Sugar induced membrane stiffening: the role of viscous fluid adlayers

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Lipid interfaces from cell membranes to the thin surfactant layers stabilizing lung alveoli are integral to living systems. Such interfaces are often subjected to mechanical forces and, due to their membrane-like geometry, can easily deform by bending into localized folds. In this paper, we explore the role of small molecule sugars like glycerol on the mechanical stability of model lung surfactant monolayers. We demonstrate that the presence of glycerol increases monolayer bending stiffness by up to 9 orders of magnitude. Our x-ray and neutron reflectivity measurements indicate that water is preferentially depleted, or that glycerol is preferentially enriched, at the lipid headgroup/solvent interface and that this glycerol enriched layer extends $O(10) \text{ Å}$ underneath the monolayer with an adsorption free energy of -2.5-4.6 kJ/mol. The dramatic change in membrane bending stiffness in the presence of the sugar adlayer is understood in terms of a continuum model of thin viscous boundary layers.

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Interfaces abound in living matter from tough outer coverings like skin, scales, and cell walls to softer ones such as lungs, blood vessels, and cell membranes. Proteins and biopolymers play a large role in the structure and function of these interfaces; however, the majority are largely composed of self-assembled lipid molecules. Roughly a few nanometers in length, cylinder like, and amphiphilic, lipids are nature’s liquid crystals providing a rich array of phases that can be tuned by temperature, pressure, and the composition of the medium in which they are embedded [1]. By changing lipid phase behavior - rendering the interface more fluid or more rigid - living matter can tune the structure, function, and mechanics of its varied interfaces [2, 3]. In this paper, we report on how lipid phase behavior and mechanical stability are highly sensitive to small sugars, which are richly abundant in the cellular environment.

Our study is motivated by the immense structural challenge posed to nature for adequate gas exchange in animals with lungs. To adequately exchange gas, the interfacial area of human lungs, for instance, must be large and easily expandable [4]. However, any such air/water interface is dominated by surface tension. Lung surface areas remain large and compliant because the effects of surface tension are minimized by a self-assembled film of lipids and proteins called lung surfactant (LS) [4]. Surfactant, while stabilizing lung tissue overall, is itself subjected to mechanical stress during the breathing cycle as lung surface area changes [2, 5]. What factors govern the response of a self-assembled lipid layer to mechanical stress is relevant not only to understanding LS but also to endothelial surfaces where lipid membranes are stretched and sheared by the pulsatile flow of blood. Moreover, virtually every epithelial lining must maintain not only global integrity but also local structure despite constant strain imposed by normal physiologic function. LS thus provides an excellent model system to study the mechanico-chemical stability of lipid films [2, 4–6].

Mechanical stability of a material is dependent on its structure and geometry. In lipid membranes, factors like surface pressure, temperature, lipid identity, and surrounding medium all play a role in the phase state, and hence structure of the membrane. An extensive literature exists on the phase behavior of different lipids as a function of pressure, temperature, and ionic strength [1]. The role of small sugary molecules on membrane structure has not been fully explored despite their supposed role in membrane stability, e.g. anhydrobiosis, and in disease processes such as diabetes [9–12] . In the case of LS, it is known that the alveolar lining fluid (ALF) is devoid of glucose through the action of
apical sodium-glucose co-transporters, yet the physiological role of this depletion is poorly understood [13–16]. The deleterious effect of high sugar levels on lung tissue is nevertheless well appreciated [17–20]. In particular, there is evidence that lungs become less compliant possibly due to a decrease in surfactant function in diabetics [17, 18, 20].

