Lipid monolayer stability: role of fluid boundary layers

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Interfaces are ubiquitous in biological systems and often occur at some solid/fluid intersection. In this paper, we explore the interface between a fluid and a lipid monolayer in the Langmuir geometry. We show that a rich set of non-trivial behavior occurs both in the fluid and the monolayer. In particular we explore the effect of glycerol.

Lung surfactant | alveolar lining fluid | lipid monolayers | collapse | neutron/x-ray reflectivity | boundary layers | elastic instability

Abbreviations: ALF, alveolar lining fluid; DPPC, dipalmitoylphosphatidylcholine; POPG, palmitoyloleylphosphatidylglycerol; XR, x-ray reflectivity; NR, neutron reflectivity; GIXD, grazing incidence x-ray diffraction; GIXOS, grazing incidents x-ray off-specular.

 Sugars are one of the chemical backbones of life. Organisms use sugar as their source of chemical energy; sugar molecules are often attached to proteins and lipids via elaborate glycosylation pathways to help in cell-cell signaling and protein trafficking; the extra-cellular matrix contains large amounts of sugar polymers (proteoglycans and hyaluronan) that provide the ever more appreciated correct environment for proper cell and tissue function [1, 2]. Sugars are also extensively used by many plant and microbial species in long term self-preservation especially in very low water environments, a phenomenon studied under the name of anhydrobiosis [30]. Cell and molecular biologists have for decades borrowed these clever tricks of nature by preserving cells and proteins in solutions rich in glycerol, sucrose, trehalose or a number of other sugars [1, 2, 30]. While sugars play a rich role in the fabric of life, they are also culprits in some of our most widespread diseases. Large consumption of sugars has played a large part in the rampantly growing epidemics of obesity and diabetes, which threaten the health and quality of life for hundreds of millions of people.

Lipid membranes are the first interface human and most animal cells have with their external environment [1]. In a dense tissue, cell membranes make tight contact with the extra-cellular matrix and each other, the space between them sometimes as little as only a few nanometers. However other interfaces are less dense, for instance the lipid membrane of blood cells spends the majority of its time making contact with the fluid plasma, likewise the apical membranes of epithelial and endothelial cells in blood vessels and in the airways and GI tracks make contact with semi-infinite fluid phases, lastly there is the alveolar lining of the lung. The lung is the largest liquid covered interface in the human body. This delicate tissue would collapse under the strength of surface tension forces were it not for a monolayer of lipid and protein located at its surface called lung surfactant. Lung surfactant lipid like the blood cell membrane and apical endothelial/epithelial tissues makes contact with a liquid layer.

The aim of this paper is to understand the interaction of lipids with solutions of sugar (in particular the sugar alcohol glycerol) in the lung surfactant type geometry.

We provide the first experimental evidence that sugars (glycerol) directly enrich a lipid covered interface. Moreover we show that the existence of these thin (on the order of ten angstroms) sugar rich boundary layers greatly changes the mechanical response of the lipid monolayer. Our work has direct consequences for the role of sugar in lung surfactant stability, moreover it has potential implication for the role of sugar in modulating cell mechanics in highly dynamic (lots of stretching and compression) tissues such as endothelial cells (the entire Constantine literature and Skip Garcia) and epithelial linings.

Glycerol is present in blood plasma at concentrations around an order of magnitude lower than that of glucose. The primary source of glycerol is from lipid breakdown by lipases. Glycerol is thought to be the primary gluconeogenic molecule used by the liver when synthesizing glucose from lipid breakdown.

GAK - glycerol metabolism problems.

Alveolar interfaces have two main components: the micron thick alveolar lining fluid [55] and the nanometer thin lung surfactant [38]. Physiologically, the lining fluid provides the necessary environment separating the delicate alveolar cells from the outside and allows for efficient gas exchange [45, 51]. Surfactant serves many roles, its main physical function being the lowering of surface tension that exists at the alveolar fluid/air interface [38]. The coupling between surfactant and the alveolar lining fluid is not well understood beyond the main postulate that the surface tension of the interface must be reduced for proper pulmonary function.

Biophysical work in lung surfactant has greatly enriched our understanding of the mechanics by which such interfacial monolayers of lipid and protein achieve the goal of low surface tension while maintaining stability in what is a very dynamic system. On average, adult humans breathe at the rate of twelve breaths a minute [3]. Each time a breath is taken the alveolar interface expands to accommodate the increase in total lung volume, likewise when we exhale the interface is compressed [3]. During the breathing cycle the interfacial tension of the alveolar surface varies from a high value on deep inhalation to a near zero value on a deep exhalation [3, 45]. Molecurally, the source of these variations comes from the change in packing density of the lipid molecules compos-
ing lung surfactant (primarily DPPC) [38]. However at very low surface tensions the surfactant covered interface begins to lose stability and leaves the interface, a process termed ‘collapse’ [4, 9, 10, 38, 26, 12, 13]. Native lung surfactant and effective surfactant replacement formulations were shown to collapse by formation of large out-of-plane folded structures extending deep in to the subphase [4, 38, 26, 39]. It is postulated that the key physiological advantage of collapse by folding is its reversibility, meaning on subsequent inhalation and alveolar expansion surfactant within the folds is capable of opening back onto the interface [4, 38, 26, 12, 39]. Thus any (bio)chemical species that effects either the ability of lipid molecules to pack tightly or the mode of collapse could seriously compromise pulmonary function.

