scale bar: 100 µm. Asterisk labeled images are zoomed in of the non-asterisk labeled images (scale: 20 µm).
Behavior of a DPPC monolayer after the introduction of 20 µM of LysoPC to its subphase. Oscillation brings the system to $\gamma=37$ mN/m. Without oscillation, the system fluctuates between low surface tension around 20 mN/m (LysoPC adsorption and mixing with DPPC) and surface tension around 35 mN/m (close to equilibrium surface tension of pure LysoPC). What affects the shift between one critical point to another is still unknown, but the system is very sensitive to environmental input, like change in stir bar position throughout its hours-long rotation, as well as small temperature fluctuation. It is likely that oscillation helps stir the system towards an equilibrium where $\gamma$ is closest to that of $\gamma_{\text{LysoPC, pure}}$.

(A) Top view and (B) Volume view of DPPC + LysoPC system left overnight with stir bar rotating, no oscillation was performed. Bright dots indicate vesicles. Volume view indicates that some vesicles leave the interface to the subphase. (C-F) Examples of domains during compression/expansion cycle which resemble those in figure 10.8.

Likely scenario by which soluble single-tailed surfactant LysoPC can replace double-tailed DPPC at the interface despite the fact that DPPC is at lower surface tension than equilibrium surface tension of pure LysoPC through interfacial monolayer solubilization. (A) initial, unperturbed insoluble monolayer coexisting with micelles and monomers in the bulk, (B) monomers adsorb to the interface, coexisting with insoluble surfactants at the interface, (C) as monomers crowd the interface, less space is available for insoluble surfactants. This is also the point where micelles get close to the interface, and (D) micelles incorporate insoluble surfactant into its structure, thereby preventing hydrophobic entropy lost from the interaction between water molecule and the hydrophobic parts of the insoluble surfactants.

Compression isotherm of DPPC:LysoPC 1:1 (mol:mol), compared to that of pure DPPC and pure LysoPC at room temperature.
10.13 Morphology of DPPC:LysoPC 1:1 (mol:mol) monolayer during compression. Unlike pure DPPC, the mixed monolayer has liquid and solid phase coexistence that is not confined to one surface pressure (due to additional degree of freedom from having two components at the interface). Thus, domains can be observed throughout a wide range of surface pressure. In the initial stage of domain nucleation, the domains appear circular. The domains later grow with some DPPC-like characteristic (chiral, multilobe), however, the sharp edges indicate that LysoPC exists at the interface and lowers the line tension between the solid and liquid phase.

10.14 Evolution of (A) $\pi - A$ isotherm and (B) dilatational modulus of DPPC:LysoPC 1:1.

10.15 (A) Surface pressure evolution as a function of time is presented for (1) DPPC:LysoPC 1:1 without LysoPC in the subphase (water subphase), and (2) DPPC:LysoPC 1:1 with LysoPC in the subphase (micellar subphase), with LysoPC being introduced at the indicated arrow. In (1), a drop of surface pressure by 30 mN/m was observed, likely correlating with the LysoPC that leaves the interface. In (2), the surface pressure does not change much and remain around 37-40 mN/m, which is around the equilibrium surface pressure of pure LysoPC. (B) The final compression isotherms of (1) and (2) 3 hours after experiment (A) starts. The isotherm of system (1) resembles that of pure DPPC, while the isotherm of system (2) resembles that of pure LysoPC.
Chapter 1

Introduction

Fluid-fluid interfaces are omnipresent in our daily lives. Many of these interfaces, such as those found in our cell membranes, dictate the very functioning of our being. Others, are important in their own unique ways: from the foams used in fire fighting, to emulsions stabilizing mayonnaise and meringue, and of course, these interfaces are even responsible for the creaminess of a gelato! Most of these interfaces exhibit complex behaviors for which surfactants or other surface active molecules are responsible for.

Surface active molecules are amphiphilic molecules that have both non-polar hydrophobic (water-fearing) parts and polar hydrophilic (water-loving) parts. To lower the energy associated with the solvation of their hydrophobic parts, they often adsorb to fluid interface and orient themselves in a fashion such that the hydrophobic part is the least exposed to water. The presence of surface-active molecules at the interface lowers the surface tension of the interface, which lowers the overall energy of the system, thus stabilizing the interface.

Despite the ubiquity of surface active materials, there remains many interfacial phenomena involving these materials that are still not well understood. An understanding of how they behave, both at the interface and at the bulk solution and what governs these behaviors is crucial in a lot of product formulation efforts, ranging from disease treatment to your daily salad dressing.

Throughout my dissertation, I will walk the reader through basic principles of interfacial phenomena, followed by our interfacial system of interest, which is the air/water
interface in our alveoli lined by lung surfactants. Afterwards, I will show how our knowledge of interfacial phenomena can be applied to solve problems relevant to respiration. In particular, I will demonstrate that life-threatening respiratory distress syndromes affecting prematurely-born infants and injured adults can be treated better and more efficiently with better understanding of interfacial and colloidal dynamics of the surface active systems in the lung.

1.1 Important surface active molecules

There are a plethora of materials that are surface active, i.e. adsorb to the interface and reduce surface tension. These materials are divided into several classes: surfactants, proteins, polymers, and particles. Surfactants typically refer to low molecular weight materials, commonly found in detergents and household products; proteins are high molecular weight materials commonly found in physiological environments, as well as in pharmaceutical and food products. Polymers are also high molecular weight macromolecules common in our life, with the most common example being plastic. In terms of adsorption properties, proteins often behave similarly to polymers as both are macromolecules, and they are often put in the same category of surface active materials as distinctions between the two are often unclear. Whereas, particles at fluid-fluid interfaces typically refer to colloid-sized particles that have certain affinities to the interface. In this dissertation, our emphasis will be on surfactants and proteins; surface-active polymers and particles will not be discussed here. Readers are encouraged to read [31, 32, 33, 34, 35] for reference on the other classes of surface active materials not discussed in this dissertation.

1.1.1 Surfactants

Based on solubility, there are two major classes of surfactants: insoluble and soluble surfactants.

Insoluble surfactants

Historically, the ability of oil to calm troubled water was common knowledge to sailors and fishermen, although it was first documented by Gaius Plinius Secundus Maior (a
Roman philosopher better known as Pliny the Elder, 23-79 AD) who noted that divers added oil to water to make it smoother and, hence, easier for them to see the bottom \[36\]. In modern days, perhaps the earliest systematic study on these phenomena was done by Benjamin Franklin in 1774 when he made a careful observation of the effects of oil on water and waves on its surface \[37, 38\].

Today, this phenomenon is well-regarded as an example of the spreading of insoluble surfactants on an air/water interface. Insoluble surfactants are highly insoluble chemicals (hydrophobic) that prefer to stay at the interface. Hence, for insoluble surfactants, there is no adsorption or desorption that takes place in observable timescale, and they form insoluble monolayers, also often called Langmuir monolayers. Because of the confinement to the interface, compressions of such monolayer result in a significant change in molecular lateral arrangements in forms of lipid tail ordering or crystalline domain formation and growth, which therefore leads to phase transitions \[39\] (more on this will be discussed in Chapter [3]). For a molecule to be an insoluble surfactant, it has to have a large hydrophobic part (larger than its hydrophilic part) and a hydrophilic part that has strong enough affinity to water to anchor the molecule to the surface. The behavior of insoluble surfactants are often studied by spreading known amount of surfactants in water immiscible solvent to a known area of air-water interface.

Examples of insoluble surfactants include phospholipids, the key component of cell membrane, and fatty acids, important building block of fats. Aside from the biological interest, insoluble surfactants are also of interest for drought prevention as it reduces evaporation of the subphase water dramatically.

**Soluble surfactants**

In contrast, soluble surfactants, as the name suggests, are soluble in water. They also readily adsorb to the interface and form a Gibbs monolayer. Many surfactants are soluble. Unlike their insoluble counterparts, soluble surfactants like to maintain the same number of molecules per area. Thus, changes in area will cause molecules to adsorb to or desorb from the interface. Here, an important timescale arises: diffusive timescale at which molecules leave/adsorb to the interface.
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Figure 1.1: Surface active molecules of interest in this dissertation: (left) insoluble surfactants, (middle) soluble surfactants, and (right) proteins, each with different solubility, adsorption properties, and surface affinity.

1.1.2 Proteins

Comprised of $L-\alpha-$amino acids linked together by peptide bonds to form chains containing up to several hundred monomer units (residues), proteins are significantly heavier than other surface-active materials. For example, sodium dodecyl sulfate (SDS), a small molecule surfactant common in household detergents, has a molecular weight of 288 g/mol, while human serum albumin, a protein found in blood that functions as hormone and fatty acid carrier, has a molecular weight of 66500 g/mol, or around 230 times heavier than SDS. Because of their weight, proteins are unique in terms of their adsorption; while they are soluble in a bulk solution, once they are adsorbed to the interface, they do not readily desorb to the subphase. In contrast to small molecule surfactants that typically form monolayer (or one molecule thick layer) at the interface, proteins tend to adsorb and create ”multilayer” at the interface, which is due to its complex 3D structure.

Proteins have multiple levels of structures: primary structure describes the covalent bonding of amino acids; secondary structure describes the local arrangement of the polypeptide backbone bound by non-covalent bonding; tertiary structure describes the complete 3D structure of the protein.

There are two kinds of tertiary structure of proteins. Flexible or disordered proteins have random native protein configuration in bulk solution, and because of that, their desorption from the interface is not affected by change in configuration at the interface. However, globular proteins, as the name suggests, has a globe-like (spherical) native structure. Upon adsorption to the interface, this structure unfolds, making desorption
back to the aqueous bulk phase unfavorable.

The complexity of these structures is what allows proteins to perform their unique function. When proteins undergo conformational change, a greater portion of the protein’s hydrophobic sequences are exposed to air or oil and thus the molecule’s free energy is lowered. When this happens, neighboring proteins can interact, often through disulfide bonds, to generate an interfacial gel that irreversibly resides at the surface, which contribute to the formation of rigid films able to stabilize liquid films resulting in long-lived emulsions and foams.

However, since proteins function optimally at its unique tertiary configuration, its adsorption to an interface and its subsequent conformational change will therefore render them "denatured". Currently, many R&D efforts, either in pharmaceutical or food and beverage industry, have great interests in using small molecule surfactants to keep proteins from adsorbing to fluid-fluid interface to protect their native configurations so that these proteins can perform their functions optimally,

1.2 Interfacial tension

Molecules at a fluid-fluid interface experience force imbalance; while molecules in the bulk phase farther away from the interface experience symmetric cohesive force from all directions (which causes net force to be zero for a molecule in the bulk), molecules at the interface experience a non-zero net force towards the fluid phase with stronger intermolecular force. Interfacial tension is thus the tendency of an interface to contract due to this inward cohesive force between molecules in and adjacent to the surface. Figure 1.2 shows the difference between neat interface vs. surfactant covered interface. At an air-fluid interface, this interfacial tension is commonly referred to as surface tension.

The stronger the cohesive force between molecules, the higher the interfacial tension will be. For example, water, with strong hydrogen bond, has a surface tension of 72 mN/m at room temperature, while hexane, a non-polar liquid with a much weaker van der Waals force, has a significantly lower surface tension of around 18 mN/m.

From a thermodynamic standpoint, surface tension is the energetic cost for a fluid-fluid interface creation of area $A$. Mathematically, this is written as:
Figure 1.2: (left) Clean air-water interface, where surface tension is that of clean water; (right) surfactant-covered air-water interface, where surface tension is the difference between the clean water surface tension and the surface pressure exerted by surfactant at the interface

\[ dG = \gamma \, dA \quad (1.1) \]

where \( G \) is Gibbs free energy of the system, and \( \gamma \) is the interfacial tension. Thus, there is a tendency for a clean interface to contract and minimize its area due to this tension.

In the presence of surface-active molecules at the interface, the interfacial tension is lowered by surface pressure:

\[ \pi = \gamma_0 - \gamma \quad (1.2) \]

where \( \gamma_0 \) and \( \gamma \) are the interfacial tensions of clean fluid-fluid interface and of non-solvent molecules-covered interface, respectively. Surface pressure is the force per unit length exerted by the surfactant to expand the interfacial area in opposition to the inward pull of the interfacial tension, which acts to decrease the interfacial area. Surface pressure can be thought of as the 2D version of the 3D pressure in bulk materials. Surface pressure is determined by the number of molecules per interfacial area, \( \Gamma \). The more surface-active molecules there are per unit area in the interface, the stronger their intermolecular attraction is, which will give a higher surface pressure, or ability to reduce the surface.
tension.

1.3 Adsorption

All systems achieve equilibrium through minimization of energy. When a surfactant molecule with long hydrocarbon chain is isolated in water (I will refer to this isolated surfactant molecule as monomer or unimer interchangeably), a cavity outlined by an ordered cage of water molecules forms and disrupts the hydrogen bonding of water molecules, leading to a decrease in the entropy of the water molecules surrounding the surfactant molecule. This effect is often referred to as the “hydrophobic effect”. While the bulk fluid offers a wide range of translational and rotational micro-states, and therefore, a larger entropy per soluble surfactant molecule, the hydrophobic effect causes a greater penalty in entropy lost \[40\]. To overcome this unfavorable interaction with water molecules, surfactants adsorb to the interface to lower their energy. Additionally, when surfactant adsorbs to the interface, they replace some of the water molecules at the interface. Since the interaction force between water molecules and surfactant is less than that between water molecules (as water molecules interact via strong hydrogen bond), adsorption of surfactants to the interface results in the reduction of surface tension. Surfactant adsorption is dependent on its bulk concentration, \(C\).

When surfactant concentration is high enough, surfactant monomers self-assemble into micelles. For dilute system, i.e. where concentration of surfactant is below its critical micellar concentration (see below, section 1.4), i.e. when micelles are not present in the system, the adsorption of soluble surfactants often follows the Gibbs adsorption equation:

\[
\Gamma = -\frac{1}{k_B T} \frac{d\gamma}{d \ln C} \tag{1.3}
\]

This equation, derived from the Gibbs-Duhem thermodynamic relations, is powerful in describing surfactant adsorption to the interface. At dilute concentration, the slope of surface tension-surfactant concentration curve gives the value of surface concentration (see Figure 1.3).

Surfactant adsorption to the interface can either be diffusion-limited, kinetic-limited, or both. In Chapter 9, we will discuss an example of a diffusion-limited adsorption process.
1.4 Self-assembly of surfactants

Once the interface to which surfactants adsorb to has been saturated, i.e. when an "equilibrium" number of surfactant per area, $\Gamma_\infty$, has been reached and the system reaches an "equilibrium" surface tension, amphiphilic molecules in the bulk can no longer adsorb to the interface. At this concentration, these amphiphilic molecules self-assemble to form aggregates (more commonly known as micelles) in order to minimize their energy. The tendency to form micelles is a manifestation of the same hydrophobic effect that leads to their adsorption at interfaces. As these aggregates form, entropy increases significantly, and the change in enthalpy is often positive, which happens because as the hydrocarbon portions of the surfactant aggregate, the cavities are melted and the hydrogen bonding of the water is restored. This increase in water entropy is greater than entropy decrease from hydrocarbon ordering in aggregates.

The concentration where this aggregation happens is called the critical aggregation concentration, and more generally known as the critical micellar concentration (CMC) for micelles formation from single-tailed detergents. With no further adsorption to the interface, $\frac{\partial \gamma}{\partial C} = 0$, i.e. surface tension does not change with any increase of surfactant concentration in the bulk, and the Gibbs adsorption equilibrium does not hold anymore. Figure 1.3 shows the concentration effects on soluble surfactant adsorption and self assembly.

Above CMC, many properties of surfactant solution, such as specific electrical conductivity, turbidity and osmotic pressure (and other colligative properties), also shows dramatic changes, often accompanied with sharp discontinuities, as seen in figure 1.4. Some of these changes are easily measurable (especially turbidity detectable by naked eyes), and thus these properties can be used as additional indicators in detecting micelle formation.

Spontaneous self-assembly of surfactants takes place if, with the addition of one surfactant monomer to the system, the overall energy of the system is reduced with the formation of aggregates. In a micellar system, the free energy of a unimer (a unbound, isolated surfactant molecule) is the same as the free energy of a bound surfactant.

Tanford [41] and Israelachvili [42] suggested that the geometry of an amphiphilic molecule is indicative of how that molecule self-assembles, as shown in Figure 1.5. Based
Figure 1.3: Surface tension vs. concentration plot for a generic soluble surfactant. Below CMC, surface tension scales linearly with logarithmic of concentration indicating adsorption following the Gibbs adsorption equation, Equation 1.3. Above CMC, surfactants cannot adsorb to interface anymore as the interface is fully saturated, and self-assembly in the bulk takes place to reduce surface tension.

Figure 1.4: Schematic of physical property changes of a sample surfactant as a function of concentration. At concentration above CMC, many physical properties change dramatically. Figure adapted from Berg [1].
on the packing parameter $p = V/A_\circ l_\circ$, where $V$, $A_\circ$, and $l_\circ$ are total volume, surface area of headgroup and tail length, respectively, surfactant molecules self-assemble into spherical micelles for $p < \frac{1}{3}$, cylindrical micelles for $\frac{1}{3} < p < \frac{1}{2}$, flexible bilayers, i.e. vesicles, for $\frac{1}{2} < p < 1$, planar bilayers for $p = 1$, or inverted micelles for $p > 1$. Micelles are transient associations of 10-1000s of lipids with lifetimes of milliseconds to minutes. Micelles are always in exchange equilibrium with its monomer. In contrast, vesicles or planar bilayers are long-lived association of between a thousand to infinitely many number of amphipiles. Bilayer structure is not in exchange equilibrium with unbound monomers. These two structures can be differentiated experimentally as micelles cannot be isolated by filtration or centrifugation, whereas, bilayers can easily be separated from the solvent.

Formation of micelles alters the dynamics of surfactant adsorption to the interface. For examples, in order to adsorb to the interface, concentrated surfactant solution with concentration above CMC adsorbs differently from dilute surfactant solution with only monomers. In Ch. 9, we will discuss the effect of micellar presence on interfacial rigidity.

1.5 Dissertation overview

The overarching goal of this dissertation is to provide a toolbox for readers to understand the complexity of the lung surfactant system which is translatable to other surface active systems. The ultimate objective of these studies is to gain insight into lung surfactant dynamic, from which novel treatments for respiratory diseases can be devised. To interrogate structure-properties relationship of the lung surfactant dynamics, I have employed many traditional materials characterization tools as well as developed novel ones in-house, and connected the properties to the structure and composition of each studied lung surfactant system.

While the main focus of this dissertation is on the lung surfactant ecosystem, a careful interpretation and further study based on observations noted in this dissertation can guide efforts on rational designs for materials and fluid handling systems.

In Chapter 2 I will introduce readers to the concept of lung surfactants and their importance to our health, focusing on current understanding of their history, properties, functions, compositions, and diseases associated with lack of functional lung surfactants.
Figure 1.5: Various self-assembly structures of amphiphilic molecules as determined by their head and tail sizes. Figure is adapted from Israelachvili [2].

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$V/A_0l_c$</th>
<th>Critical Packing Shape</th>
<th>Structure Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-chained lipids (surfactants) with large headgroups e.g. SDS (low salt)</td>
<td>$&lt; 1/3$</td>
<td>Cone</td>
<td>Spherical micelles</td>
</tr>
<tr>
<td>Single-chained lipids with small headgroups e.g. nonionic lipids, lysolipids</td>
<td>$1/3 - 1/2$</td>
<td>Truncated cone</td>
<td>Cylindrical</td>
</tr>
<tr>
<td>Double-chained lipids with large headgroups e.g. PC, PG, PS, PA</td>
<td>$\frac{1}{3} - 1$</td>
<td>Truncated cone</td>
<td>Vesicles</td>
</tr>
<tr>
<td>Double-chained lipids with small headgroups e.g. PE</td>
<td>$\sim 1$</td>
<td>Cylinder</td>
<td>Planar bilayers</td>
</tr>
<tr>
<td>Double-chained lipids with small headgroups, bulky unsaturated tails e.g POPE</td>
<td>$&gt; 1$</td>
<td>Inverted</td>
<td>Inverted micelles</td>
</tr>
</tbody>
</table>
In Chapter 3, I will discuss important physical properties of phospholipids (a major class of insoluble surfactants and the main chemical makeup of lung surfactant) as well as the traditional methods for studying them, highlighting Langmuir trough and confocal microscopy that I have employed to study thermodynamics and morphology of phospholipids at air-water interface.

To complement the understanding of the lung surfactant system derived from the classical tools, I have also used rheological tools (Chapters 5, 6, 7, 8, 9, and 10) to develop better insight of lung surfactant dynamics in non-equilibrium systems. In Chapter 5, I will provide readers with an overview of interfacial rheology. In Chapter 6, I demonstrated examples of interfacial rheology measurements of DPPC, a main lung surfactant lipid, and LysoPC, an inflammation product that inhibits lung function. I additionally compare the two measurements to a globular protein, β-lactoglobulin and note that the rheological responses of DPPC and LysoPC are distinctly different from that of β-lactoglobulin. In Chapter 7, I will show that the three clinical lung surfactants widely used in the U.S. are very different in their morphologies and responses to flow, likely contributing to the different clinical outcomes. In Chapter 8, I demonstrate that polymer can positively affect the outcome of clinical lung surfactants. In Chapter 9, I delve into the dynamics of LysoPC as a model soluble surfactant before investigating the dynamics of LysoPC/DPPC system in Chapter 10.
Chapter 2

Lung Surfactant System

In this chapter, I will delve into parts of the human lungs necessary to understand the rest of this dissertation, namely, the alveoli and the pleura (Figure 2.1). I encourage interested readers to refer to [43, 44, 45] for detailed reviews on lung physiology.

The lungs are surrounded by double layered serous membranes called the pleura. The visceral pleura covers the outer surface of the human lung, while the parietal pleura is attached to the thoracic wall. The cavity between the visceral and parietal pleura is called the pleural cavity and is lubricated by a serous fluid.

The smallest functional units of our lung are the air sacs called alveoli (plural of alveolus) where gas exchange occurs. The terminals of a 23-generation respiratory tract, there are in average 480 millions alveoli in an adult human lung. The total surface area of the alveoli is around 150 m$^2$, approximately the size of a tennis court. Each alveolus is wrapped by a fine mesh of blood capillaries to maximize the contact area for gas exchange within the confining volume of the pleural sac.

A thin aqueous layer of approximately 0.1 $\mu$m thick lines the internal alveolar surface to maintain tissue hydration [46]. This epithelial lining fluid (ELF) is primarily water, with salts and various other proteins, enzymes, sugars, etc. Consequently, there is a huge air/water (air/ELF) interface in the alveoli.

Lung surfactant (LS) is a lipid/protein complex that is secreted from a type II cells and spreads on this interface and serves major functions in breathing, and is shown in figure 2.2. In fact, its absence or displacement from this air/water interface can cause severe, potentially fatal, respiratory diseases.
Figure 2.1: Schematic diagram of the lung showing the bifurcations from the trachea through the bronchi, terminating in the alveoli. Pressure inside the alveoli: $P_{in}$, pressure in pleural sac: $P_{out}$, and ambient pressure: $P_{am}$

Figure 2.2: Schematic of lung surfactant system. Secreted by pneumocyte cell type II in forms of lamellar body, lung surfactant lines the air/water interface in the alveoli. Figure is adapted and modified from Piknova et al. [3]
2.1 Functions

With the large air-water interfacial area, there is a tendency for the alveolar interface to contract due to strong surface tension. This means that without a force opposing this surface tension contraction, alveoli can collapse.

LS assists breathing in several ways. First, its presence as surface active entities reduces the surface tension along the alveolar air-water interface, thus reducing the energetic cost of breathing. This surface tension lowering function is closely tied to several other functions of LS, as will be discussed below.

Inhalation is initiated by expanding the chest cavity via the diaphragm. As the chest cavity enlarges, the pressure in the lung pleural sac $P_{\text{out}}$ is lowered relative to the ambient pressure, $P_{\text{am}}$: $(P_{\text{am}} - P_{\text{out}})$ by approximately 1000 Pa to suck air in. For air to flow into the lungs, $(P_{\text{am}} - P_{\text{in}})$ needs to be positive. The pressure difference across the curved interface of the alveoli, i.e. capillary pressure, generated by the air-water surface tension is given by the Young-Laplace equation:

$$P_{\text{in}} - P_{\text{out}} = \gamma \left( \frac{1}{r_1} + \frac{1}{r_2} \right)$$

Since the lung alveoli are approximately spherical, $r_1 = r_2 = R$, which is the radius of the alveoli, the equation can be reduced to:

$$P_{\text{in}} - P_{\text{out}} = \Delta P = \frac{2\gamma}{R}$$

with $\gamma = 72$ mN/m and $R \sim 100 \mu$m, we have $P_{\text{in}} - P_{\text{out}} \sim 1500$ Pa, which would cause $P_{\text{am}} - P_{\text{in}} < 0$ and the diaphragm would not be able to generate the necessary pressure drop, leading to excessive work being needed to inflate the lung. This can cause insufficient air flow to the lung and an underinflated lung. The presence of LS, which can reduce the $\gamma$ to as low as 1 mN/m, is crucial because it causes a substantial decrease in $P_{\text{in}} - P_{\text{out}}$, leading to a significant decrease in work required for lung inflation and breathing in general.

From a different perspective, LS also prevents a potentially fatal lung collapse. At any given time during breathing, different alveoli will be in different states of inflation, i.e. different $R$ and thus different $2\gamma/R$ value. Without LS, the air-water interface would
have a constant $\gamma$, and heterogeneity in alveolar size would create a pressure differential between the interconnected alveoli, according to the Laplace law in equation 2.2.

Accordingly, for the same $P_{\text{out}}$, air moves from high pressure to low pressure alveolus, i.e. from small alveolus to large alveolus. Thus, one alveolus would be hyperinflated, while the other shrinks to the point of collapsing. This phenomenon is termed Laplace instability. Other than the obvious imbalance in the alveoli, collapsed alveoli also would cause inadequate gas exchange, capillary constriction, and water inside alveoli that can cause pulmonary edema. Collapse of alveoli is one of the major symptoms presented in many life-threatening respiratory diseases.

The collapse could be prevented if $\Delta P$ is the same for both alveoli, which means $\gamma$ increases with $R$, or to put it another way: $\frac{\partial \Delta P}{\partial R} > 0$. A useful parameter to quantify this is the dilational modulus, $E^* = A \frac{\partial \gamma}{\partial A}$, which is a measure of the alveoli’s resistance to dilation. For an approximately spherical alveolus, $A = 4\pi R^2$ and $dA = 8\pi R dR$, which gives $E^* = \frac{R}{2} \frac{\partial \gamma}{\partial R}$. Laplace instability can thus be prevented if:

$$\frac{\partial \Delta P}{\partial R} = 2 \frac{\partial (\gamma / R)}{\partial R} = 2 \frac{2E^* - \gamma}{R^2} > 0 \quad (2.3)$$

Equation 2.3 reduces to $2E^* - \gamma > 0$. If satisfied, alveoli will not collapse despite variation
Figure 2.4: $2E^* - \gamma > 0$ for breathing surface pressure, i.e. 20-70 mN/m for animal-derived lung surfactant, Survanta, and DPPC, a main lung surfactant component. Measurements were done in a Langmuir trough (see Chapter 6 for more details).

In Figure 2.4, we showed that $2E^* - \gamma > 0$ for breathing surface pressure, i.e. 20-70 mN/m for animal-derived lung surfactant, Survanta, and DPPC, a main lung surfactant component. This means that small changes in interfacial area led to large changes in surface tension, which in turn, stabilize alveoli against the Laplace Instability. DPPC is the main lipid responsible for this effect as the dilatational modulus of this insoluble lipid is even greater than Survanta at comparable surface pressures. This indicates that lung collapse is prevented if the alveoli are lined by either DPPC or more complex lung surfactant mixture.
2.2 Life-threatening respiratory distress syndromes

The lack of functional LS leads to neonatal respiratory distress syndrome (NRDS) and acute respiratory distress syndrome (ARDS). The physical manifestations of both diseases are similar: breathing difficulty, decreased lung compliance (change in lung volume for a given change in pressure), and liquid infiltration of the alveoli; these conditions reduce patient oxygenation and can be fatal. While NRDS is treatable by intratracheal instillation of clinical animal-derived replacement lung surfactants, the same approach has had limited success in treating ARDS, which mainly affects adults. With no known cure and the significant increase of ARDS cases due to the COVID-19 pandemic, an understanding of the role of LS in the progression of ARDS is pivotal in developing a cure for the disease.

2.2.1 Neonatal respiratory distress syndrome

Infants born before their thirty-second gestation week typically have not developed a mature respiratory system, and the absence of alveolar epithelial cells (pneumocytes) type II which secrete LS is typical [47, 48]. These infants are vulnerable to respiratory distress that manifests in these symptoms: difficulty breathing, atelectasis (alveolar collapse), decreased lung compliance (stiff lungs that require a greater pressure differential to inflate), decreased functional residual capacity (a measure of the amount of air left in the lungs after exhalation), systemic hypoxia (oxygen starvation), and lung edema (bleeding in the lungs) [49]. Collectively, the disease is called Neonatal Respiratory Distress Syndrome (NRDS). Before Avery and Mead correlated the lack of surfactant to the high surface tension in the lung and other pathophysiology of NRDS in 1959 [50, 51], the cause of NRDS was not well understood and no effective treatment was available. NRDS was the leading cause of deaths in preterm infants then and up until the 1990s.

In the late 1960s, estimates attributed NRDS as the cause of 12,000 infant deaths each year in the United States [52], with an incidence of about 25,000 cases annually [49]. The death of Patrick Kennedy, the prematurely born son of President John F. Kennedy, in 1963 due to NRDS propelled R&D efforts for NRDS treatments. The introduction of improved ventilation techniques and methods for measuring blood oxygenation in the 1970s did improve the outcome of NRDS, but it was not until Fujiwara administered
cow-derived artificial lung surfactant to neonates in 1980 that NRDS became successfully treated [53].

To this date, NRDS remains the most common complication of prematurity leading to significant morbidity in late preterm neonates and mortality in very low birth weight infants. Currently, it is estimated that 50,000-60,000 premature infants are at risk of NRDS in the United States each year. While the animal-derived LS has been successful in treating NRDS, there are still some remaining challenges associated with the use of clinical LS, which I will discuss in Chapter 7.

2.2.2 Acute respiratory distress syndrome

In adults, traumas to the lung, such as from near-drowning, car accidents, fat embolism, and pneumonia, can cause NRDS-like symptoms to manifest. This disease is called Acute Respiratory Distress Syndrome (ARDS) [54, 55, 56]. Unfortunately, ARDS does not have a cure, and animal-derived LS successful in treating NRDS has not had similar success in treating ARDS [57, 58, 59]. The mortality rate of ARDS is 40% [60].

The pathophysiology of ARDS is complex since it is a secondary condition to another underlying disease or injury, and therefore it is difficult to pinpoint a single cause of ARDS as is the case for NRDS. One symptom is diffuse inflammation in the lungs of ARDS patients, which leads to pulmonary edema (fluid in the lungs) due to increased permeability of the alveolar epithelial membrane [61]. This fluid is composed of blood serum and phospholipases from the surrounding blood capillaries (figure 2.5), which can disrupt respiration [62, 63]. The exact mechanism of ARDS progression is still unknown, but it is hypothesized that LS displacement from alveolar air-water interface is responsible. Figure 2.5 shows the difference between a healthy alveolus vs. an ARDS alveolus, where alveolar-capillary barrier becomes permeabilized as results of trauma-induced immune response. In Chapter 10, we will evaluate the alveolar mechanics evolution during ARDS and propose our hypothesis for the mechanism of ARDS progression.

2.3 Dynamics of lung surfactant

The materials making up the lung surfactant system must meet a couple of criteria. First, they must be able to reduce surface tension to lower than 20 mN/m. Second,
Figure 2.5: Comparison between healthy and ARDS alveolus. In injured lung, blood capillary lining the outside of the alveoli becomes permeabilized, resulting in fluid extravasation into the lung, which causes elevated concentration of contaminants inside the lung that can inactivate native lung surfactant.
they must adsorb quickly to the alveolar air-water interface once secreted from the pneumocyte type II cells in forms of multi-bilayer vesicle aggregates called lung multilamellar bodies of 1-3 µm diameter. After secretion, the multilamellar bodies unpack into a structure called tubular myelin, which moves across the alveolar epithelial lining fluids [64], adsorb to the air–water interface, and then transform from bilayer to monolayer and spread over the interface [47] [65] [66]. To this day, the exact mechanism by which bilayers adsorb to an interface and converts into a monolayer structure is relatively unknown, although Hall et al. [66] [67] hypothesize that lung specific proteins play a crucial role in this phenomenon.

LS monolayer must be rigid enough to promote near zero surface tensions during the alveolar compression accompanying expiration. At the same time, the monolayer needs to remain fluid enough to respread quickly and reversibly upon inhalation.

Healthy LS adsorbs and spreads at the ELF-air interface until \( \pi \geq 40 \ \text{mN/m} \) [68] [69]. Spreading of surfactant at air/water interface is governed by Marangoni flow, i.e. flow driven by surface tension gradients (usually because of surfactant concentration gradient or temperature gradient) across the interface. Marangoni flow proceeds from low surface tension to high surface tension areas. Compression during expiration increases \( \pi \) to over 65 mN/m. The minimum dynamic interfacial tension is limited by the strength and cohesion of the monolayer film. Eventually, the monolayer becomes highly unstable due to the high degree of compression and "collapses" to the subphase either via folding, buckling, squeezing out, or other mechanisms (the exact mechanism of how LS monolayer collapses is still debatable and remains an active area of research) [70] [71] [72] [73] [74] [75] [76] [77] [78] [10] [79] [80]. When monolayer collapse happens, some components of LS are removed or "squeezed-out" from the interface as expiration takes place.

After this monolayer collapse, enough lung surfactant must remain at the interface (or stay in an interface-associated “reservoir” in the vicinity of the interface to facilitate readsorption when the surface pressure is lowered on inspiration) to respread and cover

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Distinctions should be made when the term collapse is used. Monolayer collapse is a dynamic phenomenon where a monolayer is highly compressed and relaxes by exploring the third dimension. Alveolar or lung collapse is a symptom of a respiratory disease where alveoli are unable to inflate properly.
the expanding alveolar interface during inspiration to maintain low dynamic surface tension. Monolayer collapse is a dynamic process that depends on the rate of compression, as well as the monolayer composition, \([71, 78]\).