Glycerol is among the simplest carbohydrates and plays a crucial role in many biosynthetic pathways; in particular, it acts as a substrate in gluconeogenesis for generating glucose and in the synthesis of lipids. From a surface science vantage point, the availability of ultra pure glycerol, its complete miscibility with water and well-characterized physical properties of glycerol solutions make it an ideal model carbohydrate to work with. In this paper, we report that the stiffness of model LS monolayers becomes orders of magnitude greater on solutions of glycerol compared to pure water. Using x-ray and neutron reflectivity (XR and NR), we show that the perfect miscibility of water and glycerol breaks down in the adjacent layer of fluid, resulting in a glycerol enriched layer extending $O(10)$ Å beneath the interfacial lipid layer. To the best of our knowledge, this is the first experimental report of the existence of such de-mixing boundary layers for sugary molecules next to lipid layers and their profound impact on monolayer mechanical properties.

I. MATERIALS

A. Lipids, subphase and superphase

DPPC and POPG were obtained in powder form from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification as detailed in earlier publications [2, 3]. 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), D$_2$O obtained from Cambridge Isotopes (Andover, MA), or a wt/wt percentage water/glycerol or D$_2$O/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.
B. Instrumentation

Details of the Langmuir trough set-up used for isotherm collection as well as fluorescence imaging have been discussed previously [2, 3]. The details of the dedicated XR and NR beamline assemblies have been discussed previously in [44, 45] for ChemMatCARS and [48] for SPEAR. Configurations for XR and pinhole GID experiments, can be found in Lin et al [46] and Meron et al [47]. For isotherm, 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 (water) or 60 min (glycerol mixtures). Compression is carried out at 10 mm²/s until the system reaches its compression limit (isotherms and fluorescence) or the desired target pressure (scattering).

C. Image Tracking

Fluorescence images are collected from the beginning of monolayer folding until the compression limit of the trough is reached, and are used for further analysis. Details of the image tracking program have been given elsewhere [34]. The output of the program is the horizontal and vertical components of the monolayer displacement field. To distinguish different folding events and quantify 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: 

\[ x_i^f = \alpha x_{i-1}^f + (1 - \alpha) x_i. \]

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 \( (x_i^f = x_i) \), while \( \alpha = 1 \) being total smoothing where every point is equal to its preceding point and since by definition \( x_1^f = x_1 \), every point would be equal to the first point. In analyzing our data, we use \( \alpha = 0.5 \). To \( x_i^f \), we further apply a simple step filter function: if \( x_i^f \geq c \) then \( x_i^p = x_i \), however if \( x_i^f < c \) then \( x_i^p = 0 \). The cut-off for the displacement is set to \( c = 0.8 \) 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 \( x_i^p \neq 0 \), 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. \(x_i^p = 0\). The magnitude of the \(n^{th}\) folding event is calculated as the running sum \(\ell_n = \sum_{i=k}^{i=m} x_i^p\).

### D. Reflectivity Data Analysis and Modeling

Reflectivity spectra provide information about the chemical composition of an interface with angstrom level resolution [21]. To extract physical parameters such as thicknesses and refractive indices from a spectrum, it must be modeled [21, 49]. Due to the high brilliance of the x-ray beam at the APS, high quality XR data are collected for \(q_z\) values up to 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 [49]. See Supporting Material (SM) for further details.

Standard calculation was used to calculate the x-ray and neutron scattering length densities of our pure subphase components. Mixture SLD \((\rho_{mix}^s)\) can be calculated as a weighted sum of the SLD of pure components \((\rho_i^s)\) 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: \(\rho_{mix}^s = \sum_i \frac{\rho_i^m}{\rho_i^{nat}} \times \rho_i^s\). See SM for further details.