An extensive biophysical literature has explored the effect of varying agents on surfactant stability and in particular collapse. For example, tobacco smoke was shown to influence the physical properties of lung surfactant making folds less reversible and therefore compromising the ability of the surfactant to keep surface tensions low on multiple compression/decompression cycles [43, 44]. The ionic strength of a model alveolar lining fluid (called the subphase in Langmuir trough experiments) has also been shown to impact collapse: high ionic strength giving rise to more reversible collapse while low ionic strength subphases compromising re-spreadability in model systems [50]. Furthermore, the effect of blood proteins such as albumin on surfactant spreadability, stability, and collapse have been studied [47, 46]. These studies elucidated a potentially powerful mechanisms by which pulmonary compromise occurs during acute respiratory distress syndrome (ARDS), whose pathological hallmark is the loss of pulmonary capillary integrity leading to the accumulation of blood serum proteins (especially albumin) in the alveolar lining fluid [45]. Moreover, understanding the basic biophysics and surface chemistry in this case has lead to potential therapeutic interventions. In particular, the presence of hydrophilic non-adsorbing polymers within the same subphase was shown to help lung surfactant overcome the effects of albumin [47, 46, 48, 49].

While the surfactant literature has focused on questions of monolayer stability as pertaining to either surface tension or collapse, the community working on the alveolar lining fluid has dealt more with questions of clearance. The alveolar lining fluid is on average one micron thick [55]. Compositionally, the fluid is complex containing surfactant released and packaged by type-II pneumocytes for adsorption to the alveolar interface, immune cells, a variety of proteins including immunoglobulins, and a substantial amount of the non-ionic hydrophilic carbohydrate polymer hyaluronan [45, 56, 46, 49]. Proper pulmonary function from the trachea to the alveoli relies on the movement of the thin pulmonary lining fluids up the respiratory tree [45]. In the higher airways, ciliated epithelial cells give rise to the necessary flow patterns [45], however in the non-ciliated regions deeper in the lungs, physical forces generated by the oscillatory motion of lung tissue during the breathing cycle are the source of cleansing flows [52, 53, 54, 51]. While experimental study of alveolar clearance is difficult, mathematical modeling has shown that proper flow patterns are generated through a delicate balance of capillary and viscous forces [53]. So disturbances in the stability of lung surfactant which would give rise to an increase in alveolar surface tension have been predicted to compromise alveolar cleansing [53].

Both in the lung surfactant as well as the alveolar lining fluid literature, the potential role of small molecule carbohydrates such as glucose has received little attention. However, within the physiology community, it is well known that the apical cell membrane of alveolar and airway epithelial cells contains a sodium-glucose cotransporter [58, 59, 60, 57]. The presence of this transporter in a lung tissue is somewhat of a mystery given that lungs play no role in glucose absorption, unlike intestines where such transporters are also common [57]. Nevertheless, these transporters are shown to keep glucose concentrations in the alveolar and airway lining fluids markedly low [61, 62, 66]. Several physiological models link such low glucose levels as a defense mechanism against infection [65], others have proposed that regulating glucose concentrations provides the lungs a mechanism by which to control alveolar liquid clearance [57, 63, 64].

In this paper, we provide biophysical study of the effect of the simple sugar alcohol (glycerol) on lung surfactant stability, in particular collapse. We show that glycerol shows a marked adsorption to the lipid covered interface, forming a boundary layer underneath the lipids. Furthermore, the mechanical resistance of the monolayer is substantially altered. We discuss our data in light of the implications such boundary layers might have on both alveolar lining fluid clearance and lung surfactant collapse.

Sugars and sugar/lipid interactions are known to play a tremendous role in protecting living structures in low water environments. The field of anhydrobiosis has generated a decades old and rich thermodynamic literature on the interaction between sugars, poly-alcohols and lipid membranes [27, 28, 29, 30, 31, 32, 33, 34, 37]. Much of these early studies were motivated by desire to understand the molecular basis of anhydrobiosis, the preservation of living material in the absence of water commonly seen in many plant and bacterial organisms [30]. A commonly observed experimental result was the right-ward shift of Langmuir isotherms for many types of lipids when sugars such as glucose [28, 29, 37], sucrose [28, 29, 33, 37], galactose [37], threahalose [28, 29, 30, 37] or poly-alcohols (also referred to as alcohol sugars) such as glycerol [27, 29, 34, 37] were mixed into the subphase. The consensus interpretation of such isotherm shifts was that the carbohydrate additives ‘fluidized’ the lipid membranes. This interpretation of the thermodynamic data was used to hypothesize how carbohydrates might compete with and replace water in the lipid headgroup region by hydrogen bonding with its polar moieties [32, 34]. What is lacking in this literature is more direct structural studies of the water/carbohydrate/lipid interface to precisely localize the position of the different components that might put to rest the more speculative interpretations of the thermodynamic data. Our paper provides such as structural study.