The collapsed surfactant molecules that are not readorsorbed back to the interface are recycled by endocytosis into multivesicular bodies and eventually back into lamellar bodies. This process of recycling endogenous and exogenous surfactants from alveoli is responsible for maintaining the surfactant pool in a healthy adult.

### 2.4 Composition

Currently, our knowledge of the composition of native LS is evaluated from the bronchoalveolar lavage of animals. Approximately, native LS consists of \(\sim90\%\) lipids and \(\sim10\%\) proteins by mass \([81, 82]\).

#### 2.4.1 Lipids

The lipids in lung surfactant system mainly consist of double-tailed phospholipids (PL): the zwitterionic phosphatidylcholine (PC), the anionic phosphatidylglycerol (PG) and phosphatidylserine (PS) headgroups (see Figure 2.6 and 2.7) \([82]\). For most land mammals, half or more of the PC fraction is disaturated dipalmitoyl phosphatidylcholine (DPPC, \(T_m^{\text{2}} = 41^\circ\text{C}\) with 16 carbon chains on its two tails), see Figure 2.8 \([82, 83]\). DPPC has a remarkable surface tension lowering property as it can be compressed into very tight packing due to its tails saturation.

LS also contains unsaturated PLs (25% of total LS mass), and one of the main unsaturated PLs making up LS composition is palmitoyloleoylphosphatidyl-glycerol (POPG), a 16:0/18:1 phospholipid (Figure 2.8). Mainly of low \(T_m\), these unsaturated lipids reduce the overall \(T_m\) of LS mixture close to the physiological temperature of 37°C. It has been suggested that the LS composition is evolutionarily set such that the LS system is maintained at the critical point between two fluid states at physiological temperature to allow for large-scale fluctuations and long-range interactions to take place. \([84]\)

Pure DPPC can reduce surface tension to less than 1 mN/m upon dynamic compression \([85, 86, 87, 88]\). However, DPPC does not readily adsorb to the air-water interface,
Figure 2.6: Estimated surfactant composition of human bronchoalveolar lavage fluid. Note that the notation for PLs used is in the form of $a : b/c : d$ indicating that the PLs have two hydrocarbon tails. The first tail has $a$ number of carbons, with $b$ number of kink (unsaturated chain), similarly, the second tail is $c$-carbon long, with $d$ unsaturated chains. Figure is adapted from Numata et al. [4].

especially compared to various animal-derived lung surfactant extracts [68, 89]. Unsaturated lipids, like POPG, form more fluid interfacial films that do not pack as tightly as disaturated lipids, and therefore cannot achieve near-zero surface tensions [90, 91]. Despite that, these unsaturated lipids are important because they adsorb more readily than DPPC to the interface [92, 93]. It is likely that the combination of rigid, disaturated lipids with fluid, unsaturated lipids creates a lung surfactant mixture that can quickly adsorb to the interface and reduce γ sufficiently. Thus, simple mixtures based on DPPC and POPG are frequently used as loose analogs to lung surfactant [94, 95], and the recently FDA-approved replacement surfactant Surfaxin is composed primarily of DPPC, POPG, and palmitic acid [96].

Typical lavage also contains a small amount of cholesterol (Figure 2.8) [97], which is a key component of cell membranes in animals known for its ability in modulating the fluidity and rigidity of cell membranes; at high temperatures it decreases membrane
Figure 2.7: Various phospholipid headgroups to accompany Figure 2.6. Figure is adapted from [5].
Figure 2.8: Lipids found in lung surfactant mixture: (left) disaturated DPPC, (middle) unsaturated POPG, and (right) cholesterol

fluidity, and at low temperature it increases the fluidity. This property is particularly important in fine-tuning membrane permeability. Despite this importance, it is debatable if cholesterol is a natural part of LS or just a part of coextracted debris from other cells.

2.4.2 Surfactant-specific proteins

The protein constitution of LS includes four lung-specific proteins: SP-A [98], SP-B [99, 100, 101, 102], SP-C [66, 103], and SP-D that make up ~ 10% of total LS weight. The proteins were named in chronological order of their discovery. SP-A and SP-D enhance immunity during inflammation, serve as biomarkers for disease, and are also involved in homeostasis [104, 105, 106]. Unfortunately, the extraction of lung surfactants from animals still relies on a technique that excludes hydrophilic molecules [107], which means the hydrophilic SP-A and SP-D are not present in clinical lung surfactant formulation.

SP-B and SP-C proteins are crucial to LS adsorption and spreading, mainly by assisting the conversion of the bilayer to the monolayer structure and keeping the lipids
near the interface for readsorption and respreading. These proteins reside at the air-water interface and are partially immersed in the lipid monolayer or bilayer.

Due to the success of replacement lung surfactants containing SP-B and SP-C in treating NRDS, and the poor success in simpler DPPC-only (without proteins) replacement LSs, SP-B and SP-C are likely crucial for maintaining the functioning of lung surfactant. Several studies have also noted that the lack or mutation of SP-B and SP-C, both in humans and in mice can be fatal, further emphasizing the importance of SP-B and SP-C in healthy respiration. Further, SP-B supplementation of Survanta (bovine-derived clinical lung surfactant) has been shown to improve oxygenation and lung compliance in animal models of RDS.
Chapter 3

Studying Biophysics of Phospholipids

Phospholipids are essential building blocks of living cells, especially those of bacteria and eukaryotes. They are also the main component of lung surfactants, making up to 90% of lung surfactant composition (by weight). Phospholipids make up most cell membranes and act as a semipermeable barrier between the cell and its environment (see Figure 3.1). Because of the semipermeable characteristic of phospholipids, they are important in regulating cell homeostasis, i.e. materials and energy exchange with the surrounding. Cell homeostasis is governed by the fluidity of cell membranes, dictated by the composition of the cell membrane and surrounding fluids.

As a part of the homeostasis regulation, phospholipids maintain a gradient of chemical and electrical processes to ensure cell survival and functionalities. Further, phospholipids are also essential to regulate cellular processes such as: endocytosis (material insertion into cell), exocytosis (release of cell content), cytokinesis (cell division), and even phagocytosis (in some specific cells: engulfment of a large particle/bacteria). Aside from the homeostasis role, phospholipids also regulate other cellular processes related to growth, synaptic transmission and immune surveillance.

Made up of distinct hydrophilic head groups and double hydrocarbon tails (Figure 2.7), phospholipids typically assemble into lipid bilayer in the bulk phase and into lipid monolayer in the air/water interface (Figure 3.2). Colloquially and as will be done
Figure 3.1: Cell membrane is a semipermeable barrier between cell and extracellular environment that regulates materials and energy exchange with the surrounding. Phospholipids make up 80% of cell membranes and give the membranes a structural integrity.
henceforth in this dissertation, the terms phospholipid and lipid are interchangeable.

The lipid bilayer’s response to environmental stimuli and mechanical forces is integral to many roles lipids play in biological systems, and hence studies on the biophysical properties of lipid bilayer are needed to further our understanding of cellular processes. However, studying a lipid bilayer system poses some challenges. Since a lipid bilayer consists of two weakly coupled monolayers, its composition and size are hard to tune; additionally, there are less experimental probes available to investigate certain membrane properties like surface viscosity. Thus, due to practicality reasons, lipid monolayers have been traditionally, and reliably, used as an analog to extrapolate physical behaviors biological membranes [129]. In particular, to elucidate lipid-protein interactions and to mimic the surfaces of cell membranes [130, 131], lipid monolayers offer the practicality advantage.

3.1 Phospholipid in aqueous bulk phase

As discussed in Chapter 1 due to the ratio of their head and tail size, phospholipids self-assemble into bilayers. In biological systems, lipid bilayers form continuous barrier around cells; this barrier is known as the cell membrane.

At low temperatures, lipid bilayers are in gel phase, $L_\beta$, in which the lipid molecules are highly ordered and tightly packed with minimal lateral mobility.

For phospholipids like DPPC where the van der Waals interaction is strong, the gel phase bilayer is more densely packed compared to phospholipids that are unsaturated or shorter in chain length. At a temperature $T_m$, the gel transitions into a more disordered structure called $L_\alpha$ or $L_d$ or liquid disordered phase. At this temperature, the bilayer loses its ordering and the lipid molecules diffuse more freely within the bilayer.
At this transition temperature, \( L_\beta \) and \( L_\alpha \) phases coexist, creating regions of lipids with large incompatibilities in molecular packing and hydrophobic matching \[132\]. Evans et al. observed that at phase transition temperature, bilayer is highly compressible, approximately 5 times more compressible than a completely melted liquid phase membrane and approximately 40 times more compressible than the solid state membrane. \[133, 134, 135\]

Due to the intermolecular interactions between lipid molecules, \( T_m \) is affected by both lipid tail length and saturation. Lipids with shorter tails will have lower \( T_m \) than lipids with longer tails because of stronger van der Waals force experienced by longer hydrocarbon chains; similarly, lipids with unsaturated tails will have lower \( T_m \) than lipids with saturated tails because of the ability of saturated hydrocarbon chains to pack more tightly than that of unsaturated chains (which tend to create kinks in their packing). It has been observed that decreasing the overall chain length by one carbon usually alters the transition temperature of a lipid by 10\(^\circ\)C or less, but adding a single double bond can decrease the transition temperature by 50\(^\circ\)C or more \[136\].

The presence of cholesterol has been known to alter the fluidity of phospholipid membrane. Due to the rigid structure of the fused sterol ring, cholesterol molecules can be densely packed with saturated hydrocarbon chains of phospholipids, which restricts their motion. The addition of cholesterol changes both \( L_\alpha \) and \( L_\beta \) phases into an intermediate \( L_o \) ‘liquid-ordered’ phase in which acyl chains are restricted in mobility, but lipids are still able to diffuse and rotate, i.e. more ordered than \( L_\alpha \), but less ordered than \( L_\beta \) (Figure 3.3) \[135, 137, 138\].

### 3.2 Phospholipid at the interface

In an air-water interface, phospholipids form an insoluble monolayer, where the hydrophilic heads point toward the water subphase and the hydrophobic chains toward the air. This orientation gives rise to electrostatic interfacial forces \[139, 39, 140\]. Insoluble monolayers are trapped at the interface and unable to escape the adjacent fluid phases, and therefore transport of mass, charge, and energy are confined to two dimensions. The surface coverage of an insoluble monolayer is given by \( \Gamma = n/a \), i.e. number of molecules (or moles, depending on context) per interfacial area.
Figure 3.3: Lipid bilayer transition. At melting temperature, lipid bilayer transitions from rigid gel, solid-ordered $L_\beta$ phase to fluid, liquid-disordered $L_\alpha$ (also known as $L_d$) phase. The addition of cholesterol changes both $L_\alpha$ and $L_\beta$ phases into an intermediate $L_o$ ‘liquid-ordered’ phase in which acyl chains are restricted in mobility, but lipids are still able to diffuse and rotate, i.e. more ordered than $L_\alpha$, but less ordered than $L_\beta$. Figure is adapted from Eeman et al. [6].
The size of lipid monolayer depends on the type of headgroup (Figure 2.7) and on the length and saturation of hydrocarbon chains. For example, the ability to form intermolecular hydrogen bonds reduces the effective size of the headgroup. An increase in the number of unsaturated bonds in the chains increases the number of chain kinks, making the effective lipid monolayer area wider and the molecular length shorter.

3.2.1 Phase behavior of simple lipid monolayer

Analogous to the 3D system, phospholipids also undergo phase transition in 2D based on changes in the compression state of the monolayer that manifests as slope change, or even plateau, in a $\pi - A$ diagram. Upon lateral compression, the area per molecule, $A$, decreases and the surface coverage, $\Gamma$, increases. Along with the decrease in $A$, molecules pack in a more ordered way, transitioning from gas to liquid-expanded (LE) to liquid-condensed (LC) to solid (S), ultimately collapsing to the subphase [141, 142, 143, 144, 145, 146, 147, 148, 7].

A $\pi - A$ diagram is an isotherm, i.e. it is taken at one constant temperature. This isotherm can give information not only about monolayer compressibility, but also about size, shape, and molecular interaction and ordering between lipid molecules in the monolayer.

At the lowest $\Gamma$, monolayer behaves like a gas (G phase), meaning that phospholipid molecules are far apart and have a highly disordered configuration. Intermolecular interaction is very weak, giving a negligible value of $\pi$. At this gas state, a behavior similar to ideal gas in 3D can be observed:

$$\pi A = k_B T$$

With more compression, a 2D equivalent of gas-liquid condensation happens, in which a liquid expanded (LE) phase emerges. Upon compression to LE phase, the monolayer begins to "lift-off", i.e. showing non-zero surface pressure. LE phase has more ordering than the G phase although is still rather unpacked or expanded (hence the name). In both G and LE phases, no X-ray diffraction signal is detected as the heads of the molecules are translationally disordered and the chains are conformationally disordered [7]. The translational and rotational motions of the molecules in the LE phase are fast,
as the effect of the presence of neighbors is still relatively small. The enthalpy of G/LE transition follows Clasius-Clapeyron equation:

$$\Delta H = RT^2 \left( \frac{d\pi_{\text{transition}}}{dT} \right)$$

This enthalpy gives information on the tail-tail interactions energy in the LE phase.

Above the triple point temperature, further compression of an LE phase monolayer would cause the nucleation of solid-like phase called the liquid condensed (LC) phase, where the orientational order of the hydrocarbon chains increases with respect to the LE phase. The orientations of molecular axes in this phase also become more ordered. The lateral diffusion coefficient decreases by nearly two orders of magnitude, and the rotational motions of lipids become slower by several orders. The LC phase also shows an increased translational ordering of molecules with respect to the LE phase. A common structural arrangement of molecules in the LC phase is hexagonal packing. The LC phase, however, is not fully ordered and is rather similar to the $L_\beta$ in lipid bilayers.

One can think of the LC phase as a long-range ordered, semi-crystalline phase dispersed in the disordered LE matrix, and their coexistence can be thought of as 2D colloid (LC) dispersed in a 2D continuous fluid phase (LE). Throughout this coexistence, LC phase continues to grow in size and number until eventually LE phase no longer exists, and LC phase becomes the single existing phase. When it becomes a single phase, the monolayer is in its solid (S) phase, in which the area per molecule corresponds to the two dimensional packing of three dimensional crystals of the amphiphile. Some prefer to refer to the LC and S phases as the tilted condensed (TC) and untitled condensed (UC) phases, respectively. At the triple point, the LC, LE, and G phases coexist around lift off (i.e. the point where the monolayer begins to exert a measurable surface pressure). Below the triple point, no LE phase exists and the G phase transitions directly into the LC phase. S phase persists until the monolayer cannot hold further compression and fractures to the bulk phase; this phenomenon is termed the monolayer collapse, and this marks the transition of the monolayer from a 2D system to a 3D system.

With increasing temperature, the onset of the LE phase shifts toward larger molecular areas; while the LE/LC phase coexistence region becomes smaller and shifts to higher surface pressures [142, 147]. When $\pi - A$ diagrams of different temperatures are
Figure 3.4: Above the triple point, the surfactant monolayer exhibits a liquid-expanded (LE) phase between the gaseous and liquid-condensed (LC) phases. Below the triple point, the monolayer transitions directly from the gaseous to LC phase without passing through an LE phase. At high compression (low molecular area) the hydrophobic tails lose their tilt and form a solid phase. Adapted from Kaganer et al. [7]
obtained and plotted together, one can create phase diagrams and observe a critical
temperature above which the coexistence region disappears. Thus, this LE/LC coexis-
tence region is very analogous to the liquid-vapor coexistence region in bulk fluid. An
eexample of $\pi - A$ diagrams of varying temperatures is presented in Figure 4.1.

### 3.2.2 Interfacial Gibbs phase rule

Similar to the bulk system, a Langmuir monolayer also follows the Gibbs phase rule
\cite{149, 150}. For a system with $S$ types of surfaces, $\Phi$ bulk phases, $C$ chemical species,
and $\Psi$ surface phases in a flat interface, the degree of freedom is given as:

$$DOF = 2 + C - \Phi - (\Psi - S)$$ (3.3)

For example, in a single-component monolayer at a flat air/water interface, $C = 3$
(accounting for the lipid, water, and air), $\Phi = 2$, $S = 1$; if the system is at LE/LC
coexistence, $\Psi = 2$, and $DOF = 2 + 3 - 2 - (2 - 1) = 2$, meaning that as $T$ and $P$
are independent variables, there is no degree of freedom is left, and thus surface pressure is
fixed at LE/LC coexistence, hence the plateau. In contrast, as $\Psi = 1$ for non-coexistence
region, $DOF = 3$, and there is an additional degree of freedom; this means $\pi$ is a unique
function of area per molecule. This phase rule is helpful in understanding the phase
behavior of multicomponent systems like the native lung surfactant system.

### 3.2.3 Monolayer compressibility

Analogous to the 3D system, monolayer isothermal lateral compressibility $C_s$ is given
as:

$$C_s = -\frac{1}{A} \left( \frac{dA}{d\pi} \right)_{T,n}$$ (3.4)

$C_s$ describes the ease of compressing of a monolayer, thus providing information
on the physical state (thermodynamic phase) and/or packing changes of molecules as
compression takes place, with $C_s$ value being smaller for more condensed films.

One can notice that $C_s$ is, by definition from Equation 3.4, the inverse of $E^*$, di-
latational modulus, as discussed in chapter 2. The main difference between $C_s^{-1}$ and
$E^*$ is that $C_s$ is typically obtained from the slope of $\pi - A$ isotherm, i.e. by continuous
compression of the interface, while $E^*$ is obtained from oscillating the interface. $C_s^{-1}$ is often referred to as static compressional modulus, while $E^*$ is the dynamic modulus. When the term dilatational modulus is used, it strictly refers to $E^*$.

Being second derivatives of the surface free energy with respect to area (Equations 1.1 and 3.4), $C_s$ and $C_s^{-1}$ are expected to show discontinuities during both first- and second-order transitions. This is often used to check for the presence of phase transitions as well as the miscibility of components [151].

### 3.2.4 Monolayer collapse

During compression, a monolayer at an interface can only be reduced stably until a point where surfactant molecules are unable to pack into a higher density state due to the hard limit dictated by molecular cross-sectional area. Once thermodynamic phase transitions have been exhausted, i.e. when lipid monolayers are at highly compressed states, the monolayers become highly unstable and will undergo a mechanical relaxation termed as "monolayer collapse" [10, 77, 76, 78, 79, 80]. Monolayer collapse is defined as the movement of insoluble surfactant molecules from the interface into the adjacent bulk phase. Different monolayers collapse at different area densities; the exact density depends on many factors including composition, phase behavior and even rate of compression [152]. Some schematics of monolayer collapse mechanism are shown in Figure 3.5 while fluorescence micrographs of monolayer during collapse following various mechanisms are shown in Figure 3.6. Monolayer collapse is studied as it is related to various phenomena, including thin film defects and lung surfactant stability during expiration.

The surface pressure at collapse, $\pi_{\text{collapse}}$, determines the stable minimum surface tension for a monolayer, and the collapse mechanism determines its reversibility (i.e. how well the monolayer resprads upon film expansion).

Solid-like (LC or S phase) monolayers collapse via fracture followed by loss of material to the bulk phase [71, 95, 80, 10, 153, 154]. Liquid-like (LE) phase monolayers collapse by material ejection into the subphase [10, 155, 156]. Monolayers that retain a continuous liquid-expanded phase network surrounding islands of liquid-condensed or solid phase collapse at low surface tensions via a localized, large amplitude buckling. The buckled regions coexist with the flat monolayer, remain attached to the interface,
Figure 3.5: Collapse mechanisms in lipid monolayer upon lateral compression. Lipid monolayer: (a) becomes unstable at very high surface density, (b) explores a third dimension, and then collapses, (c) forming bilayer folds. (d) Bilayer folds can bend, (e) to form semivesicles, which can (f) detach from the monolayer. Figure is adapted from Baoukina et al. [8].

Figure 3.6: Fluorescence micrographs of monolayer collapse from single component films of different fluidity (a, b), and monolayer squeeze out in a binary mixture (c). (a) The collapse of the fluid film POPC on water at 25°C, at which both bilayer disks and tubes are formed during collapse. (b) The collapsed structure from a rigid palmitic acid film on water and at 16°C, at which dendritic growth of a crystalline phase is seen above the monolayer. (c) The squeeze out of the more fluid component, POPG, from a binary mixture of DPPG and POPG on water at 23°C. The scale bar is 50 µm. Figure adapted from [9] [10].
and reversibly reincorporate into the monolayer upon expansion.  

### 3.2.5 Monolayer morphology

Phase coexistence exists not only in single component monolayer systems, but also in many multicomponent monolayer and bilayer systems. In particular, many biologically-relevant natural membranes show complex phase coexistence. Lateral phase separation is the basis for the lipid raft (a system of 10-1000 nm domains in a matrix of low viscosity phase of lipid membranes) theory regarding cell membrane morphology and functions. Visualizing the phase behavior and domain morphology in an interfacial film can give valuable information about molecular arrangements of these systems in an air/water interface.

Lipid monolayer in LE/LC phase coexistence has been found to form structures, i.e. domains of one phase dispersed in another phase that can exhibit a variety of sizes and shapes that depend sensitively on chemical composition, temperature, surface pressure, compression rate, impurities, subphase pH, and the chirality of the lipid molecules. It can be thought that the domains are the ”condensed” form of the surrounding fluid phase that nucleates at the onset of phase coexistence region and grows throughout the coexistence plateau.

Domain shapes are ultimately dependent on the structure of the constituent molecules and their packing and orientation within a domain. Phospholipids with different head-groups form distinctly different domain shapes in the LE/LC coexistence region. For example, DPPC behavior differs greatly from that of DMPE, DMPA, and DLPE, despite only subtle differences in lipid tail length and headgroup.

Molecules in a lipid monolayer system at the air-water interface experience intermolecular long-range electrostatic forces. The electrostatic dipole-dipole interactions between molecules at the interface affect the size and the shape of domains. While a rough approach of estimating the size of the domains can be done similarly as in 3D based on the classic nucleation theory obtained from the balance of chemical potential and line tension energy (energetic cost of creating domain boundary, the 2D analog of surface tension), electrostatics of the domains, altogether while considering natural chirality of the molecules, can complicate this simple analysis of domains size. The balance between electrostatics, line tension, and inherent chirality, combined
3.3 Tools to study phospholipid monolayer

3.3.1 Introduction to Langmuir trough

Traditionally, phospholipid monolayers, as well as other insoluble monolayers, are studied using a Langmuir trough, which is a container for liquid that can be compressed with a uniaxially moving barrier (Figure 3.7). Despite its name, Langmuir trough was first conceived by Agnes Pockels [165, 166] in 1891 using a simple kitchen tray, a balance, and thin strips of tin. In 1917, Irving Langmuir, the head of research at General Electric (GE) adapted Pockels’s approach for his studies of oil films. Later, he and Katharine Blodgett, GE’s first female scientist, adapted the technique to withdraw monolayers from fluid interface onto a solid substrate, which is now known as the Langmuir-Blodgett deposition technique [167, 168]. In today’s convention, the experimental setup originally developed by Pockels is known as either Langmuir trough or Langmuir-Blodgett (LB) trough.

By moving the barriers, the surface area changes, which induces surfactant or protein rearrangements at the interface, which in turn leads to changes in surface tension, γ.

Connected to the trough is a Wilhelmy balance, a surface pressure sensor, from
which a plate is suspended. The plate touches the fluid interface and forms a meniscus, and the surface tension force will pull the plate downward. This positive pulling force is measured by a force transducer to give the value of surface tension of the interface:

\[
\gamma = \frac{F}{\text{wetted perimeter} \times \cos \theta_c} = \frac{F}{2(l + d) \cos 0^\circ} \approx \frac{F}{2l}
\]  

(3.5)

where \( F \) is the capillary force, \( \theta_c, l, \) and \( d \) are contact angle, plate length and plate width, respectively. For Equation 3.5 to work, we assume that the plate is very thin, i.e. \( d \to 0 \), and that the material chosen for Wilhelmy plate has complete wetting, i.e. \( \theta_c = 0^\circ \). Materials with \( \theta_c = 0^\circ \) often chosen for Wilhelmy plate materials include: platinum and filter paper.

Lipid monolayer, being insoluble in the aqueous bulk, can be studied at an air-water interface by dissolving the lipid in a chloroform (or other volatile water-insoluble solvent that spontaneously spreads, i.e. positive spreading coefficient) and then carefully depositing a known volume on the interface of a Langmuir trough using a microliter syringe.
3.3.2 Confocal fluorescence microscopy for air-water interface visualization

First conceived in 1955 and patented in the 1957 by Marvin Minsky \cite{169} for its capability to reject out-of-focus light, confocal microscopy (Figure 3.9) was designed to improve cell and tissue imaging resolution. In 2012, Shieh and coworkers successfully employed confocal fluorescence microscopy (CFM) to visualize lipid monolayer phase coexistence at air/water interface, and sharp images of the interface were obtained without out-of-focus light coming from the subphase while allowing for an in-situ, three dimensional visualization \cite{170, 171, 172}.

Overview of fluorescence microscopy

Fluorescence is the light emitted by a fluorophore (dye) after absorbing a photon of with energy $h\nu_{\text{absorbed}}$, where $h$ is the Planck's constant, $6.626 \times 10^{-34}$ m$^2$kg/s, and $\nu_{\text{absorbed}}$ is the frequency of the absorbed photon. The absorption of photon causes the dye, which initially is in its ground state $S_0$, to enter an ”excited” state $S_2$. The dye molecule will then quickly relax to the lowest energy level within the excited state $S_1$, causing a loss in energy as heat. The fluorophore then relaxes back to its ground state $S_0$ by emitting a photon of energy $h\nu_{\text{emission}}$, where $\nu_{\text{emission}}$ is the frequency of the emitted photon. This emission of photon following the series of described events is termed ”fluorescence”. The schematic of these phenomena is depicted in Figure 3.8A \cite{173}.

Because there is a loss in energy throughout the relaxation process, $\nu_{\text{absorbed}} > \nu_{\text{emission}}$. Since $\nu \sim \lambda^{-1}$, where $\lambda$ is the photon wavelength, the emitted photon has longer wavelength than the absorbed photon. This shift in wavelength is known as the Stokes shift (Figure 3.8B). Various energy levels within the ground and excited electronic states mean the fluorophore absorbs and fluoresces over a wide range of wavelengths, see the width of the spectra in Figure 3.8B. It is common practice for microscopists to pick fluorophores with suitable absorption/emission spectra based on microscopy laser specifications, where ideally the peak of the spectra should be close to the laser wavelength in use.
Overview of confocal microscopy principle

The working principle of confocal microscopy (see Figure 3.9) is to place a pinhole in front of a light source (light source pinhole aperture in Figure 3.9), thereby creating a point of light that can be focused onto a dichromatic mirror, and ultimately, onto the specimen; the light emanated from this illuminated point is then focused into another pinhole, located in front of a photomultiplier detector. This second pinhole (detector pinhole aperture in Figure 3.9) is placed in a plane confocal (meaning having the same focus as) to the first pinhole to essentially form a point detector, exclusively allowing
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Figure 3.10: Widefield vs. confocal scanning of specimens. Confocal imaging allows for sharp images with no background scattering. Figure is adapted from Nikon MicroscopyU website [12].

only the emitted fluorescent light from the focal point to pass through the detector [169, 174].

The double-pinhole strategy ensures sharply focused images as it eliminates the potential of light from planes outside the focal plane, as well as light from sample scattering to hit the detector. It is by blocking this "background" light that confocal microscopy achieves sharp, non-blurry images with increased signal-to-noise ratio, improved axial and lateral resolution, as well as optical sectioning and three-dimensional imaging; these are desirable features in imaging a 100 nm thick monolayer floating on the surface of ~1 cm deep water. A comparison between the scanning of a specimen using a traditional widefield scanning and a confocal scanning is presented in Figure 3.10 [12, 169].

3.3.3 Combining Langmuir trough and confocal microscopy

Today, Langmuir trough can easily be integrated with a microscopy setup (either fluorescence or Brewster angle) such that the insight into morphology of lipid monolayers can complement the $\pi - A$ diagram, thereby allowing for potential construction of structure-property relationship of a lipid monolayer. In our experimental systems, Langmuir trough is often integrated into confocal or multiphoton microscopy. The picture of our setup that is based in University of Minnesota Imaging Center is shown in Figure 3.11.

Our research group has traditionally relied on fluorescent probes to visualize an interface due to their ability to give contrast between solid-like and liquid-like lipid phase [170, 172]. Fluorescence creates strong contrast because the image is formed
through the detection of light emitted by dyes in the sample. Therefore, only sections of the sample containing dye will appear bright and the remaining sections will be dark. The dye typically used in our research group is Texas Red (excitation/emission maxima at 595/615 nm) conjugated to the headgroup of dipalmitoylphosphatidylethanolamine (TR-DHPE) from Invitrogen (Carlsbad, CA), as shown in Figure 3.12 which is mixed with phospholipid of interest in a chloroform-based solution to provide fluorescence contrast. The bulky structure of TR-DHPE causes it to pack inefficiently, hence it cannot be incorporated into the ordered packing of LC phase. Thus, the fluorescent contrast obtained is due to the fact that this TR-DHPE dye is excluded from the LC phase and segregates to the LE phase.
Figure 3.11: Typical setup for phospholipid visualization on Langmuir trough in Zasadzinski research group. The experimental setup is set in University of Minnesota Imaging Center. Legends: 1. magnetic stirrer, 2. Langmuir trough, 3. Wilhelmy balance, 4. Objective, 5. Scan head. The microscope used is NIKON AR-1 Multiphoton upright microscope.
Figure 3.12: (top) Structure of Texas Red DHPE typically incorporated into our lipid system (bottom) fluorescence spectra of TR-DHPE. Excitation peak at 595 nm, emission peak at 615 nm.
In this chapter, I will highlight some of the well-studied properties of DPPC, which is a disaturated 16-carbon zwitterionic phosphatidylcholine making up more than half of the lipid content of LS. As PC is also the most abundant form of phospholipid, DPPC is also often the phospholipid of choice for study of membrane biophysics. Following discussion on DPPC, I will discuss the properties of some classical models of lung surfactants.

4.1 A closer look into DPPC, a model phospholipid

DPPC is a phospholipid with 16 carbon chain long double tails. The double tails of phospholipids cause them to be extremely hydrophobic and have little solubility in water. The critical aggregation concentration for DPPC is 0.46 nM [175]. According to Figure 2.8, the cross-sectional area of the tail is small compared with that of the head for DPPC. As discussed in previous sections, DPPC self-assembles into bilayer in the bulk and monolayer in the interface. DPPC is the most studied phospholipid not only because of its role as main component in lung surfactant (50% of the phospholipid making up lung surfactant is DPPC), but also because of its wide use as a model for
Figure 4.1: Isotherms of DPPC at various temperatures. Dotted red line marks the coexistence region at various temperatures. Open circles indicate start of LE/LC coexistence, and open squares indicate end of the coexistence. The critical temperature $T_m$ is the temperature where open square and circle coincide, i.e. where the coexistence disappears. Original figure from Von Tscharner and McConnell, 1981 [13]. Reproduced figure is adapted from Jan Vermant’s lecture in a Society of Rheology short course

4.1.1 $\pi - A$ isotherms of DPPC

The $\pi - A$ isotherm of DPPC at various temperatures is presented in Figure 4.1. Zuo et al. [176] determined the triple point temperature of DPPC to be 17.5$^\circ$C, thus we note that all isotherms presented in Figure 4.1 represent those of above triple point regions of DPPC. As the critical temperature of DPPC is 41$^\circ$C, LE/LC coexistence exists in all the isotherms in Figure 4.1, with coexistence regions at higher temperatures getting
narrower than those at lower temperatures.

While DPPC monolayer can be compressed to near zero surface tension, the monolayer is unstable past $\pi = 44$ mN/m, which is the collapse surface pressure of DPPC, i.e. where DPPC is overcompressed and relaxes to the subphase. Thus, once compressed to $\pi > 44$ mN/m, DPPC monolayer cannot hold such a high, unstable $\pi$, and $\pi$ will start to decay instantaneously (see Figure 4.2), and this relaxation process has been studied extensively by Tabak, Notter, and coworkers [88, 92]. This behavior is also apparent when compression-expansion cycles are performed on DPPC monolayer as there is a hysteresis between the compression and expansion [86, 88, 92]. Compression-expansion isotherms of DPPC performed in our KSV-NIMA Langmuir trough with Teflon-based ribbon are shown in Figure 4.3.

Clements and coworkers [177] have shown that lung surfactant extract from animal
demonstrates hysteresis in $\pi - A$ compression/expansion cycle as well, indicating that this hysteresis might have physiological relevance, and how DPPC plays a crucial role as a main component of lung surfactant system.

At $\pi = 52$ mN/m, Teflon undergoes a wetting transition [151, 178]. Due to this, the meniscus between teflon and water changes from convex to concave (sometimes called "creepage"). In our experimental setup, the physical dimensions of the monolayer, when it is close to collapse, are considerably small, making it sensitive to perimeter effects. The change in meniscus leads to a non-monotonic error in area during compression, which manifests as a shoulder around $\pi = 52$ mN/m and a corresponding change in slope between $\pi = 50$ and 60 mN/m. This phenomenon is commonly reported in the literature [151, 179, 180] and recognized as an artifact of the Langmuir trough for high $\pi$ studies. If readers encounter DPPC isotherms without such shoulders [170], it should be noted that the trough (and barrier) materials are likely different, and creepage might not take place in those systems.

4.1.2 Morphology of DPPC

The morphology of DPPC monolayer has been studied extensively [161, 181, 182, 183, 184, 185] and has been shown to be sensitive to subphhase, compression speed, thermal fluctuation, relaxation process, and contaminants. DPPC morphology displays characteristic chiral shapes due to a twist of the in-plane orientation of the tilted hydrocarbon chains [186].

In an ideally clean interface, when a DPPC monolayer is compressed at a speed between 0.2 to 8.0 Å²/molecule.min, the domains start nucleating in the form of bean-like shapes (Figure 4.4a), which grow into S-like appearance (Figure 4.4b), and eventually into multilobe shapes (Figure 4.4c). The three lobes structure shape is often called triskelion. If the system is allowed to rest for a prolonged period (hours), the domains relax into circular domains (Figure 4.4d).