Our NR data extend only to \(q_z = 0.12\) Å\(^{-1}\). StochFit is not well equipped to handle data with such a low spectral range. Instead, we use the well validated box-model approach [21, 49]. We improve the reliability of our modeling by using the model independent XR analysis to further constrain the parameter space over which the Parratt recursion and subsequent minimization are done within the framework of Motofit [50]. In particular, the lengths of tail and headgroup boxes extracted from StochFit electron density profiles are used as a starting point in modeling the neutron data for both DPPC-d62 and DPPC-d75. See SM for further details.
II. RESULTS

A. Effect of Glycerol on Lipid Monolayer Folding

When highly compressed (as upon the end of exhalation), the locally flat LS membrane moves out of the interfacial plane into deep folds [2–5]. We study the folding of model LS monolayers composed of DPPC and POPG in a 7:3 molar ratio at 25° C on water and glycerol mixtures (see movies S1 and S2 in Supplemental Material). By tracking surface displacement fields of a monolayer undergoing folding, we measure the distribution of fold amplitudes as a function of glycerol content and time. On pure water, fold amplitudes for the model LS system span from 2 – 20 µm, with the most probable size being 2 µm followed by an anomalously broad decay in probability for larger amplitudes (figures 1A and 2). On a subphase containing 40 wt% glycerol, the most probable fold amplitude remains 2 µm; however, the probability distribution has an even broader tail spanning into the very high amplitude region. As figures 1B and 2 show, fold amplitudes several orders of magnitude larger than those seen on water are observed on glycerol mixtures. Moreover, changes in fold size distribution as a function of time are seen only on subphases containing glycerol (see figure S1). Monolayers on water fold rather homogeneously throughout as evidenced by the even distribution of events in figure 1A. However on glycerol, very high amplitude folds tend to occur at the beginning of collapse and the monolayer begins to respond nearly identical to that seen on water toward the end of collapse (see supplemental figure 1). Our prior work has shown that monolayer fold amplitude (ℓ) is proportional to the ratio between the elastic bending stiffness (B) of the lipids and the gravitational weight of the subphase fluid: ℓ ∼ (B/ρg)^1/4, with a proportionality coefficient O(1) [3, 5]. As the equation clearly relates fold amplitudes to mechanical properties of the system, data in figure 2 demonstrates that a lipid monolayer can be orders of magnitude stiffer with glycerol present in the subphase than on water alone. To understand the origin of this dramatic mechanical change, we probe the interactions between the surfactant lipids and the water/glycerol subphase at the molecular level.
B. Effect of Glycerol on Lipid Monolayer Isotherms

Figure 3A shows a set of 14 DPPC isotherms taken at 25° C on subphases with increasing amounts of glycerol; these measurements focus on changes in the lift-off areas with glycerol content. The area per molecule where surface pressure is first generated (called lift-off) corresponds to the minimal surface density where lipid molecules begin to interact [1]. The rightward shift of lift-off in figure 3A with increasing subphase glycerol indicates that the lipids begin interacting at increasingly lower surface densities. At such low densities, the largest contribution to lipid-lipid interaction comes from the subphase embedded headgroups, which includes the hydrophilic part of the lipid molecule and any associated solvation shell [1]. Assuming ideal surface mixing of the subphase components, the lift-off area on mixed subphases (a) is a linear combination of the lift-off areas on the two pure subphases (a$_{glyc}$ and a$_{h2o}$): $a = \theta \cdot a_{glyc} + (1 - \theta) \cdot a_{h2o}$, where $\theta$ is the mole fraction of glycerol in the solvation shell. Using our measured $\theta$ and the known mole fraction of glycerol in the bulk subphase ($x$), the adsorption isotherm for glycerol to a DPPC monolayer is constructed in Figure 3B; its convex shape indicates that glycerol is enriched in the DPPC solvation shell compared to its bulk concentration. Fitting to the Gibbs equation gives an equilibrium partition constant of 2.8 and an adsorption free energy of -2.5 kJ/mol ($2.5 k_B T$/molecule at 25° C). Also plotted is the surface excess isotherm ($\theta - x$ versus $x$) showing the largest concentration difference between solvation and bulk glycerol existing at $x = 0.3$ or 70 wt % glycerol. At higher compression, as the interaction between molecules become dominated by hydrocarbon tail-tail interactions, the large difference in Langmuir isotherms seen above diminishes. To probe the structure of the glycerol enriched solvation shells at higher lipid surface densities, XR and NR experiments were performed.