Results

Folding Analysis. As a fold forms, material is drawn from the interface, however the folding event also generates an in-plane displacement of the monolayer still remaining on the surface. Representative movies of DPPC:POPG 7:3 folding at 25°C on water and glycerol mixtures are provided in the supplemental materials (see movie 1 and movie 2). Surface jerking motion is easily seen with the naked eye. These displacement fields can be tracked (see Materials and Methods: Image Tracking) to provide quantitative information about the amount of surface material pulled into a given folding event [21, 13]. Figure 1 is a seismogram of sorts showing the amplitude of surface displacement (fold sizes) as a function of compression time. On a pure water subphase, the monolayer folds extend from a couple of microns to a few tens of microns, the events show no particular time clustering and are regular. This is in agreement with a previous extensive study of DPPC:POPG 7:3 on water [21].
The seismograms are markedly different when the monolayer is on a subphase containing glycerol (see figure 1B). While no folding events with amplitudes greater than 20 microns are observed on water, events extending into the hundreds of microns regularly occur on mixed subphases. The data also shows a clear time dependence of the large events. While small amplitude events occur throughout the compression (see supplement figure 1), large amplitude events are clustered in the first half of the seismogram. The average data can be collapsed to consider only the frequency and amplitude of given events (see figure 2). Both sets of subphase conditions show the largest number of events clustering around a couple of microns with an exponential decay in the frequency of larger amplitude events. However on mixed subphases, the exponential tail is very long, extending outwards to events several orders of magnitude larger than the most frequent event. To probe the origin of such large differences in the mechanical stability of lipid monolayers on water and water/glycerol mixtures, we explore the molecular structure of the lipid/glycerol/water interface using surface thermodynamics and scattering.

Isotherms. Pressure versus area isotherms provide a rich source of information about Langmuir monolayers. Figure 3A shows a set of fourteen DPPC isotherms taken at 25°C on subphases with increasing amounts of glycerol. As more glycerol is incorporated into the subphase, the isotherms shift to the right. The area per molecule where surface pressure first begins to be generated is called the lift-off, which molecularly it is thought to correspond to a surface density where lipid molecules begin to interact [7]. The rightward shift in figure 3A indicates that the lipid molecules begin to feel each others presence at smaller and smaller densities as glycerol is added to the subphase. This shift is not unexpected, since a large part of the lipid molecule’s size at low densities is contributed by the solvation shell [8]. Thus as more bulky glycerol molecules incorporate into the solvation shell the lift-off will shift to the right. However a more careful analysis (see figure 3B) shows that the amount by which the lift-off areas shift to the right increases more rapidly than expected if the solvation shell had a composition similar to that of the bulk subphase. Figure 3B is a Gibbs adsorption isotherm generated from the lift-off data of the Langmuir isotherms. It shows that an excess amount of glycerol seems to partition into the lipid covered interface, thus leading to the conclusion that glycerol is driven into the lipid headgroups by a decrease in the free energy of the interface.

X-ray Reflectivity. Figure 4 shows specular x-ray reflectivity data for DPPC:POPG 7:3 at 30 mN/m at 25°C on four different subphase conditions: water, 20, 40, and 64 wt% glycerol. An observation of the spectra shows a global shifting of the reflectivity fringes to lower q values with the addition of glycerol into the subphase. Generally such a leftward shift indicates an increase in the thickness of the interface [22]. To accurately determine the structure of the interface, the spectra were modeled using a multi-box ‘model independent’ fitting routine (see Materials and Methods: XR Modeling for details). The model electron density profiles which give the best fits to the measured reflectivities are shown in figure 4B. The electron densities of the lipid tails and headgroups are relatively the same on all four subphase conditions, indicating that glycerol does not penetrate into the headgroup or tail regions. On the pure water subphase, the electron dense phosphate headgroup region connects smoothly to the lower electron density water underneath. However, as glycerol is added, the smoothness disappears within a region extending around ten angstroms below the headgroup (see inset of figure 4B). As more glycerol is added, this region becomes more well defined and begins to separate the lipids from the bulk subphase. The increased electron density within this boundary layer is indicative of glycerol enrichment given that glycerol is slightly more electron dense than water. However the contrast between the two molecules is small and to determine the precise composition of this layer neutron reflectivity was done.