The multilobe shape does not often appear as it is not the most stable form of DPPC domains (the bean shape is the most stable form [184]); the shape typically occurs early in the coexistence region and will not develop once domains have grown to occupy most of the monolayer (latter stage of coexistence) [184], thus the amount of DPPC that is dispersed in the interface pre-compression affects the existence of such
Figure 4.3: $\pi - A$ isotherms of compression-expansion cycles of DPPC performed in KSV-NIMA Teflon ribbon Langmuir trough at room temperature. $C_x$ and $E_x$ refer to $x$th compression and expansion of the monolayer. The compression/expansion cycles are mostly identical except for the shift in area as well as smaller hysteresis with each consecutive cycle. Hysteresis is observed between every compression/expansion cycle, indicating that there might be monolayer collapse associated with compression past its collapse pressure of 45 mN/m. This argument is also supported by shift in the area with consecutive cycles. For example, in the first compression, end of LE/LC coexistence happens at 72% of trough area, in the second cycle, at 55%, and in the third cycle, at 48%, indicating that there are less materials at the interface and more compressions are needed to achieve the same surface pressure.
structure. The factors that can cause a bean to transform into a multilobed structure are also quite subtle: changes in compression speed or slight variations in subphase conditions are sufficient to drastically affect the appearance of multilobe shapes [184]. In all cases, however, multilobed domains relax over time, reverting back to the original bean domains.

When DPPC is compressed past its coexistence region, existing domains persist and pack very crowdedly such that the boundary between domains become imperceptible, and often one cannot visualize the interface past this region. The use of insoluble dye like Texas Red allows for the visualization of the interface past this stage if one adjusts for change in brightness and contrast. For example, while in Figure 4.5 no contrast was visible to eyes past $\pi = 12 \text{ mN/m}$, adjustment of contrast and brightness allows for us to discern segregation between dye-rich domains to dye-less domains (more solid domains), as shown in Figure 4.6. In Figure 4.6 a brightness and contrast enhancement was performed.

4.1.3 Examining LE/LC coexistence from thermodynamic perspective

The nature of LE/LC phase transition of DPPC monolayer has been much discussed. Based on Ehrenfest’s classification [187], first-order transition exhibits discontinuity in the first derivative of the free energy with respect to a thermodynamic parameter, and thus, it was previously argued that a first-order phase transition should have a zero $\pi - A$ slope. Since the $\pi - A$ isotherm of DPPC shows non-zero slope (albeit really small), the LE/LC transition was often thought to be of a higher order, and not that of a first order transition [188, 148]. However, observations of the coexistence of two distinct phases under a microscopy has strengthened the arguments that indeed the transition is a first order.

Pallas and Pethica hypothesized that monolayer impurities (uncleanliness, either in trough or in prepared chloroform-based sample) are the source of this thermodynamic irregularity observed in monolayers [189]. They provided experimental evidence of the first-order character of the LE/LC transitions, although they also recognized the fact that under lateral compression, markedly nonzero slopes are systematically obtained, even for the highest-purity samples.

When a two-phase monolayer is compressed, condensed phase grows at the expense
Figure 4.4: Characteristic DPPC domain shapes: (a) beans ($\pi = 3.8$ mN/m, 72.7 $\AA^2$/molecule); (b) S shapes ($\pi = 3.9$ mN/m, 69.5 $\AA^2$/molecule); (c) multilobes ($\pi = 4.1$ mN/m, 53.5 $\AA^2$/molecule); (d) circles (aged for 10 hours, 53.5 $\AA^2$/molecule) obtained from fluorescence microscopy. Scale: 1.5 cm = 27 $\mu$m. The fluorescence contrast was obtained from C6-NBD-DPPC dye. Adapted from Klopfer and Vanderlick [13].
Figure 4.5: DPPC morphology taken with a confocal microscopy setup. Compression rate = 5 mm/minute. No correction was performed. To naked eyes, above $\tau = 11$ mN/m, nothing is discernible and the interface looks homogeneous.
Figure 4.6: DPPC morphology taken with a confocal microscopy setup. Compression rate=5 mm/minute. Brightness and contrast were adjusted.
of expanded phase via molecular exchange, equilibrating over some time scale $x$. At co-
existence plateaus (when isotherms are horizontal), $A(d\pi/dA) = 0$, and the dilatational
modulus will be dominated by a viscous dissipation caused by this lateral molecular
diffusion, which will have its own intra-monolayer exchange kinetics \[190\].

In Chapter 6, we show that DPPC dilatational modulus can be tied to the kinetics
of LE/LC coexistence. Additionally, we also will demonstrate that interfacial curvature
complicates this analysis.

## 4.2 Lung surfactant isotherms

While DPPC makes up the majority of phospholipids in a lung surfactant system, DPPC
alone cannot capture the richness of complex behaviors in a real, multi-component lung
surfactant system. In this section, I will examine the properties of Survanta, a bovine-
derived clinical lung surfactant used to treat NRDS in infants. Survanta has a high
content of DPPC, and its formulation includes palmitic acid and tripalmitin which
have been known to have condensing (or rigidifying) effects on monolayer \[191\]. $\pi - A$
isotherms for Survanta films spread from saline solution onto saline buffer at room
temperature adapted from Alonso et al. \[15\] are shown in Figure 4.7.

In Figure 4.7, four consecutive compression-expansion cycles are shown, labeled from
1 to 4. All cycles exhibit relatively the same features, except for shift in area to the left,
indicating that materials are lost during compression in each cycle. The shift from the
first to second cycle is the greatest as refinement of interfacial composition might still
be taking place following surfactant adsorption to the interface (unlike DPPC, Survanta
is an aqueous solution, hence it is soluble and can adsorb to the interface in a timely
manner).

The compression cycles were also divided into five different zones of different $\pi - A$
slopes (marked with dashed lines). In zone A, below $\pi = 10$ mN/m, the slope of the
isotherm is small, signifying a film of high compressibility, typical of LE phases, while
in zone B, $10 < \pi < 40$ mN/m, slope increases, indicating a less compressible film. At
room temperature, pure DPPC monolayers undergo a LE/LC phase transition at $\pi \sim
7 - 12$ mN/m, close to the onset of the transition between zones A and B. The decrease
in compressibility is likely related to the formation of condensed domains of DPPC
cocrystallizing with PA or tripalmitin (additives in Survanta) \[192, 193\], coexisting with LE phase (see Figure 4.8). A plateau appears at $\pi = 38$ mN/m (zone C) consistent with the partial removal or “squeeze-out” of the fluid phase \[73, 194, 3\]. Unsaturated lipids are generally unable to sustain high surface pressures, and are pushed out of the interface toward a reservoir of material located underneath the film (see bright spots on Figure 4.8) \[195, 196, 197\]. Zone D corresponds to a less compressible film than in zone B, illustrating the enrichment in saturated lipids and fatty acids that form the most condensed phases in monolayers \[192, 198\]. The film undergoes collapse to the subphase as shown in zone E at $\pi = 66$ mN/m. After being compressed to the collapse point, the interface is expanded. Upon expansion there is a large hysteresis manifested by a rapid drop in surface pressure to 20 mN/m at almost constant area on the initial expansion. The rate of surface pressure decrease changes abruptly below 20 mN/m as material is adsorbed or reincorporated into the film \[199\]. The surface pressure at full trough expansion drops to a value lower than the starting point of the cycle, indicating a net loss of material from the interface, likely from collapse. Subsequent cycles are more similar to one another than to the first, but in each consecutive cycle, the trough has to be compressed more than previous cycle to achieve the same surface pressure. At surface pressures $\pi > 40$ mN/m, the cycles closely superimpose. At higher surface pressures, the film is enriched in solid phases (see Figure 4.8); this shows that DPPC and PA (and likely the tripalmitin) are retained in the film after collapse and subsequent cycling. This isotherm demonstrates that Survanta lowers surface tension and respreads well after collapse, two essential requirements for a lung-surfactant replacement.
Figure 4.7: $\pi - A$ isotherms of Survanta adsorbed from saline suspension onto a saline buffer at 25°C. The first compression-expansion cycle is labeled 1. Consecutive compression-expansion cycles are labeled 2-4. The lower surface pressure at the beginning of the second cycle suggests that material is lost during the first compression. Dashed lines identify several zones common to all isotherms. Zone A is a homogeneous LE phase at low surface pressure. Zone B corresponds to phase coexistence between an LC phase and the LE phase. Zone C shows the squeeze-out plateau, starting at 38 mN/m, which accompanies the removal of the LE phases from the film, which leads to a less compressible condensed phase (zone D). At E, the film collapses; after the first cycle, the high surface pressure zones are reproducible, suggesting a minimal loss of solid phase. Figure is adapted from Alonso et al. [15].
Figure 4.8: Fluorescence microscopy images of Survanta throughout multiple cycles of compression/expansion. The texture of the interface changes due to refinement following adsorption from the bulk phase. Survanta undergoes domain growth at $\pi \sim 13$ mN/m, and domains continue growing until the interface becomes relatively packed at $\pi \sim 43$ mN/m. The domain starts undergoing collapse at $\pi \sim 55$ mN/m, where bright dots and bright lines are present at the interface, indicating that monolayer relaxes to the bulk phase. Figure is adapted from Alonso et al. [15].
Chapter 5

Deformation in Fluid-Fluid Interface

There is a plethora of fluid-fluid interfaces in our lives; many of which undergo routine deformation that we usually take for granted. For example, in a syringe filled with vaccine or other medication, there is an air-water interface that continuously changes size and shape depending on the motion involved in its handling. Another example, the continuous inflation and deflation of our alveoli, the air sacs in our lungs, is essential for the functioning of our lung. Depending on the species present at the interface during these deformations, as well as the unique physical and chemical environment of the interface, the mechanical response of the interface to these disturbances are also different and can affect the stability of the interface. The study of how a fluid-fluid interface responds to deformations is called interfacial rheology.

Understanding the relation between interfacial rheology and interfacial microstructure can give rich information about macroscopic aspects such as the stability of thin films as well as intermolecular interactions and dynamic evolution of the films as they age. The knowledge is crucial not only in solving biophysical problems mentioned above, but also in tuning the stability of foams and emulsions, controlling the efficiency of liquid-liquid separations, enhancing mineral recovery, and development of pharmaceutical and food products.

In this chapter, I will start by informing readers on the background of the rheology
field. As many rheological studies focus on bulk deformation, the background will be described in terms of the more familiar terrain of bulk rheology, which shares many parallels to the interfacial rheology. This introductory section also serves for guidance for readers while encountering Chapter 7, where bulk rheology of various clinical lung surfactant suspensions was studied to understand factors affecting their delivery and efficacy. Following the general background to rheology, I will provide a more specific discussion on interfacial rheology, and the two major modes of interfacial deformation: shear and dilatation, after which I will discuss the challenges associated with independent measurements of each mode of interfacial rheology. I will conclude this chapter by showing our group’s efforts in creating effective dilatational rheometers.

5.1 Background on rheology

Derived from the Greek word ρειo 'rheo' meaning to flow, rheology is the study of flow and deformation of matters. When a deformation or strain ($\epsilon$) is applied to a material, the material will revert this deformation and return to its original conformation within response time $\tau$ after said deformation is applied.

An elastic material, like a solid, has $\tau \sim 0$ and follows Hooke’s relations: $\sigma = G\epsilon$, where $\sigma$ is stress, $\epsilon$ is strain, and $G$ is modulus. It means that an elastic material will revert to its original structure as soon as the stress is removed. On the other hand, a viscous material, like a Newtonian liquid, has $\tau \to \infty$; viscoelastic material, which encompasses many soft matters, has a finite response time and responds to deformation with some combination of both "viscous" and "elastic" characteristics.

Classically, the field of rheology studies shear deformation more than other form of deformation. An easy way to understand a shear deformation is by imagining two parallel plates with a material sandwiched between them, where one plate (let’s say the top one) is moving and thereby shearing the material sandwiched between the two plates (see Figure 5.1 for pictorial depiction). The strain rate, $\dot{\epsilon}$, is the rate of deformation and proportional to the speed of the plate, and the viscosity of a material, $\eta$ is given by $\eta = \sigma/\dot{\epsilon}$, where $\sigma$ is the shear stress applied.

Newtonian fluids are fluids with shear stress linearly proportional to the shear rate, and hence the viscosity is constant and invariable with shear rate or shear stress. Typical
Newtonian fluids include water, honey, simple organic solvents, and dilute colloidal dispersions. Non-Newtonian fluids are those whose viscosities are a function of shear rate or stress. The shear stress is not linearly proportional to the shear rate, and this non-linearity is often associated with microstructures formed in the fluid. There are two types of non-Newtonian fluids. Shear thinning fluids are fluids with viscosities that decrease with increasing shear rate, the example being our blood. In contrast, cornstarch, when mixed with water, will create a shear-thickening fluid, i.e. fluid whose viscosities increase with increasing shear rate. In Chapter 7 I will discuss shear thinning behavior of clinical lung surfactant, and how it can affect lung surfactant efficacy and delivery time.

It is often of interest to understand how a material responds to an oscillatory shear deformation. Small amplitude oscillatory shear (SAOS) is typically performed in the linear viscoelastic (LVE) region, i.e. range of applicable strains above which materials behave non-linearly, i.e. a small increment of strain would cause a dramatic jump in viscosity and modulus. In linear region, the viscoelasticity of the materials is probed without breaking down the structure, while in the non-linear region, structural breakdown takes place irreversibly.

The linear region is obtained by conducting an amplitude sweep at a constant frequency to find a critical strain $\epsilon_c$. After $\epsilon_c$ is determined, SAOS is performed by running frequency sweeps at $\epsilon < \epsilon_c$. 

Figure 5.1: Shear deformation depiction. (a) shows a daily life example of how spreading butter on a bread using a knife involves a shear movement, and (b) shows the schematic of how one might picture a shear sandwich of height $h$ with a top plate moving at the speed $v$. The shear rate is given by $\dot{\gamma} = v/h$. Figure is adapted from [16].
Figure 5.2: (A) Materials response to sinusoidal deformation where stress $\sigma$ is applied, and the response is strain $\epsilon$ for (i) Hookean, (ii) Viscoelastic, and (iii) Newtonian materials. (B) Depiction that complex modulus, $G^*$, is the absolute value of the complex number $\sqrt{G'^2 + G''^2}$

When a shear strain $\epsilon(t) = \epsilon_o \sin \omega t$ is imposed on a material, the shear rate is defined as the time derivative of $\epsilon$: $\dot{\epsilon}(t) = \epsilon_o \cos \omega t$, and the resulting shear stress is measured: $\sigma = \sigma_o \sin(\omega t + \delta)$. Algebraic manipulations lead to: $-\sigma/\epsilon_o = G' \sin \omega t + G'' \cos \omega t$, where $G' = (\sigma_o/\epsilon_o) \cos \delta$ is the elastic (storage) modulus, and $G'' = (\sigma_o/\epsilon_o) \sin \delta$ is the viscous (loss) modulus. When combined, $G'$ and $G''$ give the complex shear modulus, $G^* = G' + iG''$, which is a material function comparable to $G$, the equilibrium shear modulus.

tan $\delta = G''/G'$ estimates the lag in response; tan $\delta < 1$ indicates an elastic system, i.e. more energy is stored than dissipated during the deformation, and vice versa. These definitions of storage and loss modulus also apply to other modes of deformations, such as those happening at the interface. Some readers might also recognize this form of storage and loss relationship from other system, such as power loss from electrical resistance in a capacitor.

Viscoelastic materials are often described as a spring-dashpot system, where the spring represents the elastic part of the material, and dashpot represents the viscous part. The most basic forms of this spring-dashpot model are the Maxwell model, which
involves the connection of a spring and a dashpot in series, and the Kelvin-Voigt model, which describes viscoelastic solids, where springs and dashpots are connected parallelly. More complex systems usually involve forms of permutation of the two models.

Bulk shear rheology is often probed using a shear rheometer (figure 5.3A), which is a device capable of measuring both stress and strain independent of the sample being tested, for variable stress and strain (cf. viscometer that is only capable of measuring Newtonian fluid). A rheometer operates based on shear drag flows created between plates of various geometries (Figure 5.3B). The geometries are chosen based on a couple of considerations: sample volume, range of expected viscosity, as well as the kind of measurement one looks to perform [16].

5.2 Interfacial rheology background

5.2.1 Brief history

In 1869, upon comparing the damping of a magnetic compass needle on a bare and on a surfactant-laden interface, Joseph Plateau reported the studies in a series of papers published in 1873 [200], where he suggested that molecular forces act in a thin surface layer and claimed the existence of a surface viscosity. Carlo Marangoni argued that surface tension gradient [201], instead of surface viscosity, could be responsible for Plateau’s observation. To resolve the issue, Lord Rayleigh designed an experiment with a ring and found that both arguments had their scientific merits and highlighted the significance of reducing the area of the measurement geometry in contact with the subphase and avoiding concentration gradients (see next sections) [202, 203].

In 1914, Joseph Boussinesq [204] proposed that the interface is a 2D analog of the bulk fluid, and hence can be characterized by surface shear and dilatational viscosities. Later, Oldroyd [205], Scriven [206], and others extended the theory developed by Boussinesq. To this day, the Boussinesq-Scriven equation [206] is one of the most important equations to describe the dynamics of 2D complex fluid.
Figure 5.3: Examples of (A) a rheometer and (B) common geometries used to probe materials viscosity. The choice of geometry is dependent on the type of materials that is being probed, as well as the amount of sample available.
5.2.2 Overview of complex interfaces

Unlike 3D fluid, fluid-fluid interfaces are not always able to support shear and normal stresses. However, terminologies in describing stress-strain relationship of an interface are similar to those used to describe a 3D system, as will be seen throughout this section.

A "simple" interface, such as that of a single component liquid (surfactant-free), can not support straining stresses, and it can be described fully by its scalar, static interfacial tension, $\gamma$, which is an intrinsic thermodynamic property [206].

Complex interfaces develop when the concentration of surface active species in the interface increases to a point where the interface can support both shear and normal stresses that result from the presence of interfacial rheological properties. Such interfaces are influenced by mass and momentum transfer during formation, deformation, and breakup of liquid interfaces [207]. For these complex interfaces, the surface tension depends on the surface coverage, $\Gamma$, and there could be an extra stress contribution to the interface. For complex interface, $\gamma(\Gamma)$ depends on many interfacial phenomena that can affect $\Gamma$, ranging from Marangoni flows originating from spatial gradients in surface concentration or temperature, capillary flows resulting from normal stress differences on a curved surface, and Gibbs elasticity arising from gradients in equilibrium surface tension due to interfacial compressibility.

A "Newtonian" complex interface may support surface stress, with the stress proportional to strain or strain rate. More complexity arises as the surface-active species concentration on the interface increases, or if the surfactant forms long-lived structures at the interface. These complex interfaces show a nonlinear or non-Newtonian relationship between surface stress and strain or strain rate.

While some interfacial phenomena can be understood by going back to their 3D analogies, we need to note that phenomena at an interface are often coupled to the adjacent bulk phase. This means that the measurement and analysis of the interfacial response are complicated by transport phenomena of the materials at the adjacent bulk system, as well as transport of molecules between interface and bulk phase [207]. To this day, the techniques in measuring and analysing an interfacial response to deformation have been continuously perfected and almost reach maturity stage where data obtained are mostly separable from bulk contribution and variation in data across different techniques can be explained by geometric arguments.
Deformation at fluid/fluid interface is often complex, and can involve combinations of shear, dilatation, and possibly bending and torsion components. As the deformations occurring in droplet (bubble) collision and film drainage involve both shear and dilatation, both dilatational and shear rheological properties are responsible for many interfacial phenomena that we encounter \cite{208}. Additionally, despite the presence of out-of-plane deformations in many interfaces, the bending modulus of our interface of interest (DPPC and small MW surfactants) is usually negligible. As an estimate, the bending modulus of DPPC (from DPPC lipid bilayer bending modulus) is approximately $\sim 10^{-20} \, k_B T$, which is around $10^{-20} \, \text{J}$, a very small, negligible value as compared to DPPC interfacial shear modulus, $\sim 10^{-6} \, \text{J/m}^2$ and dilatational modulus, $\sim 10^{-1} \, \text{J/m}^2$. Hence, in this dissertation, only shear and dilatation modes (Figure 5.4) of interfacial deformation will be discussed.

Interfacial rheology from the two modes can give rich information about the interface; shear deformation is sensitive to conformational changes and the resulting intermolecular interactions between the adsorbed molecules \cite{209}. Dilatational deformation measures the relaxation of molecules undergoing compression or expansion and informs us of the intrinsic softness or hardness of the molecules at the interface, in addition to their dynamic rearrangement. \cite{210, 211, 212}.

### 5.2.3 Shear deformation at the interface

Interfacial shear rheology shares many parallels with bulk shear rheology as interfacial flows have parallels to bulk flow kinematics, except that the interfacial flow is coupled to that of the bulk \cite{207}.

Typical settings for interfacial shear rheometry involve the measurement of either torsional stress, angular displacement, or velocity of a probe at the interfaces that occurs due to surface flow. The probe could be a particle, a disk, a knife-edge, a needle \cite{213}, a rod \cite{214}, a microbutton \cite{215}, and a ring \cite{216}, while the surface flow could be generated from either rotation of the geometry or controlled oscillation imposed by the probes. The geometry involved includes: movable barriers (Langmuir trough), rotational (double wall ring, DWR), etc. Examples of interfacial rheometry setup can be found in Figure 5.5. Rotational rheometers, which are classically used to study bulk shear rheology as a system, can be employed to create a shear deformation at an interface
Figure 5.4: A 2-D shape deformation at constant area, $A_0$, is pure shear. An area change from $A_0$ to $A_0 \pm \Delta A$ at constant shape is pure dilatation. A general deformation is a combination of these two. Designing experiments to probe the individual shear and dilatational moduli requires creating imposed shape deformations at constant area (shear) or constant imposed area deformations at constant shape (dilatation). While shear is important in both 2D and 3D flows, dilatational kinematics are only observed in 2D for incompressible liquids.
Figure 5.5: Various interfacial shear fixtures: (a) double-wall ring geometry rheometer, (b) bicone rheometer (cross section), (c) interfacial rod rheometer, and (d) microbutton rheometer. Adapted from Fuller and Vermant [17], Zell et al. [18], and Tein [19] on 2D equivalents of Couette geometry (see Figure 5.3B2 for cup and bob, an example of a bulk Couette geometry). A popular Couette geometry that has garnered interest in recent years for interfacial shear rheometry is the double-wall ring (DWR) Couette geometry [216] (see Figure 5.5A) due to its high sensitivity; a more traditional probe used for interfacial shear rheometry is the bicone geometry, another form of Couette geometry, shown in Figure 5.5B. In Figure 5.5C, a modern interpretation of Plateau’s original needle probe is shown, where a rod is placed at the interface and forced to translate by magnetic coils such that it is equivalent to the sliding plate rheometer in 3D [217]. The microbutton probe shown in Figure 5.5D is similar to the rod geometry, except that its circular symmetry ensures that the deformation created is uniform, and the holes in the microbutton can additionally be used to track the probe during deformation using a microscope [218].

A key design consideration for an interfacial shear fixture is that the contribution
from the surface should dominate the force on the measurement probe. Boussinesq number ($Bq$) is a dimensionless number used to describe the contribution of bulk viscosity to measured interfacial viscosity, given by:

$$Bq = \frac{\text{surface drag}}{\text{subphase drag}} = \frac{\eta_s}{\eta l}$$  \hspace{1cm} (5.1)

where $\eta_s$ and $\eta$ are the surface viscosity and bulk viscosity, respectively, while $l$ is characteristic length scale given by the ratio of the contact area between the measuring geometry and the subphase and their three-phase contact line. Equation 5.1 is thus sometimes broken down into probe dimensions:

$$Bq = \frac{\eta_s P_c/L'_c}{\eta A_c/L''_c}$$  \hspace{1cm} (5.2)

where $P_c$ is the probe perimeter in contact with the interface, $A_c$ is the surface area of the probe in contact with the bulk, and $L'_c$ and $L''_c$ are characteristic length scales for shear in the surface and subphase. As $Bq \to \infty$, the surface drag dominates, while if $Bq \to 0$, the bulk drag dominates, while an intermediate value of $Bq$ would indicate a hydrodynamic coupling between the bulk and surface [207]. According to some reported measurements, $Bq = 100$ is the lower bound for optimal sensitivity [219]. To maximize the sensitivity of the probes, the interfacial viscous force should be significantly larger than that of the bulk to the interfacial flow, i.e. a high $Bq$ is desired. Thus, a maximal amount of perimeter for a given contact area would give an optimal sensitivity. However, the smaller probe would then require a larger oscillatory force to shear the interface, which is ineffective for a highly viscous surface. Due to the limit imposed by its physical dimension and Boussinesq number (Equation 5.1), it is often the case that one probe size cannot study a system in its entirety, and a combination with other instrument or probe of different dimensions is required.

For monolayer studies, where surface pressure is a thermodynamic property of interest that changes with compression and expansion, we are interested in studying interfacial shear deformation as function of surface pressure, a relationship that is not easily obtained by geometries mentioned above. Thus, we have adapted Vermant’s group approach in combining Langmuir trough and double wall ring [220], and the setup looks like Figure 5.6. Figure 5.7 shows a measurement of DPPC interfacial shear rheometry
using the setup in Figure 5.6. In Figure 5.7, it is shown that the complex viscosity of DPPC at LC phase spans an order of magnitude, further highlighting the importance of the trough/DWR setup.

5.2.4 Dilatational deformation at the interface

In contrast to the bulk system of incompressible fluid, i.e. where the volume of a system is always preserved (a consequence of the continuity equation), an interface can experience dilatational deformation, where the area of an interface changes at constant shape, for an example through compression or expansion. The consequence from this argument is that materials are often not conserved at the interface as they can move to and from the adjacent bulk phases, which is particularly true for soluble surfactants.

Experiments on the surface dilational rheology of an interface covered by surface-active molecules provide important information on surfactant adsorption/desorption dynamics and on relaxation mechanisms in the adsorption layers. The knowledge can be extended to understanding stability of foam and emulsions.

Dilatational modulus \( E^*(\omega) \) is a measure of an interface’s resistance to dilatational deformation, by relating the change in surface stress, \( \tau \), to the change in interfacial area, \( A \), at an oscillation frequency \( \omega \):

\[
E^*(\omega) = \frac{d\tau}{d \ln A} = A \frac{d\tau}{dA}
\]  

However, as our systems of interest that will be discussed in this dissertation exhibit no apparent non-isotropic and/or deviatoric stress, i.e. \( \tau = \gamma \), Equation 5.3 can be reduced to:

\[
E^*(\omega) = \frac{d\gamma}{d \ln A} = A \frac{d\gamma}{dA}
\]  

Experimentally, the apparent transient dilatational modulus \( E(t) = \Delta \gamma(t) / (\Delta A/A_o) \) is usually measured by recording the temporal change in surface tension \( \Delta \gamma(t) \) following change in area at time \( t \). \( E(t) \) is related to the complex modulus \( E^*(\omega) \) via a Fourier transform.

As dilatational modulus describes interfacial resistance to area change, large dilatational modulus is often tied to stable interfaces. For example, as discussed in Chapter 2, high dilatational modulus correlates with stable alveoli. Another example, emulsifier
Figure 5.6: Langmuir trough integrated with double wall ring rheometer to allow for study of surface pressure - surface shear viscosity relationship. (A) Schematics: (a) TA-Instruments DHR-3 rheometer, (b) Langmuir trough, (c) cup and (d) ring with openings openings to allow uniform compression/expansion of monolayer. (1) is the regular DWR setup without modification. (B) Photo of real setup in UMN Polymer Characterization Facility
Figure 5.7: Interfacial shear rheometry as a function of surface pressure of DPPC using DWR/trough setup
with large dilatational moduli are the more effective ones.

5.2.5 Challenges in dilatational rheometry

Principally, from Equation 5.4, any apparatus that can change an area of an interface while simultaneously recording the change in surface tension can be used for dilatational rheometry. However, area change is often accompanied by shape change in a lot of system. Hence, more often than not, interfacial deformations are a combination of dilatation and shear. Thus, the greatest experimental challenge in measuring $E^*$ is to create an isotropic, purely dilatational deformation without any changes in shape, which would contribute a shear deformation component.

Classically, interfacial dilatational rheometry used to be measured on the Langmuir trough. However, compression using Langmuir trough changes the shape of the interface as well, which means that it is not purely dilatational. Some efforts have been focused on changing the geometry of Langmuir trough to have a radial symmetry, which ensures a uniform and radial expansion, i.e. a purely dilatational deformation. Some groups developed Langmuir trough that enables a centro-symmetric, isotropic compression by simultaneously withdrawing the bulk fluid while rising the cups [221]. Another approach to improve Langmuir trough is by using independently movable barriers, such that an interface can be purely sheared or purely dilated [222]. However, this method would still give shear localization, i.e. at different location from the barrier, the local values of strains vary [223]. These local deformations also propagates as a longitudinal surface compression wave until it reaches the Wilhelmy plate. A very viscous subphase alters the dampening of this disturbance [207, 223]. This effect is especially strong in a rigid interface, as shown by Aumaitre et al [224].

Another method that is commonly used is the oscillating pendant drop, in which a drop of a material is generated from the tip of a needle, and the shape is analysed, typically by matching it to Bond number, $Bo$:

$$Bo = \frac{\text{gravitational forces}}{\text{surface tension forces}} = \frac{\Delta \rho g R^2}{\gamma}$$

(5.5)

in which $\Delta \rho$ is the density difference between the bubble and the suspending fluid, $R$ is the radius of the bubble and $g$ is the gravitational constant (9.8 m/s$^2$). For $Bo \ll 1$,
gravity does not distort the spherical bubble shape \[225, 226\]. However, for \( Bo > 1 \), gravity alters the shape of the bubble or drop from spherical, and the deformation has elements of shear and dilatation \[227, 228, 210\]. As the surface tension is inferred from shape changes in the pendant drop, the deformation can never be purely dilatational \[30, 29, 229\].

5.2.6 Development of capillary pressure microtensiometer

A recent development of capillary pressure microtensiometer (CPM) by Alvarez et al. \[230\], relies on creating a hemispherical bubble at the end of a hydrophobic glass capillary tip, from which the pressure is controlled by a water column. An objective is situated underneath the cell such that the bubble generated can be visualized, allowing for calculation of surface tension, interfacial area, as well as fit for Bond number. Since the bubble is of micron size, the bubble shape is relatively undistorted by gravity, so \( Bo > 1 \), thus ensuring a more shear-free measurement \[230, 231\].

CPM allows examination of interfacial properties on highly curved interfaces, at much smaller length scales, while using significantly less materials than other common methods. Other than its use as a dilatational rheology probe at a curved interface, it also allows for a careful observation of adsorption of surface-active materials to a highly curved interface.

After bubble generation, the bubble radius is adjusted by controlling the applied capillary pressure difference across the hemispherical interfaces. The contact line of the bubble at the capillary end is pinned by a hydrophobic treatment applied to the capillary (Figure 5.8). Following equilibration, small amplitude \((\delta P/\Delta P_{eq} < 0.05)\) pressure oscillations are applied to the bubble to induce small changes in bubble area, \( \Delta A \), which in turn, induce small changes in surface tension, \( \Delta \gamma \). Air bubbles of these dimensions have a Bond number \( Bo = \frac{\Delta \rho g b^2}{\gamma} < 0.01 \) such that gravity does not distort the spherical shape. Hence, the pressure oscillations result in a nearly perfect dilatational deformation \[232\] that is assessed by fitting a circle to microscope images of the bubble taken with a high-speed camera to determine the bubble radius as a function of time as well as any deviations from circularity (See red circle in Figure 5.8C). For small amplitude pressure oscillations, \( E^* \approx A_{eq}(\Delta \gamma/\Delta A) \) in which \( A_{eq} \) is the bubble surface area at the equilibrium surface tension, \( \gamma_{eq} \), for a capillary pressure, \( \Delta P_{eq} \).
Our group has improved the CPM design to incorporate microfluidic pump for model-based pressure, bubble curvature, or bubble area control based on the Laplace equation, which extended the capability of the CPM (Figure 5.8) [20]. In earlier versions of the CPM, the capillary pressure difference between the inside and outside of the bubble was controlled via a water column and oscillatory syringe pump [230], which then only allows for pressure control mode. With these new modifications, the measurement and control precision and response time are greatly enhanced, capillary pressure variations can be applied and controlled in milliseconds, and area-control as well as curvature-control modes are readily available.

Additionally, we also combined CPM with a confocal microscope setup (CFM), which allows for real-time observation of interfacial structure as the interface undergoes deformation [20]. Using combined CPM-CFM, we can connect morphological phenomena to dynamic and equilibrium interfacial properties to establish structure-function relationships of highly curved interface, allowing for new exploration of factors governing interfacial morphology previously only studied on flat interfaces.
Figure 5.8: (A) Schematic of CPM/CFM, not to scale. (1) the CPM cell, (2) the capillary tube with a bubble at the tip, (3) confocal microscope objective, (4) microscope camera objective with filter, (5) CPM light source, (6) microfluidic pump, (7) safety valve, (8) fluid exchange inlet, (9) fluid exchange outlet, (10) peristaltic pump, (11) exchange fluid reservoir, (12) fluid exchange waste, (13) direct to cell syringe, (14) temperature control jacket inlet and outlet, and (15) temperature-controlled reservoir and pump. (B) CPM reservoir in which the capillary is exposed to an aqueous surfactant solution. The solution can be exchanged through the ports on either side. (c) Schematic diagram of the bubble in the glass capillary of inside diameter 100 µm. Microscope images of the bubble shape are fit to a circle to determine the bubble radius and the surface tension, $\gamma = R\Delta P/2$. Figure is adapted from Iasella et al. [20] and Narayan et al. [21]
Chapter 6

Langmuir Trough for Dilatational Rheometry

A portion of this chapter is adapted from Ciutara et al. [26].

A typical Langmuir trough is a rectangular container for liquid with one or more barriers that move along a single axis to expand or compress the interfacial area (Figure 6.3). By moving the barriers, the surface area changes, which induces surfactant or protein rearrangements, which in turn leads to changes in surface tension.