C. X-ray and Neutron Reflectivity

Figure 4 shows specular XR data for DPPC:POPG 7:3 at 30 mN/m at 25° C on four different subphases: water with 0, 20, 40, and 64 wt% glycerol. The XR spectra show a global shifting of the reflectivity fringes to lower values of the wavevector transfer in the $z$ direction, $q_z$, with the addition of glycerol into the subphase. Generally such a shift indicates an increase in the thickness of the interface [21]. The electron scattering length
density profiles which give the best fits to the measured reflectivities are shown in figure 4B. The density of the lipid tails and headgroups are rather similar 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 decays smoothly to the lower electron density water subphase. However, in presence of glycerol, there is approximately a 10 Å region, around \( z = 75 \), of elevated density between the headgroup and the subphase (see inset of figure 4B). Upon increase in the fraction of glycerol, this region becomes more pronounced and develops into a third layer separating the lipid headgroups from the bulk subphase. Since the electron scattering length density of glycerol (11.36\( \times 10^{-6} \) Å\(^{-2} \)) is higher than water (9.37\( \times 10^{-6} \) Å\(^{-2} \)), our data suggest that this layer is enriched in glycerol. The existence of this layer and its composition can be better determined from NR experiments performed on fully deuterated DPPC monolayers with a subphase containing \( \text{D}_2\text{O} \) and hydrogenated glycerol.

Figure 5 shows NR data for such systems on subphases containing \( \text{D}_2\text{O} \) with 0, 20, and 60 wt% glycerol. Since neutron scattering length densities (SLD) are highly sensitive to isotopic composition we can distinguish the relative amounts of the two subphase components within the glycerol enriched layer. The best-fit model on the \( \text{D}_2\text{O} \) subphase does not require the existence of an intermediate layer between the headgroups and the subphase. However, as glycerol is added, a layer with low SLD becomes necessary to fit the data. Previous data showed minimal H/D exchange between glycerol and \( \text{D}_2\text{O} \) [22], therefore the low SLD of this layer can be explained by enrichment in hydrogenated glycerol. Inverting the mixture density equation (see Materials and Methods), the amount of glycerol and \( \text{D}_2\text{O} \) in the enrichment layer can be determined. On both 20 wt\% (\( x = 0.05 \)) and 60 wt\% (\( x = 0.25 \)) glycerol, the enrichment layer contains three times more glycerol (\( \theta = 0.15 \) and 0.8) as compared to that in the bulk subphase.

A second set of data collected using DPPC molecules with only the alkyl tails deuterated (DPPC-d62) is shown in Figure S2 and the same trend is observed. In the case of a DPPC-d62 monolayer measured on a pure \( \text{D}_2\text{O} \) subphase, no intermediate layer between the headgroups and the subphase is required, but such a layer is necessary for subphases containing glycerol. It is important to note that best fits were obtained only when the headgroup of DPPC-d62 contained 2-3 \( \text{D}_2\text{O} \) molecules. Using the mole fraction of glycerol in the bulk subphase and our NR results, a glycerol adsorption isotherm can be generated similar
to that carried out for low lipid densities described above (see figure 3C). Our NR results hence unequivocally show the presence of a thermodynamically stable glycerol enriched layer extending 10 Å below the headgroups with an equilibrium constant of 6.6. Correspondingly, the adsorption free energy for glycerol is found to be -4.6 kJ/mol.