Neutron Reflectivity. Figure 5 shows neutron reflectivity data for monolayers of fully deuterated DPPC-d75 on several subphases containing D2O and glycerol. XR spectra showed that as glycerol was mixed into the subphase a boundary layer began to appear underneath the lipid monolayer. This boundary layer was slightly more electron dense and extended around ten angstroms beneath the lipids. Since neutron scattering length densities are highly sensitive to isotopic composition by using heavy water and hydrogenated glycerol allows us to distinguish the relative amounts of the two subphase components within the boundary layer. Figure 4 shows that the best fit model on a D2O subphase contains no boundary layer whose composition is different from bulk subphase. However as glycerol is added into the subphase, the best fit density profiles show a boundary layer whose scattering length density is lower than that of the bulk subphase mixture. By inverting the weighted average in equation two of the Materials and Methods, the amount of glycerol and D2O in the boundary layer was determined. On both 20 wt% glycerol (x = 0.05 in subphase) and 60 wt% (x = 0.25), the boundary layer contains three times the amount of glycerol (θ = 0.15 and 0.8 respectively) contained in the bulk subphase. Note that by the time glycerol makes up a quarter of all molecules in the subphase, the boundary layer is nearly all glycerol. A second set of data was collected using a different lipid deuteration scheme (DPPC-d62) where only the tail hydrogens were replaced with deuterium. The spectra and corresponding scattering length density models are shown in supplemental figure 2. The same trend as with DPPC-d75 is observed, on a pure D2O subphase there is no additional layer, however on mixed subphases the best fit models to the reflectivity spectra contain a glycerol enriched boundary layer. It is important to note that best fits to the spectra were obtained only when the headgroup of DPPC-d62 contained 4-6 deuterium atoms, the significance of this will be explained in the discussion. Using the mole fraction of glycerol in the bulk subphase and the NR measured concentration of glycerol in the boundary layer, an adsorption isotherm can be generated much like that done for the above isotherm data (see supplemental figure 3). Neutron reflectivity therefore shows the presence of a thermodynamically stable glycerol enriched boundary layer with an equilibrium constant of 6.63.

Higher Temperature Data. DPPC:POPG 7:3 has been shown to melt at 33°C [26]. The transition is characterized by the disappearance of elastic folds in-favor of more fluid like structures such as vesicles, tubes, and disks underneath the monolayer [26]. Supplemental video 3 shows such fluid collapse structures for a monolayer of DPPC:POPG 7:3 at 37°C spread on a water subphase. However spreading the monolayer on a 64 wt% glycerol subphase at the same physiological temperature recovers folding (jerking) behavior even though vesicle formation is not completely suppressed (see supplemental video 4). To study the molecular structure of the interface at the higher temperature, where the monolayer is unstable, special scattering techniques are needed which allow for fast collection of data [18].
GIXOS is a special reflectivity technique which allows one to capture a snap-shot of the interfacial structure factor [18]. Spectra obtained for DPPC:POPG 7:3 at 25°C and 37°C were normalized to remove contributions from surface capillary waves to the off-specular signal, leaving only the interfacial structure factor, which was modeled with a standard two-box model (see Materials and Methods: GIXOS Modeling). On water, the lipid monolayer when heated to 37°C becomes thinner (see supp. figure 4E). On 64 wt% glycerol, no change in monolayer thickness was observed, the monolayer electron densities overlapping nearly perfectly at both temperatures.

Pinline GIXD data were also taken at the above conditions (see supplemental figure 5). At low temperature, both monolayers showed a Bragg peak indicating that in-plane crystalline order exists within the monolayer at both subphase conditions. The Bragg peak on the mixture subphase is slightly to the right of its counterpart on water, leading to the conclusion that on glycerol at the same surface pressure the lipid lattice structure is more tightly packed. However upon heating both systems to 37°C, the monolayer on water lost its Bragg peak while the monolayer on 64 wt% glycerol retained the signature of crystalline order. There is a leftward shift of the peak position at 37°C, indicating that while on water crystalline order is completely destroyed by heating to physiological temperature on glycerol the structure is preserved yet the lattice opens up somewhat.

Discussion
In folding analysis mentioned displacement however did not discuss how it is believed that displacement is linked to amount of material pulled into a given fold. The DPPC-262 data could only be fit when deuterium was present in the headgroup, increasing the scattering length density of the headgroup from 1.2×10⁻⁶ Å⁻² to 3×10⁻⁶ Å⁻². This distinction was not seen in DPPC-475 because the scattering length density of the fully deuterated headgroup is already very high, such that small changes in deuterium content in the sea of deuterium made little difference. NMR studies of D₂O/glycerol solutions have shown that virtually no chemical exchange occurs between hydrogens in the glycerol hydroxyls and deuteriums in D₂O [67]. We therefor conclude that based upon our data the core solvation shell of the lipid headgroup remains populated with water molecules. This is in agreement with a recent thermodynamic study of DPPC on sugar solutions, which concluded that while headgroup atoms remained hydrogen bonded to first shell water molecules sugar molecules penetrated into the headgroup region and formed hydrogen bonds with the solvation waters [68]. Kreseva et al use this argument to explain their isothermal data where, as with out glycerol data in figure 3, a pronounced effect of the sugar is seen only at low surface pressures; the isotherms approaching and joining once lipids undergo phase transitions into condensed phases. In other words, as lipids become packed more tightly the solvation shell surrounding the headgroup is depleted of all the sugars. However what our reflectivity data show is that these sugars do not simply disperse back into the subphase, yet they from a boundary layer which is enriched in them. Given that there must be continuity between the still bound waters in the headgroup and the subphase molecules underneath, we conjecture that the boundary layer sugars remain hydrogen bonded to the hydration shell. A recent molecular simulation of disaccharide solutions and lipid bilayers shows a similar trend, with a higher concentration of sugar molecules underneath the headgroups than in the bulk solvent [69]. Sum et al used the disaccharide molecules trehalose and sucrose while we use the sugar alcohol glycerol, however a recent diffraction paper studying the effect of glycerol and trehalose on monoolein (a natural lipid) has shown that all these molecules have similar modes of interaction with the lipid [70].