While the uniaxial barrier motion in a rectangular trough involves both dilatational and shear deformations (Figure 6.1), Langmuir trough remains the most reliable tool in understanding other properties of a monolayer like its $\pi - A$ isotherm and morphology (as it can be combined to a microscopy system). Additionally, it is challenging to directly deposit phospholipids at air-water interface typical curved fluid-fluid interface experimental setup such as capillary pressure microtensiometer (CPM) due to the insolubility of phospholipids in water and the relatively complicated (and not fully understood) kinetics of DPPC adsorption involving converting a bilayer (the form a phospholipid takes in an aqueous environment) to a monolayer, see Figure 6.2. While there are ways to circumvent this, such as using solvent exchange, the deposition of phospholipid into an air/water interface is nonetheless difficult to achieve. Langmuir trough, on the other hand, gives free access to the interface (see Figure 6.2), and therefore remains uniquely suited as an experimental setup to study complex phospholipid systems.
Figure 6.1: Uniaxial compression of an interface in a Langmuir trough by an areal strain \( \epsilon \). This unidirectional compression can be written as the sum of pure dilatation and equal-area shear. Figure adapted from Lin et al. [22].

\[
\begin{pmatrix}
-\epsilon & 0 \\
0 & 0 \\
0 & 0
\end{pmatrix} = \frac{1}{2} \begin{pmatrix}
-\epsilon & 0 \\
0 & -\epsilon
\end{pmatrix} + \frac{1}{2} \begin{pmatrix}
-\epsilon & 0 \\
0 & \epsilon
\end{pmatrix}
\]

Figure 6.2: Introducing phospholipid to the (a) curved and (b) flat air/water interface.
Motivated by these reasons, in this chapter, I will revisit the use of Langmuir trough for dilatational rheometry for three classes of surface-active materials: soluble surfactant, insoluble monolayer, and proteins.

Extracting both $E^*$ and $G_s^*$ from single surface pressure measurements in a Langmuir trough was proposed by Petkov et al. [233] and Cicutta et al. [23]. They used two Wilhelmy plates parallel and perpendicular to the uniaxial compression direction in a Langmuir trough to try to separate the shear and dilatational components of complex interfaces, see Figure 6.3. Their analysis shows that:

$$|E^* + G_s^*| = A_o \frac{\Delta \pi_{\text{par}}}{\Delta A}$$

$$|E^* - G_s^*| = A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$$

in which $|E^*|$ and $|G_s^*|$ are the surface dilatational and shear moduli, and $\Delta \pi_{\text{par}}$ and $\Delta \pi_{\text{perp}}$ are the changes in surface pressure measured parallel and perpendicular, respectively, to the uniaxial compression direction for an oscillatory change in area, while $A_o$ is the area at which the interface is oscillated.

For the remainder of this chapter, I will compare values of $E^*$ and $G_s^*$ measured with the Langmuir trough to independent measurements of $E^*$ determined using a capillary pressure microtensiometer (CPM) and $G_s^*$ determined using the double-wall ring apparatus (DWR) [216] or microbutton shear rheometer [218, 234] for soluble palmitoyl lysophosphatidylcholine (LysoPC), the insoluble surfactant dipalmitoylphosphatidylcholine (DPPC), and the globular protein $\beta$–lactoglobulin. The work on this chapter is based on Ciutara et al. [26].

For LysoPC and DPPC, $A_o \frac{\Delta \pi_{\text{par}}}{\Delta A} \approx A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$ at all oscillation frequencies tested consistent with $E^*$ being at least 4 orders of magnitude greater than $G_s^*$. The values of $E^*$ for LysoPC and DPPC determined using the LB trough showed quantitative agreement with $E^*$ determined in the CPM when corrected for the bubble curvature [228]. Hence, $E^* = A_o \frac{\Delta \pi_{\text{par}}}{\Delta A} = A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$, and only a single surface pressure measurement is necessary to determine the dilatational modulus of these surfactants in an LB trough. The LB trough provides good estimates of $E^*$ for LysoPC and DPPC with $E^* \gg G_s^*$, (and by inference, we expect that pendant and sessile drop measurements of systems in which $E^* \gg G_s^*$ should also be in quantitative agreement with CPM).
Figure 6.3: Schematics of Langmuir trough setup to obtain $\pi_{\text{par}}$ and $\pi_{\text{perp}}$. Adapted from [23].
For $\beta$-lactoglobulin films in the trough, $A_0 \frac{\Delta \pi_{\text{cap}}}{\Delta A} \gg A_0 \frac{\Delta \pi_{\text{exp}}}{\Delta A}$. Double wall ring shear measurements showed that $G^*_{s}$ was of the same order as $E^*$ measured with the capillary microtensiometer. However, the calculated values of $E^*$ and $G^*_{s}$ from the trough were only in qualitative rather than quantitative agreement with the independent measurements. This may be due to the original assumption underlying Equations 6.1 and 6.2 that the protein interface behaves as a purely elastic sheet, or, more likely the development of anisotropic deviatoric stresses as the protein ages and unfolds at the air-water interface [235, 236, 23]. Protein films are quite sensitive to sample preparation and aging, and there has not been any systematic comparison of $E^*$ and $G^*_{s}$ on surface with varying curvature [237, 238, 239].

6.1 Origin of dilatational modulus

When the interfacial area available for soluble surfactants is changed, the surfactants can leave the interface with a characteristic frequency that depends on the diffusive transport on and off the interface as well as the molecular solubility and surface activity.

If the initial interfacial area is $A_0$, a sinusoidal area oscillation of magnitude $\Delta A$ gives the relative area deformation:

$$\ln A = \ln (A_0 (1 + \Delta A e^{i\omega t})) \cong \ln A_0 + \Delta A e^{i\omega t}$$

The surface area oscillations induce corresponding changes in the surface concentration, $\Gamma$, around the equilibrium surface concentration, $\Gamma_\infty$: $\Gamma = \Gamma_\infty + \Psi e^{i\omega t}$, which in turn generate oscillations in the bulk concentration: $C = C_0 + f(r) e^{i\omega t}$, which are governed by the spherical diffusion equation (for small amplitude oscillations, convection is negligible):

$$\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial r^2} + \frac{2}{r} \frac{\partial C}{\partial r} \right)$$

A general solution of Equation 6.4 for a system oscillating at frequency $\omega$ with a bulk concentration $C_0$ is:

$$C = C_0 + \frac{\alpha}{r} e^{-nr} e^{i\omega t}, \quad n^2 = \frac{i\omega}{D}$$
Figure 6.4: Schematic diagram of the surfactant exchange at a planar (as in the Langmuir trough) air-water interface undergoing periodic oscillations in surface area of frequency, \( \omega \). Surfactant has a characteristic exchange frequency, \( \omega_o = D/L_d^2 \) in which 
\[ L_d = \Gamma_\infty/C_o \] is the depletion depth needed to fully saturate the interface to a surface concentration of \( \Gamma_\infty \) from a bulk solution of concentration \( C_o \). If the alveolar surface oscillation frequency, \( \omega > \omega_o \), surfactant remains at the interface and the dilatational modulus remains high. If \( \omega < \omega_o \), the surfactant has sufficient time to diffuse off the interface, the surface concentration and surface tension remain roughly constant, and the dilatational modulus goes to zero.
A mole balance at the interface equating the diffusive flux to the change in surface concentration is applied to solve for $\alpha$ [228]:

$$\frac{d(\Gamma A)}{dt} = AD \left( \frac{\partial C}{\partial r} \right)_R$$

and when combined with Equation 6.5 and linearized gives: [240] [228]:

$$\frac{d\gamma}{d(\ln A)} \approx \frac{E^*_0}{\Delta A} \frac{d\gamma}{d(\ln \Gamma)} = \frac{E^*_0}{1 + (\frac{D}{\omega R}) \frac{dC}{dt}} \left( 1 + \frac{nR}{2} \right)$$

(6.7)

$E^*_0 = d\gamma/d(\ln \Gamma)$ is the high frequency limit of $E^*$.

From Equation 6.7, there are two characteristic frequencies for adsorption, $(\frac{Dn}{\omega R}) \frac{dC}{dt} = \sqrt{\frac{D}{\omega R}}$ and $nR = (\frac{\omega}{\omega R})^{1/2}$ and $\omega R = (\frac{\omega}{\omega R})^{1/2}$. The first is $\omega_o = \frac{D}{(dC/dt)^2}$ or the diffusivity timescale, in which $D$ is the surfactant diffusivity. The ratio between the equilibrium surface concentration, $\Gamma_\infty$, at a given bulk concentration, $C_o$, for a particular surfactant determines the planar diffusion length scale, $L_d$. This length scale can also be thought of as a depletion depth, or the volume of solution of concentration $C_o$ per unit area of interface that contains sufficient surfactant to saturate the surface to $\Gamma_\infty$ (Figure 6.5). Both $L_d$ and $\omega_o$ depend on the nature and concentration of the surfactant being adsorbed. The second frequency describes the effects of interfacial curvature, $\omega_R = D/R^2$; $R$ is the curvature radius of the interface. For our CPM system, $R$ is then the radius of the hemispherical bubble. As shown in Figure 6.5B, the depletion depth around a spherical surface, $L_{ds}$, is smaller for a given interfacial area than a planar interface because the volume of the fluid contained within the dotted line increases faster with distance than in the planar case [30] [29] [231].

Joos [228] derived a diffusion model for $E^*$ based on surface area oscillations of frequency $\omega$ that induce corresponding changes in the surface concentration, $\Gamma(t)$, around the equilibrium surface concentration, $\Gamma_\infty$, which in turn generate oscillations in the bulk concentration. The bulk concentration profiles are governed by the spherical diffusion equation (for small amplitude oscillations, convection is negligible [210]). Defining
Figure 6.5: Schematic representation of depletion depths: (A) for a planar interface, the depletion depth, \( h_p = \Gamma / C_0 \), is the thickness of the volume of concentration \( C_0 \) required to cover the interface of area \( dA \) with a surface concentration, \( \Gamma \). (B). The depletion depth for a spherical interface is \( h_{ds} \). \( R \) is the radius and \( A_s \) is the surface area of the sphere. As the volume of the fluid contained within the dotted line increases faster with distance than in the planar case, the spherical depletion length is less than \( h_p \). (Adapted from Alvarez et al. [24])

\[
\zeta = \sqrt{\omega_o / 2\omega}
\]
gives:

\[
E^*(\omega) = \frac{E_o^*}{\sqrt{1 + 2\zeta + 2\zeta^2(1 + \frac{\omega R}{\omega} + \sqrt{\frac{2\omega R}{\omega}})}}
\] (6.8)

The same characteristic timescale that governs dynamic surfactant adsorption also governs the dilatational modulus and this characteristic time can be related to the surfactant equilibrium properties. The effects of curvature are important when \( \frac{2\omega R}{\omega} \sim 1 \), which occurs for small bubbles and low frequencies.

As \( \omega R \to 0 \), the equation becomes:

\[
E^*(\omega) = \frac{E_o^*}{\sqrt{1 + 2\zeta + 2\zeta^2}}
\] (6.9)

This equation is the same as the original equation derived by Lucassen and van den Tempel [226] to describe dilatational modulus of a flat interface, which means that Equation 6.8 can universally describe dilatational modulus in both flat and curved
6.2 Materials and methods

6.2.1 Materials

Lysophosphatidylcholine (LysoPC, MW=495.63 g/mol, CMC = 6 µM), a saturated 16 carbon single-tail lipid, was purchased in powder form from Anatrace and was used as received. Dipalmitoylphosphatidylcholine (DPPC, MW=734.04 g/mol), a saturated 16 carbon double-tailed phospholipid, was purchased in powder form from Avanti Lipids and was used as received. β-lactoglobulin (18.4 kDa) was purchased in powder from Sigma Aldrich and was used as received. Water with a resistivity of 18.2 MΩ·cm at 25°C was purified with a Millipore Direct Q 3UV-R (Millipore, Billerica, MA) system. Sodium chloride (NaCl) and phosphate buffer were purchased in powder form from Sigma-Aldrich (Saint Louis, MO, USA) and used to prepare phosphate buffered saline (PBS) solutions (150 mM NaCl and pH=7.0). The lipid dye, Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt, (TR-DHPE) was purchased from Life Technologies Corporation, CA, USA and used as received.

6.2.2 Methods

Langmuir trough

A rectangular continuous ribbon KSV-NIMA (Model KN 1007) Langmuir trough constructed from polytetrafluoroethylene (PTFE) that provides leak-free uniaxial compression of the monolayer was used. The filter paper Wilhelmy plate surface pressure system was also from KSV-NIMA. Before each set of experiments, a new filter paper for the Wilhelmy plate is exchanged and the pressure sensor is calibrated on pure water. The reproducibility of the surface pressure sensor is ± 0.5 mN/m. Prior to any film deposition, the interface was aspirated several times to remove any surface contamination using a sterile pipette tip connected to a vacuum pump. The trough interface is determined to be clean if the surface pressure of the air-water interface is zero (within ± 0.3 mN/m) following full compression of the trough barriers. A stock solution of 1000 µM LysoPC in phosphate buffered saline (PBS, pH=7.40) was stored at 4°C until use.
A 5 µM solution was prepared by dilution of the stock solution with PBS to remain below the CMC of LysoPC (6-7 µM) to simplify the analysis of the results [227, 228]. All prepared samples were used within two weeks. 250 mL of the desired LysoPC solution was poured directly into the trough and the LysoPC adsorbed spontaneously to the interface. Rheological measurements were performed once the equilibrium surface tension of 43 mN/m or lower (which depends on LysoPC concentration below the CMC) was achieved [30].

For DPPC monolayers, 25 µL of a 1 mg/mL DPPC in chloroform solution was deposited dropwise on the air-water interface using a Hamilton microsyringe and 10-20 minutes was allowed for the chloroform to evaporate. Surface pressure area isotherms for DPPC were consistent with previous measurements and literature values [172, 186].

A 10 µM solution of β-lactoglobulin was poured directly into the trough. Rheological measurements were performed one hour later to allow the protein to adsorb to and equilibrate at the interface. The surface pressure at this concentration varied from 13 to 18 mN/m depending on the age of the film [241].

To estimate the dilatational modulus, the trough was allowed to equilibrate at a fixed area, \( A_0 \) to reach a steady surface pressure, \( \pi_0 \). \( \pi_0 \) was the same for both parallel and perpendicular orientations of the Wilhelmy plate in all cases. The barriers were oscillated with an amplitude, \( \Delta A/A_0 = 0.01 \) at angular frequencies from 0.01-1.0 rad/s to determine \( \pi_{par} \) and \( \pi_{perp} \) from which \( E^* \) and \( G^*_s \) were determined from Equations 6.1 and 6.2.

**Double wall ring (DWR)**

Independent measurements of the surface shear modulus of β-lactoglobulin were done using a TA Instruments interfacial rheology device [216]. A 1 mm sharp edged square platinum-iridium ring of inner radius 34.5 mm and outer radius 35.5 mm is suspended by a wire frame and attached to a conventional TA DHR-3 rheometer [216]. The ring is placed into the sample solution contained within a PTFE cup with a 3.5 mm gap for the inner and 4 mm outer gap. The cup is placed within the KSV Langmuir trough and the sample solution in the cup is connected to that in the trough via channels to control the surface pressure. The ratio of contact area, \( A_c \), to wetted perimeter, \( P_c \), in Equation 5.2 is \( \sim 0.7 \) mm. After each experiment, we processed the raw data in MATLAB code.
(courtesy of Jan Vermant [210]) to correct for the subphase contribution to the measured interfacial shear modulus. The DWR apparatus was not sensitive enough to measure the shear modulus of DPPC or LysoPC over this frequency range [242, 218, 243]. Previous microbutton measurements of $G^*_s$ for DPPC from Kim et al. were used to compare with data from the trough measurements [242]. $G^*_s$ for LysoPC was below the resolution limit of both the DWR and the microbutton, similar to other soluble single-chain surfactants [18].

**Capillary pressure microtensiometer**

In the capillary pressure microtensiometer (CPM), a hemispherical air bubble is pinned at the end of capillary in a 1 mL volume liquid reservoir. Capillaries of 100 µm diameter were pulled from 1.5 mm OD, 1.1 mm ID, 10 cm long borosilicate glass capillaries in a Sutter Instrument P-1000 micropipette puller (Novato, CA). Capillaries were cleaned with Alnochromix and sulfuric acid (Millipore-Sigma) and rinsed with Millipore water. The capillaries were immersed in 5% Xiameter OFS-6124 Silane (Dow Chemical) in ethanol solution and cured by heating under house vacuum at 100°C for one hour [30]. The capillary pressure across the bubble interface is controlled via a computer controlled commercial microfluidic Fluigent Lineup Flow EZ pressure-based flow controller that allows for model-based pressure, bubble curvature, or bubble area control based on the Laplace equation, $\Delta P = 2\gamma/R$; $\Delta P$ is the applied capillary pressure difference, and $R$ is the bubble radius [20]. The dynamic response of the bubble interface is visualized via a second optical lens and camera system as the bubble expands and contracts. The bubble contour is fit to a circular profile to determine the bubble curvature radius, $R$, as well as any deviations from circularity that would invalidate the results [30, 20]. The Laplace equation is used to determine the dynamic surface tension of the interface using a custom Labview program. Following equilibration, small pressure oscillations can be imposed by the computer-controlled microfluidic pump to oscillate the bubble area (frequencies of 0.001-100 cycles/min) to determine the dilatational modulus, $E^* = A_0 \frac{d^2}{dA^2}$. From Equation 5.5 the Bond number, $Bo \sim 0.0005$ for bubble radii, $R = 50$ µm, hence the surface tension dominates gravitational forces. The uniform stress imposed by the isotropic capillary pressure produces an equal strain in both principle directions, leading to a purely dilatational strain except in the immediate vicinity of the pinning line at the
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Figure 6.6: The variation in surface pressure for LysoPC measured with a Wilhelmy plate parallel, and perpendicular, to the trough barriers are the same within experimental error. Hence, $E^* + G^*_s \approx E^* - G^*_s \approx E^*$ from Eqns. 6.1 and 6.2. $E^*$ ranges from 40 - 75 mN/m, while $G^*_s < 0.1$ mN/m. Double wall ring (DWR) shear measurements show that $G^*_s$ is below the sensitivity of the DWR suggesting that $G^*_s < 0.1$ mN/m.

capillary tip \[24\], which can be confirmed by interface images showing that the bubble remains hemispherical during oscillations \[20, 30, 29\].

6.3 Results and discussions

6.3.1 Soluble surfactant – LysoPC

Figure 6.6 shows the measured values of $\frac{A_0 \Delta \Pi_{par}}{\Delta A}$ and $\frac{A_0 \Delta \Pi_{perp}}{\Delta A}$ for 5 µM LysoPC for 1% area strain in the Langmuir trough. The values are the same within experimental error, suggesting that $E^* \gg G^*_s$ and $E^* + G^*_s \approx E^* - G^*_s \approx E^*$. The shear modulus determined independently by the DWR apparatus is apparently independent of frequency, but is below the resolution limit of the instrument as the Boussinesq number is less than one and the measured stress is due to the drag from the bulk water. Hence, $G^*_s$ is less than 0.1 mN/m or about 500 times smaller than $E^*$. Zell et al. \[18\] have shown using the most sensitive microbutton shear rheometers available that for a number of low molecular
Figure 6.7: Dilatational modulus, of 5 µM LysoPC measured with the Langmuir trough and the capillary pressure microtensiometer (CPM) using an 80 µm diameter bubble. The solid lines are fit to Equation 6.8 with the same value of \( \omega \), which depends only on the surfactant type and concentration. The curvature of the interface is important for (dotted red line) for the bubble. Curvature causes to decrease faster with decreasing frequency than for a flat trough surface (Equation 6.8 and Figure 6.5). The trough data is somewhat below the CPM data at high frequency, which may be related to packing differences at equilibrium.

weight, soluble surfactants (including ionic, non-ionic, and polymeric surfactants), that the interfacial shear modulus is less than 0.01 \( \mu \text{N/m} \).

Figure 6.7 shows a comparison of the dilatational modulus measured in the Langmuir trough compared to that measured using an 80 µm diameter bubble in the capillary pressure microtensiometer based on fits from Equation 6.8. For soluble surfactants, the interfacial tension is directly related to the dynamic surface concentration, \( \gamma(t) \), of the surfactant, which in turn depends on the rate of exchange of surfactant between the surface and the bulk solution of concentration \( C_0 \).

In a Langmuir trough, \( R \rightarrow \infty \) and \( \omega_R \rightarrow 0 \). In the CPM, the bubble radius \( R \) corresponds to the hemispherical interface of initial area \( A_0 \) and is fixed for a given set of experiments. The effects of curvature are important when \( \frac{2 \omega R}{\omega} \sim 1 \), which occurs for small radius bubbles and low applied frequencies. \( E^*_o = \frac{d^2 \gamma}{d (\ln \Gamma) \omega} \) is the high frequency
The solid lines in Figure 6.7 show that Equation 6.8 gives excellent fits to both the trough and CPM dilatational modulus data for identical values of $\omega_0 = 0.02$ rad/sec. As $\omega_0$ should be the same for LysoPC at the same concentration on the trough and on the bubble, this gives confidence that the measurements in the trough and the CPM are the same within experimental error. The estimated value of $\omega_R = 0.14/s$ for the bubble was based on an estimated $D \approx 2 \times 10^{-10}$ m$^2$/s \cite{20} and a bubble radius of $\sim 40\, \mu$m. $\omega_R \approx 0$/sec ($R \to \infty$) for the trough measurements. The vertical dotted line in Figure 6.7 shows that for $\omega < \omega_R$, the dilatational modulus decreased faster with frequency for the bubble than for the flat interface consistent with Equation 6.8.

For the trough data, $E^*_{o} \approx 75$ mN/m, and for the CPM, $E^*_{o} \approx 80$ mN/m. It is not clear if this deviation is significant as variations in the magnitude of $E^*(\omega)$ are typically $\pm 3$ mN/m. However, as we show below (Figure 6.10) for DPPC and other insoluble monolayers \cite{245}, the equilibrium organization of the monolayer can be influenced by the interfacial curvature, which might be responsible for the variation in $E^*_s$. Hence, when curvature corrections are accounted for, the trough and the CPM give similar qualitative and quantitative results for $E^*$ and are consistent with a negligibly small value of $G^*_s$ for the soluble surfactant LysoPC.

### 6.3.2 Insoluble surfactant – DPPC

Figure 6.8 shows the measured values of $A_o\pi_{\text{par}}/\Delta A$ and $A_o\pi_{\text{perp}}/\Delta A$ for the insoluble surfactant DPPC spread from chloroform solution for 1% area strain at 0.6 rad/sec in the Langmuir trough, along with CPM data taken from Kotula et al. \cite{225} taken at 1 rad/sec for comparison. In the CPM, DPPC was spread by first dissolving the DPPC in isopropanol. The DPPC/isopropanol solution was exchanged with the saline in the solvent reservoir. After sufficient time for DPPC adsorption to the bubble interface, the isopropanol was exchanged for saline. The mean molecular area was approximated via the value of the surface pressure in reference to a surface pressure-area isotherm as in Figure 6.9A. Figure 6.8 shows that on the trough, $A_o\pi_{\text{par}}/\Delta A$ and $A_o\pi_{\text{perp}}/\Delta A$ are equal within the experimental error consistent with $E^* \gg G^*_s$ and $A_o\pi_{\text{par}}/\Delta A \approx A_o\pi_{\text{perp}}/\Delta A \approx E^*$. Independent measurements of $G^*_s$ using the microbutton surface rheometer show that $G^*_s$ is roughly four orders of magnitude less than $E^*$ for DPPC \cite{242}. The trough measurements agree with $E^*$
Figure 6.8: Comparison between dilatational rheometry measurement for the insoluble surfactant DPPC spread from chloroform solution for 1% area strain at 0.6 rad/sec in the Langmuir trough at 22°C vs. CPM data (green circles) at ambient temperature performed by Kotula et al. [25] measured by Kotula et al. [25] using the CPM system except over the 6-10 mN/m surface pressure range associated with the LE-LC coexistence plateau (Figure 6.9A).

Figure 6.9A shows a surface pressure vs. molecular area isotherm for the insoluble surfactant DPPC spread from a chloroform solution. One of the important benefits to the Langmuir trough is the ability to quantitatively deposit insoluble surfactants from volatile, immiscible solutions. In this way, the mass deposited is known, allowing for quantitative evaluation of the area/molecule at gas to liquid expanded (LE), liquid expanded-liquid condensed (LC) coexistence, and even higher order tilt transitions such as the solid or S phase at higher surface pressures [7, 148]. The limiting area/molecule at high surface pressures for DPPC isotherms and other insoluble surfactants agrees with that measured by grazing incidence X-ray diffraction [193, 246, 247].

The coexistence plateau occurs at nearly constant surface pressure (Figure 6.9A). Figure 6.9B shows the “static” dilatational modulus, $E^* = A \frac{d\gamma}{dA}$, determined from the slope of the surface pressure area isotherm in Figure 6.9A. $E^*$ reaches a minimum value of < 1 mN/m at the coexistence plateau; the small value of $E^*$ is consistent with a first order phase transition.

Figure 6.10A shows $E^*$ determined from trough measurements of $\frac{A_o \Delta \pi_{\text{par}}}{\Delta A}$ on the flat
 trough interface are $< 10 \text{ mN/m}$ for low frequencies, compared to $\sim 100 \text{ mN/m}$ for surface pressures above and below coexistence (Figure 6.8). The mean molecular area, $A_M$, at coexistence varies due to changing fractions, $f$, of LC phase, as both LC and LE phase have a fixed area per molecule at the coexistence surface pressure:

$$A_M = f A_{LC} + (1 - f) A_{LE}$$  \hspace{1cm} (6.10)

Hence, area oscillations at coexistence modify the fraction of LC and LE phase:

$$\frac{dA_M}{dt} = (A_{LC} - A_{LE}) \frac{df}{dt}$$  \hspace{1cm} (6.11)

at nearly constant surface pressure ($\Delta \gamma \to 0$), which means $E^* = A_o \frac{\Delta \gamma}{\Delta A} \to 0$. Figure 6.10B shows that the LE phase (red) is continuous on the flat trough surface, and the LC phase (black) forms discrete islands. The area fraction of either phase can change continuously as the trough area is changed. However, $E^*$ will remain low at coexistence only at sufficiently low frequencies that the monolayer can dynamically adjust the fraction, $f$, of LC and LE phases (Equation 6.11). That is, if $\frac{df}{dt} < \omega$, the applied oscillation frequency, the fractions of LC and LE phases can adjust themselves at constant surface pressure and $E^*$ remains small. Figure 6.10 shows that for frequencies
Figure 6.10: A) Average trough value of $E^*$ at coexistence for DPPC as a function of applied frequency. For frequencies below 2 rad/sec, $E^*$ is low and independent of frequency. For 0.3 Hz and higher, $E^*$ increases and approaches that of the LC phase. B) On the Langmuir trough, the LE (red) phase is continuous, while the LC phase (black) forms discrete domains. Expanding the trough area causes the black domains to decrease in area fraction, while compressing the trough area causes the black domains to increase in area fraction. C) On a highly curved bubble interface in a hydrophobic capillary in the CPM, the LC phase (black) forms an interconnected network separating the red LE phase (3). This LC phase network morphology may be responsible for the larger value of $E^*$ measured in the CPM compared to the trough.

above 2 rad/sec, the dilatational modulus at coexistence increases, approaching that in the LC phase (Figure 6.9), suggesting that for $\frac{df}{dt} > \omega$, the system lags and $E^*$ approaches the LC phase.

However, Figure 6.10C shows an additional complication on the highly curved bubbles in the CPM. Curvature on the 50-micron length scale of the domain sizes in Figure 6.10B dramatically alters the LE-LC morphology; the LC phase forms an interconnected network over the entire hemispherical interface. As the bubble area is expanded and compressed, the LC network must expand and compress, which requires elastic energy, which may increase $E^*$ [245]. Future comparisons of the detailed values of $E^*$ as a function of frequency on the flat trough interface and on bubbles of various radii may reveal the contribution of this network structure to the dynamic properties of surfactant coated bubbles, which may be important to the conditions existing in lung surfactant covered, highly curved alveoli in the lungs.
6.3.3 Soluble protein - β-lactoglobulin

β-lactoglobulin contains 162 amino acids and has a molecular weight of about 18 kDa, in comparison to 734 Da for DPPC and 496 Da for LysoPC. The molecule contains 2 disulfide and 1 free sulfhydryl groups, and in bulk solution is globular with a well-defined secondary structure [23]. β-lactoglobulin and other proteins combine feature of both soluble and insoluble surfactants. Proteins can change configuration at the interface by partially unfolding and entangling, which gives distinct differences to their interfacial rheological properties [23, 249, 250, 251, 208, 210, 238]. Figure 6.11A shows the measured values of \( \frac{A_0 \Delta \pi_{\text{par}}}{\Delta A} \) and \( \frac{A_0 \Delta \pi_{\text{perp}}}{\Delta A} \) for 10 μM β-lactoglobulin solution in the Langmuir trough. Unlike LysoPC and DPPC, \( \frac{A_0 \Delta \pi_{\text{par}}}{\Delta A} > \frac{A_0 \Delta \pi_{\text{perp}}}{\Delta A} \) and the values are significantly different. Figure 6.11B shows \( E^* \) and \( G^*_s \) determined from Eqns. 6.1 and 6.2 compared to \( E^* \) and \( G^*_s \) measured with the CPM and DWR respectively. The trough results for \( E^* \) and \( G^*_s \) are in qualitative agreement with the direct measurements of \( E^* \) and \( G^*_s \) and with previous literature values [236]. The trough value for \( E^* \) is underestimated relative to the CPM, while the trough value for \( G^*_s \) is over-estimated relative to the DWR.
Figure 6.12: Compressing the protein film from the initial 13 mN/m to 23 mN/m increases both $E^*$ and $G^*_s$ similar to insoluble DPPC. β-lactoglobulin has a dilational modulus similar to soluble LysoPC but has a shear modulus orders of magnitude greater than LysoPC or DPPC. B) Following an initial compression of β-lactoglobulin in the trough to a surface pressure of 23 mN/m, both $\pi_{par}$ and $\pi_{perp}$ decay with time. $\frac{A_o \pi_{par}}{\Delta A} < \frac{A_o \pi_{perp}}{\Delta A}$ due to the rearrangement of β-lactoglobulin at the air-water interface inconsistent with Eqns. 6.1 and 6.2. Proteins unfold due to the change in the hydrophobic environment at the interface.

Globular proteins can unfold and rearrange at the hydrophobic-hydrophilic interface making the interfacial behavior of β-lactoglobulin and other structured proteins much more complex than small molecular weight soluble or insoluble surfactants. Aging protein films can lead to entanglements and gelation which does not occur in surfactants. After the 10 µM β-lactoglobulin solution was added to the trough, the surface pressure spontaneously increased to ~ 13 mN/m as the protein adsorbed to the interface, similar to the soluble surfactant LysoPC. However, unlike LysoPC, compression of the adsorbed β-lactoglobulin film caused the surface pressure to increase from 13 to 23 mN/m, similar to the insoluble surfactant monolayer DPPC (Figure 6.8). However, continued compression did not increase the surface pressure, consistent with some exchange of protein between the surface and bulk or a folding or collapse of the protein film. $E^*$ increases with surface pressure for β-lactoglobulin, which is similar to DPPC (Figure 6.9), but remains more similar in magnitude to soluble LysoPC than insoluble DPPC. $G^*_s$ also increases with increasing surface pressure, consistent with greater entanglements and molecular interactions for the more concentrated film.
However, as shown in Figure 6.12B, both $\Delta \pi_{\text{par}}$ and $\Delta \pi_{\text{perp}}$ decay with time, likely due to the rearrangement of $\beta$-lactoglobulin at the air-water interface. This means that $\frac{A_o \Delta \pi_{\text{par}}}{\Delta A} < \frac{A_o \Delta \pi_{\text{perp}}}{\Delta A}$, which is incompatible with Eqns. 6.1 and 6.2 as this would suggest a negative shear modulus. This is more likely due to the formation of an anisotropic, elastic gel network as the protein unfolds. For LysoPC and DPPC, the surface pressure is constant with time after the initial adsorption and does not depend on the age of the interface. Figure 6.12B suggests that small differences in the surface pressure or age of $\beta$-lactoglobulin might account for the quantitative differences between the direct CPM and DWR values and those determined from the LB trough.

### 6.4 Conclusions

Accurate and repeatable measurements of the shear and dilatational moduli of interfacial films is essential to characterization of their dynamic rheological behavior and provides interesting information on interfacial morphology. The Langmuir trough has several advantages for interfacial studies such as the ease of dealing with large, flat interfaces which makes quantitative depositions possible, direct surface pressure measurements using a Wilhelmy plate, and precision control of the interfacial area using mobile barriers. The interface can be readily visualized with confocal fluorescence microscopy. However, uniaxial barrier motion in a rectangular trough involves both dilatational and shear deformations and both the shear and dilatational moduli are involved in interfacial deformations. Petrov et al and Cicuta et al. postulated that $|E^* + G_s^*| = A_o \frac{\Delta \pi_{\text{par}}}{\Delta A}$ and $|E^* - G_s^*| = A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$ in which $\Delta \pi_{\text{par}}$ and $\Delta \pi_{\text{perp}}$ are the variations in surface pressure parallel and perpendicular to the barrier axis in the trough for small sinusoidal area changes, $A_o \pm \Delta A e^{i\omega t}$.

Here we determined values of $E^*$ and $G_s^*$ with the Langmuir trough and independent measurements of $E^*$ determined using a capillary pressure microtensiometer and $G_s^*$ determined using the double-wall ring apparatus and the microbutton shear rheometer for soluble palmitoyl lysophosphatidylcholine (LysoPC), insoluble dipalmitoylphosphatidylcholine (DPPC), and the globular protein $\beta$-lactoglobulin. For LysoPC and DPPC, $A_o \frac{\Delta \pi_{\text{par}}}{\Delta A} \approx A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$ at all oscillation frequencies consistent with $E^* \gg G_s^*$. 
The values of $E^*$ for LysoPC and DPPC determined using the trough showed quantitative agreement with $E^*$ determined in the capillary microtensiometer when corrected for the bubble curvature. DWR and microbutton measurements showed that $G_s^*$ was at least 4 orders of magnitude smaller than $E^*$ for both LysoPC and DPPC. Hence, $E^* = A_o \frac{\Delta \pi_{\text{par}}}{\Delta A} = A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$, and only a single surface pressure measurement is necessary to determine the dilatational modulus of these surfactants in an LB trough. The LB trough provides good estimates of $E^*$ across a range of frequencies for LysoPC and DPPC (and by inference, we expect that pendant and sessile drop measurements of systems in which $E^* \gg G_s^*$ should also be in quantitative agreement with microtensiometry). The ease of use of the LB trough for insoluble surfactant depositions makes it a valuable addition to surface rheology studies.