D. GID Probed Glycerol Induced Ordering

Figure 6 shows pinhole GID spectra for DPPC:POPG 7:3 as a function of temperature and subphase composition. At 25°C, the monolayer has in-plane crystalline order on both a water subphase and 64 wt % glycerol. Using the standard relation \(d\)-spacing \(= 2\pi/\theta_{xy}\), the lattice packing on water and glycerol at the lower temperature is quite similar: 4.24 Å and 4.18 Å, respectively, with a slightly higher packing seen on the glycerol subphase. Upon heating to 37°C, no crystalline order remains in the monolayer on water as indicated by the lack of a Bragg peak. However, crystallinity is retained on the glycerol solution. Note that at higher temperature on glycerol, the left shift of the Bragg peak gives rise to a \(d\)-spacing of 4.21 Å, indicating that the lattice opens up.

III. DISCUSSION

Our isotherm data show a right-ward shift in lift-off area for DPPC on glycerol solutions. This is in agreement with much earlier isotherm-based studies of glycerol solutions [9, 12]. The conventional interpretation of such isotherm shifts is that glycerol exerts a fluidizing effect on the lipid monolayer, because of their similarity to isotherm scaling with increasing temperature. In light of the condensing and stiffening effects we observe at higher packing densities, we provide an alternative to this ‘fluidizing’ model. By allowing for free diffusion of glycerol or water molecules into and out of the solvation shell, the right-ward isotherm shifts with increasing glycerol can be accounted for by the increasing size of the solvation shell as bulkier glycerol molecules are incorporated. Moreover, the non-linear shift in lift-off area with glycerol concentration is explained by a preferential enrichment of glycerol in the solvation shell even at low surface densities, which provides thermodynamic evidence \(\Delta G_{\text{ads}} = -2.5\) kJ/mol) for the earlier hypothesis that carbohydrates might compete with and replace water in the lipid headgroup region by hydrogen bonding with its polar moi-
eties [12]. More recent monolayer work with DPPC on fructose and sucrose using a 2D Clausius-Clapeyron equation shows that the presence of subphase carbohydrates leads to the formation of up to six additional hydrogen bonds per lipid molecule [23, 24]. Their interpretation, based on the change in latent heat for the liquid-expanded to condensed phase transition as a function of subphase composition, is that carbohydrate molecules bind to the hydration shell of DPPC but do not actually displace water from the headgroup. Our NR data supports the idea that the hydration shell retains 2-3 water molecules per lipid headgroup (as on a pure water subphase) despite high subphase concentrations of carbohydrate.

Most of the previous work on lipid/carbohydrate systems has focused on the effect of sugars at low lipid surface densities. Our isotherm data, in agreement with literature, show little change as a function of subphase composition at higher densities. Furthermore, GID for DPPC on fructose and sucrose shows that once the lipid enters the condensed phase, the in-plane lipid packing structure is not influenced by the presence of subphase carbohydrate [23, 24]. Our data further show very minimal change in d-spacings for model LS lipids on water or glycerol solutions at 25° C. However at physiologic temperatures, the presence of glycerol prevents the melting of in-plane ordered structures, signifying that glycerol exerts a significant film stabilizing effect even at high lipid packing density.