Previous work has argued that there is impact of sugar molecules on lipid condensed phase structure [68]. Our work at physiological temperatures with the lung surfactant system shows that indeed glycerol stabilizes condensed phases (the GIXD peak does not disappear) and allows for collapse via folding.

We hope not to add to the somewhat heated and apparent disagreements about the nature of the sugar lipid interaction that have played out in the literature over the past several years. The early literature in this field form the 70’s and 80’s was largely done by work with lipid monolayers in Langmuir trough geometries. The conclusions from this work, though at times somewhat clouded by arguments of surface active contaminants in the sugars used, are that lipids are expanded by the presence of many different types of sugars. The data, being primarily in the form of surface compression isotherms, could only provide what has been argued as a ‘qualitative’ interpretation about the location of sugar or water molecules with respect to the interface. Nevertheless, the interpretations of the observed isothermal right-ward shifts by those early studies as evidence for sugar partitioning into the interface is well justified. Such shifts would easily be predicted by very quantitative isotherm equation of states.

More later work, also on Langmuir monolayers by very noted lipid scientists further showed that sugar partitioning into the interface is thermodynamically justified [68]. These authors did mention that it is unlikely that the very tightly held hydration shell of the lipid molecule would be replaced by sugars. However their analysis shows that sugars should be located within the larger hydration shell making hydrogen bonds with the tightly help first layer and perhaps even with the lipids themselves at times. Molecular dynamics simulations of lipid bilayers surrounded by semi-infinite half-planes of liquid have born out what Langmuir monolayer studies have shown. Sugars partition to the interface and according to several of the studies even enrich at the interface. The work we present here is a first detailed experimental study which shows such enrichments in a system which more closely resembles the simulations. Indeed the Langmuir monolayer sitting a top a semi-infinite subphase is much more like the embedded simulated bilayers than experiments done on bulk lipid phases where confinement could play a large role.

Experimental work on bulk lipid systems, ULV, MLV, and even morphologically uncharacterized samples, seems to be in stark disagreement with the large simulation and Langmuir monolayer literature. We would like to point out that in fact comparing the two is a bit like comparing apples and oranges. The bulk lipid experiments are done in systems where the degrees of freedom available to the lipids and solvent molecules are tremendous. Indeed much of the work done in these systems is based upon measuring separations between different bilayers within the complex solution. When lipid densities are so high such that bilayers are only tens of nanometers apart, the effects of confinement can not be ignored. In the end, we believe the systems are different, yet both have biological counterparts. In bulk tissue, cells are highly confined and so the effect and location of sugars in such systems might be more appropriately represented by the bulk system studies. Also, in the study of anhydrobiosis where densities become high the later might be more appropriate. However in trying to understand the impact of sugars on lung surfactant or endothelial cells the monolayer approach is more appropriate. In
these cases, the membrane is in contact with a semi-infinite half-plane of liquid.

Viscous Boundary Layer. Equally important to consider in such mathematical models is the elasticity of surfactant films. The resistance of the surfactant to stretch would produce a similar force as surface tension. Thus well packed surfactant films and even more importantly films possibly containing the glassy boundary layers seen in our work might react as rigid boundaries within the context of the hydrodynamic clearing models present in the literature and so substantially compromise alveolar clearance [53]. Moreover, presence of sugars in the ALF of diabetics may via such mechanisms be one of the causes of underlying pulmonary compromise seen in such patients.

Anti-plasticizer. Freed and Douglas

Biological implications. Simple model lung surfactant systems as well as clinically used surfactant replacement formulations like Surfactant undergo a jamming or glass-like transition where surface molecular viscosity diverges when the monolayer surface fraction covered by condensed phase exceeds 0.5-0.6. [39, 40]. The divergent viscosity and presumed glassy nature of the monolayer has led to modeling the lung surfactant membrane as a solid elastic sheet [12, 13].

Role of polyol and sugar like molecules and their interactions with membranes.

anhydrobiosis - cell preservation diabetes lung surfactant and albumin or alveolar lining fluid disturbances that are non-ionic

Such boundary layers are extremely thin requiring molecular level techniques to detect them such as neutron and x-ray reflectivity. They seem to be removable with rather gentle motion such as the self-jerking of the monolayer, thus their stability in real biological systems might be highly dynamic. However their ability to greatly perturb the underlying mechanical properties of biological membranes highlights how they might play a critical role in regulating the properties of membranes.