We found substantially different results for the globular protein $\beta$-lactoglobulin. For $\beta$-lactoglobulin films in the trough, $A_o \frac{\Delta \pi_{\text{par}}}{\Delta A} > A_o \frac{\Delta \pi_{\text{perp}}}{\Delta A}$. Double wall ring shear measurements showed that $G_s^*$ was of the same order of magnitude as $E^*$ measured with the capillary microtensiometer. $G_s^*$ for $\beta$-lactoglobulin was 4-6 orders of magnitude greater than $G_s^*$ for the soluble and insoluble surfactants, while $E^*$ for $\beta$-lactoglobulin was of the same magnitude for soluble LysoPC. The trough values of $E^*$ and $G_s^*$ were in qualitative rather than quantitative agreement with the CPM and DWR. These quantitative differences are likely the result of the complex evolution of globular proteins at the air-water interface. Aging experiments showed that $\beta$-lactoglobulin continued to evolve over hours, resulting in changes in surface pressure and the development of anisotropic deviatoric stresses as the protein unfolds and gels at the air-water interface. High molecular weight structured proteins at interfaces have fundamentally different behavior than small molecular weight surfactants, but both systems are amenable to rheological measurements in a conventional LB trough. This validation of the LB trough measurements of $E^*$ and $G_s^*$ may make evaluating the rheological effects of curvature and finite interface size possible by comparing the similarities and differences of $E^*$ on flat and highly curved interfaces.
Chapter 7

Outlooks on Clinical Lung Surfactants Elasticity

Results of this work were adapted from [253]

7.1 Background

In 1996, NRDS was the leading cause of infants death, with the mortality rate of 51.6%. [254] As the SRT and neonatal intensive care have improved, death due to NRDS has dropped to 2.1% in 2016. [255] In contrast, ARDS mortality rate remains high, as no effective treatment or even understanding of the origins of ARDS have been developed. The clinical LSs that work for NRDS have had low success in treating ARDS. It is likely that the current SRT efforts have not succeeded as ARDS is not due simply to a lack of surfactant as is the case for NRDS, but the inactivation of a sufficient quantity of surfactant. New formulation should be based on this idea of reversing the surfactant inhibition and delivery optimization. I explored the bulk rheology of three clinical LSs and the effects of nonadsorbing polymer to study the effects of various formulation in delivery. The results, as discussed in the next section, confirmed that the different formulation affects LS viscoelasticity and likely contributes to the differing efficacy.

In the United States, the three clinically approved replacement lung surfactants are Survanta (minced bovine lung extract), Infasurf (calf lung lavage extract), and Curosurf (minced porcine lung extract). Although replacement surfactant therapy has been
quite successful in treating NRDS, the composition, bilayer and monolayer properties of these three surfactants varies widely [256, 257, 82]. In addition, as these surfactants are animal-sourced, all clinical surfactants suffers somewhat from batch-to-batch variability and potential viral or prion contamination. Animal-derived surfactants have not been effective in treating ARDS; in fact, there are no generally accepted treatments or mechanistic understanding of the breathing instabilities associated with ARDS [258].

The composition of replacement LS are extrapolated from both bronchoalveolar lavage and pharyngeal aspirates of animals and humans, which were presumed to closely represent the composition of in vivo lung surfactant. By weight, extracted lung surfactant consists of 85% glycerol-phospholipid and 10% lung-specific proteins [82]. The remaining fractions are cholesterol and other neutral lipids such as triglycerides.

The differences in lipid composition between the clinical lung surfactants likely play a major role in the differences in their suspension properties. Both Survanta and Curosurf remove any cholesterol from their formulations, Infasurf retains 5-8 wt% cholesterol (10-15 mol:mol% relative to the phospholipids) [256, 257]. Cholesterol is ubiquitous in cell membranes and inevitably appears in the lavage material extracted from animal lungs prior to purification. It is still an open question if cholesterol is present in native human lung surfactant or what its role may be [97, 259]. Even small mole fractions of cholesterol decrease the ordering of DPPC at physiological temperatures; at sufficiently high cholesterol concentrations, the DPPC lattice is disrupted, and the mixture forms a liquid disordered ($L_o$) phase, which incorporates both cholesterol and DPPC in a single phase [260, 261]. As its name suggests, the $L_o$ phase is disordered and fluid at room temperature and above. In addition to the extracted lipids, between 6-14 wt% saturated palmitic acid (PA) is added to Survanta [256, 257]. PA co-crystallizes with DPPC, which increases the solid to fluid melting temperature to 50-60°C for the mole ratios of DPPC:PA in Survanta [262, 263]. Hence, in Survanta, and to a lesser extent Curosurf and Infasurf, multiple solid and fluid phases can coexist in each bilayer at physiological temperature. The effects of these components on the bilayer aggregates in the clinical suspensions is not well understood nor is how the phase behaviour of the bilayers in these aggregates might influence the suspension viscosity. DPPC, and other saturated and unsaturated phosphatidylcholines, phosphatidylglycerols, and phosphatidylserines in LS, self-assemble into lipid bilayers in saline. In the lungs, LS bilayers are further
organized into “multilamellar bodies” that are extruded from the type II pneumocyte cells that line the alveoli into the alveolar line fluid [264, 265]. These multilamellar bodies resemble multilamellar liposomes that form spontaneously on hydration of phospholipids [266]. On contact with the air-water interface in the lung, the multilamellar bodies transform into monolayers that spread to cover the alveolar interface. Previous optical microscopy and freeze-fracture transmission electron microscopy (FFTEM) results [266, 267, 268] show that the bilayer aggregates of the three clinical surfactants are polydisperse and each has a unique internal structure and ordering, with aggregate sizes varying from 100 nm-10 \(\mu\)m, although the lipid bilayers are organized differently in the different surfactants.

For NRDS treatment, the surfactant suspension is instilled via an intratracheal tube and must rapidly flow through the multitude of airway bifurcations, and upon reaching the lung periphery, swiftly spread to cover the alveolar air-water interface as monolayers. Gravity is typically not the main driving force for such flows at the 1- 100 \(\mu\)m length scales in the lung alveoli epithelial lining fluids; rather surface tension gradients drive such flows from areas of low surface tension to higher surface tension via Marangoni flow [269]. But whatever the driving force, the suspension viscoelasticity resists the flow. If the suspension has a yield stress or substantial elasticity, flow can be completely arrested. Rapid and complete spreading is also essential for drug delivery via surfactant suspensions to treat other bronchoalveolar diseases in preterm infants. Lung surfactants are well tolerated drug carriers as they are physiologically compatible to the airway and promote spreading of the drug to the distal portions of the lung [269, 270].

In practice, Survanta takes longer to restore breathing in NRDS patients compared to Curosurf and Infasurf, although there is little correlation between this initial lag time and long term complications or survival [271]. The Grotberg group has studied the flow of lung surfactant mixtures through airway-like bifurcations using microfluidic channels and theoretical modelling [272, 273, 274]. Their work is primarily concerned with the effects of local environment and geometry on the flow profile; the effects of different LS compositions on the flow properties of surfactant suspensions have not been examined. Here, we investigated the bulk rheology of the three commonly used clinical surfactant suspensions. We find that the differences in lung surfactant composition dictate the structure of the bilayer aggregates in suspension, which in turn, dictate the rheological
properties of the suspensions. At low shear rates, all three clinical surfactants are soft viscoelastic solids, with the elastic modulus greater than the loss modulus. The rigid, high aspect ratio aggregates formed by Survanta have a significant apparent yield stress at physiological temperature, while the spherical Curosurf and Infasurf have a smaller apparent yield stress. Increasing the temperature of Survanta above the bilayer solid-liquid transition lowers the suspension viscosity to that of Curosurf and Survanta as the Survanta aggregates melt into spherical liposome shapes. Our results parallel the surface rheology of the three surfactants; Survanta has a significantly more viscoelastic interface compared to Infasurf and Curosurf [275, 276, 15]. This suggests that the monolayer and bilayer composition and phase behaviour govern the viscoelasticity from suspension to monolayer, and likely also governs the rate of surfactant spreading in the premature lung.

7.2 Experimental details

7.2.1 Materials

Three clinical lung surfactants: Survanta (minced bovine lung or beractant, AbbVie Inc., 25 mg/mL), Infasurf (chloroform-methanol extract of calf lung lavage or calfactant, ONY Inc., 35 mg/mL), and Curosurf (minced porcine lung surfactant or poractant alpha, Chiesi USA Inc., 80 mg/mL) were purchased from the pharmacy at University of Minnesota Boynton Health Service and kept in a refrigerator at 4°C until the time of use. The concentration refers to the PL concentration in buffered saline solution. The (known) composition of these surfactants, as well as the comparison to estimated composition of native lung surfactant, is presented in Figure 2.6.

For the study of the effects of nonadsorbing polymer, polyethylene glycol (PEG), Mn = 20,000 kDa, was purchased from Sigma Aldrich. PEG is mixed with Curosurf and Infasurf at 5% w/v, while Survanta at 1% w/v, due to reasons explained in section 4. The mixture was then vortexed for about 30 seconds until the PEG crystals had dissolved. Rheology measurements of the PEG-based mixture were performed on the same day the mixture is made.
7.2.2 Cone and plate rheometry

The rheological results presented here are obtained from stress-controlled rheometer AR-G2 from TA Instruments at University of Minnesota Polymer Characterization Facility. We used cone and plate fixture with a 4 mm diameter, 2° angle, 49 µm gap. The schematic is shown in Figure 7.1. The plate is a Peltier plate system that allows a sensitive control of the temperature. The choice of cone and plate probe is due to the expensive cost of the clinical lung surfactants, and using cone and plate requires less sample (∼0.6 mL) than other geometry like concentric cylinder (∼10 mL).

Steady shear rheology measures the fluid viscosity as a function of shear rate: \( \eta(\dot{\epsilon}) \), with shear rate range set to be: \( 10^{-3}/s \leq \dot{\epsilon} \leq 10^{2}/s \) after proper calibrations and rotational mapping were performed. Banerjee et al. [277] estimated, based on geometrical arguments, that the shear rate experienced by the surfactant flowing through the airway varies from 0.002/s to 1.4/s, so our chosen range of measurements covers the shear rate of interest. Unfortunately, due to the rheometer torque limitation, the regime at \( \dot{\epsilon} \leq 10^{-2}/s \) for Infasurf and Curosurf cannot be trusted and thus not included in the data presentation. The wait time between each subsequent shear rate is 10 seconds. To ensure that evaporation is minimum, we created a cover that encloses both the lateral side and the top side of the cone, with hydrated paper towel near the sample. To obtain information on the dynamic behavior of the surfactants, we induced small amplitude oscillatory shear (SAOS) on the samples at their linear viscoelastic regime where the viscoelastic moduli \( G' \) and \( G'' \) are independent of the applied strain at frequency relevant to breathing, i.e. between 0.1-1 Hz, corresponding to 6-60 breathes per minute. A healthy individual breathes 12-40 times per minute, and thus the selected range is appropriate for the system of interest.

7.2.3 Differential scanning calorimetry (DSC)

To probe thermodynamic phase changes, differential scanning calorimetry measurements were performed using TA Instruments Q1000 DSC by increasing the temperature from 10°C to 65°C with 2°C/min heating rate after samples were loaded at 23°C and equilibrated at 10°C for 120 s.
Figure 7.1: Experimental setup for bulk rheometry using cone and plate

7.2.4 Small angle X-ray scattering

Small angle X-ray scattering experiments were performed on a custom-built instrument with an 18 kW Rigaku rotating anode source (CuKα, λ = 1.54 Å) and 2-D area detector at room temperature. The instrument is optimized for small angle work (5-60 nm), has an Osmic Confocal Maxflux double focusing multilayer mirror, an 11 cm Bruker HI-STAR multiwire area detector, and a 1.5 m sample to detector distance. The FWHM was less than ~0.005 Å⁻¹. In all experiments, the surfactant dispersion in saline were loaded into 1.5 mm borosilicate glass capillaries (Charles Supper, Mass) and flame sealed. Temperature was controlled at 37 °C via circulating heated or cooled fluid through an aluminium sample holder block, monitored by a thermistor located adjacent to the capillary.

7.2.5 Freeze-fracture microscopy

Freeze-fracture samples were prepared by first depositing a film of sample liquid approximately 100 microns thick between two copper planchettes. The samples were frozen by plunging the sample into a liquid propane bath cooled by liquid nitrogen. The frozen
sample was transferred under liquid nitrogen to the sample block of a JEM Cryofract freeze-fracture device. The sample was fractured, and the two resulting surfaces were replicated with 1.5 nm platinum deposited at 45°, followed by 15 nm of carbon deposited normal to the surface. The copper planchettes were dissolved in chromerge (a mixture of chromic acid, sulfuric acid and water), and then the carbon-platinum replicas were washed in water. The replicas were collected on formvar-coated TEM grids (Ted Pella, Redding, California)[278]. A Gatan CCD camera was used to record digital bright field images using a FEI Technai transmission electron microscope at 100 keV. More details and additional images are available in Braun et al.[266]

7.2.6 Double wall ring

The surface rheology measurement was performed using a TA Instrument double wall ring setup. The lung surfactant suspension was contained in a double-walled circular Teflon trough positioned on top of a Peltier plate, and a platinum-iridium wire ring was attached to a TA Instrument DHR-3 rheometer 45. The cross section of the ring is square-shaped with a sharp edge to create a planar interface and is ∼1 mm in width. After the interface was found, proper calibration was performed before each experiment.

7.2.7 Centrifuge

To obtain a qualitative estimate of the volume fraction of LS suspension, the suspensions were centrifuged using a benchtop IEC Micromax RF centrifuge at 13600 RPM (∼15000 ×g) for 60 minutes at 37°C. This was sufficient to cleanly separate the lipid fraction from the solvent fraction and make approximate estimates of the surfactant particle volume fraction.

7.3 Experimental Results

7.3.1 Steady shear rheology

Figure 7.2 shows the steady shear viscosity of Survanta, Infasurf and Curosurf at 37°C. Banerjee et al. [277] estimated, based on geometrical arguments, that the shear rate experienced by the surfactant flowing through the airway varies from 0.002/s to 1.4/s.
Higher shear rates may be experienced during injection via intratracheal intubation, so our chosen range of measurements covers $10^{-3} / s$ to $10^3 / s$. Surprisingly, the steady shear viscosity of Survanta is roughly two orders of magnitude higher than Infasurf and Curosurf. All three surfactants are strongly shear-thinning; the viscosity of Survanta decreases by more than four orders of magnitude over the tested shear rates, while that of Infasurf and Curosurf decrease by two orders of magnitude. All three suspensions have a Newtonian plateau (arrows in Figure 7.2) at shear rates of $10^{-2} / s$ and below which can be interpreted as wall slip, which is common in highly concentrated suspensions [270, 280]. For shear rates of 1-100/s, Infasurf and Curosurf are less shear thinning and asymptote to a nearly constant viscosity of $4 \times 10^{-3}$ Pa.s similar to the continuous saline suspending fluid of $10^{-3}$ Pa.s. This high shear viscosity regime is similar to measurements by Thai et al. [267] and Lu et al. [268]. Shear thinning at low shear rates was observed previously in LS dispersions [268, 281] and in other bilayer liposome systems [282, 283]. However, most previous work with LS suspensions only considered shear rates greater than $\sim 1 / s$ at which the surfactant suspensions were close to Newtonian [267, 268].

The shear thinning of the three surfactants follows a power-law relationship $\eta = a\dot{\epsilon}^m$ over shear rates from $10^{-2}$ to $10^2 / s$ with $-1 \leq m \leq -0.8$ for the three surfactants. Hence, at low shear rates, the shear stress, $\sigma = \eta \dot{\epsilon}$, is roughly constant, suggesting that these suspensions are sufficiently concentrated to have a yield stress $2$. For practicality, we define yield stress fluids as power law fluids with $m$ values close to -1, instead of fluids that do not flow at all at low shear.

The yield stress in densely packed suspensions must be overcome to initiate flow by deforming the individual particles or by breaking down any long-range structure in the suspension. Figure 7.3 shows the viscosity as a function of shear stress, $\sigma(\dot{\epsilon}) = \eta \dot{\epsilon}$. All three surfactant suspensions show a nearly constant, Newtonian viscosity below the suspension yield stress: $\sigma_{yield} \approx 1$ Pa for Survanta and 0.01 - 0.03 Pa for Curosurf and Infasurf (arrows in Figure 7.3). At the yield stress the viscosity drops by orders of magnitude, followed by shear thinning at high shear stresses. Similar results were obtained for Survanta using a sweep at constant stress (open symbols). For both systems, the constant viscosity plateau at low shear stress is consistent with wall slip below the yield stress, which is typical for soft viscoelastic solids [279]. The wall slip likely causes the
Figure 7.2: Steady shear viscosity vs. shear rate for the clinical surfactants Survanta, Curosurf and Infasurf at 37°C. The three surfactants follow a power-law relationship $\eta = \sigma \dot{\varepsilon}$ with $m \approx -1$ over shear rates from $10^{-2}$ to $10^2/s$. The viscosity of Survanta is two orders of magnitude greater than Curosurf or Infasurf although the lipid mass loading of Survanta is 25 mg/mL, while 35 mg/ mL for Infasurf and 80 mg/mL for Curosurf. At the lowest shear rates, a Newtonian plateau (arrows) indicates slip of the concentrated suspension at the walls of the cone and plate. Except where noted, the error was smaller than the symbols.

The magnitude of the yield stress to be under-estimated [279]. The two orders of magnitude difference in yield stress carries over from the two orders of magnitude difference in viscosity between Survanta and Infasurf and Curosurf (Figure 7.2).

Suspension rheology is typically explained by Equation 7.1, the phenomenological Krieger-Dougherty relationship for concentrated (non-interacting) suspensions as a function of effective particle volume fraction $\phi$:

$$\eta = \eta_m \left(1 - \frac{\phi}{\phi_c}\right)^{-[\eta]\phi_c}$$  \hspace{1cm} (7.1)
Figure 7.3: Viscosity vs. shear stress. All three surfactants exhibit the features of yield stress fluids. The Newtonian plateau at shear stresses below the yield stress (arrows) is indicative of wall slip, which is common to concentrated suspensions.1 The viscosity drops by more than an order of magnitude at the yield stress, and the fluid is weakly shear thinning at higher stresses. The error in measurements is within the size of the symbols used unless noted.
\[ \eta, \eta_m, [\eta], \text{and } \phi_c \text{ are the suspension viscosity, medium viscosity, intrinsic viscosity (for hard spheres, the value is 2.5), and maximum random packing fraction. For polydisperse hard spheres, } \phi_c \approx 0.7, \text{ which gives the exponent, } [\eta]\phi_c \approx 1.8. \text{ Equation } 7.1 \text{ predicts that the suspension viscosity is sensitive to small changes in the effective particle volume fraction, } \phi, \text{ near the maximum packing fraction, } \phi_c. \text{ Hence, if the bilayer aggregate structures of all three lung surfactants were simple hard sphere particles of equal density, } 7.1 \text{ suggests that Curosurf, with 80 mg/mL lipid loading, should have the highest } \phi, \text{ Infasurf, with 35 mg/mL lipid loading should be intermediate, and Survanta, with 25 mg/mL loading, should have the lowest } \phi. \text{ Therefore, Equation } 7.1 \text{ would predict that Curosurf should be the most viscous and Survanta the least viscous which is in direct contrast with the results in Figures 7.2 and 7.3.} \\

7.3.2 Surfactant aggregate imaging

Optical and freeze-fracture electron microscopy (Figure 7.4) show that the lipid aggregates in Curosurf and Infasurf are multi-layered and spherical. Curosurf (Figure 7.4B, E) is more polydisperse with particle sizes ranging from 100 nm to 10 \( \mu \)m \[267, 284\], while Infasurf (Figure 7.4C, F) is more monodisperse with particles around 3 \( \mu \)m. The freeze-fracture transmission electron microscopy (FFTEM) images show that the internal organization of the bilayers in the aggregates is quite different (For additional FFTEM images, see \[266\]). Curosurf aggregates are organized in onion-like multilayers, with concentric lamellar layers with few internal saline-containing voids (Figure 7.4E). Infasurf particles are agglomerations of single bilayer vesicles; the agglomerates also contain multiple water-filled voids (Figure 7.4F). Hence, the lipid mass per volume of suspension, \( c_m \) and the bilayer aggregate volume fraction, \( \phi = c_m/\rho \), depends on \( \rho \), the total lipid mass per total particle volume. This modifies Equation 7.1 to be:

\[ \eta = \eta_m \left( 1 - \frac{c_m/\rho}{\phi_c} \right)^{-[\eta]\phi_c} \tag{7.2} \]

The FFTEM shows that \( \rho \) is quite different between Infasurf and Curosurf. Curosurf packs more lipid into its onion-like multilamellar aggregates than Infasurf with its unilamellar vesicle aggregates, and hence has a higher total lipid mass per particle volume, \( \rho \). Centrifuging the three surfactants shows that the effective surfactant volume fraction
Figure 7.4: Optical micrographs (top row (A–C)) and freeze-fracture transmission electron micrographs (bottom row (D–F)) of Survanta, Curosurf and Infasurf showing the differences in bilayer aggregate microstructures. (A) Survanta consists of 5–20 µm jagged, prolate bilayer aggregates (arrows) with length to width aspect ratios, $p \sim 3–6$. (D) The corresponding FFTEM image shows one such flat aggregate surface with multiple bilayer steps. (B) Curosurf and (C) Infasurf form spherical multilayered structures of 1–10 µm in diameter. However, FFTEM images show (E) Curosurf has close packed concentric lamellae that are organized like an onion. (F) Infasurf consists of aggregated unilamellar vesicles and water-filled void spaces. Hence, Curosurf packs more lipid mass within each aggregate than Infasurf so that the volume fractions are similar for these two surfactants.
$\phi \sim 0.5$ (Figure 8.7) for Survanta, Curosurf and Infasurf. Hence, from Equation 7.2, Curosurf and Infasurf suspensions should have similar viscosities, as we observe.

Survanta aggregates, however, have jagged, prolate shapes, with length to width aspect ratios, $p = 3 - 6$ (Figure 3A, arrows). This is due to the stiffening effect of palmitic acid on the gel phase bilayers of DPPC at and below physiological temperature [262, 198]. PA co-crystallizes with DPPC, which increases the solid to fluid melting temperature to 50-60°C for the mole ratios of DPPC:PA in Survanta [262, 198] (See Figure 7.9). Recent studies by Nakagawa et al. [285, 286] showed that mixtures of hexadecanol with saturated phospholipids change the bilayer morphology from spherical vesicles or liposomes to flat, multilamellar sheet-like structures similar to those seen in Survanta. Both palmitic acid and hexadecanol have a small headgroup size that can easily insert in between bulkier headgroups of DPPC, allowing for a stronger van der Waals attraction among the hydrophobic tails, leading to rigid bilayers with greatly increased bending moduli [263, 276, 192]. If the resistance to bilayer bending is sufficient, as is also found in the interdigitated phase of saturated phosphatidylcholines, bilayers form stacks of flat sheets rather than closed liposomes [287, 288].

The higher aspect ratio $p = 3 - 6$ of Survanta aggregates leads to both a higher intrinsic viscosity $[\eta]$ and a lower critical volume fraction $\phi_c$ in Eqns. 7.1 and 7.2 compared to the spherical aggregates in Curosurf or Infasurf. Kuhn and Kuhn [289] proposed for moderate aspect ratios that:

$$[\eta] \approx 2.4 + 0.4(p - 1)^{1.5}$$  \hspace{1cm} (7.3)

For $p = 3$, $[\eta] \approx 3.6$. For rod-like structures, the critical packing fraction, $\phi_c$ of non-Brownian (larger than 1 $\mu$m) particles is also a function of the aspect ratio: [290]

$$\phi_c = 0.54 - 0.0125p$$  \hspace{1cm} (7.4)

which reduces $\phi_c$ to about 0.5. As a result, there is little change in the exponent in Eqns. 7.1 and 7.2 as $[\eta]\phi_c \approx 1.8$. However, from centrifuge experiments with Survanta, the particle volume fraction, $\phi = 0.5 \sim \phi_c$, or the Survanta volume fraction is close to the critical packing fraction. Hence, the non-spherical shapes of Survanta aggregates lead to the highest viscosity of the three clinical surfactants.
7.3.3 Thixotropy of Survanta

Dense suspensions are also sensitive to shear history. In thixotropic materials, the viscosity decreases with increasing shear rate, but recovers after a certain rest period. Figure 4 shows the hysteresis of Survanta viscosity for both ascending (filled squares) and descending (open squares) shear rate ramps. The descending shear rate ramp was begun after the sample was allowed two minutes to recover following the ascending ramp. For the first measurement, the shear rate was increased from 0.001/s to 1000/s, and the descending ramp was performed from 1000/s back to 0.01/s. As the shear rate was decreased to 0.001/s on the descending ramp, the Survanta viscosity decreased by about an order of magnitude relative to the ascending ramp (Compare closed black squares to open black squares).

In measurement 2, the Survanta sample was subjected to a second shear ramp from 0.01 to 100/s. The ascending branch of the second sweep (filled red squares) was very similar to the ascending sweep of the first measurement (filled black squares). However, there was less hysteresis on the descending sweep (compare open red squares to open black squares). The difference in the hysteresis suggests that the structure becomes more deformed as the shear rate increases.

The two-minute window between ascending and descending ramps was not sufficient for the suspension structure to rebuild. This behavior may be due to some alignment of the prolate Survanta particles at high shear rates that take some time to equilibrate. Thixotropy might also explain discrepancies in viscosity measurements in the literature, especially if high shear rates are examined first by doing the descending shear sweep [267, 284].

7.3.4 Oscillatory shear rheology

The shear thinning and thixotropic nature of LS suspensions suggests an underlying microstructure that responds to shear. To examine this, oscillatory linear viscoelasticity of the three lung surfactants was probed at 37°C (Figure 7.6). Amplitude sweep experiments at 0.2 Hz (≈ 1 rad/s) were done to determine the linear viscoelastic regime for each material, that is how much deformation can be applied before the moduli become dependent on strain (See Supplementary Information Figure 7.7).
Figure 7.5: Survanta viscosity shows a significant hysteresis following high shear rates. The descending shear rate ramp (open black squares) was initiated after allowing the suspension to rest for 2 minutes following the ascending ramp to 1000/s. The viscosity was reduced by almost an order of magnitude at low shear rates (open black squares). The hysteresis was less pronounced if the ascending ramp was terminated at 100/s before initiating the descending ramp after a 2 minutes rest. The Survanta structure takes more than 2 minutes to recover its original viscosity. Errors in the measurement are smaller than the size of the symbols used.
Figure 7.6: Linear shear rheology of Survanta, Infasurf and Curosurf at oscillatory strains determined to be in the linear regime for each material. All three materials had $G' > G''$ consistent with significant microstructure in the suspension that could store elastic energy over this frequency range. $|\eta^*| = \sqrt{G'^2 + G''^2}/\omega$ for Survanta is also around two orders of magnitude larger than that of Curosurf or Infasurf, similar to the trend in steady shear viscosity (Figure 7.2). Standard deviation of multiple measurements is smaller than the symbols used.
Figure 7.7: Strain amplitude sweep for linear oscillatory shear experiments. The critical strain is determined as being the strain amplitude that causes the maximum $G'$ to decrease 10%.
Figure 7.6 shows that all three demonstrate significant viscoelastic behaviour with the elastic modulus, $G'$, larger than the viscous modulus, $G''$ at all frequencies tested. When $G'$ is larger than $G''$, the material can store energy and can return to near its initial configuration like a soft solid. Survanta has both $G'$ and $G''$ higher by $\sim 2$ orders of magnitude than Curosurf and Infasurf, meaning that the corresponding complex viscosity, $|\eta^*| = \sqrt{G'^2 + G''^2}/\omega$ of Survanta is also two orders of magnitude larger than that of Curosurf and Infasurf, similar to the trend in steady shear viscosity. Concentrated emulsions, which are somewhat similar in structure to concentrated surfactant suspensions are known to yield at applied stresses such that $\sigma_{\text{yield}} \approx G'/10$.\cite{291, 292}

For Survanta $G' \approx 10$ Pa and $\sigma_{\text{yield}} \approx 1$ Pa, while for Infasurf and Curosurf, $G' \approx 10^{-1}$ Pa and $\sigma_{\text{yield}} \approx 0.01$ Pa, surprisingly consistent with this prediction for emulsions.\cite{215}

### 7.3.5 Temperature effects on suspension rheology

Eqns. 7.1, 7.2, 7.3, and 7.4 suggest that the prolate structure of Survanta is responsible for the 2 orders of magnitude difference in steady and oscillatory shear viscosity compared to Infasurf and Curosurf. Palmitic acid increases the gel to liquid crystalline transition temperature of DPPC as well as making the bilayers rigid enough that they cannot bend to form spherical structures (Figure 7.4A, D). Differential Scanning Calorimetry (DSC) (Figure 7.9) shows a broad peak centered at 52°C for Survanta, similar to simple mixtures of DPPC and palmitic acid.\cite{202} Curosurf and Infasurf have broad peaks between 25 and 30°C\cite{284, 293} showing the lipids are in the fluid state at physiological temperatures. Figure 7.8A shows the effect of temperature on the Survanta reduced viscosity, $\eta/\eta_m$. The reduced viscosity eliminates the effects of temperature on the solvent (here saline) viscosity, $\eta_m$, and highlights the effects on changing the shapes of the Survanta aggregates as manifested in $\phi_c$ and $[\eta]$ in Eqns. 7.1, 7.2, 7.3 and 7.4. Raising the temperature from 5 to 37°C has a minimal effect on the Survanta reduced viscosity over this range of shear rates. However, at 60°C, which is above the gel-liquid crystalline transition temperature of Survanta, the reduced viscosity decreases by more than an order of magnitude. Optical microscopy images (Figures 7.8B, C) show that the prolate Survanta aggregates melt into spherical particles when heated above 60°C. Under flow, the Survanta aggregates distort into extended shapes, which is common to liposome structures under flow. This transition from prolate to spherical aggregates
Figure 7.8: (A) Survanta reduced viscosity, $\eta/\eta_m$ as a function of shear rate. No difference is observed between 5 and 37°C, which are below the gel-liquid crystal transition of Survanta. However, the viscosity drops by more than an order of magnitude at 60°C, above the transition temperature of 52°C. (B) Optical microscopy shows that as Survanta is heated above 60°C, the prolate aggregates (black arrow) melt into spherical shapes (white arrow), similar to those of Curosurf and Infasurf in Figure 7.4B and C. (C) On gentle shear, the Survanta aggregates stuck to the cover slip distort and form extended tubular structures (arrow), similar to bilayer liposomes. This temperature induced change in morphology is responsible for the drop in viscosity of Survanta on heating.
Survanta shows a broad endothermic peak from 50-55 °C due to the stabilizing effect of palmitic acid on the dipalmitoylphosphatidylcholine gel phase. Infasurf has a very broad, small endotherm from about 28-40 °C. Curosurf has a broad peak similar to Survanta but from 25-30°C.

$p \rightarrow 1$ in Eqns. 7.3 and 7.4 reduces the intrinsic viscosity and increases the critical volume faction, $\phi_c$, both of which lead to a decrease in the suspension viscosity.

### 7.3.6 Interfacial shear viscoelasticity effects on suspension viscosity

As all lung surfactant suspensions are surface-active, lung surfactant will form films at the air-water interface of the cone and plate rheometer (Figure 7.10). If this surface film is sufficiently elastic, an additional, potentially dominant, contribution to the measured torque may arise, especially as torque scales with distance from the center of the cone [294, 295, 296]. In surface-active protein suspensions of albumin or monoclonal antibodies, this interfacial elastic contribution can dominate the bulk contribution in cone
and plate measurements and could make the measured suspension viscosity appear as highly viscous and shear-thinning, even if the bulk suspension viscosity was Newtonian and small \[294\, 295\, 296\]. Distinguishing between the bulk and interfacial contributions to the cone and plate results can be made by measuring the suspension viscosity with a Couette rheometer or other geometry with a much smaller air-water interfacial area per sample volume than the cone and plate geometry \[294\, 296\]. Sharma et al. \[294\] derived two expressions to describe the effects of interfacial stress on the cone and plate measurements: \[294\, 296\]

\[
\eta_{CP}(\dot{\varepsilon}) = \eta_\infty + \frac{3\sigma_s}{R\dot{\varepsilon}} = \eta_\infty + \frac{3\eta_s}{R}
\] (7.5)

\(\eta_{CP}\) is the suspension viscosity determined with the cone and plate, \(\eta_\infty\) is the suspension viscosity at high \(\dot{\varepsilon}\) (\(\sim 0.002\) Pa.s for the diluted Survanta in Figure 7.11B), \(\sigma_s\) and \(\eta_s\) are the surface stress and surface viscosity of the Survanta monolayer\(\eta_s\dot{\varepsilon} = \sigma_s\), and \(R\) is the radius of the cone and plate. A second model for cone and plate experiments was proposed for surface-active albumin and monoclonal antibody proteins that form elastic, gel-like films with a yield stress \((G'_s > G''_s\) for all frequencies tested in small amplitude oscillatory shear) at interfaces \[294\, 296\]:

\[
\eta_{CP}(\dot{\varepsilon}) = \eta_\infty + \frac{3\sigma_{s,yield}}{R\dot{\varepsilon}}
\] (7.6)

Sharma et al. defined \(\sigma_{s,yield} = G'_s\dot{\varepsilon}_{s,yield}\) to be the crossover stress at which \(G'_s < G''_s\) in a non-linear strain sweep measurement of interfacial moduli at a fixed frequency of \(\omega = 1\) rad/s \[294\, 296\, 19\]. Eqns. 7.5 and 7.6 give similar predictions for highly elastic protein films \[296\, 19\]. However, all the protein films were highly elastic, with \(G'_s > G''_s\) up to strains of more than 100% \[294\, 295\, 296\, 19\]. Figure 7.11A compares the steady shear viscosity measured in a cup and bob Couette geometry for ten-times diluted Survanta with the viscosity measured with the cone and plate. The interfacial viscoelastic contribution can be identified using rheological probes with different ratios of interfacial area to suspension volume. Unlike the protein suspensions \[294\, 296\, 295\], the measured viscosities of Survanta are indistinguishable using the two methods. Previous measurements by Lu et al. \[268\] using a capillary viscometer, for which the interfacial effect is also minimal, gave comparable values to the cone and plate measurements.
Figure 7.11: (A) Diluted Survanta suspension viscosity measured with a Couette viscometer compared to a high interfacial area cone and plate rheometer. No differences were found. (B) Survanta surface viscosity obtained with a DWR apparatus. The surface viscosity, like the suspension viscosity (Figure 7.2) is shear thinning. (C) Comparison of diluted Survanta suspension viscosity using the cone and plate (black squares) compared to the predicted viscosity using eqn 7.5 (blue circles) and eqn 7.6 (pink triangles). Eqn 7.6 suggests a minimal effect of the surface on the cone and plate rheology as observed in (A).
The similarity in measured values across the different rheological probes with different ratios of interfacial area to suspension volume therefore suggests that no with different ratios of interfacial area to suspension volume. Unlike significant interfacial effects were present do not play a significant role in the measured Survanta bulk viscosity using cone and plate geometry. This is in contrast to protein suspensions whose rheology is dominated by the interfacial elasticity [294, 296, 295].