The XR and NR data show that at packing densities well into the lipid condensed phase, the lipid/subphase interface on glycerol-containing subphases remains substantially different from that on pure water, with a ~ 10 Å adlayer enriched in glycerol (θ ≈ 3x). The thermodynamic driving force for such de-mixing is the surface energy difference between water and glycerol: $h \sim (k_B T)^{-1} \left[ (\mu_{\text{glyc}} - \mu_{\text{glyc}}^s) - (\mu_{\text{D2O}} - \mu_{\text{D2O}}^s) \right]$, where μ is the chemical potential of each species in the bulk and at the surface (s superscript) [25–27]. This surface energy reduces to the difference in the surface tension of the two subphase components. When such difference is ≥ 6 mN/m, a binary liquid is considered to exist in the strong field regime with saturation of the lower surface tension component at the surface [26]. P.G. de Gennes was the first to consider how such concentration gradients would change as one moved away from the surface, proposing the algebraic scaling law $\theta(z) - z \sim (z/\xi)^{-1/2}$ where z is distance from surface and ξ is the bulk correlation length of the binary mixture [25]. Taking ξ as the cube root of the glycerol molecular volume ($\xi \sim 5$ Å), the predicted surface concentration should approach the bulk within 10-15 Å, in agreement with our measurements.
At low lipid densities, the surface field driving de-mixing and glycerol enrichment has contributions from glycerol-air, water-air, glycerol-lipid, water-lipid, and glycerol-water interactions. Without lipids at the interface, the surface field is approximately the difference in pure water-air and glycerol-air surface tensions, which, at 25° C, comes to 8 mN/m, placing a water-glycerol solution in contact with air into the strong field regime. Spectroscopic work on pure glycerol-water/air interfaces has shown an enrichment of glycerol at the interface [28–30]. Moreover, they have obtained a glycerol surface excess free energy of $-1.5\text{--}3.5 \text{ kJ/mol}$, in agreement with our isotherm-derived adsorption free energy at low lipid density [28]. The question of whether the dominant surface field contributions are from glycerol/air or glycerol/lipid interactions becomes irrelevant at higher lipid densities because the hydrophobic lipid tails screen any subphase liquid/air contact. A parsing of the molecular contributions to the surface tension in a lipid-covered interface becomes exceedingly complex; however, molecular dynamics simulations of DPPC bilayers in the presence of trehalose and sucrose show sugar enrichment near the headgroups and strong hydrogen bonding between sugar hydroxyls and the lipid phosphate [31, 32]. These simulations are in qualitative agreement with the above mentioned thermodynamic data of Krasteva et al. [23, 24]. Structurally, glycerol, like the disaccharides, has a carbon backbone enriched in hydroxyl groups capable of hydrogen bonding, making it possible for our experimentally observed water/glycerol de-mixing to be driven by the energetics of glycerol/phosphate hydrogen bonding.

Our XR and NR data on DPPC and DPPC:POPG 7:3 lipid monolayers on subphases of water and water/glycerol provide the first experimental evidence for the existence of carbohydrate enrichment and water exclusion in a thin layer adjacent to the phospholipid headgroups. Above, we show that the existence of this layer is consistent with binary fluid de-mixing next to a surface. We now turn to understanding how the presence of this adsorption layer alters the lipid membrane mechanical response. The size distribution of folds in our model LS system on water is in agreement with previous studies [2, 34]. By inverting the scaling law for folding introduced in the results section, we can measure the distribution of monolayer bending stiffnesses: $B = \left(\frac{\ell}{2\pi}\right)^4 \times \rho g$, where $\rho$ is subphase density, $\ell$ is fold amplitude, and $g$ is the acceleration of gravity [3, 5]. On water, the distribution of fold sizes gives bending stiffness of $O(0.1 \text{--} 10 \text{ kT})$, in agreement with diffuse x-ray scattering measurements of condensed monolayer stiffness [5, 33]. Incredibly on glycerol solutions, the measured bending stiffness in any one monolayer spans over nine orders of magnitude,
We focus the following analysis not on the largest or the rarest folds, but rather on the 20 $\ell \leq 40 \, \mu m$ folds with $B \sim O(1000 \, kT)$ commonly seen on glycerol containing subphases but never on water (see figure 2). As a membrane folds, the principle forces generated are bending moments arising from the variation of stress across the membrane:

$$M \approx \int_{-t/2}^{t/2} \sigma_{xx} dz,$$

where the integration of the in-plane stress tensor $\sigma_{xx}$ is carried out across the membrane thickness $t$ [8, 35–38]. Treating the lipid monolayer as an incompressible elastic solid ($\sigma_{xx} \sim E u_{xx}$) and the glycerol adlayer as an incompressible Newtonian fluid ($\sigma_{xx} \sim \eta u_{xx}/d\tau$), the corresponding bending moments are $M_{\text{lipid}} \sim Et^3 \times \kappa$ and $M_{\text{adlayer}} \sim \eta t^3 \times d\kappa/d\tau$, where $\kappa$ is curvature and $u_{xx}$ are in-plane strains. By definition, the respective bending stiffnesses are $B_{\text{lipid}} \sim Et^3$ and $B_{\text{adlayer}} \sim \eta t^3/\tau$, where $E$ is the lipid monolayer Young’s modulus, $\eta$ is glycerol viscosity within the adlayer, and $\tau$ is the characteristic time scale over which the fold forms. Assuming the bending stiffness that enters the fold scaling law is to first order $B \sim B_{\text{lipid}} + B_{\text{adlayer}}$, the subsequent discussion focuses on the interfacial physics that influences the cohesion of the lipids and glycerol.

The magnitude of the Young’s modulus is a measure of the cohesive strength between lipid tails. While our GID data show that on glycerol the packing of DPPC hydrocarbon tails is slightly denser than on water, it is unlikely that this small density change could lead to a 100-fold increase in cohesive strength. Another intriguing possibility is that the glycerol adlayer has an antiplasticization effect on the lipids. Recent work with glassy polymers showed that antiplasticization not only increases local elastic moduli but render the distribution of moduli across the material more heterogeneous [39]. The increase was shown to be less than 10 fold, thus possibly increasing the overall bending stiffness of the monolayer from $O(10 \, kT)$ to $O(100 \, kT)$.

Using our measured adlayer thickness of 10 Å and $\tau \sim O(100 \, \text{ms})$ from the fold analysis, $B_{\text{adlayer}}$ approaches 1000 kT only if its viscosity diverged to $10^{10} \, \text{Pa} \cdot \text{sec}$, which is equivalent to its vitrification [40]. The glass transition temperature of bulk glycerol is -80°C [40], however when confined in thin layers its $T_g$ can increase [41–43]. Work with glycerol in nano-porous silica shows that increases in glycerol glass transition temperature in such geometries is a function of strong hydrogen bonding between the glycerol hydroxyls and those of the silica surface [41, 43]. Moreover, the dynamically supercooled interfacial layer is shown to extend on the order of 10 Å from the silica surface [41, 43]. In light of this literature, it is plausible
that the large folds seen with LS on glycerol solutions are due primarily to the glassy nature of the adsorbed glycerol owing to its strong hydrogen bonding with the phosphate headgroups. This again points to the nature of the glycerol/lipid interaction. Interestingly, this interaction drives both the de-mixing of the glycerol/water solution adjacent to the lipid headgroups and dramatically slows the dynamics within the glycerol layer, which ultimately affects the mechanics of the entire lipid interface and its response to lateral stress.

We now return to the role of small carbohydrates in biological systems. While our work has been with the simplest of these, glycerol, the similarity of our structural data with simulations [31, 32] and preliminary experiments in our lab with sucrose and glucose showing similar behavior allow the conclusion that most small, strongly hydrogen-bonding molecules should follow trends presented in this report. Given the dramatic impact that carbohydrates can have on the mechanical stability of LS, it is possible that the reason nature keeps glucose concentrations low in the ALF is to prevent the large scale folds described here. Such large scale folds could potentially lead to a very rapid depletion of surfactant from the alveolar interface, leading to increased work of breathing. Lipid membrane mechanics [2, 5, 6], cell mechanics [51], mechanical stresses impacting cell differentiation and signaling [52–54] are all evolving if not exploding fields. In this work, we add viscous adlayers to the array of physical fields like composition, pressure, and temperature that nature has at its disposal to shape the structure and mechanics of lipid membranes.

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**Figure 1:** Displacement field seismogram for fold formation on (A) pure water subphase and (B) 40 wt% glycerol subphase (red and black indicate two separate monolayers). The y-axis is the amount of material displaced in the direction parallel to compression ($\vec{x}$) during a given folding event and on the x-axis is a running time log with 0 corresponding to the first folding during collapse.