Materials and Methods

Lipids, subphase and superphase. DPPC and POPG were obtained in powder form from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. The lipids were dissolved in chloroform (HPLC grade, Fisher Scientific, Pittsburgh, PA) to make 5 mg/ml stock solutions. Lipid mixtures were prepared in a 7:3 DPPC:POPG molar ratio to mimic lung surfactant [9, 10, 7]. All solutions were diluted to obtain 0.1 mg/ml spreading solutions. The fluorescent probe used for visualization at 0.05 mol % with fluorescence microscopy was Texas Red, 1,2-dihexadecanoyl-sn-glycerol-3-phosphothanolamine (TR-DHPE) (Molecular Probes, Eugene, OR). For all Langmuir trough experiments, the subphase was ultrapure water (resistivity 18 MΩ−cm) processed by a Milli-Q ultra-purification set-up (A-10 gradient, Millipore, Bedford, MA), D2O obtained from Cambridge Isotopes (Andover, MA), or a wt/wt percentage water/glycerol or D2O/glycerol mixture. The glycerol used was spectrophotometric grade (Sigma-Aldrich, Missouri) with no further purification; however each batch was tested with surface tension measurements to assure no surface active contaminants were present. When fluorescence experiments were performed on monolayers containing POPG, ultra high purity argon (Airgas, Chicago, IL) was used to minimize oxidative damage to the unsaturated oleoyl chain and improve fluorescence; for x-ray experiments the superphase was ultra high purity helium (Airgas, Chicago, IL) to minimized oxidative radiation damage; and, in the case of neutrons the superphase was air.

Instrumentation. Details of the Langmuir trough set-up used for isoflurane collection as well as fluorescence imaging were discussed previously [26, 13]. Briefly, the home-build trough is mounted on a vibration isolation table (Newport, Irvine, CA) and allows for symmetric compression of the surface via two coupled teflon barriers; subphase temperature is maintained within 0.5°C of target (25 or 37°C for experiments in this paper). A stationary Wilhelmy balance (Regeler and Kirstein, Berlin, Germany) is used to measure surface pressure. Fluorescence imaging is performed using a 20X extra-long working distance objective (Nikon Y-FL, Fryer Company, Huntley, IL) with surface illumination carried out via a high-pressure mercury lamp (Osram Sylvania, Danvers, MA). Emitted light is gathered with a dichroic mirror/filter cube (Nikon Y-FL, Fryer Company, Huntley, IL) and collected at a rate of 30 frames/s using a CCD camera (Stanford Photonics Inc., Palo Alto, CA), and recorded on a Sony digital video cassette with a recorder (B-H Photo-Video, New York, NY).

Both at ChemMatCARS (Advanced Photon Source, Argonne National Lab) where XR, GIXOS, and GIXD were performed and at SPEAR (Lujan Center, LANLCE, Los Alamos National Lab) where NR was performed, the dedicated beamline Langmuir trough assemblies were used. The details of these set-ups have been discussed previously in [14, 15] for ChemMatCARS and in [19] for SPEAR. In short, both set-ups contain a trough trough, with one barrier capable of surface compression, surface pressure readings are measured with a Wilhelmy plate (both facilities have feedback loops capable of maintaining constant surface pressures), and subphase temperature control is possible.

The liquid surface spectrometer at ChemMatCARS is a versatile machine allowing us to perform specular XR, off-specular (GIXOS) reflectivity, as well pinhole GIXD [18]. In all experiments, the XR-rays used are produced by an undulator insertion device placed into the electron beam. The white XR-ray beam is modulated by optics upstream from the experimental hutch including a high heat load diamond monochromator. In the experimental hutch, the final x-ray energy (\( \lambda = 1.23960 \)) is selected using a Si(1 1 1) monochromator that is also used to steer the XR-ray beam onto the liquid surface. A pair of vertical (s1v) and horizontal (s1h) slits shapes the beam as it reflected from the steering crystal, defining the x-ray footprint on the lipid monolayer covered surface. For XR, the following slit settings were used s1h 2 µm and s1v varied from 0.02 to 0.2 µm during the experiment, for GIXD s1h 0.08 µm and s1v 0.12 µm, for GIXOS s1h 3 µm and s1v 0.12 µm. The reflected beam is collected in a fast pixel array detector PILATUS 100K [17, 18], which is mounted at the end of the output arm. Two sets of horizontal slits called s4h and s5h on the output arm select the acceptance angle of the detector; s4h and s5h are 8 µm and 24 µm, 0.2 µm and 0.2 µm, and 1 µm and 20 µm for XR, GIXOS, and GIXD, respectively. The spectrometer allows for the control of three angles important in defining the part of reciprocal space probed in a given experiment: \( \alpha \), angle between incident beam and monolayer surface; \( \beta \), angle between reflected beam and monolayer surface; and, \( \theta \), angle made between the detector and the long axis of the spectrometer (defined as the horizontal component of the incident beam). In XR experiments, \( \alpha = \beta \) and an angular range of 0.3 to 9 degrees was used, \( \theta = 0^\circ \). The same angular settings are used in GIXOS, with the exception that \( \theta = 0.3^\circ \). For GIXD, \( \alpha = \beta = 0.3^\circ \) and \( \theta = 16-18^\circ \). Further details about the geometry and configuration of the ChemMatCARS spectrometer can be found in Lin et al [16] and Meron et al [18].

The SPEAR neutron liquid surface spectrometer is a time-of-flight instrument using neutrons produced by spallation when high energy protons hit a tungsten target (the target is pulsed at 20 Hz) [20]. A liquid hydrogen moderator at 20 K and a pair of Fermi choppers select an energy band with wavelengths between 1 - 16 Å making SPEAR ideal for soft-matter atomic scattering [19]. For our experiments, the neutron beam is incident onto the monolayer covered liquid surface at a glancing angle of 0.9 degrees. The reflected neutrons are detected using a single 2HE linear position-sensitive detector.

For isothem, fluorescence, x-ray, and neutron experiments a similar method of preparing the lipid interface is followed. The Langmuir monolayer is spread at the (Ar,He,air)/water interface by drop-wise addition of the lipid spreading solution and allowed to equilibrate for 20 min on water and 60 min on glycerol mixtures to ensure evaporation of organic solvent and equilibration of interface. The barriers are then compressed with a linear speed of 0.1 mm/s and isotherm data in the form of surface pressure \( \Pi \) (mN/m) versus area per lipid molecule \( A^*/molecule \) are collected at 1-second intervals until the system reaches its compression limit (isotherms and fluorescence) or desired target pressure (x-ray and neutron scattering).

Image Tracking. Fluorescence images collected from the beginning of monolayer folding to the compression limit of the trough are used for further analysis. Typically this allows for four to five minutes of image collection or on the order of ten-thousand frames. The details of the image tracking program are given elsewhere [21]. The output of the program is the horizontal and vertical components of the monolayer displacement field. To distinguish different folding events and quantitate the amount of material displaced during each event, each vector component is processed in the following way. The data are first smoothed using a simple exponential moving average: \( x_i^f = \alpha x_i^{f-1} + (1 - \alpha) x_i \). Here \( x_i \) is the \( i \)-th data point from the displacement vector, \( x_i^f \) is the corresponding smoothed point,
and $\alpha$ is a smoothing parameter with $\alpha = 0$ implying no smoothing ($x_i' = x_i$), while $\alpha = 1$ being total smoothing where every point is equal to its preceding point and since by definition $x_i' = x_i$ every point would be equal to the first point. In analyzing our data, we use $\alpha = 0.5$. To $x_i'$ we further apply a simple step filter function:

1. $x_i' \geq c$ then
   
   $x_i' = x_i$

2. $x_i' < c$ then
   
   $x_i' = 0$

The cut-off for the displacement is set to $c \equiv 0.8$ pixels, this corresponds to the pixel limited resolution of the CCD camera. The filter allows us to easily sum the vector components of the displacement field that contribute to one folding event. We define the start of a folding event ($i = k$) as the first point where $x_i' \neq 0$, all subsequent consecutive points fulfilling this condition are considered part of the same folding event. The end of the folding event ($i = m$) is defined as the first point in the sequence where the above condition fails, i.e. $x_i' = 0$. The magnitude of the $n^{th}$ folding event is calculated as the running sum $F_n = \sum_{i=k}^{m} x_i'$

Reflectivity analysis. Reflectivity spectra provide information about the chemical composition of an interface with angstrom level resolution [22]. The general principles behind the experiment assume that the surface is composed of thin layers with finite thicknesses and indices of refraction. The variation in thickness and refractive index behind the experiment assume that the surface is composed of thin layers with finite thicknesses and indices of refraction. The fits presented in this paper were obtained, which will define the particular scattering signature of this layer.

Ultimately, the reflectivity detected is related to the refractive index of the layers, their thickness, and the angle of incidence via Snell’s law and the Fresnel equations [22]. By collecting the reflected radiation at multiple angles or wavevector transfer values ($q = 4\pi/\lambda \sin \alpha$), the reflectivity spectrum is generated. To extract physical parameters such as thicknesses and refractive indices from the spectrum, it must be modeled. In general, modeling proceeds by generating a function representing how electron density or scattering length density varies across the interface. The model density profile is then used to calculate a theoretical reflectivity. The calculated reflectivity is compared to the measured spectrum and if needed the model is modified to improve the fit. Below we outline the different modeling approaches we took for XR, NR, and GIXOS.

XR modeling. Due to the high brilliance of the x-ray beam at APS, high quality XR data are collected out to $q_x = 0.75$ Å$^{-1}$. The extended spectral range allows us to use a modeling approach (StochFit) developed at the University of Chicago where a stochastic Monte Carlo algorithm is used to search through the solution space of all possible electron density profiles [23]. Details of the method and the program have been published previously [23]. In short, StochFit generates an initial electron density profile using three input parameters: one, the average electron density of the interface (9.38 × 10$^{-6}$ Å$^{-2}$ for our systems); two, the electron density of the subphase (see below); three, an estimated upper bound to the total interfacial thickness (35-40 Å). StochFit takes the initial electron density profile and divides it into many boxes, typically on the order of 0.5-2 Å in thickness. The fits present in this paper were calculated using 80 boxes. The goodness of any particular model is determined by using the Parratt method ([22, 23]) to calculate a model reflectivity and calculating a $\chi^2$ value for the model reflectivity and the actual data. The benefit of this method is that it is a very thorough search of the parameter space is performed in an efficient and unbiased manner.

To calculate the electron density of our pure subphase components, we note that a water molecule has 10 electrons while a glycerol molecules has 50 electrons. Multiplying these values by 2.82 × 10$^{-10}$ Å (classical radius of an electron), and dividing by their molecular volumes 30 Å$^3$ (water) and 124 Å$^3$ (glycerol), gives the electron densities 9.37 × 10$^{-6}$ Å$^{-2}$ (water) and 11.36 × 10$^{-6}$ Å$^{-2}$ (glycerol). If the assumption of ideal mixing is applied to a water/glycerol mixture away from the interface, then the mixture electron density ($\rho_{mix}$) can be calculated as the weighted sum of the electron densities of the pure components ($\rho_i$) with weighting factors defined as the ratios of the partial mass density ($\rho_i^m$) of a component in the mixture divided by the natural density ($\rho_i^{nat}$) of that component:

$$\rho_{mix} = \sum_{i} \frac{\rho_i^m}{\rho_i^{nat}} \times \rho_i^m$$

Using equation 2, we calculate the electron densities of our water/glycerol mixtures: 9.7 × 10$^{-6}$ Å$^{-2}$ (20 wt% glycerol), 10.23 × 10$^{-6}$ Å$^{-2}$ (40 wt% glycerol), and 10.76 × 10$^{-6}$ Å$^{-2}$ (64 wt% glycerol).

NR modeling. Neutron beams unlike x-rays can not be bent nor are they as brilliant, the consequence of which is that our neutron reflectivity data extend only to $q_x = 0.12$ Å$^{-1}$. StochFit is not well equipped to handle data with such low spectral range. Moreover, the lack of a well defined critical angle (angle below which total external reflection occurs) for some samples calls for a more model depended approach in analyzing the NR spectra. Traditionally, lipid covered interfaces have been divided into two layers of uniform scattering length density corresponding to the hydrocarbon tails and phosphate headgroups [24]. The lipid layers sit upon an infinitely deep layer representing the bulk subphase. In the framework of this model each slab has three fitting parameters: scattering length density ($\rho_i$), length ($\ell_i$), and standard deviation of the the Gaussian used to convolute the interface between slab $i$ and $i + 1$ that is often referred to as the layer roughness ($\sigma_i$). Added to these parameters is the scattering length density and roughness of the infinitely deep subphase. Such simple ‘box models’ are based upon physical intuition about the structure of the interface and have been shown to model Langmuir layers well [22, 23]. We improve the reliability of this modeling approach by using our higher-order XR analysis to further constrain the parameter space over which the Parratt recursion and subsequent minimization are done within the framework of Matsufi [25]. In particular, the length of tail and headgroup boxes extracted from StochFit electron density profiles is used as a starting point in modeling the neutron data for both DPPC-d62 and DPPC-d75.

The box-model approach also requires that good initial guess values for the scattering length densities of the different layers be provided. A well established literature exists on how to perform such calculations [24], which involve summing up the scattering length densities of all atoms in the tail and headgroup boxes of one molecule and then dividing by the corresponding molecular volume fractions of the two layers. The molecular volume is obtained by using the projected lipid area per molecule from the isotherms, and assuming a rectangular volume element, multiplying the areas by headgroup and tail lengths measured with x-ray reflectivity. This procedure produces reasonably accurate averages for the tail layers, however the headgroup values are too low. Indeed neutron studies have shown that the presence of hydration shell atoms must be taken into account to accurately predict headgroup scattering length densities [8]. For the scattering length densities of the subphase an approach similar to the one discussed in the XR section is taken. This gives the following subphase scattering length densities: 6.35 × 10$^{-6}$ Å$^{-2}$ (D$_2$O) and 0.60 × 10$^{-6}$ Å$^{-2}$ (glycerol), while for the mixtures 5.3 × 10$^{-6}$ Å$^{-2}$ (20 wt% glycerol in D$_2$O) and 2.97 × 10$^{-6}$ Å$^{-2}$ (64 wt% glycerol in D$_2$O).

GIXOS modeling. The key to the GIXOS measurement is moving the detector slightly out of the specular plane to $\theta = 0.3$ degrees [18]. The intensity of the reflection falls sharply with increasing $\theta$ ($q_y = 4\pi/\lambda \sin \theta$). Moving the detector slightly away from the specular peak reduces the large dynamic range of the reflectivity intensity allowing the entire spectrum to be collected in one shot with a linear detector. In short, GIXOS measurements use the strong fluctuations of the surface to carry the surface structure factor in a region of the spectrum where Fresnel decay has greatly weakened. The difficulty lies in having to explicitly take the surface fluctuations into account, otherwise the data cannot be properly normalized. Assuming the surface fluctuations due to thermal capillary waves, the analytical form of these fluctuations is used to normalize the GIXOS reflectivity spectrum, thus returning the pure interfacial structure factor [22].

The structure factors were modeled using the reflectivity master formula [22]:

$$|F(q_x)|^2 = \frac{R(Q_x)}{R(Q)} = \int_0^\infty \left( \frac{dp}{dz} \right) e^{i q x z} dz$$

The master formula is valid only in regions where $q_x \gg q_s$ and so the fits were constrained to parts of the spectrum five times the critical $q_s$ values, which are calculated above for the two subphase conditions. The electron density profile is constructed based upon the simple box models discussed in the neutron section: a box of uniform density for the tails and one for the headgroups. There are three interfaces:
The amplitude reflectivity is obtained by taking the absolute square of equation 4.

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