Figure 7.11B shows the measured steady shear interfacial viscosity of Survanta, $\eta_s$, as a function of shear rate using a double wall ring apparatus [297, 298]. To form the monolayer on the double wall ring apparatus, Survanta was diluted to 0.8 mg/mL in phosphate buffered saline (PBS), and was loaded onto the circular trough and equilibrated for one hour. The equilibrium interfacial tension of Survanta does not change with concentration above a minimum level of $\sim 0.1$ mg/mL; once the interface is saturated, the area/molecule and surface pressure of Survanta is fixed, as is the interfacial viscosity [15, 276, 299]. Like the bulk rheology measured with the cone and plate, the surface viscosity of Survanta was shear thinning with a power law relationship: $\eta_s = a\dot{\epsilon}^n$ with $n \sim 0.6$, which is significantly different power law than the measured Survanta bulk viscosity in Figure 7.2A that showed $m \sim 1$.

Figure 7.11C (blue circles) shows the predicted cone and plate viscosity using Equation 7.5 and the surface viscosity measured for the diluted Survanta from Figure 7.11B. The black squares in Figure 7.11C show that the suspension viscosity of the diluted Survanta measured with the cone and plate is roughly an order of magnitude less than Equation 7.5 predicts. As Figure 7.11A shows, there is a minimal contribution to the suspension viscosity from the interface. This may be because surfactant monolayers are brittle and yield easily. Micro-button surface rheometer measurements showed that the monolayer yield stress for pure dipalmitoylphosphatidylcholine (DPPC is the dominant component in Survanta, Curosurf and Infasurf) is $\sigma_{s,yield} \approx 0.01$ mN/m [215]. This low yield stress is due to the fracturing of the monolayer at crystalline grain boundaries between ordered DPPC domains [215]. Hermans et al. showed that for Survanta, $G'_s < G''_s$ in linear oscillatory surface viscometry measurements [275], suggesting that $\sigma_{s,yield}$ was very small and likely beyond the resolution of the double-wall ring (DWR) surface viscometer. Small strains of 1 - 2% shatter the DPPC monolayer and $G'_s < G''_s$ and the interface cannot apparently couple to the bulk and contribute to the torque on
the cone and plate. Using $\sigma_{s,yield}$ in Equation 7.6 shows a negligible surface contribution to the cone and plate rheology (pink triangles), which means that the bulk suspension is responsible for the rheological properties we observe. Curosurf or Infasurf have much lower interfacial shear viscosities than Survanta, which is also likely due to the differences in composition and phase behavior. Protein films act more like a crosslinked gel network and do not yield until large strains of 100% or more are applied [296, 19]. Our results suggest that the interfacial film must be highly elastic and maintain its integrity at large strains to influence the cone and plate measurements.

7.4 Conclusion

An efficient lung surfactant replacement needs to flow rapidly throughout the airways due to differences in surface tension between the trachea, bronchi and alveoli. During these flows, the shear rate experienced by the surfactant flowing through the airway is estimated to vary from 0.002/s to 1.4/s. Over these shear rates, Curosurf, Infasurf and Survanta are strongly shear thinning as expected for high volume fraction suspensions; all three surfactants flow like soft solids as shown by $G' > G''$ over a wide range of frequencies in oscillatory viscosity measurements. All three surfactants have an apparent yield stress with Survanta having $\sigma_{yield} \approx 1$ Pa, and Curosurf and Infasurf with $\sigma_{yield} \approx 0.02$ Pa. The two orders of magnitude difference in both shear viscosity and yield stress may be due to the rigid, asymmetric, high aspect ratio particles formed by Survanta compared to the spherical particles of Curosurf and Infasurf. The properties of the suspension viscosity were consistent with the phenomenological Krieger-Dougherty relationship (Equation 7.1) for concentrated suspensions of effective volume fraction, $\phi$. However, the different surfactant aggregates had quite different internal bilayer organization, which gave quite different relationships between the mass loading given by the manufacturers, and the volume fraction of the particles in the suspension. Both Curosurf and Infasurf formed spherical particles; Curosurf bilayers were organized in concentric layers, like onions, while Infasurf bilayers were organized as agglomerations of unilamellar vesicles. Hence, the density of lipids was lower for Infasurf than Curosurf. This means that the higher mass loading in Curosurf gave a similar volume fraction as the lower mass loading in Infasurf. Survanta consisted of higher aspect ratio, jagged
bilayer crystals, likely due to the addition of palmitic acid to the extracted lipids which stabilizes the crystalline packing in the bilayer. Higher aspect ratio particles decrease the maximum packing fraction in the Krieger-Dougherty model, and for a given particle volume fraction, greatly increase the suspension viscosity, as we observe. Raising the temperature of the Survanta suspensions above the melting temperature of the bilayer crystals caused a dramatic drop in the suspension viscosity. The high aspect ratio crystals melted into spherical bilayer aggregates similar to Curosurf and Infasurf, which increased the critical packing volume fraction, leading to an order of magnitude decrease in the suspension viscosity.

Clinical observations suggest that Survanta takes longer to treat neonatal respiratory distress syndrome following intra-tracheal delivery of the suspension than Curosurf or Infasurf. There is also a higher incidence in deoxygenation following surfactant instillation compared to Infasurf and Curosurf [300, 301, 302, 303]. We speculate that these differences are related to our observations that Survanta is a hundred times more viscous with a significantly higher yield stress, which can inhibit spreading by surface tension gradients in the lung. The interfacial viscosity of Survanta is also significantly greater than that of Curosurf or Infasurf, which likely also contributes to slower spreading over the alveolar interfaces. Curosurf delivers the greatest mass of surfactant with the same suspension viscosity as Infasurf.
Chapter 8

Lung Surfactant Inactivation and Reversal by Non-Adsorbing Polymer

A portion of this chapter is adapted from Ciutara and Zasadzinski [253].

8.1 Inactivation of lung surfactant

Surfactant inactivation is a qualitative term for the inability of nominally sufficient amounts of lung surfactant to promote proper lung function [47, 84, 304, 305]. Surfactant inactivation likely contributes to the severity of ARDS in both children and adults. ARDS [54], was first described in 1967 and shares many symptoms with NRDS, although ARDS occurs as a rapid onset of respiratory failure that can affect patients regardless of age [304, 305, 306, 307, 308]. Prior to the COVID-19 pandemic, ARDS had an incidence of 150,000 cases per year (U.S.) and a mortality rate as high as 50% [308, 309]. It has been reported that 67% of COVID-19 patients with severe illness have developed ARDS [310], and that 90% of COVID-19 non-survivors suffered from ARDS [311].

The pathophysiology of ARDS involves injury to the alveolar-capillary barrier, lung inflammation, atelectasis, surfactant dysfunction, and intrapulmonary shunting. The disorder typically appears within 12 to 24 hours of an identifiable clinical event such as
gastric content aspiration, pneumonia, near-drowning, toxic gas inhalation, or chest/lung trauma. In addition, ARDS may be associated with systemic processes such as sepsis, non-thoracic trauma, acute pancreatitis, major surgery, multiple blood transfusions, fat embolism, or shock. No specific therapy for ARDS currently exists.

There are various ways LS can be inactivated at various points in its life cycle; from during transcription and protein translation, lamellar body formation in the type II pneumocytes [264, 265], secretion into the alveolar subphase from the type II pneumocytes, transformation from lamellar bodies to tubular myelin to membrane vesicles [47], re-uptake by type II cells or macrophages, losses due to transport out of the alveoli to the airways [47], or chemical degradation. However, most of these mechanisms should respond favorably to surfactant replacement therapy. Additionally, these forms of inactivation are often slow to develop. However, in ARDS, inactivation is rapid upon injury. Understanding how this inactivation occurs might explain why exogenous animal-based surfactant, which has shown success in treating NRDS, does not have the positive effect in treating lung injuries in ARDS.

Surfactant inactivation in ARDS may be induced by extensive inflammation as well as damage to alveolar type II cells where lung surfactant is made and stored [312, 310, 313]. Inflammation also causes high permeability of alveolar-capillary barrier, causing lung to be flooded by fluid and contaminants, which include serum proteins, phospholipases, and lysolipids. Bronchial fluid extracted from ARDS patients contains elevated levels of serum proteins such as albumin and fibrinogen [314, 315, 316, 317], as well as phospholipases, lysolipids, cholesterol and fatty acids [318, 319, 320], and decreased amounts of lung surfactant phospholipids: phosphatidylcholine (PC) and phosphatidylglycerol (PG). These biochemical abnormalities correlate with the severity of respiratory failure. An increase in the fraction of the lung with impaired ventilation in which normal inflation/deflation is inhibited, known as the ”dead space” fraction, is also associated with an increased mortality [321].

Each time inhalation takes place, a new air-water interface is created that must be lined by LS to ensure proper lung functioning. The presence of surface-active serum proteins and lysolipids likely slows LS adsorption to air/water interface through competitive adsorption. This is consistent with the clinical observation that ARDS severity increases with increasing serum protein concentration in the alveolar fluids. The barrier
Figure 8.1: ARDS feedback loop. Initial trauma or injury leads to inflammation whereby innate immune response leads to permeabilization of alveoli, leading to flooding of alveoli with fluids and contaminants that can alter the dynamics of lung surfactant at the interface and create lung heterogeneity in forms of dead spaces where lung cannot inflate. This leads to decrease in lung dilatational modulus, increase in surface tension, and LS inactivation. Eventually, Laplace instability happens and the alveoli loses compliance, exacerbating the initial injury and one’s ability to breathe.
to surfactant adsorption imposed by the serum proteins also explains why replacing surfactant, as in NRDS treatment, which results in more subphase surfactant but not necessarily more surfactant at the interface, has not been an effective treatment for ARDS. Reversing surfactant inactivation requires a better understanding of how nearly insoluble bilayer aggregates of lung surfactant and a soluble, surface-active contaminant (e.g. lysolipids, serum proteins, etc.) compete for space at the air-water interface [322].

8.2 Thermodynamics or kinetics?

At constant temperature $T$, pressure $P$, and number of molecules $n$, $\pi_{eq} = -\left(\frac{\partial G}{\partial A}\right)_{T,P,n}$, a high $\pi$ is equivalent to a low Gibbs free energy of the interface [323]. LS can be compressed to $\pi = 65$ mN/m or even higher. Serum proteins such as albumin or soluble lipids such as lysolipids only reach $\pi_{max}$ at 25–30 mN/m due to their tendency to maintain the same surface coverage, $\Gamma$ at all times. This implies that LS adsorption should be preferred at thermodynamic equilibrium in the presence of albumin and lysolipids. However, in a continuously expanding and contracting lung, it is unlikely that equilibrium is ever established. Previous studies as well as our current investigation have suggested that both DPPC-only vesicle and animal-derived LS mixture need hours to adsorb to air-water interface and achieve equilibrium surface tension [89], which is significantly longer than our breathing frequency, suggesting that kinetics, instead of thermodynamics, may be the determining factor of materials occupying the interface.

As ARDS progresses, the concentration of serum proteins and PLA$_2$ (an enzyme that cleaves a PL into a lysolipid) increases. The soluble serum proteins and lysolipids (10-100 nm) are smaller than LS bilayer aggregates (1-10 $\mu$m). As diffusivity of spherical particles of radius $a$ can be estimated by the Stokes-Einstein equation:

$$ D = \frac{k_B T}{6\pi \eta a} \quad (8.1) $$

the diffusivity of LS vesicles is thus approximately 100 times lower than that of the inflammatory products, suggesting that the inflammatory products are adsorbed more easily than LS. Once adsorbed, these protein and lysolipid contaminants leave less space for LS to be adsorbed, and due to the lack of LS, $\pi$ is not reduced to the required level. These contaminants follow Gibbs adsorption isotherm for soluble surfactants,
where \( \pi \) scales logarithmically with the bulk concentration up to a saturation level (Equation 1.3). Not only will the equilibrium \( \pi \) be lower than LS, but also the \( \pi \) depends on the protein and lysolipid concentration. There is a continuous dynamic exchange between the bulk and interface, keeping \( \pi \) relatively constant at 25-35 mN/m.

These contaminants further inhibit LS diffusion by creating an electrostatic energy barrier, \( \Phi_{DLVO} \) between two spheres of radius \( a \), as well as between a sphere and a wall (interface):

\[
\Phi_{DLVO}(a) = 32\pi \epsilon \epsilon_0 \left( \frac{k_B T}{e z} \right)^2 a \tanh \left( \frac{e z \psi_s}{4 k_B T} \right) \exp(-\kappa r) - \frac{a A_H}{12r} \quad (8.2)
\]

Where \( A_H \) is the Hamaker constant that gives the magnitude of the molecular attractive dispersion force \[326\], \( \psi_s \) is the surface potential, and \( \kappa^{-1} \) is the Debye length, i.e. electrostatic screening length, given below with \( z_i \), the valence of the ionic species \( i \), \( e = 1.6022 \times 10^{-19} \) C the electron charge, \( \epsilon_0 \) the permittivity of the vacuum, \( \epsilon \) the solution dielectric constant, \( c_i \) the electrolyte bulk concentration,

\[
\kappa^{-1} = \sqrt{\frac{\epsilon \epsilon_0 k_B T}{\Sigma_i e^2 z_i^2 c_i}} \quad (8.3)
\]

Thus, for LS to adsorb to the interface, a repulsive energy \( \Phi_{adsorption} \) would have to be overcome:

\[
\Phi_{adsorption} = \Delta G_{bilayer} - \Delta G_{monolayer} + \pi \Delta A + \Phi_{DLVO} \quad (8.4)
\]

where \( \pi \Delta A \): the work to clear the interface from inhibitors. Taesch et al. have shown that \( \Phi_{adsorption} \sim O(5k_B T) \), while the ratio of the actual adsorption flux \( J \) to the uninhibited flux \( J_o \) follows Equation 8.5, meaning that the LS adsorption is reduced by 150 times!

\[
\frac{J}{J_o} = \exp \left[ - \frac{\Phi_{total}}{k_B T} \right] \quad (8.5)
\]
8.3 Polymer pushes LS aggregates to the interface

The electrostatic energy barrier to LS adsorption (Equation 8.2) is the same as the energy barrier that stabilize colloidal dispersions and prevents them from coagulating \[65, 27, 330, 331, 332, 333, 334\]. Colloidal dispersions should coagulate at equilibrium due to strong, short range interactions, yet some remain stable for years, even decades \[2, 335, 336, 337\].

In 1857, Michael Faraday reported the first scientific study of the effects of albumin and other proteins on colloid stability \[338\]. In the absence of protein, a gold sol could be induced to aggregate by the addition of sodium chloride; aggregation was not observed when protein was present \[338\]. Some of Faraday’s original gold colloids remain dispersed and stable today.

Based on this colloidal stability analogy, the barrier of LS adsorption might be reduced significantly if flocculation can be induced, for example, by adding salts or polymeric flocculants. Of our particular interest is non-adsorbing polymer flocculant, which will be discussed in this chapter.

Previous studies have shown that the addition of polyethylene glycol (PEG), hyaluronan, dextran and other non-adsorbing hydrophilic polymers to the LS suspension induces a depletion attraction that causes the surfactant to aggregate and to adsorb more rapidly to air-water interfaces \[331, 339, 332, 27, 333\]. This effect has been shown to enhance oxygenation in animal models with ARDS and meconium aspiration syndrome \[340, 341\].

Non-adsorbing polymers adsorb to neither surfactant aggregates nor interface. The mechanism by which they reverse LS inhibition is through depletion attraction. Generally, introducing non-adsorbing polymer of volume fraction \(\phi_p\) and gyration radius \(R_g\) induces depletion attraction between two colloids of radius \(a\) separated at distance \(r\). In our LS system, this attraction leads to entropic aggregation of colloid-like vesicles and pushes the aggregates to the interface, as \(\Phi_{DLVO}\) is negated by \(\Phi_{dep}\) \[327, 342, 343\]. These phenomena are depicted in figure 8.2.

\[
\Phi_{dep} = -3\phi_p k_B T \frac{a}{R_g} \left(1 - \frac{r}{2R_g}\right)^2
\]

(8.6)
Figure 8.2: Origin of depletion forces in a binary mixture of colloid and non-adsorbing polymer. (Left) The centers of the small spheres are excluded from the hatched regions within one small sphere radius ($R_g$) of the larger spheres (radius $R$) or the interface. (Right) When the larger spheres move to the interface or toward each other, the hatched regions overlap, and the total volume accessible to the small spheres increases by this amount times the number of large spheres (total increase in volume in the bottom right-hand corner). The increase in the volume accessible to the polymer increases the entropy of the system, resulting in a net “depletion” force pushing the large spheres toward the interface or each other. Figure is adapted from [27].
for \( r < 2R_g \), and \( \Phi (r) = 0 \) for \( r > 2R_g \). The depletion potential is independent of the chemistry of the colloids and polymers, as long as the polymer does not adsorb to surfactant or interface. The depletion potential is always negative (attractive) so depletion attraction always enhances colloid flocculation or surfactant adsorption. The magnitude of this attraction force depends on the Debye length, the surfactant aggregate size, the polymer molecular weight, and the polymer volume fraction, as dictated by Equation 8.6.

### 8.4 Polyethylene glycol effects on viscosity

Despite the potential benefits of adding non-adsorbing polymer to the LS system in reversing LS inactivation and enhancing lung functions [340, 341], the effects of those polymers on LS viscosity is still unknown. Typically, polymer would increase the viscosity of a surrounding liquid, as seen in figure 8.4, when we added 20 kDa polyethylene glycol (PEG) to water. As discussed in the previous chapter, viscosity plays an important role in LS instillation along the airway, where a higher viscosity is deemed less desirable; thus, the evaluation of polymer effects on LS viscosity is crucial in determining its suitability for additives in clinical LS system. In this chapter, we investigated effects of PEG, a hydrophilic, non-adsorbing polymer, on three clinical LS suspensions.

Figure 8.5 shows that adding 20 kDa molecular weight polyethylene glycol (Figure 8.3 reduces the suspension reduced viscosity (\( \eta/\eta_m \)) for all three clinical surfactants. PEG and other non-adsorbing hydrophilic polymers are added to clinical lung surfactants to promote adsorption to air-water interface in the alveoli that have been contaminated by surface-active serum proteins or lysolipids [328]. Figure 8.5 shows the reduced viscosity decreased by an order of magnitude or more on addition of PEG compared with neat clinical lung surfactants at 37°C. Adding PEG increases the viscosity.
of the solvent, \( \eta_m \), roughly six-fold from 0.7 mPa.s for saline to 4 mPa.s for the PEG-saline at 37\(^\circ\)C (Figure 8.4), even though the suspension viscosity decreased for all three surfactants.

The added PEG has two effects on the surfactant suspensions, both of which contribute to a decrease in the effective particle volume fraction, \( \phi \), in Equation 7.1 and 7.2. Macromolecules such as PEG cannot enter the aqueous spaces between bilayers within the surfactant aggregate [266]. This generates a PEG concentration difference between the inside and outside of the aggregate, which in turn, generates an osmotic pressure difference that dehydrates the aggregate, causing a decrease in the bilayer d-spacing, which in turn increases \( \rho \), the total lipid mass per particle volume, and decreases \( \phi = c_m/\rho \) at constant \( c_m \). [266]

Small angle X-ray scattering (SAXS) shows that adding PEG dehydrates Survanta and Infasurf aggregates leading to a decrease in the d-spacing between the bilayers (Figure 8.6). SAXS of both Infasurf (Figure 8.6A) and Survanta (Figure 8.6B) without PEG showed a number of relatively weak reflections that varied slightly between samples. As
Figure 8.5: Reduced viscosity, $\eta/\eta_m$ as a function of shear rate on addition of polyethylene glycol (PEG) of 20 kDa. The reduced viscosity decreases by an order of magnitude or more on addition of PEG, consistent with a decrease in the particle volume fraction in the suspension caused by the combined effects of osmotic pressure induced dehydration of the surfactant aggregates and a loss of excluded volume due to the depletion attraction.
both Infasurf and Survanta are multicomponent products derived from organic extraction of bovine lung surfactant, small variations in composition and bilayer spacing are to be expected. The d-spacing for neat Infasurf varied from 7.6 - 7.8 nm, depending on the sample. Neat Survanta had 3 repeat spacings of 8.8, 8.3, and 7.8 nm, indicative of possible lateral phase separation within the bilayers due to segregation of the palmitic acid and DPPC [293]. Similar phase coexistence in bilayers with different d-spacings has been observed in other lung surfactant formulations [344, 345]. Previous work has shown that neat Curosurf has a bilayer d-spacing of ~ 11 nm, [293, 266] that decreases with PEG concentration in a similar fashion. The d-spacings in Figure 8.6 are the combined thicknesses of the lipid bilayer and the intercalated saline layer that separates the bilayers in the aggregates.

Adding PEG to Infasurf causes the d-spacing to decrease to 7.1 nm. The osmotic pressure difference imposed by the PEG is balanced against the inter-bilayer repulsion, leading to the decrease in the water thickness separating the lipid bilayers [266]. This dehydration brings the lipid layers closer together and causes much better correlations between the lipid layers. This is indicated by the order of magnitude increases in the peak intensities with added PEG. This enhanced internal ordering of the bilayers is confirmed by the freeze-fracture TEM image of Infasurf with PEG. The freeze-fracture inset in Figure 8.6A shows that the internal organization of Infasurf changed to concentric, tightly packed bilayers with no internal water-filled voids as in Figure 7.4F [266]. The d-spacing of Survanta decreases to 7.5-7.6 nm and the correlations between layers also increase. Dehydrating the surfactant particles leads to a substantial reduction in the effective particle volume fraction, $\phi$, and a corresponding decrease in the reduced viscosity according to Equations $7.1$ and $7.2$. Centrifuging the suspensions also showed this decrease in the particle volume fraction on the addition of PEG compared to the neat suspensions (Figure 8.7).

The second effect of PEG is to generate weak flocs in the suspension due to the depletion attraction, as discussed in previous section [266, 268]. A mixture of micron sized surfactant particles with nanometer sized macromolecules maximizes its entropy by maximizing the volume accessible per particle [342]. Here, the small particles are the PEG polymers with radius of gyration, $R_g$ (5-10 nm), and the large particles are the surfactant aggregates of radius $a$ (typically microns, See Figure 7.4A-C). Each large
Figure 8.6: (A) Small angle X-ray diffraction patterns of Infasurf and (B) Survanta with and without added polyethylene glycol polymer (PEG). Without PEG, both Infasurf and Survanta had broad, weak reflections. The d-spacing for Infasurf varied from 7.6-7.8 nm and Survanta had 3 repeat spacings of 8.8, 8.3 and 7.8 nm, indicative of lateral phase separation within the bilayers. Adding PEG greatly increased the peak heights for both Infasurf and Survanta, indicating better correlations between the bilayers. The d-spacing for Infasurf decreased to 7.1 nm and 7.6 nm for Survanta. The freeze-fracture inset in A shows that the internal organization of Infasurf changed to concentric, tightly packed bilayers with no internal water-filled voids as in Figure 7.4F.
particle immersed in a polymer solution experiences the osmotic pressure induced by the exclusion of PEG from the interior of the surfactant bilayers, acting normal to its surface. For an isolated particle, this pressure is distributed homogeneously over the entire surface, so the net force in any direction is averaged out to zero. However, when two surfactant particles approach each other closer than the effective size of the polymer, $2R_g$, the polymer can no longer fit into the gap between the large spheres. Hence, in the gap between the surfactant particles, the polymer concentration is reduced, resulting in a lower osmotic pressure in the gap. Consequently, the pressure on the surfactant particles due to the polymer osmotic pressure becomes unbalanced, leading to a force that pushes the large particles toward each other, leading to flocculation of the large particles. This is known as the depletion attraction. Moving two surfactant aggregates together decreases the free energy by $3a\phi_p kT/2R_g$ as given in Equation 8.6, in which $\phi_p$ is the volume fraction of polymer in the solution. The depletion potential is independent of the chemistry of the surfactant and the polymer, as long as the polymer does not adsorb to surfactant or interface. The depletion attraction decreases the volume fraction occupied by the surfactant particles and has the largest effect on Survanta at low shear rates. By aggregating, the prolate particles of Survanta take up less excluded volume as rotation is inhibited; the flocculated Survanta particles become more spherical \cite{266}. The depletion attraction provides a more efficient packing in the Survanta suspension, reducing the effective particle volume fraction, $\phi$. Previous work has also shown that
adding PEG can flocculate and reduce the viscosity of latex suspensions \cite{346}. The difference in reduced viscosity between the neat and PEG Survanta decreases with increasing shear rate as the Survanta flocs are broken up at the higher shear rates.

8.5 Conclusion

When lung surfactant is inactivated, both endogenous and exogenous lung surfactant cannot function properly in facilitating breathing. This means that surfactant replacement therapy using clinical lung surfactant has had limited success in treating ARDS. In this chapter, we have shown that the addition of polyethylene glycol (PEG) reverses the inactivation through depletion attraction forces. Additionally, we also demonstrated that adding polyethylene glycol to the suspensions caused significant decreases in the suspension viscosity of all three suspensions, even though adding PEG led to a 6-fold increase in the solvent viscosity. SAXS and freeze-fracture imaging showed the PEG generated osmotic pressure difference between the aggregate interior and exterior, which dehydrated the aggregate bilayers. The bilayer d-spacing decreased for Infasurf and Survanta by $\sim 10\%$; previous results show a similar decrease for Curosurf \cite{246}. PEG also led to depletion-induced flocculation of the 3 suspensions; this led to an even larger decrease in viscosity as flocculating the high aspect ratio particles provided additional free volume to the suspension. The decrease in suspension volume fractions led to the decrease in suspension viscosity as shown qualitatively by centrifugation, consistent with the Krieger-Dougherty model.
Chapter 9

Dynamic Behavior of Lysolipids: A Case Study into The Dynamics of A Soluble Surfactant Created during Lung Inflammation

This chapter discusses the behavior of Lysolipid, a soluble surfactant created during lung inflammation. The first four sections here are adapted from Barman et al. [29]. The Section 9.5 is an adaptation of Dr. David Morse’s interpretation of Lucassen’s model for dilatational modulus above surfactant’s CMC [317], incorporating polydispersity effects. The data discussed here are obtained either from Langmuir trough (Figure 3.5) or microbubble tensiometer (Figure 5.8). The system of interest here is lysophosphatidylcholine (LysoPC 16:0), the same soluble surfactant studied in Chapter 6. The terms lysolipid, LPC, LysoPC16, or LysoPC will henceforth be used interchangeably to describe this soluble surfactant.

9.1 Overview

As lung inflammation occurs, the increased alveolar-capillary permeability causes increase in PLA$_2$ concentration in the lung. Part of the innate immune system, PLA$_2$
Phospholipase A$_2$ (PLA$_2$) catalyzes the hydrolysis of double-chain phospholipids such as DPPC to form single chain lysopalmitoylphosphatidylcholine (LysoPC) and palmitic acid (PA).

Figure 9.1: Phospholipase A$_2$ (PLA$_2$) catalyzes the hydrolysis of double-chain phospholipids such as DPPC to form single chain lysopalmitoylphosphatidylcholine (LysoPC) and palmitic acid (PA).

acts to catalyze the hydrolysis of double-tail lipids making up bacterial, viral, and fungal cell membranes, resulting in the creation of lysolipid and fatty acid. The orders of magnitude greater solubility of single chain lysolipids relative to double-chain phospholipids leads to lysolipid desorption from pathogen cell membranes into the surrounding solution following lipase action, leading to membrane defects, which in turn, lead to pathogen cell lysis and death. A consequence of pathogen lysis by PLA$_2$ is a substantial increase in the concentration of lysolipids with a variety of headgroups and alkyl chain lengths and saturations in the alveolar fluids.

As the phospholipids making up LS are also double-tailed, they are also hydrolized in the presence of PLA$_2$ (Figure 9.1), which causes a significant decrease in LS lipid concentration and further increase in lysolipid concentrations. While previous work suggested that PLA$_2$ degradation of lung surfactant drove the development of ARDS, inhibition can result from the overall increase in lysolipid concentration in the alveolar fluids during inflammation, both from the breakdown of LS lipid and from lysis of pathogen cell membranes. With increasing concentration, lysolipid competes for and alters the interface even in the presence of a lung surfactant coated interface.
9.2 Implications of lysolipid dynamics to lung interface

During the continuous expansion and contraction of alveolar area during breathing, lysolipids may enter and leave the interface due to their higher solubility. DPPC and other phospholipids are "trapped" at the interface as they are effectively insoluble in the alveolar fluids. Compressing the DPPC and other insoluble molecules at the interface increases the surface concentration, which reduces interfacial tension. These changes in surface concentration and interfacial tension, increase the dilatational modulus and stabilize the interface against the Laplace Instability (Equation 2.2).

Figure 6.4 shows schematically that when the interfacial area available for soluble surfactants like lysolipid is changed, the soluble surfactant can leave the interface with a characteristic frequency, \( \omega_0 = D/L_d^2 = D/\left(\frac{\Gamma_\infty}{C_0}\right)^2 \) in which \( D \) is the diffusivity, \( \Gamma_\infty \) is the equilibrium saturation surface concentration for a surfactant of bulk concentration \( C_0 \). \( L_d \), the depletion depth, is the thickness of the volume adjacent to the interface containing sufficient lysolipid to saturate the interface to a surface concentration of \( \Gamma_\infty \) (Figure 6.5). Thus, depending on the bulk surfactant concentration and the frequency \( \omega \) at which an interface is deformed, the surface coverage, \( \Gamma \), and consequently \( \gamma(\Gamma) \) will be different. If \( \omega < \omega_0 \), the surface concentration of lysolipids, \( \Gamma \), remains roughly constant, which in turn, keeps \( \gamma \) constant, and \( E^* = A \frac{d\gamma}{dA} \to 0 \) and \( (2E^* - \gamma) < 0 \), which could lead to the Laplace instability. However, if \( \omega > \omega_0 \), soluble surfactants do not have sufficient time to diffuse off the interface, \( \Gamma \) increases and \( \gamma \) decreases as the surface area decreases, and \( E^* = A \frac{d\gamma}{dA} \neq 0 \) and \( (2E^* - \gamma) \) remains large.

If the same scenario plays out in the lung, there will be a range of lysolipid concentrations where lysolipid could occupy alveolar interface (if the diffusive timescale is at the same order of magnitude as breathing frequency), and \( E^* \) of the interface could decrease to the point of \( 2E^* - \gamma < 0 \).

In addition to lysolipids, the ARDS lung contains a number of surface-active, relatively soluble, serum proteins in the epithelial fluid that may contribute to the variations in dilatational modulus. Here we review the dynamic adsorption, surface activity and frequency dependent dilatational modulus of lysolipid and its effects on lung surfactant dynamics as representative of inflammation induced mechanical instabilities that may be associated with ARDS.
Figure 9.2: (a) Surfactant coated interface with surface concentration $\Gamma_0$ undergoing oscillations in area, $\delta A$, at frequency $\omega$ in a liquid containing soluble surfactant. (b) for $\omega \tau_s \gg 1$, surfactant exchange is slow, $\Gamma_0 \rightarrow \Gamma_0 - \delta \Gamma_0$, and $\gamma \rightarrow \gamma + \delta \gamma$ resulting in a large $E^* = A\delta \gamma / \delta A$. (c) for $\omega \tau_s \ll 1$, surfactant exchange is fast, leaving $\Gamma_0$ and $\gamma$ unchanged, resulting in $E^* \rightarrow 0$. Figure adapted from Manikantan and Squires [28].

9.3 Diffusion-limited surfactant adsorption

Figure 9.2 shows schematically that when the interfacial area available for soluble surfactants like lysolipids is changed, the surfactants can leave the interface with a characteristic frequency that depends on the diffusive transport on and off the interface as well as the molecular solubility. The characteristic diffusion time scale for soluble surfactant transport is $\tau = h^2 / D$, in which $h$ is a characteristic length scale over which diffusion occurs for adsorption to a surface, discussed in detail in Ferri and Stebe [351], and $D$ is the surfactant diffusivity.

For a planar interface, a natural length scale for diffusion process is $L_d = \Gamma / C_0$, or the depletion depth over which a subsurface concentration gradient would develop.
as shown in Figure 6.5A. \( L_d \) is the thickness of the volume element adjacent to an interface with the necessary bulk concentration \( C_0 \) (mol/m\(^3\)) required to provide a surface concentration of \( \Gamma \) (mol/m\(^2\)). Rewriting the time scale for a planar diffusion as \( \tau_{D,p} = L_d^2/D \) where both \( L_d \) and \( \tau_{D,p} \) depend only on the nature and concentration of the surfactant being adsorbed. To evaluate \( L_d \), we have used Langmuir adsorption isotherm to relate \( \Gamma \) to \( C_0 \) [30, 24]:

\[
\Gamma = \frac{\Gamma_\infty K C_0}{1 + KC_0} \tag{9.1}
\]

\( \Gamma_\infty \) is the maximum surface concentration, taken to be the saturation surface concentration at or above the critical micelle concentration of the particular surfactant, and \( K \) is a measure of the relative affinity of a surfactant molecule for the interface, which is equivalent to the ratio of the adsorption to desorption rate constants in the Langmuir model of adsorption kinetics [28, 315, 316]. For this model:

\[
L_d = \frac{\Gamma}{C_0} = \frac{\Gamma_\infty K}{1 + KC_0} \tag{9.2}
\]

To evaluate the parameters \( \Gamma_\infty \) and \( K \), it is necessary to relate the surface concentration to the measured surface tension for a given bulk concentration via the Gibbs adsorption equation (eq. 1.3) and the Langmuir isotherms [315, 316],

\[
RTT = RT \left( \frac{\Gamma_\infty K C_0}{1 + KC_0} \right) = -C_0 \left( \frac{\partial \gamma}{\partial C_0} \right)_T \tag{9.3}
\]

Integrating Equation 9.3 gives a Szyszkowski equation of state relating the surface tension to the bulk concentration or the surface concentration with \( \Gamma_\infty \) and \( K \) as parameters:

\[
\pi = \gamma_0 - \gamma = RT \Gamma_\infty \ln (1 + KC_0) \tag{9.4}
\]

\[
\pi = -RT \Gamma_\infty \ln \left( 1 - \frac{\Gamma}{\Gamma_\infty} \right) \tag{9.5}
\]

\[
\Gamma = \Gamma_\infty \left[ 1 - \exp \left( -\pi / RT \Gamma_\infty \right) \right] \tag{9.6}
\]
Figure 9.3: Equilibrium surface tension vs concentration of LysoPC fit to Equation 9.8 to extract $\Gamma_\infty, K$. Inset: Plot of surface tension vs ln (LysoPC) can provide $\Gamma_\infty$ from the slope of the curve near the CMC concentration of 7 $\mu$M. Best fit values were $\Gamma_\infty = 1.7 \mu$mol/m$^2$ and $K = 330 \mu$M$^{-1}$.

Fitting Equation 9.4 to the equilibrium interfacial tension vs concentration data for LysoPC in Figure 9.3 gives values for $\Gamma_\infty$ and $K$. $\Gamma_\infty$ can also be independently determined as the slope of the equilibrium surface tension, $\gamma_{eq}$ vs ln $C_0$ curve (Equation 1.3 and Figure 9.3 inset) as the CMC concentration is approached 315. Best fit values were $\Gamma_\infty = 1.7 \mu$mol/m$^2$ and $K = 330 \mu$M$^{-1}$, which are used in all calculations here. From Equations 9.4, 9.5 and 9.6 the relationships between surface concentration, surface pressure, and bulk concentrations can be determined for surfactants consistent with simple Langmuir isotherms without using more complex isotherms for which the resulting equations of state are not usually analytical 28.

Curving the interface introduces a second length scale, the bubble curvature radius, $R$, which can modify the characteristic time scale for diffusion (Figure 6.5B). Jin et al. 352 suggested a characteristic time scale of $\tau_{D,s} = L_d R/D$ for $R/L_d \ll 1$ or that the appropriate length scale for the diffusion time is $h = \sqrt{L_d R}$, the geometric mean of the planar depletion depth and the curvature radius. Taking the ratio of $\tau_{D,s}$ to the planar diffusion time gives $\tau_{D,s} / \tau_{D,p} = R/L_d$. This reflects the observation that diffusion is faster
Figure 9.4: A. A generic schematic of adsorption to a curved surface being faster than to a flat surface. B. Curvature effect on the adsorption of 1 µM LysoPC.

to a spherical interface than a planar interface, as discussed briefly in Chapter 6. In Figure 9.4, we also showed the comparison of adsorption for 1 µM LysoPC to interfaces of different curvature and noted that the adsorption to more curved interface occurs faster.

As shown in Figure 6.5B, the number of molecules necessary to reach a surface concentration on a spherical interface of radius, R, is $4\pi R^2 \Gamma$, while the number of molecules in the volume extending a distance $L_{ds}$ from the sphere is given by $\frac{4\pi}{3} C_0 \left[(R + L_{ds})^3 - R^3\right]$. Comparing these two terms shows that the equivalent depletion depth around a sphere, $L_{ds}$, is reduced compared to a planar interface, $L_d$:

$$\frac{L_{ds}}{L_d} = \left(\frac{R}{L_d}\right) \left[\left(\frac{3L_d}{R} + 1\right)^{\frac{1}{3}} - 1\right]$$

(9.7)

For $L_d \ll R$, $L_{ds} \rightarrow L_d$ and the spherical depletion depth reduces to that of a planar surface, which is the case for high surfactant concentrations (small $L_d$) or large spheres. The resulting spherical depletion depth depends on both the nature and concentration of the surfactant and the radius of curvature of the interface.

For purely Fickian diffusion, the time scale should be $\tau_{D,s} = \tau_s = \frac{L_{ds}^2}{D}$ with the
single characteristic depletion depth of \( L_{ds} \):

\[
\frac{\tau_{D,s}}{\tau_{D,p}} = \left( \frac{L_{ds}}{L_d} \right)^2 = \left( \frac{R}{L_d} \right)^2 \left[ \left( \frac{3L_d}{R} + 1 \right)^{\frac{1}{3}} - 1 \right]^2
\] (9.8)

Alvarez et al. [24] suggested that if the flux boundary condition is scaled using the spherical depletion depth, the characteristic diffusion timescale would be \( \tau_1 = L_{ds}L_d/D \), which differs from the Fickian timescale of \( \tau_s = L_{ds}^2/D \). As both timescales have their merits, Alvarez et al. chose to use the geometric mean for the spherical diffusion time:

\[
\tau_{D,s} = \sqrt{\tau_1 \tau_s} = \left( \frac{L_{ds}^3}{L_d} \right)^{1/2}/D
\] (9.9)

which gives a characteristic depletion depth of \( (L_{ds}^3L_d)^{\frac{1}{4}} \).

The models from Jin et al. [352], Alvarez et al. [24], and Fick’s law for characteristic diffusive timescale relative to the planar diffusion time are all functions only of \( R/L_d \). Distinguishing the correct model requires matching the various scaling laws to experimental data for dynamic surfactant adsorption.

### 9.3.1 Experimental validation of diffusive scaling below CMC

The rate of surfactant adsorption from a large reservoir of constant composition to a clean bubble interface experiments has been measured by Barman et al. [29] and is shown in Figure 9.5. The equilibrium surface tension values are the long-time values to determine \( \Gamma_\infty \) and \( K \) from fits to Equations. 9.5, 9.4, and 9.6 which were plotted in Figure 9.3. Over this concentration range, \( R/L_d \ll 1 \), especially for the smaller 40 \( \mu \)m bubble. \( \Gamma(t) \) is calculated from the values of \( \gamma(t) \) plotted in Figure 9.5 using Equation 9.6 with \( \Gamma_\infty \) being the value of surface coverage at equilibrium coverage at long times. Figure 9.6 shows this relationship for 1 \( \mu \)M LysoPC. The fractional surface coverage reaches relatively high values before the surface tension changes appreciably; \( \Gamma/\Gamma_\infty \) must reach 0.9 for a surface tension reduction from 72 to 65 mN/m, which corresponds to only 30% of the eventual surface tension reduction at 1 \( \mu \)M. \( L_d = \Gamma(t)/C_0 \) was determined from Figure 9.5 and Equation 9.6 to relate \( \tau_{exp} \) to \( R/L_d \) as in Alvarez et al.
Figure 9.5: Adsorption of Lysolipid from bulk lysolipid concentrations ranging from 0.01 – 10000 µM. The dynamic surface tension vs time is plotted for bubble radii of 40 µm (A) and 135 µm (B). The long-time minimum values of surface tension plateau at 38 mN/m for concentrations at or above the lysolipid CMC of 7 µM. For a given concentration, the surface tension decreases more slowly for the larger bubble radius. 175 sec were required to reduce the surface tension to 60 mN/m for a 10 µM solution on the 40 µm bubble, while the same reduction required 350 s on the 135 µm bubble.

Adsorption data for both large and small capillaries follows the Fickian diffusion-limited scaling behavior \( \frac{\tau_{D,s}}{\tau_{D,p}} = \left( \frac{L_{ds}}{L_{d}} \right)^2 \) below the CMC (Equation 9.8), in contrast with Alvarez et al.\[353\], whose data was better fit by the \( \frac{\tau_{D,s}}{\tau_{D,p}} = \left( \frac{L_{ds}}{L_{d}} \right)^{3/2} \) model (Equation 9.9). However, the data diverges from any simple diffusion model above the critical micelle concentration, which for the data in Figure 9.7 are associated with values of \( R/L_d > 1 \). This means that there are processes other than diffusion also governing surfactant transport to and from the interface. In Section 9.5 I will discuss how micellar kinetics can affect this phenomenon.

9.4 Dilatational modulus of LysoPC as a function of concentration, frequency, and curvature

Figure 9.8 shows the frequency and curvature dependence of the dilatational modulus, \( E^*(\omega) \), of LysoPC at the equilibrium surface tension for various concentrations above
Figure 9.6: Surface concentration, $\Gamma(t)$ calculated from Equation 9.6 from the dynamic surface tension data in Figure 9.5 for 1 $\mu$M LysoPC. $\Gamma_\infty$ is the equilibrium surface concentration at a given bulk concentration, $C_0$. $L_d = \Gamma/C_0$ is determined from these values.

and below the CMC for 40 $\mu$m, 135 $\mu$m, and infinite curvature interfaces. The curvature of 40 and 135 $\mu$m radius bubbles were chosen to represent small and large alveoli and were generated using capillary microtensiometer (Figure 5.8), while the data for infinite curvature interface were obtained from a Langmuir trough.

In figure 9.8, we divided the frequency range of curved interface LysoPC dilatational modulus plots into three regions of different colors. Normal breathing ranges from 6 breaths per minute to about 40 breaths per minute (yellow). High frequency ventilation, which has shown some benefit for ARDS patients is $> 50$ breaths/minute [354, 355, 356, 357] (green). In many ARDS patients, parts of the lung are unable to perform normal respiratory expansions and compressions; these parts are known as dead spaces (red) and correlate with adverse outcomes and higher mortality [321, 358, 359]. A dotted red line was drawn on each figure to mark $2E^* - \gamma = 0$ for LysoPC, based on our knowledge of the equilibrium surface tension value of LysoPC at sufficiently high concentration from Section 9.3.1.

At the lowest LysoPC concentrations $\leq 0.01$ mM that may arise in normal lungs from the chemical hydrolysis of DPPC and other lipids, the dilatational modulus decreases slowly with decreasing frequency and $2E^* - \gamma > 0$ over the range of normal
Figure 9.7: Experimental characteristic times for diffusion, $\tau_{D,s}$, scaled by the planar diffusion time, $\tau_{D,p} = L_d^2 / D$ determined from Figures 9.5, 9.6 and Equations 1–5 for the small and large capillary adsorption as a function of $R/L_d$ in which $L_d = \Gamma / C_0$ and $R$ is the radius of curvature of the bubble interface. Solid grey line is a plot of Equation 7, $(L_d / L_a)^2$ and solid black line is Equation 8 $(L_d / L_a)^2$ the predicted scaling relationships for the adsorption time. Filled symbols are the characteristic timescales extracted from fits to the dilatational rheology data. Above $R/L_d \sim 1$, the data no longer fit either diffusion model as the surfactant concentration is above the critical micelle concentration. Figure is adapted from Barman et al. [29].
Figure 9.8: Dilatational modulus of LysoPC at various curvature: (A) 40 \( \mu \text{m} \), (B) 135 \( \mu \text{m} \), and (C) infinite curvature (flat surface) for various concentration. Dotted red line marks Laplace instability boundary: \( 2E^* - \gamma = 0 \). The frequency range was divided into colored regions: red indicates restricted breathing, likely also happens when parts of alveoli are unable to inflate due to inflammation; yellow indicates healthy breathing frequency; green indicates frequency associated with high frequency ventilation. (A) and (B) were adapted from Barman et al. [30].
breathing frequencies (yellow region) across all curvature size. Hence, the Laplace Instability is preventable, and normal lung inflation occurs. For concentrations from 0.01 to 0.1 mM, $E^*$ decreases at lower frequencies and may lead to difficulties in areas of the lung that are cut off from normal inspiration during ARDS, i.e., the dead spaces. Increasing the lysolipid concentrations to 1 mM, $(2E^* - \gamma) < 0$ over the entire range of breathing frequencies. For frequencies $> 10$ rad/sec, which correspond to high frequency mechanical ventilation, $E^*$ remains large and well above the $(2E^* - \gamma) > 0$ cut-off for the Laplace Instability for all concentrations tested. High frequency tests in flat interface were not included due to the limitation of performing high frequency experiments in a Langmuir trough.

Figures 9.8B and C show that the dilatational moduli for $R_{eq} = 135 \, \mu m$ radius bubbles corresponding to the larger alveoli are similar to that for $E^*(\omega)$ for $R_{eq} = 40 \, \mu m$, although the decrease in $E^*(\omega)$ is shifted to somewhat lower frequencies for a given LysoPC concentration. As is the case for the dynamic surfactant adsorption, interfacial curvature increases the net rate of adsorption and desorption (Figure 9.5). For the 40 \, \mu m bubbles in Figure 9.8A, $\omega_R = D/R^2 \sim 0.1$ rad/sec, decreasing to $\omega_R \sim 0.01$ rad/sec for the 135 \, \mu m bubbles in Figure 9.8B, assuming $D \sim 2 \times 10^{-10}$ m$^2$/s for LysoPC diffusivity. As the effects of curvature are important for $2\omega R/\omega \sim 1$, curvature effects become important in Figure 9.8B for $\omega < 2\omega_R \approx 0.2$ rad/sec for 40 \, \mu m bubbles but not until $\omega \approx 0.02$ rad/sec for the 135 \, \mu m bubbles in Figure 9.8B. For example, at $\omega < 0.2$ rad/s, $E^* = 6$ mN/m for the 40 \, \mu m bubbles, while $E^* = 12$ mN/m for the 135 \, \mu m bubbles at 0.1 mM LysoPC concentration. At frequencies $\omega > 0.2$ rad/sec the values of $E^*$ are similar for both large and small bubbles in Figures 9.8A and B.

For $C_{LysoPC} > 0.1$ mM, the dilatational modulus decreases with decreasing frequency, but not as rapidly as for the smaller capillary, especially at lower frequencies (Figure 9.8B). Even for the larger bubble, $E^*$ for 1.0 mM LysoPC drops below the cut-off for the instability, demonstrating that larger alveoli are also at risk of succumbing to the Laplace instability at normal breathing frequencies. For 10 mM LysoPC, $(2E^* - \gamma) < 0$ over the entire range of breathing frequencies. Increasing the bubble radius decreases the net rate of LysoPC exchange with the subphase, which leads to a lower frequency crossover.

For any concentration of LysoPC, even for concentration where $2E^* - \gamma < 0$ within
Figure 9.9: Dilatational modulus vs. concentration of LysoPC across different frequencies measured on Langmuir trough.
Figure 9.10: Dilatational modulus data for LysoPC obtained from Langmuir trough with fit from Lucassen equation (Equation 6.9). The fit does not work well at higher concentration.
breathing frequency, there will be a cutoff frequency above which \((2E^* - \gamma) > 0\). Here, we provide an additional argument as to why high frequency ventilation on ARDS patients has positive impacts on their breathing and oxygenation, in addition to the already known capability of the ventilation in assisting inflation/deflation of patient’s lungs.

To compare dilatational modulus of LysoPC to other systems and known models, multiple dilatational modulus vs. concentration curves for LysoPC across different frequencies in a flat interface are presented in Figure 9.9. The trend is similar to nonionic surfactants originally studied by Lucassen [347], where a maximum point in dilatational modulus is observed right before CMC. This signifies that the dilatational modulus dependence on frequency changes as soon as micelles are created.

In Figure 9.10, an example of dilatational modulus vs. frequency plot of LysoPC in Langmuir trough is shown, where each curve is fitted into Lucassen and van den Tempel model for flat interface dilatational modulus (Equation 6.9). From the figure, one can see that while at lower concentration the fit works well, at higher concentration the fit starts failing. This is also due to the presence of micelles in the solution, which calls for a new model to describe the dilatational modulus dependence on frequency above CMC.

### 9.5 Dilatational modulus model for above CMC system

As discussed in previous sections, surfactant adsorption plays an important role in its dilatational modulus. Because the presence of micelles in the bulk has an impact on the adsorption dynamics (as shown in Section 9.3.1 and Figures 9.7, 9.9, and 9.10), the dilational elasticity is thus also significantly affected by micellar presence. The seminal analysis on dilatational modulus of micellar solution was published by Lucassen more than 40 years ago [360, 347], and was further investigated in many subsequent studies [361, 362, 363, 364, 365, 366, 228, 367, 368]. Of particular interests are the kinetics of micelle creation, dissociation, diffusivity, and adsorption properties in relations to their monomer counterpart, which determine how fast surfactant molecules can adsorb to the interface.

Above CMC, the monomer surfactant concentration is regulated by the presence of
micelles, and this likely changes the dependence of the depletion depth on the bulk concentration as monomers and micelles are ”reacting”, as shown schematically in Figure 9.11. The micelles act as a reservoir of surfactant monomers near the interface. During surfactant adsorption, only monomers can adsorb to the interface, while micelles cannot. Instead, micelles have to breakdown into monomers before being able to adsorb to the interface. This dynamic of micelles/monomers reaction thus governs the rate of surfactant adsorption to the interface, and consequently, the dilatational modulus dependence on frequency. Because only monomers can adsorb to the interface (this monomer diffusivity timescale is $\tau_{ap}$), any change in subsurface monomer concentration resulting from interfacial area changes will disrupt monomer/micelle equilibrium, and to reestablish the equilibrium, the system relaxes through either monomer incorporation to micelles, monomer desorption from micelles, micellization or demicellization process.

Relaxation process involving monomer incorporation to micelles and monomer desorption from micelles is a fast relaxation process, and during this process the number of micelles remains constant, while the number of monomers changes. The monomer exchanges in and out of a micelle with reaction rate $k$. The timescale for this is $\tau_1$ and is generally much less than a microsecond for common surfactants. $\tau_1$ is longer the less soluble a surfactant is. The model that will be discussed here assumes that the system
is in partial equilibrium, i.e. when $\omega \ll \tau_1^{-1}$. In contrast, relaxation process where micelles are created or destroyed is a slow process, and the timescale associated to this process is $\tau_2$, and can vary from miliseconds to seconds or longer in the case of sparingly soluble surfactants.

Micellar kinetics also involve the following parameters: $C_n$ to describe the number of $n$–mers (micelles with aggregation number $n$), where $C_1$ is unimer concentration. The total number concentration of surfactant is given as:

$$\rho = \sum_{n=1}^{\infty} nC_n$$  \hspace{1cm} (9.10)

From here, it is assumed that the number of micelles of aggregation number smaller than $b$ is negligible. Then, the micelle concentration is defined as $C_m$:

$$C_m = \sum_{n=b}^{\infty} C_n$$  \hspace{1cm} (9.11)

And the concentration of surfactant in micelles are given as $\rho_m$:

$$\rho_m = \sum_{n=b}^{\infty} nC_n$$  \hspace{1cm} (9.12)

As polydispersity may be present, the variance of micelle aggregation number is defined as $\sigma_m^2 = \langle n^2 \rangle_m - \langle n \rangle_m^2$. We also define $q = \langle n \rangle_m = \rho_m/C_m$ to be the average micelle aggregation number. We will also denote equilibrium state parameter with asterisk (*) as a superscript. Under a small perturbation, $\rho$ changes slightly, and the change is given as:

$$\delta \rho = \delta C_1 + \delta(q^*C_m) = (\delta C_1 + C_m\delta q^*) + q^*\delta C_m = \psi_1 + \psi_m$$  \hspace{1cm} (9.13)

where $\psi_1$ term (parenthesized term in Equation 9.13) is proportional to $\delta C_1$, and $\psi_m$ is proportional to $\delta C_m$. Defining dummy variables $\kappa_p$ and $\kappa_e$ as in Equations 9.14 and 9.15:

$$\kappa_p = C_m \frac{(\sigma_m^*)^2}{C_1}$$  \hspace{1cm} (9.14)
allows for simplifications of $\psi_1$ and $\psi_m$ definitions:

$$\psi_1 = (1 + \kappa_p)\delta C_1 \quad (9.16)$$

$$\psi_m = (\kappa_e - \kappa_p)\delta C_1 \quad (9.17)$$

The diffusivity of micelle, given as $D_m$, while that of monomer is given as $D_1$. An effective diffusivity for $\psi_1$ (for coupled evolution of $C_1$ and $q^*$) is defined as:

$$D_p = \frac{D_1 + \kappa_p D_m}{1 + \kappa_p} \quad (9.18)$$

As the derivation leading up to the full solution of the micellar solution dilatational modulus is tedious and has been discussed elsewhere [347], here I will just present the solution that has been derived for flat interface:

$$E(\omega) = E_o \left(1 + \frac{D_p K_s}{i\omega L_{ap}}\right)^{-1} \quad (9.19)$$

where $L_{ap}$ is the effective planar diffusion length for the monomer,

$$L_{ap} = \frac{d\Gamma^*}{d\psi_1} = \frac{L_d}{1 + \kappa_p} \quad (9.20)$$

and $L_d$ is determined from the ratio of $d\Gamma/dC_1$. Additionally,

$$K_s = \frac{K_+ + \frac{F}{E} K_-}{1 + \frac{F}{E}} \quad (9.21)$$

where,

$$\frac{F}{E} = -\frac{K_+ D_p K^2_+ + i\omega - kg}{K_- D_p K^2_- - i\omega - kg} \quad (9.22)$$

and $K_+$ and $K_-$ are the positive and negative roots to:

$$K^2 = \frac{1}{2} (A_m + A_p \pm R) \quad (9.23)$$
Figure 9.12: Simulations of dilatational modulus of systems where $C > CMC$. Depending on the timescale values in relation to one another, the regions where dilatational modulus value changes in respect to frequency also changes. For example, in (A), $\tau_{ap}^{-1} > \tau_2^{-1} > \tau_b^{-1}$, while in (B), $\tau_{ap}^{-1} > \tau^{-1} > \tau_2^{-1}$. The MATLAB code is courtesy of Dr. Steven Iasella.

The rest of the parameters are defined as follows:

$$R = \left[(A_m - A_p)^2 + 4k^2D_p^{-1}D_m^{-1}g\right]^{1/2}$$  \hfill (9.24)

$$A_m = D_m^{-1}(k + i\omega)$$  \hfill (9.25)

$$A_p = D_p^{-1}(kg + i\omega)$$  \hfill (9.26)

$$g = \frac{\psi_m}{\psi_1} = \frac{\kappa_e - \kappa_p}{1 + \kappa_p}$$  \hfill (9.27)

Additionally, from eigenvector expansion leading to Equation 9.19, another timescale is also defined to describe micelle diffusive timescale:

$$\tau_b^{-1} = \frac{k(1 + g)}{g^2} \frac{D_p}{D_m}$$  \hfill (9.28)

### 9.5.1 Asymptotic analysis

Equation 9.19 can be rewritten into

$$E(\omega) = \frac{E_o}{1 + R(\omega)}$$  \hfill (9.29)
where
\[ R(\omega) = \frac{D_p K_s}{i\omega L_{ap}} \]  
(9.30)

\( R(\omega) \) is a monotonically decreasing function of \( \omega \). At high frequencies where \( R(\omega) \ll 1 \), we obtain a high frequency plateau regime of \( E(\omega) \approx E_o \). This is equivalent to the high frequency regime observed in submicellar system. The timescale where this transition to plateau is observed is \( \tau_{ap} \).

At lower frequencies where \( R(\omega) \gg 1 \), \( E(\omega) \approx E_o / R(\omega) \). At these frequencies, there are three processes that can be rate determining: unimer diffusion, micellar exchange reaction, and monomer diffusion. Thus, the modulus can exhibit up to three different regimes within this lower frequency region.

Unimer diffusion regime occurs where \( \tau_{ap}^{-1} \ll \omega \), giving:
\[
\frac{E(\omega)}{E_o} \sim \left( \frac{i\omega L_{ap}^2}{D_p} \right)^{1/2}
\]  
(9.31)

where \( E \sim \omega^{1/2} \), meaning that at this region, the slope of \( E(\omega) \) is \( \frac{1}{2} \).

Reaction controlled regime occurs where \( \tau_b^{-1} \ll \omega \ll \tau_2^{-1} \), giving:
\[
\frac{E(\omega)}{E_o} \sim i\omega \left( \frac{\tau_2 L_{ap}^2}{D_p} \right)^{1/2}
\]  
(9.32)

where \( E \sim \omega \), and the slope of \( E(\omega) \) is 1.

Micelle diffusion regime occurs when \( \omega \ll \tau_b^{-1} \), giving:
\[
\frac{E(\omega)}{E_o} \sim \left( \frac{i\omega L_{ap}^2}{D_m g^2} \right)^{1/2}
\]  
(9.33)

where \( E \sim \omega^{1/2} \), meaning that at this region, the slope of \( E(\omega) \) is \( \frac{1}{2} \).

In Figure 9.12 we present an example of a micellar system that has dilatational modulus varying with \( \omega \) following Equation 9.19. In Figure 9.12A, the system timescale varies as: \( \tau_{ap}^{-1} \gg \tau_2^{-1} \gg \tau_b^{-1} \), the frequency range can be divided into 4 as the slope changes, which is the maximum number of regimes. In a different system, like in Figure 9.12B, depending on the value of each timescale in relations to each other, as well as the instrument frequency range, some of the regions might not be present, or the change of
9.5.2 Model evaluation on LysoPC system

Figure 9.13 shows a 1000 µM LysoPC dilatational modulus vs. frequency plot, divided into three different regions (marked with differently colored points). While it is known that high frequency regime where the dilatational modulus plateaus as a function of frequency always exists in every system, none is observed in this figure. This suggests that the measurement was not performed at high enough frequency, i.e. $\tau_{ap}^{-1}$ is not within the range of our measurements. However, since Figure 9.13 displays three regions with different slopes (of 0.45, 0.8, and 0.6 in the order of increasing frequency), all three low frequency regimes exist in LysoPC 1000 µM system, with $\tau_{ap}^{-1} > \tau_{2}^{-1} > \tau_{b}^{-1}$. Comparing this data with Figure 9.12A, it is determined that $\tau_{b}^{-1} \sim 0.12 \text{ rad/s}$, and $\tau_{2}^{-1} \sim 1.5 \text{ rad/s}$. 
9.6 Conclusion

Lysolipid solubility affects the dilatational modulus in a concentration and frequency dependent fashion and is much different that the dilatational modulus of healthy lung surfactants, which are effectively insoluble. Figure 9.8 shows the frequency dependence of dilatational modulus as a function of frequency. It is shown that below the CMC, the dilatational modulus is relatively high and does not have strong dependence on frequency; above CMC, due to micellar exchange kinetics, the number of monomers in the bulk is a strong function of frequency. At high enough concentration above CMC, the dilatational modulus is low such that Laplace instability can be triggered in the breathing frequency.

Why some lung injuries or diseases progress to ARDS, while other do not, is unknown. However, inflammation generally accompanies the underlying lung disease or trauma which can trigger the innate immune system. This results in the recruitment of phospholipases to the lung capillaries and alveolar lining fluid to degrade bacterial and viral membranes, leading to cell lysis, thereby eliminating the bacteria or virus. This increases the lysolipid concentration in the alveolar fluid. While phospholipases also degrade the lung surfactant phospholipids, lowering the available concentration of lung surfactant, this is likely not the origin of most of the lysolipids. Lysolipids are orders of magnitude more soluble in bodily fluids than phospholipids but remain surface active and can compete for the alveolar interface even in the presence of lung surfactant. Clinical and model lung surfactant monolayers have a large (> 100mN/m) dilatational modulus that is stable for hours to days on a saline subphase. For these monolayers, \((2E^* - \gamma) > 0\) for any value of \(\gamma\) in the lung, making lung inflation stable against the Laplace Instability. However, the dilatational modulus of clinical and model lung surfactants degrade over the course of hours in the presence of micromolar LysoPC concentrations, eventually lowering the dilatational modulus such that \((2E^* - \gamma) < 0\) at breathing frequencies. In dead spaces in the lung cut off from normal respiration, the effect of lysolipids on the dilatational modulus may be more severe, which might explain recent reports that the poorly ventilated "dead space" fraction is associated with an increased rate of mortality [321]. At high frequencies, even at high lysolipid concentrations, \((2E^* - \gamma) > 0\), which correlates with the benefits obtained by high-frequency
oscillatory mechanical ventilation \cite{60, 354, 355}. This is a completely new physical mechanism for the origin of ARDS and a direct link from inflammation products to the development of ARDS. If this idea is correct, it is easy to understand why conventional surfactant replacement therapy has been ineffective in treating ARDS as even adequate amounts of healthy lung surfactant would not be resistant to lysolipid altering the dilatational modulus and inducing the Laplace Instability \cite{65, 305, 307, 308, 369, 370}. 
Chapter 10

Dynamics of Lung Surfactant Monolayer In The Presence of Lyoslipid

This Chapter is part of Manuscripts in preparation by Clara Ciutara, Steven Iasella et al.

Interaction between soluble surfactants and insoluble monolayers is often of interest to the cell biology community due to the fact that many membrane perturbants are small soluble molecules capable of altering the packing of the cell membranes, causing disruption in cell dynamics, alteration of protein configuration in the cell membranes, as well as change in cell membrane permeability [371, 372, 373, 374, 375, 376, 377].

To study these phenomena, researchers have employed lipid monolayer techniques to tackle difficulty in creating an ideal lipid bilayer model system. Thus, the approaches employed in this chapter can be translated to gain new insights into cell dynamics.

In our system of interest, lung surfactant, a lipid monolayer is subject to exposure to small soluble surfactants like LysoPC due to our innate immune response during disease progression. From discussions in Chapter 9, it is clear that above a certain threshold concentration, LysoPC, on its own, can cause Laplace instability to interfaces within frequency relevant to our breathing, which can be fatal. However, despite the ability of LysoPC to trigger Laplace instability in one-component system, it is still unknown
if Laplace instability can be triggered by LysoPC if an existing rigid DPPC monolayer is present at the interface. In this chapter, we investigate the dynamics of DPPC monolayer when LysoPC is introduced to its subphase, highlighting the possibility of DPPC replacement by LysoPC at the interface. We use a Langmuir trough system, a simple *in vitro* lung model that can mimic compression and expansion process in the lung.\footnote{Despite it being a flat interface system, the Langmuir trough measurements are highly relevant as the thickness of ELF is in the range of 0.1 to 0.5 μm, whereas the mean diameter of the alveolus is 200 μm, meaning that one can treat the thin layer of surfactant at the alveolar interface as flat.} To complement the understanding of physical properties evolution, we have also employed a confocal microscopy setup to visualize the dynamic of the interface during these changes.

10.1 Experimentals

10.1.1 Materials

Dipalmitoylphosphatidylcholine (DPPC, MW=734.04 g/mol) and Lysophosphatidylcholine (LysoPC, MW=495.63 g/mol) were purchased in powder form from Avanti Lipids. A 1 mg/mL solution of DPPC in chloroform was made and stored in -20°C freezer until time of use. A 1000 μM solution of LysoPC in a phosphate buffered saline (PBS, pH=7.40) was made and stored in a 4°C fridge until time of use. For mixed monolayer of DPPC:LysoPC 1:1 (mol:mol) experiment, the same volume of 1 mg/mL of DPPC in chloroform and 1 mg/mL of LysoPC in chloroform were mixed together and stored in a 4°C fridge until time of use.

10.1.2 Langmuir trough

A continuous ribbon KSV-NIMA Langmuir trough constructed from polytetrafluoroethylene (PTFE) that provides leak-free uniaxial compression of the monolayer was used. The Wilhelmy plate surface pressure system was also purchase from KSV-NIMA. Before each set of experiments, a new filter paper for the Wilhelmy plate is exchanged and the pressure sensor is calibrated. The sensitivity of the surface pressure sensor is 0.1 mN/m (manufacturer’s specification). The interface was aspirated to remove any surface contamination several times using a sterile pipette tip connected to a vacuum.
pump. The trough interface is clean if the surface pressure of the air-water interface is zero (within ±0.3 mN/m) following full compression of the trough. In all figures, the standard deviations are smaller than the symbols used.

**Sample deposition**

A gas-tight Hamilton syringe was used to disperse desired amount of 1 mg/mL DPPC solution on a 250 mL of clean milli-Q water surface inside the Langmuir trough.

Upon solution dispersion on the water surface, the monolayer was let to sit still for 20 minutes to ensure complete evaporation of chloroform. The monolayer is then compressed at 5 mm/minute to a desired surface pressure. Afterwards, a desired volume of the 1000 µM LysoPC was introduced to the subphase outside of the barrier (Figure 10.1) using a serological pipette. The volume of LysoPC to be deposited is determined by: $M_1 V_1 = M_2 V_2$, where $M_1$ and $M_2$ are the molarity of LysoPC in the stock solution and the desired molarity of LysoPC in the trough, respectively, while $V_1$ is the volume of the stock solution to be introduced to the trough, and $V_2$ is 250 mL (the initial volume of the trough). To ensure that the height of the water level remains the same (so that it does not interfere with Wilhelmy balance measurement), before the introduction of LysoPC to the system, $V_1$ amount of water is drawn from outside of the barrier. As shown in Figure 10.1, material exchange happens outside of the barrier.

To accelerate LysoPC introduction to the system, a 0.8 cm long magnetic stir bar is placed in the middle of the trough, and a magnetic stirrer is positioned underneath the trough. The stir bar is rotated at 50 RPM during LysoPC introduction until surface pressure reaches a plateau between 40-42 mN/m.

**Barrier Oscillation Experiment**

A barrier-oscillation technique was employed to study the dilatational response of the monolayer. As the name indicates, the barrier of a Langmuir trough is continuously oscillated, which thus results in the area ($A$) oscillation. The change in surface tension ($\gamma$) due to the oscillated monolayer area is measured by a platinum Wilhelmy balance. The dilatational modulus is obtained from: $E^* = A\left(\frac{d\gamma}{dA}\right)_T$. Unless otherwise specified, the frequency at which the barrier is oscillated is 0.1 Hz. Due to the physical limitation of the trough, non-stop continuous oscillation cannot be performed without errors in
the software. For the sake of discussion, when the term continuous oscillation is used in this chapter, the oscillation refers to the barrier being oscillated at least once every 20 minutes for 9 full oscillations.

**Surface pressure-area isotherm**

In this experiment, surface pressure-area isotherm is obtained by continuously compressing (or expanding) the barrier of a Langmuir trough. Unless otherwise specified, the compression speed of the barrier is 5 mm/minute.

**10.1.3 Confocal microscopy**

NIKON AR1-multiphoton confocal microscope setup is used at the University Imaging Center at University of Minnesota with a Nikon Plan Apo objective 20x with numerical aperture of 0.75 and working distance of 1 mm. The images were obtained using a resonant scanning mode.
10.2 Results and discussions

10.2.1 Introduction of LPC into the subphase of a continuously-oscillated DPPC-covered interface: effects on physical properties

Following the introduction of LysoPC to the subphase of DPPC monolayer, the surface tension and dilatational evolution was monitored by oscillating the interface every 10-20 minutes, the results are presented in Figures 10.2 and 10.3. Additionally, the surface tension evolves in the following manners: (1) Reduction in surface tension indicates that lysoPC adsorbs to existing DPPC interface; (2) plateau indicates that the adsorption of LysoPC slows down; the interface might be undergoing mixing between DPPC and LysoPC; and (3) surface tension increases from this plateau point to the equilibrium surface tension of pure LysoPC. While (1) and (2) have been observed by Holm et al. [378], where LysoPC help reduce the surface tension of existing lung surfactant film, the latter phenomenon (3) is never observed before, where the system prefers a higher surface tension (higher surface energy) state than a lower surface tension state. This indicates that there are other forces governing the equilibrium of the interface.

We notice a significant behavioral difference when the introduced LysoPC is at \( C_{\text{LysoPC}} < C_{\text{MC LysoPC}} \) compared to when \( C_{\text{LysoPC}} > C_{\text{MC LysoPC}} \), as depicted in Figure 10.3. Below CMC, the dilatational modulus of such system shows relatively no change from DPPC’s initial dilatational modulus value of 250 mN/m, indicating that despite the presence of LysoPC at the interface, the interface maintains its rigidity. This is consistent with clinical observation that small amount of LysoPC exists in a healthy lung, which does not alter lung’s mechanical properties.

The thermodynamic of an interface is usually studied by probing how its surface pressure changes with change in area. The \( \pi - A \) compression/expansion isotherms of these systems after equilibrium are presented in figure 10.4 while \( \pi - t \) evolution throughout the cycles is presented in Figure 10.5. Along with these systems, we also included the isotherms for pure DPPC and pure LysoPC. When above CMC concentration is introduced, the system becomes mostly LysoPC-like, where it maintains a relatively constant surface pressure. The isotherms coincide for the most part with pure LysoPC, except when compression reaches 50% of the trough area, in which the surface pressure starts to climb up, mimicking the compressed state of pure DPPC. When below CMC
Figure 10.2: A) Surface tension and B) dilatational modulus evolution of DPPC-covered interface following 20 µM and 40 µM LysoPC introduction to the subphase. Following the introduction, there is a decrease in surface tension, which indicates that there is an adsorption of LysoPC to the interface. The surface tension eventually reaches a plateau, after which the stir bar is turned off and the interface is oscillated once every 15 minutes to obtain surface tension and dilatational modulus data. Following the oscillation, the surface tension increases to around 37 mN/m, which is the equilibrium surface tension of pure LysoPC. The dilatational modulus also drops gradually as surface tension increases, terminating in values close to $E^*$ of pure LysoPC of introduced concentration. The label in (A) corresponds to the point in time when images in figure [10.6] were obtained.
Figure 10.3: Dilatational modulus of DPPC interface after LysoPC is introduced and the system equilibrates. Below CMC of LysoPC (6-7 µM), the dilatational modulus of the system is relatively high and close to the value of $E^*$ of DPPC. Above the CMC, the dilatational modulus drops dramatically close to $E^*$ of LysoPC of introduced concentration.
concentration is introduced, the compression and expansion cycles do not resemble that of pure LysoPC or pure DPPC, indicating that its behavior is a mix between the two systems.

10.3 Morphological changes of the interface with LysoPC introduction

In a pure DPPC interface, the only regions where structure can be observed is the LC/LE coexistence, which at room temperature occurs around 7-12 mN/m in room temperature. During the early stage of the coexistence, solid-like LC domains start nucleating and growing among liquid-like continuous LE phase. At the end of the coexistence region, the boundary between solid-like and liquid-like domains becomes indiscernible. Far past coexistence when \( \pi > 20 \) mN/m, one usually can no longer observe any discernible structure, the region is typically homogenous except for some possible defects. When LysoPC is added to the subphase, the texture of the interface changes slowly while still maintaining a relatively homogenous morphology (figure 10.6). This is a trend for both above and below CMC LysoPC introduction to DPPC monolayer. We note here that we have enhanced the contrast to see if the insoluble dye spreads uniformly past the coexistence.

When subjected to compression and expansion, distinct domains start to develop, despite the surface pressure being above 35 mN/m, i.e. far past the coexistence region of pure DPPC. The morphologies are shown in Figures 10.7 and 10.8. Following equilibration after LysoPC introduction and slow compression/expansion cycle, the interface starts showing various morphologies coexisting at the same time. We also note that upon compression, these domains grow and form networks by fusing together (Figures 10.7 and 10.8).

Above CMC system during compression/expansion cycles

Firstly, we note that while we have shown that the DPPC and LysoPC 40 µM system can be compressed up to \( \pi = 70 \) mN/m, when the Langmuir trough is placed under confocal microscope, we cannot compress the interface as much anymore because of the space constrains imposed when placing a surface pressure sensor and the microscope.
Figure 10.4: Compression (C) / Expansion (E) isotherms of the interface once surface tension reaches a relatively stable plateau after oscillations. Compared to DPPC isotherm, the system with 40 µM of LysoPC (dark and light blue) has a plateau of around $\pi = 35-40$ mN/m for most of its area, sharing a great similarity with that of pure LysoPC isotherm (purple and pink). However, the system still retains some DPPC as $\pi$ starts to jump when the trough is compressed past 50% of its area. In contrast, for the system with 3 µµM of LysoPC (red and orange), the compression and expansion cycles do not resemble that of pure LysoPC or pure DPPC, indicating that its characteristic is a mix between the two systems.
Figure 10.5: Compression/Expansion cycles of the interface of DPPC+LysoPC of (A) 40 µM and (B) 3 µM concentration once surface tension reaches a relatively stable plateau after oscillations as a function of time. The alphabet labels in (A) and (B) are guides for morphological evolution in figures 10.7 and 10.8 respectively.

scan head side by side. Thus, the interfacial images reported for Figure 10.7 span only for $35 < \pi < 40$ mN/m (Figure 10.5A), meaning that the change in surface pressure is minimal throughout the cycles.

To ease the understanding the compression/expansion cycle for the DPPC and LysoPC 40 µM system is presented as a function of time in Figure 6A, with the labels to guide readers through the morphology presented in Figure 10.7.

While remaining relatively uniform throughout the first two cycles of compression/expansion, at the end of second expansion cycle, small domains start to grow (some are pointed in the arrow in Figure 10.7D). More compression causes growth of domains (arrow 1 on Figure 10.7E). The domains are similar to cusp or bean-shaped (arrow 4, Figure 10.7F) and triskelion (arrow 5, Figure 10.7F) domains of pure DPPC at its coexistence, except that the edges are sharper than typical morphology in DPPC. This is likely due to LysoPC’s known effect to alter line tension (a two dimensional equivalent to surface tension, or the energy per length of perimeter of two-phase boundaries) at the interface. However, smaller domains tend to fuse with each other (arrow 2 in Figure 10.7E) to form fractal networks (arrow 3 in Figure 10.7E).

When the fractal networks become so fine, the region becomes uniform, with the
Figure 10.6: Evolution of interfacial morphology following the introduction of 40 \( \mu \text{M} \) of LysoPC. Brightness and contrast were enhanced to demonstrate the evolution. Each figure is labeled based on the corresponding points in Figure 10.2. The scale bar in figures A-H indicates 20 \( \mu \text{m} \), while the scale bar in the lower row for figures D*-H* is 100 \( \mu \text{m} \). While not depicted in this chapter, 3 \( \mu \text{M} \) LysoPC creates similar changes to the interface following its adsorption and oscillation, resulting in a uniform interface.
Figure 10.7: The interface of DPPC+LysoPC 40 µM during compression/expansion cycles. Labels are according to figure 10.5A. Scale bar is 100 µm.
network still being formed at the boundary (see arrows 6 and 10 in Figures 10.7G, H, I). In Figure 10.7G and H, it is likely that the network formed from fusion was made of really small domains, which create really fine networks such that no structure is discernible from the uniform region. However, in arrow 7 in Figure 10.7I, the network forms a region with the fractal structure being visible to the eyes. The difference is likely related to the fact that while the majority of domains fusing to form network are those of smaller size, throughout the third cycle of expansion, larger domains also start to fuse (arrows 8 in Figures 10.7J, K). In Figure 10.7L, arrow 9 points out the rough uniform region at the boundary of the regions with some large domains. We also note that when larger domains form part of the network, there often are some domains trapped within the networks, as shown in Figures 10.7L, K, L, M. As fusion between fractals is happening (arrows 11 and 13, Figures 10.7O and P), the uniform regions with darker shade grows (example: arrow 15 Figure 10.7R). Some fractals never form uniform regions and they become giant networks (arrows 12, 14, 17 in Figures 10.7O, Q, T) where some regions of the networks would be darker or brighter than others around them.

**Below CMC system during compression/expansion cycles**

To ease the understanding the compression/expansion cycle for the DPPC and LysoPC 40 µM system is presented as a function of time in Figure 10.5B, with the labels to guide readers through the morphology presented in Figure 10.8.

Below CMC, the first compression/expansion cycle also similarly shows a relatively uniform interface. During the second cycle, the texture of this interface starts to change, where the relatively smooth interface starts to roughen. The roughness start to manifest as a network of domains (black regions in Figures 10.8G-T) growing in a continuous LE phase (red background). As the compression goes on, the network grows larger and replace the LE phase. In some LE regions completely surrounded by networks, some individual domains grow (arrows in Figures 10.8K, L, M, O, also enlarged in Figures 10.8K* and M*). At point O, the peak of the second compression cycle, the interface is mostly dark with some small bright patches trapped in the giant network. Expansion past point O causes the continuous phase to grow, likely due to the “melting” of some part of the networks. However, the morphology of the interface during expansion is
Figure 10.8: The interface of DPPC+LysoPC 3 µM during compression/expansion cycles. Labels are according to figure 10.5B. Scale bar: 100 µm. Asterisk labeled images are zoomed in of the non-asterisk labeled images (scale: 20 µm)
slightly different from the ones observed during compression. In figures 10.8P, Q, R, the domains become more organized, likely because after being at a highly compressed state, the domains like to be closer to each other. Eventually, at the most expanded state, at point S, the morphology looks comparable to point I, which is the expanded state before the compression/expansion cycle leading to point S. When compressed again, the network grows similarly to the previous compression cycle (compare Figures 10.8J and 10.8T).

10.3.1 Oscillation effects

The surface tension evolution of a DPPC monolayer following LysoPC introduction is sensitive to small perturbations. For example, while the surface tension tends to plateau around 37 mN/m for a continuously oscillated interface, the surface tension would follow one of two different paths when no oscillation is performed. First, the surface tension would continue decreasing and relatively stabilize (red in Figure 10.9). Second, the surface tension can fluctuate between two equilibriums: one with lower surface tension and one with high surface tension corresponding to pure LysoPC surface tension (blue in figure 10.9). We hypothesize that the plateau that is first reached after LysoPC is introduced is a metastable point that is sensitive to a lot of factors, including oscillations, stir bar location (stir bar can move a little during the course of hours of rotations), and temperature fluctuations. Temperature is likely to play a role in this sensitive change as we observe bright spots of various shapes: circular, tubular, as well as irregular (figure 10.10A) in the morphology in hours following LysoPC adsorption. These bright dots are monolayer-associated vesicles (compare to Figures 3.5 and 3.6) that start to nucleate when surface pressure is relatively high [10, 379]. The formation of these monolayer-associated vesicles is a monolayer collapse mechanism that this particular interface explores as the monolayer starts becoming unstable with the high concentration of molecules at the interface. It has been shown that even with smallest departure in temperature (starting from 3°C increase from room temperature), the vesicles appear more abruptly and can detach from the interface to the subphase [95, 10]. In our system, this is shown in figure 10.10B where the volume (of depth 280 µm) below the interface has high concentration of fluorescence materials that escaped the interface.

The morphology throughout the compression/expansion cycle of DPPC/LysoPC 40
Figure 10.9: Behavior of a DPPC monolayer after the introduction of 20 µM of LysoPC to its subphase. Oscillation brings the system to $\gamma=37$ mN/m. Without oscillation, the system fluctuates between low surface tension around 20 mN/m (LysoPC adsorption and mixing with DPPC) and surface tension around 35 mN/m (close to equilibrium surface tension of pure LysoPC). What affects the shift between one critical point to another is still unknown, but the system is very sensitive to environmental input, like change in stir bar position throughout its hours-long rotation, as well as small temperature fluctuation. It is likely that oscillation helps stir the system towards an equilibrium where $\gamma$ is closest to that of $\gamma_{\text{LysoPC, pure}}$. 
Figure 10.10: (A) Top view and (B) Volume view of DPPC + LysoPC system left overnight with stir bar rotating, no oscillation was performed. Bright dots indicate vesicles. Volume view indicates that some vesicles leave the interface to the subphase. (C-F) Examples of domains during compression/expansion cycle which resemble those in figure 10.8.

µM system that has been sitting overnight without oscillation is similar to that of oscillated DPPC/LysoPC 3 µM, whereby initially no structure forms, but with consecutive cycles, network-like structure forms (Figures 10.10C-F). Noting that DPPC/LysoPC 3 µM needs significantly longer time to reach a surface tension plateau following LysoPC introduction, it is likely that this network like structure develops as an interface of DPPC/LysoPC mixture ages and the interface is compressed/expanded slowly.

Nguyen et al. [380] have also demonstrated that oscillation of an interface can cause desorption of molecules from the interface, as well as adsorption of other surface active molecules to the same interface, indicating that oscillation provides additional energy for the system to mix to achieve an equilibrium state.
Analysis on LysoPC adsorption to preexisting DPPC monolayer

The fact that LysoPC adsorbs to the interface following its introduction to subphase, even when the surface tension is already lower than its equilibrium surface tension suggests that there is another force other than surface tension lowering that drives LysoPC adsorption to the interface. For example, the mixing between LysoPC and DPPC could be a driving force.

Sundaram and Stebe [372] derived the relationship for monolayer penetration of insoluble monolayer of component 1 by soluble surfactant (component 2) for non interacting system:

\[ \Delta \pi = RT \Gamma_{2\infty} \ln(1 + K_2 C_2) \]  

(10.1)

Equation (10.1) is based on Langmuir adsorption isotherm modified for 2 components system to describe adsorption of the soluble surfactant to the interface, thus, to compare that to our system, we must only use \( \Delta \pi = \pi_{\text{max}} - \pi_{\text{initial}} \), i.e. to not include the part where surface tension starts increasing. In Table 10.1 we compared the value of measured \( \Delta \pi \) vs. calculated \( RT \Gamma_{2\infty} \ln(1 + K_2 C_2) \), with known \( K = 330 \, \mu \text{M}^{-1} \) [29], and there is a great mismatch between the two values for all measured concentration, meaning that Langmuir isotherm cannot describe LysoPC adsorption, both for \( C < \text{CMC} \) and \( C > \text{CMC} \), to DPPC covered interface, even though it has been shown to work for LysoPC adsorption to clean interface. This means that there is an interaction between DPPC and LysoPC that affects the rate of LysoPC adsorption.

After reaching a surface tension minimum point, the surface tension slowly increases again. The change in \( \gamma \) is counterintuitive to what would be expected from a thermodynamic perspective. From Equation (1.1) a system with low \( \gamma \) is desired to reduce the

<table>
<thead>
<tr>
<th>( C (\mu \text{M}) )</th>
<th>Measured ( \Delta \pi ) (mN/m)</th>
<th>Calculated ( RT \Gamma_{2\infty} \ln(1 + K_2 C_2) ) (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>12.5</td>
<td>39.96</td>
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<td>31.97</td>
</tr>
<tr>
<td>3</td>
<td>9.8</td>
<td>29.06</td>
</tr>
</tbody>
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Table 10.1: Measured \( \Delta \pi \) vs. calculated \( RT \Gamma_{2\infty} \ln(1 + K_2 C_2) \).
total free energy of a system. Thus, the increase in $\gamma$ in the latter time in Figure 10.2A suggests that there is some other contribution to the preferred state of the interface; likely, this involves mixing or rearrangement energy of the molecules at the interface. This gradual increase subsequently plateaus at 37 mN/m, the equilibrium $\gamma$ of LysoPC, suggesting that LysoPC occupies a big portion of the interface. This phenomenon of increased $\gamma$ following a decrease in $\gamma$ after a soluble surfactant introduction has never been observed in literatures studying soluble surfactant introduction to a monolayer-covered interface [372, 373, 374].

While $\Gamma_{\text{LysoPC}} = 1.7 \mu\text{mol}/\text{m}^2$ for at its equilibrium surface pressure, $\pi_{\text{eq,LysoPC}} = 35$ mN/m, $\Gamma_{\text{DPPC}} = 4.6 \mu\text{mol}/\text{m}^2$ at the same surface pressure. This means $\Gamma_{\text{DPPC}} \sim 2.5\Gamma_{\text{LysoPC}}$ at that surface pressure, despite the fact that both LysoPC and DPPC have the same headgroup size.

For DPPC+LysoPC 40 $\mu$M system, a rough estimate predicts that since the interface needs to be compressed 17% more than pure DPPC to reach $\pi = 40$ mN/m, which means that at least 17% of the area is occupied by LysoPC. Since the full trough area is $\sim 0.015$ m$^2$, the area approximately occupied by LysoPC is 0.0026 m$^2$, giving at least 0.0044 $\mu$mol of LysoPC at the interface (total LysoPC concentration in the bulk is 40 $\mu$M, equivalent to 10 $\mu$mol), meaning that despite the overwhelming amount of LysoPC available in the bulk, there is still a significant amount of DPPC left at the interface (inferred from isotherm). Likely, the equilibrium dictates that some portion of DPPC be left at the interface.

As the behavior changes dramatically around CMC, the concentration of the LysoPC in the subphase is critical in whether or not the displacement of insoluble surfactant takes place. We hypothesize that the solubilization of interfacial monolayer takes place, as depicted in Figure 10.11. Figure 10.11 depicts: (A) initial, unperturbed insoluble monolayer coexisting with micelles and monomers in the bulk, (B) monomers adsorb to the interface, coexisting with insoluble surfactants at the interface, (C) as monomers crowd the interface, less space is available for insoluble surfactants. This is also the point where micelles get close to the interface, and (D) micelles incorporate insoluble surfactant into its structure, thereby preventing hydrophobic entropy lost from the interaction between water molecule and the hydrophobic parts of the insoluble surfactants.

Phospholipid solubilization is a common phenomenon in the lipid bilayer of cell...
membrane and has been studied extensively to understand the roles of proteins and small molecules in cell signaling and homeostasis. However, this is the first reported study of a lipid monolayer solubilization by small molecules. Our hypothesis is supported by the z-stack image in Figure 10.10B, where portions of lipid monolayer is being solubilized to the bulk phase.

10.4.1 DPPC/LysoPC mixed monolayer

In addition to understand LysoPC effect to a preexisting DPPC monolayer, it is also important to understand how DPPC and LysoPC can coexist in a monolayer if such a scenario is to take place. In this section, I will discuss the behavior of DPPC:LysoPC 1:1 (mol/mol) monolayer. Figure 10.12 shows the initial compression isotherm of such monolayer at room temperature, compared to pure LysoPC and pure DPPC. The isotherm of the mixed monolayer is very different from either pure DPPC or pure LysoPC, and it resembles the one obtained by McConlogue and Vanderlick. The isotherm shows no plateau, as expected from equation as $DOF = 3$ as the system is a binary mixture, meaning that $\pi$ changes with area. The morphology of such monolayer is presented in Figure 10.13. Unlike pure DPPC, the mixed monolayer has liquid and solid phase coexistence that is not confined to one surface pressure (due to the additional degree of freedom). Thus, domains can be observed throughout a wide range of surface pressure. During the nucleation stage ($\pi = 9 \text{ mN/m}$), the domain initially starts out as a circular structure. However, as the domains grow, the distinction from pure DPPC morphology becomes more apparent. While still maintaining some characteristic structure of bean-like and multilobed DPPC domains, the domains have sharper edges, indicating that the line tension between the liquid condensed and liquid expanded phases is reduced from that of typical DPPC monolayer. The same effect of LysoPC on DPPC morphology has also been suggested by McConlogue and Vanderlick. In a broader application, LysoPC has also demonstrated line tension changing capability in lipid membrane, as suggested by recent studies.

Due to the solubility of one component of the system (LysoPC), the properties changes were also observed over time. Figure 10.14A shows that repeated compression/expansion cycles of the mixed monolayer leads to dramatic changes in the monolayer $\pi - A$ isotherm, terminating in an isotherm similar to that of pure DPPC. Similarly,
Figure 10.11: Likely scenario by which soluble single-tailed surfactant LysoPC can replace double-tailed DPPC at the interface despite the fact that DPPC is at lower surface tension than equilibrium surface tension of pure LysoPC through interfacial monolayer solubilization. (A) initial, unperturbed insoluble monolayer coexisting with micelles and monomers in the bulk, (B) monomers adsorb to the interface, coexisting with insoluble surfactants at the interface, (C) as monomers crowd the interface, less space is available for insoluble surfactants. This is also the point where micelles get close to the interface, and (D) micelles incorporate insoluble surfactant into its structure, thereby preventing hydrophobic entropy lost from the interaction between water molecule and the hydrophobic parts of the insoluble surfactants.
Figure 10.12: Compression isotherm of DPPC:LysoPC 1:1 (mol:mol), compared to that of pure DPPC and pure LysoPC at room temperature.
Figure 10.13: Morphology of DPPC:LysoPC 1:1 (mol:mol) monolayer during compression. Unlike pure DPPC, the mixed monolayer has liquid and solid phase coexistence that is not confined to one surface pressure (due to additional degree of freedom from having two components at the interface). Thus, domains can be observed throughout a wide range of surface pressure. In the initial stage of domain nucleation, the domains appear circular. The domains later grows with some DPPC-like characteristic (chiral, multilobe), however, the sharp edges indicate that LysoPC exists at the interface and lowers the line tension between the solid and liquid phase.
Figure 10.14 also demonstrates the evolution of dilatational modulus of such system as a function of surface pressure. As \( t \to \infty \), the dilatational modulus values tend to those of pure DPPC. This indicates that while DPPC and LysoPC can coexist together in the interface, when the interface is deformed repeatedly, LysoPC can leave the interface, and the long time behavior of the monolayer will be that of pure DPPC. However, as discussed in Subsection 10.2.1, the behavior of monolayers in presence of high concentration of LysoPC (above CMC) will have a high characteristic of pure LysoPC. Thus, in Figure 10.15A, the surface pressure evolution as a function of time is presented for (1) DPPC:LysoPC 1:1 without LysoPC in the subphase (water subphase), and (2) DPPC:LysoPC 1:1 with LysoPC in the subphase (micellar subphase), with LysoPC being introduced at the indicated arrow. Figure 10.15A shows a significant difference between system (1) and (2), where in (1), a drop of surface pressure by 30 mN/m was observed, likely correlating with the LysoPC that leaves the interface. In (2), the surface pressure does not change much and remain around 37-40 mN/m, which is around the equilibrium surface pressure of pure LysoPC. The final compression isotherms of both system (1) and system (2) are shown in Figure 10.15B following experiment in 10.15A (after 3 hours). The isotherm of system (1) resembles that of pure DPPC, while the isotherm of system (2) resembles that of pure LysoPC. These observations indicate that LysoPC concentration in the subphase determines whether or not the desorption of LysoPC from the interface takes place.

### 10.5 Conclusion

In this chapter, we have systematically studied the effects of soluble surfactant inflammatory product like LysoPC into a monolayer of LS lipid. Here, we report that the dilatational modulus of the system evolves to the final value close to the dilatational modulus of pure LysoPC of introduced concentration when the concentration of introduced LysoPC is above its critical micellar concentration. We hypothesize that this is strongly related to the fact that micelles are capable of solubilizing phospholipid monolayer.

As the lung can undergo Laplace instability when \( 2E^* - \gamma < 0 \), or where \( E^* < 20 \) mN/m, there is a concentration range of LysoPC that can trigger this instability within
Figure 10.14: Evolution of (A) $\pi - A$ isotherm and (B) dilatational modulus of DPPC:LysoPC 1:1.

Figure 10.15: (A) Surface pressure evolution as a function of time is presented for (1) DPPC:LysoPC 1:1 without LysoPC in the subphase (water subphase), and (2) DPPC:LysoPC 1:1 with LysoPC in the subphase (micellar subphase), with LysoPC being introduced at the indicated arrow. In (1), a drop of surface pressure by 30 mN/m was observed, likely correlating with the LysoPC that leaves the interface. In (2), the surface pressure does not change much and remain around 37-40 mN/m, which is around the equilibrium surface pressure of pure LysoPC. (B) The final compression isotherms of (1) and (2) 3 hours after experiment (A) starts. The isotherm of system (1) resembles that of pure DPPC, while the isotherm of system (2) resembles that of pure LysoPC.
breathing frequency. While previously hypothesized, no previous study has shown that LysoPC can trigger this instability in the lung because the lung alveoli are lined with insoluble monolayers with $E^* > 200$ mN/m during breathing process. In this chapter, we systematically showed that even with the presence of such insoluble monolayer, LysoPC can adsorb the interface and cause $E^*_{\text{interface}} \rightarrow E^*_{\text{pure LysoPC of introduced concentration}}$. 

Chapter 11

Concluding Remarks

Throughout my PhD, I have studied the dynamics of lung surfactant and its inhibitor, namely LysoPC, using various materials characterization techniques such as microscopy, rheology, X-Ray scattering, and thermal analysis, as well as comparison to existing transport models to understand what governs lung surfactant functionalities and how these functionalities evolve as lung injury occurs. In this chapter, I will revisit some of the highlights of this dissertation, focusing on the materials science contribution to the treatment efforts of RDSs. Further, I will also highlight other potential applications of the knowledge I have built throughout my PhD. Finally, I will conclude this dissertation with thoughts on the future directions this area of research could potentially go.

11.1 Materials science and RDSs

The major theme of this dissertation is to utilize materials science to give new insight into the lung surfactant system in order to tackle various life-threatening respiratory distress syndromes.

The understanding developed from Chapters 7 and 8 on lung surfactant structure, rheology, and thermodynamics can attack RDSs from the standpoint of clinical lung surfactant formulation. As the components of LS mixtures strongly determine the structure and viscosity of the system, an effective formulation that can minimize the viscosity of LS mixture is highly preferred for an effective surfactant replacement therapy.

In Chapters 9 and 10 I demonstrated that the stability of an interfacial system is
achieved only when $2E^* - \gamma > 0$. While our native lung surfactant system easily meets this requirement, the change in interfacial composition in the alveoli during lung injury and trauma can lead to an unstable lung prone to mechanical collapse. Specifically, changes that lead to a higher surface tension system in the lung occur, suggesting that some insoluble components of the lung surfactant mixture can be displaced from the interface. While the phenomenon of insoluble monolayer desorption has not been observed in literature, I hypothesize that this occurrence takes place following a mechanism similar to that of bilayer solubilization by detergents. This is the first ever reported study on lipid monolayer solubilization.

With this new understanding, the medical community can approach through: (1) high frequency respirator, and (2) reducing LysoPC concentration in patients’ lung. While approach (1) has been used in hospitals to help patients breathe, here we provide a different perspective of why it can help patients. While high concentration of LysoPC can cause $2E^* - \gamma < 0$ in breathing frequency, it is possible to use high frequency respirator to regions where $2E^* - \gamma > 0$. On the other hand, approach (2) relies on significant reduction of LysoPC concentration in the lung to below CMC. This might take place by introducing Lysophosphatidylcholine Acy1transferases, an enzyme that can catalyze the transition of lysolipid back to phospholipid (a reaction called Lands cycle) to the patients’ lung system. However, in vivo study of this reaction in lung system has not been well investigated, and a future interrogation of this approach should be explored.

11.2 Contributions to wider discipline

11.2.1 Drug formulation and delivery

Surfactant transport to the interface is of many interest in drug formulation, especially those delivered via injection. Many proteins and antibodies prefer to adsorb to the interface and form aggregates. When this happens, drugs lose their therapeutic functions. Thus, adding components that are more surface-active can help combat this lost of functionality, and the study of the transport of surfactant to the interface becomes crucial. Furthermore, determining factors governing proteins/surfactant dynamics in such systems, i.e. what drives surfactant adsorption and protein displacement relies on
understanding of competitive adsorption between two surface active systems. During my PhD, I have studied such dynamics extensively, as discussed in Chapters 8 and 10, which can be translated into these other systems.

On the other hand, viscoelasticity plays a role in drug delivery performance as the drug vehicles (mostly made of DPPC and other phospholipids) need to maintain an elastic enough structure to be unperturbed before reaching target tissue or cell; my paper on factors affecting LS viscoelasticity [253] discussed in Chapter 7 contributes to this advancement.

Additionally, knowledge obtained from how small soluble surfactants can alter the functionality of lipid bilayer and monolayer gives information on the leakiness of a designer drug delivery vehicle, and thereby the timescale associated with the delivery and drug uptake in the body.

11.2.2 Cell membrane study

In a similar way soluble surfactants can alter dynamics of drug delivery vehicles, soluble surfactants can also disrupt many cellular functions by interacting with cell membranes. Of particular interest, the dynamics between cell membranes and antimicrobial peptides, as well as toxins, are pivotal in building better understanding about our own host defense system in physiological environment.

On the other hand, the many aspects of monolayer dynamics that I have investigated during my PhD are directly translatable to cell membrane dynamics. For example, further studies of domains in LC/LE coexistence can inform us further about lipid rafts structures believed to be vital for intracellular transport, signal transduction, and entry of pathogens.

11.2.3 Expanding current understandings to foam stability

Laplace instability, which happens when $2E^* - \gamma < 0$, is also a marker for foam instability in general. Thus, knowledge that has been built in this dissertation regarding factors governing $E^*$ can be used to create stable foams. For example, to create a stable foam of soluble surfactants like LysoPC, one could adapt knowledge discussed in Chapter 9 about factors governing dilatational modulus, including surfactant transports.
to and from the interface, surfactant concentration, curvature effects, as well as micellar exchange dynamics. From a different perspective, basing on my observation that $2E^* - \gamma > 0$ for insoluble surfactants like DPPC for most frequencies due to negligible transport from the interface, we also believe that insoluble surfactants can make stable foams.

11.3 Future directions

11.3.1 Modeling adsorption and desorption of LysoPC to and from interface

Understanding adsorption behavior of LysoPC to a preexisting DPPC monolayer can shed light into the rate by which LysoPC can alter the monolayer. Similarly, the desorption behavior of materials (either LysoPC, DPPC, or both) from the interface due to such adsorption is also crucial.

Currently, there are few existing models that describe these phenomena; and these models are usually modeled based on assumptions such as: non-interacting, low initial starting surface pressure, below CMC concentration of penetrating soluble surfactants. Unfortunately, these assumptions are far from our physiological systems where high concentration of LysoPC penetrate DPPC monolayer at high enough surface pressure, and likely, where LysoPC and DPPC interact, as shown from creation of fractal networks in the interface morphology (Chapter 10).

Going forwards, describing the dynamics of the two systems is fundamental not only ARDS treatment efforts, but also for extending knowledge for drug delivery and cell membrane studies, as discussed in above sections. The point of interest that I think should be addressed is to firstly understand the nature of DPPC/LysoPC interactions, from which one may gain insight into (in orders of determination): 1) fractional area occupied by each molecule, 2) area per molecule occupied by LysoPC and DPPC molecules during this process, 3) number of molecules of each species at the interface, and finally, 4) relevant adsorption isotherms. To determine fractional area occupied by each molecule one might start from thermodynamics relationship to see if DPPC/LysoPC forms an ideal mixture.
11.3.2 Investigating effects of other inflammatory products

Other than LysoPC, there are many other inflammatory products that enter the lung during pulmonary edema, starting from serum proteins like albumin, fibrinogen, and IgG, to enzymes like PLA$_2$, and finally to other lysolipids of different headgroups and tail lengths. Each of these inflammatory products is surface-active and much more soluble in an aqueous bulk phase than DPPC.

In addition to expanding our understanding of mechanism of ARDS progression, close interrogation of the dynamics of these other inflammatory products and DPPC can benefit the studies on cell membrane and drug delivery applications. For example, studies of the various family of serum proteins can explain how cell membranes and drug delivery vehicles react to various shapes of protein configurations and molecular weight. Similarly, lysolipids with different head groups and tail groups represent the many families of hydrolized phospholipids during inflammation, which can inform us the how surfactants of different head group charge and tail length, and hence solubility and CMC, could alter lipid bilayer/monolayer ecosystem.

11.3.3 Comparing curved and flat interface morphologies

As shown in Figure 6.10, domains form differently in highly curved interfaces compared to flat interfaces. In flat interface, individual domain can grow up to 20 $\mu$m in size. However, curving the interface, for example, to a 100 $\mu$m-sized bubble, imposes strain on crystalline growth. For a DPPC monolayer, this results in creation of rigid interfacial network. The factors driving this transition are still unknown.

Going forward, developing models to understand this transition is important as highly curved interfaces are physiologically relevant. The approach that might be ideal is to adapt the perspective of Meng et al. [388], to incorporate elastic stress effects on lattice packing frustration at curved interfaces.
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