**Figure 2:** Distribution of folding events represented in figure 1 for water (black) and 40 wt % glycerol (red) subphases. The largest number of events (N) occur for $\ell \leq 10\,\mu$m, where the data for water and glycerol overlap rather well. Beyond this point, the number of folding events on water decays to zero with no events recorded for $\ell \geq 20\,\mu$m, while on the glycerol subphase large amplitude events continue to occur.

**Figure 3:** (A) DPPC isotherms on increasing amounts of glycerol from 0 wt% (mole fraction $x = 0$), including 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100 wt% ($x = 1$), with isotherms shifting to the right and lift-off occurring at greater areas per molecule. (B) Adsorption isotherms based upon the $\Pi$-$A$ isotherms in (A). $\theta$ is mole fraction of glycerol at surface and $\theta - x$ the amount of excess glycerol at surface. Points on the adsorption isotherm are generated using the lift-off area per molecule (measured when $\Pi=0.5\,\text{mN/m}$) and an ideal surface mixing model. The adsorption isotherm shows that an excess amount of glycerol is present on the surface. The equilibrium constants for the adsorption of glycerol to the interface (K) are measured by fitting the data to a Gibbs adsorption isotherm (black) and excess adsorption isotherm (red). (C) Adsorption isotherms constructed from NR data for DPPC-d62 and DPPC-d75. The scattering length density of the boundary layer from the best fit profiles is used to back calculate the mole fraction of glycerol ($\theta$) and D$_2$O ($1-\theta$) in the layer. The points at $x = 1$ are not measured but assumed based on conservation of mass. The data are fit to Gibbs adsorption isotherms (black formula) and the excess adsorption isotherm (red).

**Figure 4:** (A) XR spectra for DPPC:POPG 7:3 on water (blue), 20 wt% (magenta), 40 wt% (black), and 64 wt% (red) glycerol. The x-axis $q_z$ normalized by the critical $q_c$ calculated for each subphase mixture. The y-axis is the reflectivity intensity multiplied by $(q_z/q_c)^4$. The arrows indicate the overall shift of the spectra to lower $q$ values with the addition of glycerol. (B) X-ray SLD of best fits to data in (A) generated via StochFit. Yellow region: lipid tails; blue region: headgroups; boxed region: subphase adjacent to the monolayer. The most notable difference in the profiles on the four subphase conditions occurs in a boundary
layer region extending ten angstroms underneath the headgroups. As the inset in (B) shows, on water there is a smooth connection between the electron rich phosphate headgroup layer and the bulk subphase, however with increasing amounts of glycerol a more electron dense third layer appears.

**Figure 5:** NR data for DPPC-d75 at Π=30 mN/m (25° C) on (A) pure D₂O subphase, (C) 20 wt% glycerol/D₂O, and (E) 64 wt% glycerol/D₂O. (B), (D), and (F) show scattering length density profiles calculated using a three-box model with the Parratt method. The best fit models (solid red in the profiles) give rise to the solid black model reflectivities. The dashed profiles are for boundary layers whose composition is identical to that of the bulk subphase. In (C) and (D), the ratio of χ² values of the dashed model to the best fit is 1.23, and similarly for (E) and (F) it is 16.6. See SI Neutron Reflectivity Data for all values.

**Figure 6:** Pinhole (GID) spectra for DPPC:POPG 7:3 (Π= 30 mN/m) on (A) water and (B) 64 wt% glycerol at 25 and 37° C. The presence of a Bragg peak for both subphases at the lower temperature (red) indicates that in-plane crystalline order exists within the monolayer at both conditions. At 37° C (blue), the monolayer on water melts, losing its Bragg peak while the monolayer on the glycerol mixture retains its signature of crystalline order.
FIG. 1:

FIG. 2:
FIG. 3:

FIG. 4:
FIG. 5:

FIG. 6: