Adsorption of Egg Phosphatidylcholine to an Air/Water and Triolein/Water Bubble Interface: Use of the 2-Dimensional Phase Rule To Estimate the Surface Composition of a Phospholipid/Triolein/Water Surface as a Function of Surface Pressure

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Phospholipid monolayers play a critical role in the structure and stabilization of biological interfaces, including all membranes, the alveoli of the lungs, fat droplets in adipose tissue, and lipoproteins. The behavior of phospholipids in bilayers and at an air−water interface is well understood. However, the study of phospholipids at oil−water interfaces is limited due to technical challenges. In this study, egg phosphatidylcholine (EPC) was deposited from small unilamellar vesicles onto a bubble of either air or triolein (TO) formed in a low-salt buffer. The surface tension (γ) was measured using a drop tensiometer. We observed that EPC binds irreversibly to both interfaces and at equilibrium exerts ∼12 and 15 mN/m of pressure (Π) at an air and TO interface, respectively. After EPC was bound to the interface, the unbound EPC was washed out of the cuvette, and the surface was compressed to study the Π/area relationship. To determine the surface concentration (Γ), which cannot be measured directly, compression isotherms from a Langmuir trough and drop tensiometer were compared. The air−water interfaces had identical characteristics using both techniques; thus, Γ on the bubble can be determined by overlaying the two isotherms. Both TO and EPC are surface-active, so in a mixed TO/EPC monolayer, both molecules will be exposed to water. Since TO is less surface-active than EPC, as Π increases, the TO is progressively ejected. To understand the Π/area isotherm of EPC on a TO bubble, a variety of TO-EPC mixtures were spread at the air−water interface. The isotherms show an abrupt break in the curve caused by the ejection of TO from the monolayer into a new bulk phase. By overlaying the compression isotherm above the ejection point with a TO bubble compression isotherm, Γ can be estimated. This allows determination of Γ of EPC on a TO bubble as a function of Π.

Introduction

Phospholipid monolayers play a critical role in biology by stabilizing the interface of two immiscible phases. Due to their amphipathic nature, phospholipids lower the interfacial tension (γ) between two immiscible phases, allowing both emulsification and extension of the surface area. Phospholipids contain a hydrophilic headgroup, which in some cases is charged, and two hydrophobic fatty acid chains. Two important biological interfaces that are stabilized by a monolayer consisting predominately of phospholipids are an air−water (A/W) interface in alveoli and triacylglycerol (TAG)−water (TAG/W) interface on lipid droplets; for example, adipose tissue and extracellular lipoprotein particles.[1−3] The focus of this study is to understand the behavior of PC at a TAG/W interface and use the A/W interface as a model system to test our methodology. The phospholipids of most biological emulsions, such as egg yolk droplets, fat droplets, and triacylglyceride-rich lipoproteins are PC-rich and have an unsaturated acyl chain in the sn-2 position, which keeps the acyl chains in the liquid state at ambient temperature.

Biological PC having at least one unsaturated chain form a gaseous state at a low surface concentration (Γ). As the surface density increases, the phospholipid monolayer condenses to a liquid state. The liquid state has a compressibility $[-(1/A)(\delta A/\delta\pi)_T]$ which is significantly lower than the gaseous state due to the fatty acid chains interacting with one another. As the surface is compressed, there is less available area per molecule and the fatty acid chains align roughly parallel to one another but are still in the liquid state. When the chains are compressed to a minimum area, further compression expels phospholipid from the surface, forming a new bulk phase of multiple layers, similar to a lamellar liquid crystal.[4] As a liquid crystal, the fatty acids chains remain fluid, are roughly parallel to one another, and are approximately perpendicular to the plane of the water interface.[4] This is an irreversible (or very slowly reversible) change to the monolayer and is called the collapse point.[5]

Triacylglycerols (TAGs) are important energy storage molecules composed of three fatty acid chains esterified to a glycerol. TAGs are both ingested as fat and produced in body tissue. At physiological temperatures, TAGs are in a liquid state and are stored principally in adipose tissue. Since TAGs are not soluble in water, they must be packaged into lipoprotein particles for transport and compartmentalized within adipose tissue for storage and utilization.[3,6] Phospholipids, as well as protein and cholesterol, are present on the surface of TAG droplets and prevent coalescence of lipid particles by reducing the γ. Although TAGs are hydrophobic molecules, they have a surface solubility and positive spreading pressure of ∼12 mN/m. They interact with the aqueous surface by rearrangement of their fatty acid chains from a tuning fork conformation in liquid oil to a conformation in which all three ester groups are at the aqueous interface and the 3 chains extend upward into the air or oil.[4]

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The phase rule is used to predict equilibrium phases in multicomponent systems. The phase rules states that the degrees of freedom (F) of a system are equal to the number of components (C) minus the number of phases (P) plus 2 intensive variables (i.e., temperature and pressure), summarized as[7]

$$F = C - P + 2 \quad (1)$$

If temperature and ambient pressure are fixed, then the phase rule simplifies to

$$F = C - P \quad (2)$$

When one or more components are confined to an interface, which is generally the case for long-chain phospholipids on water,[4] the interface becomes equivalent to a separate phase. To examine phase changes of insoluble monolayers, a 2-dimensional phase rule has been derived for gas/liquid and liquid/liquid interfaces by Crisp.[8] At constant temperature and ambient pressure, the 2-dimensional phase rule for a system with a single surface simplifies to

$$F = C - P^S - P^B + 1 \quad (3)$$

where P^S and P^B are the number of surface phases and bulk phases, respectively, and C is $C^B + C^S$, where C^B is the number of components equilibrated through the system and C^S is the number of surface confined components. The resultant equation (eq 3) is similar to the classical phase rule (eq 2) with a slight modification to account for an independent intensive variable linked to the free energy of the surface. Since the surface free energy is related to the interfacial pressure, the surface pressure (Π) can be used as an intensive variable to define surface energy.

Compression isotherms of surface films composed of two surface components can be classified into two types: 2-dimensional immiscible and miscible films. An immiscible film occurs when two surface-active molecules are immiscible in 2-dimension and separate into two surface phases. In the liquid state, there will be only one degree of freedom ($C = 3$, $P^B = 1$ (bulk liquid), $P^S = 2$ (two immiscible surface phases)). As the surface is compressed, there will be an abrupt change in the slope of the Π/area curve called the envelope point, which occurs at the collapse pressure (Π_C) of the less surface-active molecule. At the envelope point, which is equivalent to the Π_C, the less surface-active molecule begins to be expelled from the monolayer, forming a new bulk phase, regardless of the starting composition. Further compression has little effect on Π as the less surface-active molecule is being expelled; thus, $F = 0$ because P^B is now equal to 2. After the interface has been depleted of one molecule, P^S now becomes 1 and $F = 1$; thus, compression will cause the Π to rise until the collapse point of the second component is reached. An example of this type of film would be a mixture of polyvinyl acetate and polyvinyl alcohol and possibly a film of cholesterol and triolein (TO).[9]

A miscible film is made when two surface-active molecules are spread at low Π, forming a single homogeneous surface phase. When the monolayer is in the liquid state, it will have 2 degrees of freedom ($C = 3$ (2 surface components, 1 bulk phase), $P^B = 1$ (bulk phase), $P^S = 1$ (homogeneous surface phase)). The envelope point occurs at Π higher than the Π_C of the less surface-active molecule and corresponds to the initial formation of a new bulk phase. At the envelope point, $P^B = 2$; thus, $F = 1$. Compression beyond the envelope point causes the Π to rise, resulting in more molecules' being expelled from the surface and incorporated into the new bulk phase.

The Π and area that defines the envelope point depends on the composition of the monolayer. As the starting composition of the spread mixed layers becomes enriched in the more surface-active component, its envelope point will be at a higher Π and lower area. *In fact, the composition of the spread monolayer defines the composition at the envelope* Π *and area.* It follows, then, that the common curve that connects all the envelope points can be used to define the monolayer composition. By fixing Π (or area), the system is invariant, and the composition can be deduced. At a higher Π, there will be an abrupt second change in slope, called the collapse point, which indicates the final collapse of the monolayer. This type of behavior has been observed for mixed fluid monolayers of various triacylglycerols and diacyl phosphatidylcholines (PCs), as well as mixtures of diacylglycerols and PCs.[10,11]

Studies of fluid−fluid interfaces, such as a TAG−water interface, have been limited because of difficulties with traditional surface chemistry techniques. The advent of high-speed charged coupled device (CCD) cameras and modern computing have made oil-drop tensiometry a practical alternative to a Langmuir trough for fluid−fluid interfacial studies.[12−16] In this technique, a drop (or bubble) of either a gas or liquid is made at the tip of a needle attached to a syringe, which is submerged in water (Figure 1). The profile is recorded by a CCD camera, which records the bubble volume, area, and γ (Figure 1). Surface-active molecules, such as peptides and lipids, can be injected into the buffer, and γ followed as they adsorb. The syringe is attached to a motor, which changes the bubble volume, allowing compressions, expansion, and oscillations of the volume, and therefore, the surface area.

Three techniques have been used to deposit phospholipids on a bubble surface: (1) A drop of organic solvent containing phospholipid is made in water.[17,18] The phospholipid in the solvent forms a monolayer on the surface, which allows the study of phospholipids at the organic solvent−water interface. (2) A small amount of organic solvent containing phospholipid is deposited by syringe at the surface of an air bubble formed in water where the phospholipids spontaneously spread.[19] When DPPC is spread by this method on an A/W bubble interface, the characteristic liquid expanded/liquid condensed conversion[5] is absent or severely dampened, indicating that chloroform may remain in the interface.[20] (3) Phospholipid-TAG emulsions (generally Intralipid (a soy bean oil emulsion) that has been enriched in phospholipid via centrifugation) is injected into the bulk, and then the phospholipid transfers slowly to the bubble surface with time.[16,21,22] This technique has not been thoroughly examined and introduces additional lipids in the emulsion to the system, which could confound the experiment.[23] In all these techniques, additional components are added to the system, which might interfere with the molecule of interest, making the experiments more complex and less biologically relevant. In this study, we introduce a modified technique to deposit phospholipid, particularly EPC, to an A/W and TO/W interface using small unilamellar vesicles (SUVs). Then by applying the Gibb's 2-dimensional phase rule (eq 3),[8] phase changes can be observed in the monolayer and the Γ can be estimated.

Methods and Materials

Phospholipid Preparation. Chicken egg L-α-phosphatidylcholine (EPC) was used in all the experiments (Avanti Polar Lipids Inc.; lot: EPC-522). The sample was dissolved in chloroform and stored at −20 °C at a concentration of 21.4

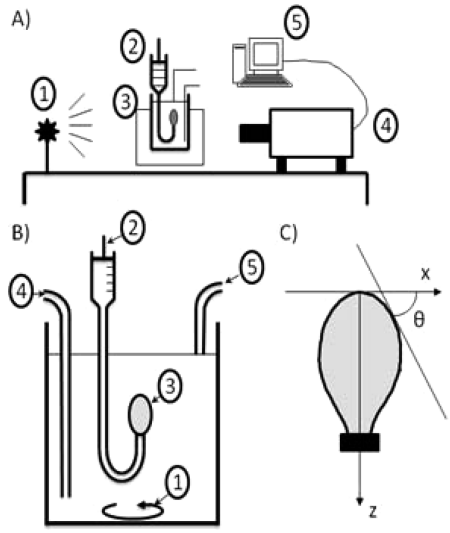

Figure 1. Diagram of a drop tensiometer shows the basic features of the apparatus. (A) The bubble outline is illuminated by a light source (1). The bubble is made at the tip of a J-tube connected to a syringe filled with oil or air (2). This is submerged in a thermostatted cuvette filled with 2 mM sodium phosphate buffer (3, see Figure 1B for more details). The volume of the bubble can be adjusted by a motor attached to the syringe, which automatically makes changes in the volume >0.02 μL (2). The profile of the bubble is collected by a high-speed CCD camera (4) which outputs to a computer that reports the surface tension (γ) according the Laplace equation adapted for a bubble (5). (B) An enlarged image of the thermostatted cuvette shows important details. The cuvette is filled with buffer and is stirred by a magnetic stir bar (1). The volume of the bubble is controlled by a motor attached to the syringe (2), which can make small, fast, or calibrated slower changes to the volume of the bubble (3). To exchange the buffer, a tube attached to a reservoir of lipid-free buffer was placed near the bottom of the cuvette (4), and a tube attached to a vacuum was placed directly on the surface of the water to remove buffer as the buffer level rose (5). (C) The bubble profile is analyzed according to the Laplace equation adapted for a bubble.[12,16] The *x*- and *z*-axes are the Cartesian coordinates. Θ is the angle between the tangent to the bubble and the *x*-axis, and b is the radius of curvature at the point of the tangent. This figure was adapted from Labourdenne et al.[16] and Benjamins et al.[12]

mg/mL. For Langmuir trough experiments, the EPC was diluted in chloroform (Fischer Scientific; ACS grade) to a final concentration of 1 mg/mL. The concentration was confirmed by the dry weight of the lipid. For drop tensiometry experiments, the EPC was constituted into SUVs. The SUVs were made using sonication according to the protocol of Jiang et al.[24] To confirm the size of the vesicles, some of the sample was observed with an electron microscope after being negatively stained with phosphotungstic acid. Over 300 particles were measured, and a particle diameter of 230 $\pm$ 44 Å (mean $\pm$ SD) was found, which is characteristic of SUVs. The purity of the EPC SUVs after both of these procedures was confirmed using high dose thin layer chromatography.

Langmuir Trough Experiments. For all Langmuir trough experiments, a dual barrier minitrough (KSV; Helsinki, Finland) with dimensions of 35 cm $\times$ 7.5 cm was used. Π was measured with a Wilhemy plate (2 cm $\times$ 1 cm) attached to a KSV microbalance. All experiments were performed at a temperature of 22.8 $\pm$ 0.6 °C. Two sets of experiments were performed. In the first set of experiments, a compression isotherm of EPC or TO at an air/water (A/W) interface was collected. The experiment was performed by slowly dripping 10 μL of 1 mg/mL EPC/chloroform or TO/chloroform solution from the tip of a 10 μL Hamilton syringe onto the surface of the bulk phase (2 mM PB, pH 7.4). Solvent was allowed to evaporate for 10 min, then the surface was compressed at a rate of 3.75 cm^2/min from an area of 260 cm^2 to 50 cm^2. This same experiment was repeated 10 times. In some experiments, the surface was reexpanded to test the reversibility of the isotherm.

In the second set of experiments, the properties of a mixed film of EPC and TO were observed. For these experiments, TO (NU-CHEK PREP Inc.; Elysian, MN; lot T-235-JY9-S, > 99.0% pure) and EPC were dissolved in chloroform at molar ratios of 0.2:1, 0.5:1, 1:1, 2:1, and 3.5:1 (TO/EPC) and were spread at an A/W interface. The bulk buffer was 2 mM PB, pH 7.4. At molar ratios >1:1 (TO/EPC), only 5 μL was deposited because of the higher lipid concentration; otherwise, 10 μL was deposited. The surface was then compressed at a rate of 3.75 cm^2/min from an area of 260 to 40 cm^2. This experiment was repeated five times at each molar ratio with similar results. In some experiments, the surface was reexpanded to test the reversibility of the isotherm. In other experiments, a 1 μL drop of pure TO was added at an interface at a starting ratio of 3.5:1 TO/EPC at either a Π of 20 or 5 mN/m. During some experiments, a small amount of talc was added to the surface as a light jet of air was blown on the surface to confirm that the surface remained fluid through-out the experiment.[25] The talc powder had no effect on the Π during the compression.

Drop Tensiometer Experiments. Drop tensiometry is a surface chemistry technique that is well-suited for studying biological molecules (see the Introduction and Figure 1 for a description of the technique). In these experiments, γ was measured using an I.T Concept (Longessaigne, France) Tracker oil-drop tensiometer.[16] In all experiments, the bulk buffer was 2 mM PB, pH 7.4. The temperature for all experiments was 25.0 $\pm$ 0.1 °C. The experiments were conducted by injecting 500 μg of EPC SUVs into the bulk buffer, after which a bubble was made. The bubble was made of either TO or air, depending on the experiment. The system was then left for more than 10 000 s for equilibrium to be approached. After >10 000 s, the bulk buffer was exchanged with 2 mM PB, pH 7.4, that was devoid of EPC. This was done by flowing 250 mL of lipid-free buffer through the cuvette over the course of ~600 s. The volume of the cuvette was kept constant by placing a needle attached to a vacuum directly at the surface (Figure 1). This process removed >99.9% of the original buffer (unpublished results). After the exchange, the volume of the bubble was changed so that the relationship between the area and Π could be established. This was done in 3 ways: (1) constantly changing the volume by $\pm$0.02 μL/s, (2) oscillating at a period of 128 s (0.0078 Hz), and (3) stepping up or down and holding the volume constant.

For the A/W system, the initial γ was 72 mN/m, and the volume was initially 8 μL. The Π_C for EPC at an A/W interface was about 40 mN/m. Because of this, the reversibility was checked both above and below Π = 40 mN/m. For the TO/W system, the initial γ was 32 mN/m, and the bubble volume was initially 16 μL. When the γ was lowered below ~4 mN/m, the bubble released, giving an effective range of Π between 0 and 28 mN/m, which could be examined.

Results

Compression Isotherm of EPC and TO at an A/W Interface Using a Langmuir Trough. The Langmuir trough experiment, shown in Figure 2A, shows a typical compression isotherm of EPC at an air−water interface. At a large area per molecule (~170 Å^2/molecule), the EPC exerted a very small force on the surface, which was measured at less than 1 mN/m. When each molecule occupied ~140 Å^2, Π increased more

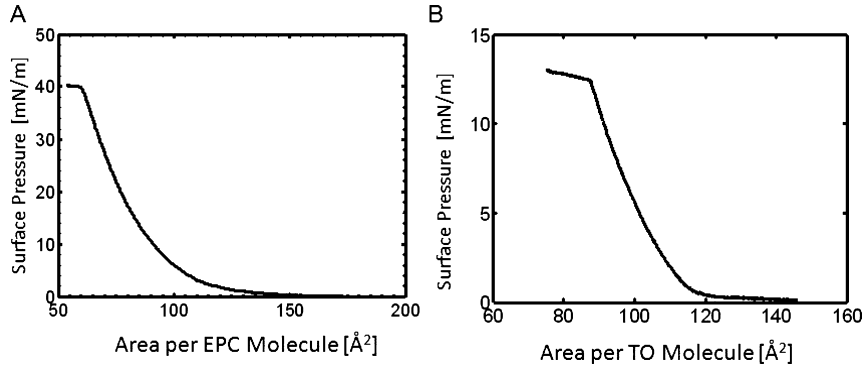

Figure 2. Compression isotherms of EPC (A) and TO (B) monolayers spread on a Langmuir trough. EPC showed a collapse point at an area per molecule of 58 ± 3 Å^2/molecule and a Π of 38.9 ± 0.8 mN/m (mean and standard deviation; $N = 9$). Π deviates from 0 at an approximate area per molecule of 140 Å^2, and the extrapolated deviation from Π = 0 is 90 Å^2/molecule. The surface was compressed at a rate of 3.75 cm^2/min. The isotherm was reversible when reexpanded from 37 mN/m, but when it was reexpanded beyond Π = 40 mN/m, it was irreversible. Compression isotherm of TO spread on a Langmuir trough showed a break in the curve at a pressure of 12.1 mN/m (at 23 °C and a compression rate of 3.75 cm^2/min). At an area smaller than 90 Å^2/molecule and Π >12.1 mN/m, some of the TO is expelled from the monolayer and forms a new bulk phase, which is in equilibrium with the TO monolayer at its minimum area (∼90 Å^2/molecule).

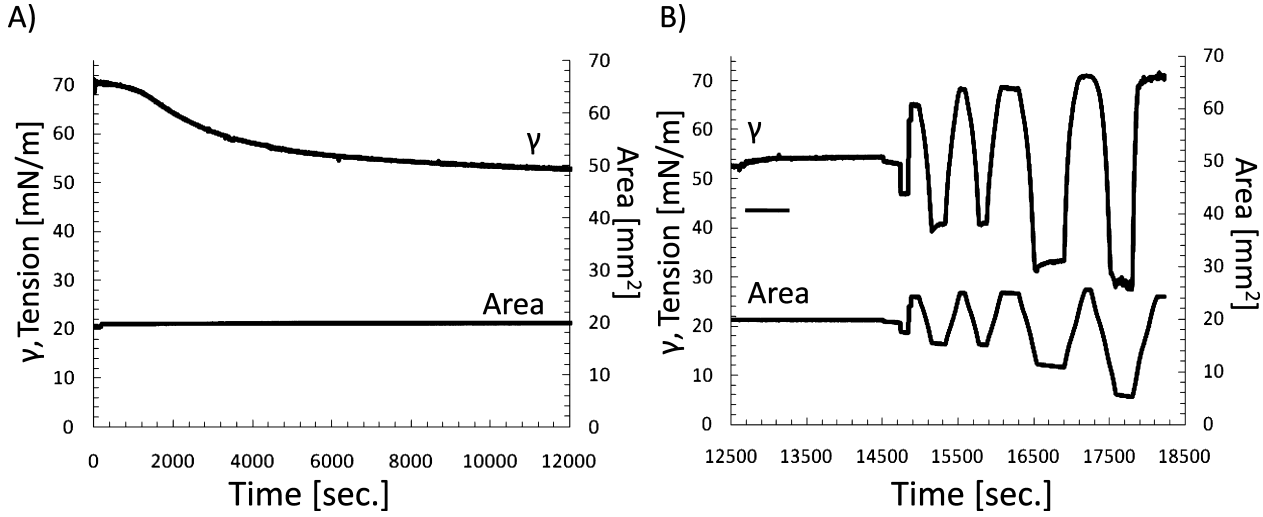

Figure 3. Drop tensiometer experiment of EPC binding to an air−water interface showing the relationship between γ (top) and bubble surface area (bottom) verse time. (A) At time point zero, a bubble was created in a suspension of EPC SUVs at a concentration of 0.17 mg/mL. During the adsorption, there is a short time lag and a rapid adsorption phase, and finally, it approached an equilibrium value of 52 ± 1.7 mN/m. (B) After equilibrium γ was approached, the lipid was removed from the surrounding suspension by flowing 250 mL of lipid-free buffer through the cuvette from the time period 12 610 to 13 140 s (bold line). After the buffer exchange, γ remained constant. Then the bubble volume was slowly changed at a constant rate of ±0.02 μL/s to vary the bubble area and generate data to construct a Π/A curve (see Figure 4).

rapidly and clearly deviated from the baseline. As the area per molecule decreased, Π continued to steadily increase at an accelerating rate until it reached an area per molecule of 58 ± 3 Å^2 and Π of 38.9 ± 0.8 mN/m. At this point, there was an abrupt change in the slope of the isotherm, called the collapse point. After the collapse point, decreasing the area had very little further effect on Π. At Π lower than 40 mN/m, the isotherm was reversible, but after the collapse point, it was irreversible (data not shown).

A TO monolayer at an area per molecule >125 Å^2 exerted less than 0.5 mN/m, shown in Figure 2B. Between an area per molecule of 125 and 90 Å^2, the Π rose. At ∼90 Å^2/molecule and Π of 12 mN/m, there was a change in the slope, indicating the expulsion of bulk TO from the monolayer. Any further compression caused very little change in the Π. When a 1 μL drop of TO was added to the surface at an area per molecule <90 Å^2, the drop stayed as a lens and did not change Π. However, when the drop was added at an area per molecule >90 Å^2, the Π immediately rose to 12 mN/m, indicating that 12 mN/m is the spreading Π of TO. The excess TO remained as a lens.

Deposition and Compression of EPC at an A/W Interface Using a Drop Tensiometer. The SUVs of EPC are adsorbed to the surface of an air bubble. The values of the area and γ during a typical experiment are shown in Figure 3. At the beginning of the experiment, γ was equal to 72 mN/m and rapidly lowered to 52.7 ± 1.7 mN/m, which corresponds to a Π of 19.3 mN/m (Π = 72-γ). There were three distinct phases to the adsorptions. Initially, there was a lag phase in which there is very little change in γ, followed by a rapid reduction in γ, then a gradual lowering of γ. The length of time for each segment of adsorption depended on the amount of EPC added to the surrounding buffer (data not shown). At high concentration, γ changed faster than at low bulk concentration.

After equilibrium was approached during the third part of adsorption, the SUVs in the bulk surrounding the bubble were removed by flowing lipid-free buffer through the cuvette and removing the excess from the surface to keep the cuvette volume constant. During the exchange, γ rose between 0.5 and 3 mN/m without any change in the bubble volume. This slight lowering of Π was possibly caused by the release of some reversibly

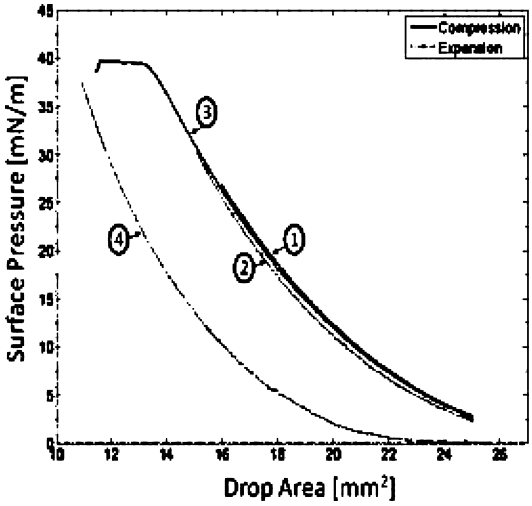

Figure 4. Π/area isotherm of an EPC monolayer at an A/W interface using a bubble tensiometer. When the bubble is compressed (1) and reexpanded (2) to Π = 27 mN/m, the reexpansion is reversible and follows the same isotherm as the initial compression. The compressions were done at a rate of 0.02 μL/sec, corresponding to a change of ~4.1 mm²/min. When the bubble is recompressed (3) to Π > 39 ± 1.3 (n = 9) mN/m, the slope of the curve becomes dramatically smaller, indicating the collapse point. When the surface is reexpanded after the collapse, it follows a different isotherm from before the collapse (4). When the surface is recompressed, it follows the post collapse reexpansion isotherm. Because the amount of phospholipid that binds to the surface is unknown, the area per molecule cannot be directly calculated.

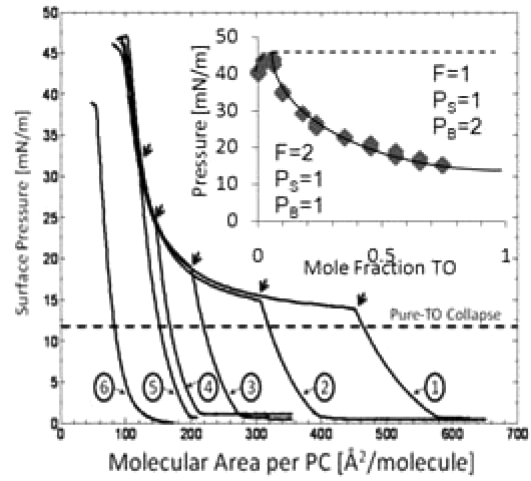

Figure 5. An example of Π/area compression isotherms of EPC/triolein (TO) mixtures (1−5) and pure EPC (6) spread on a Langmuir trough at an A/W interface, expressed as area per EPC molecule. The mixture that was initially spread contained a TO/EPC ratio of 3.5:1 (1), 2:1 (2), 1:1 (3), 0.5:1 (4), 0.2:1 (5), and pure EPC (6, equivalent to Figure 2). Mixed TO/EPC monolayers (1−5) show two distinct changes in the slope of the compression isotherms: one at Π = ~45 mN/m and area of 100 Å²/molecule and the other, called the envelope point, occurring at varying Πs and areas, depending on the ratio of TO/EPC added. The collapse Π for the pure TO monolayer is 12.1 mN/m and is shown as a broken line. All experiments happened at a temperature of 22.8 ± 0.6 °C. Below the envelope points (marked by arrows), Π is independent of the area per EPC molecule. Above the envelope Π, all five isotherms align. The envelope Π in all cases is higher than the collapse Π of a pure TO monolayer (12.1 mN/m, indicated by the dotted line). Both the collapse Π and area of mixed monolayers of TO/EPC are higher than the collapse Π and area of a pure EPC monolayer (6). Each molar ratio was repeated at least three times, yielding similar results. (Inset) The derived surface phase diagram of a mixture of TO and EPC as a function of mole fraction. The solid line and diamonds (♦) represent the envelope point, and the dashed line represents the collapse point. At a high mole fraction of EPC or low Π, the TO and EPC are miscible in the monolayer and have 2 degrees of freedom (F). At high Π and low mole fraction of EPC, a portion of the TO is in the monolayer, and the remainder forms a bulk TO phase. When there is a bulk phase of TO, the number of bulk phases (P_B) increases to 2, and the number of surface phases (P_S) remains at 1 and F reduces to 1.

bound molecules from the surface. The buffer exchange continued until γ remained constant.

After γ stabilized from the buffer exchange, the bubble was slowly shrunk at a constant rate of 0.02 μL/s, which is the slowest rate the volume can be continuously changed. As the volume decreased, the surface area became smaller; thus, the area per molecule also became smaller (Figure 3B). After a small compression, the volume was increased at 0.02 μL/s, whereby the area was expanded and the γ rose toward 72 mN/m. Increasingly larger compressions were carried out and from the Π/A relationship, the curves in Figure 4 were produced, which show the relationship between the area of the bubble and Π. When the surface was compressed, there was an increase in Π. Compression of a monolayer of EPC at an A/W interface on the Langmuir trough was reversible at Π < 38.9 mN/m and irreversible at Π > 38.9 mN/m. Because of this, the bubble was reexpanded at Πs both above and below 38.9 mN/m. When reexpanded below the Π_C, it followed the same isotherm back to low Π. Once the surface was compressed beyond 39 ± 1.3 (n = 9) mN/m, Π essentially stopped rising, similar to the post collapse isotherm using the Langmuir tough (Figure 2A, far left). When the surface was reexpanded from Π > 39 mN/m, the Π fell more rapidly than the precollapse reexpansion, and when Π equals zero, the area is smaller (curve 4, Figure 4). When the surface was recompressed, it followed the same isotherm as the post collapse reexpansion until Π reached ~40 mN/m, where the slope abruptly flattened. When the surface was reexpanded, Π fell even more rapidly than it did after the postcollapse reexpansion and reached low Π at an even smaller area than any of the previous re-expansions (data not shown). This shows that the EPC monolayer was reversibly compressible below the Π_C, but after the Π_C was reached, there were irreversible changes in the monolayer within the time frame of the experiment.

Properties of EPC and TO Mixed Monolayers Using a Langmuir Trough. Mixed monolayers of EPC and TO have characteristics very different from a monolayer of only EPC. Figure 5 shows the compression isotherms of mixed monolayers of EPC and TO spread on a Langmuir trough at different molar ratios. There are many distinct features of the curve that are not found in a monolayer of only EPC (see Figure 2A). The most noticeable was that there was a second point where there was an abrupt change in the slope, which happened below the Π_C of ~45 mN/m. This point is called the envelope point. As the molar ratio of TO/EPC decreases, the Π at which the envelope point occurs increased. For example, at a ratio of TO/EPC of 3.5:1, 1:1, and 0.2:1, the envelope Π was at ~15, 20, and 37 mN/m, respectively.

Another feature is that Π deviates from zero at a larger area per EPC molecule than in the case of a pure EPC monolayer, meaning that the TO must be present on the surface when Π first deviates from zero. The final collapse point is also at both a large final area per EPC molecule of 100 Å²/molecule and a higher Π of 45 mN/m. This is as opposed to the area of 58 Å² and Π of 38.9 mN/m for the EPC monolayer at the collapse point (Figure 2). The Π_C of pure TO is 12.1 mN/m. For all the ratios of TO/EPC the isotherm was reversible when Π does

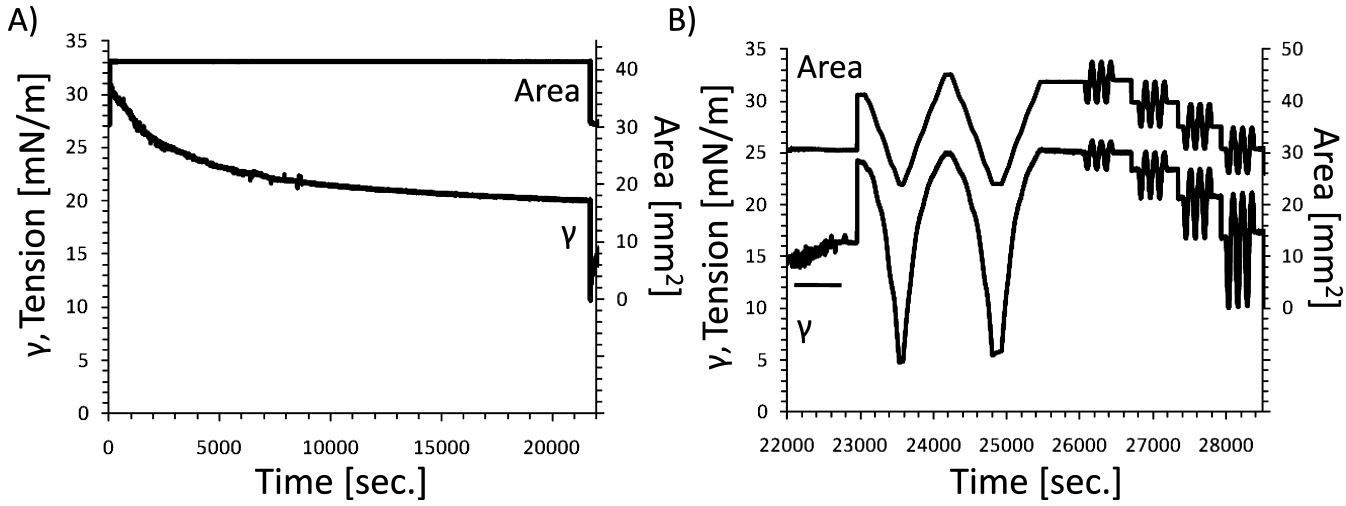

Figure 6. Bubble tensiometer experiment of EPC binding to a TO/W interface showing the relationship between γ (bottom) and bubble surface area (top) versus time. At time point zero, a 16 μL bubble was created in a suspension containing EPC SUVs. The phospholipid adsorbed, lowering γ to 20 $\pm$ 0.8 mN/m (panel A). The adsorption occurred without any lag period. After equilibrium γ was approached, the bubble volume was lowered from 16 to 8 μL. This prevented the bubble from falling off during the buffer exchange. The lipid was removed from the surrounding solution by flowing 250 mL of lipid free buffer through the cuvette from 22 070 to 22 664 s (bold line in panel B). There was a slight increase in γ during the exchange. During the exchange, there is an increase in the noise of the data caused by the bubble's moving due to turbulence in the cuvette. After the buffer exchange, the bubble volume was either expanded or reduced at a constant rate of $\pm$0.02 μL/s (panel B). After 2 compressions and expansions, the bubble was oscillated at a period of 128 s and amplitude of 2 μL centered on different areas, which show the relationship between Π and the bubble area.

not exceed 45 mN/m (data not shown). By overlaying the isotherms, it becomes clear that the low Π parts of the isotherms below the envelope point do not overlay one another, whereas the isotherms above the envelope point do, as shown in Figure 5; in other words, once the envelope point is reached and the slope dramatically changes and the curves overlay with one another, regardless of the starting concentration. When Π is above the envelope point, there is the same Π at a given area per EPC molecule, regardless of the starting concentration of the monolayer. However, below the envelope point, the area will be less when there is less TO added to the monolayer. The inset of Figure 5 shows the isothermal Π/composition phase diagram derived from the EPC/TO compression isotherms (Figure 5). This phase diagram is similar to the 1-palmitoyl-2-oleyl PC/Triolein phase diagram given by Smaby and Brockman.[11] When a 1 μL drop of pure TO was added to the surface at a Π greater than the envelope point the drop remained as a thin lens and had no effect on Π. When the surface was reexpanded after a drop of pure TO was added, the isotherm was reversible until the envelope point ($\sim$450 Å^2/molecule and Π = 15 mN/m). When the area was increased beyond 450 Å^2/molecule the envelope point vanished, there was no break in the isotherm, and the Π approached 12 mN/m, the Π of a pure TO interface (data not shown). The excess TO remained as a lens on the surface.

Deposition and Compression of EPC at a TO/W Interface Using a Drop Tensiometer. When EPC was adsorbed to the surface of a TO bubble, it lowered γ from 32 to 20.8 mN/m (i.e., Figure 6). There were two distinct phases to the adsorption. There was a rapid adsorption phase in which γ rapidly decreased, then a slow equilibration phase in which γ gradually decreased. Unlike the air bubble, there was no lag phase when EPC was adsorbed to a TO interface. After the EPC had adsorbed, the bubble was compressed to about 2/3 of the original area (Figure 6a, far right). This was done to ensure that the bubble was not released during the buffer exchange. Once equilibrium was reached, the EPC SUVs were removed from the bulk buffer and replaced with lipid-free buffer. During the exchange interval, there was a small increase in γ, possibly caused by the release of partially spread SUVs from the interface. After the exchange was complete and the γ was equilibrated, the bubble volume was compressed and expanded. When the area decreased, Π (Π = 32 $-$ γ) increased. The surface was also slowly oscillated at a rate of 0.0028 Hz around different starting areas to show the effect of changing the area on the Π. Figure 6 shows a representative TO bubble experiment.

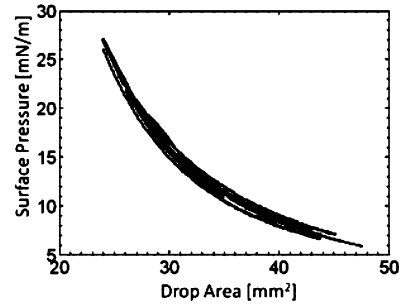

Figure 7. From the drop tensiometer data (Figure 6), a Π/area compression isotherm was constructed. The compressions and reexpansions (black), and oscillations (gray) all overlay one another, both within the same experiment and between experiments. This shows that compressions of EPC at a TO/W interface are reversible and shows little hysteresis. The range of Π for this experiment is limited between 0 and 28 mN/m. Above Π = 28 mN/m (below γ = 4 mN/m), the bubble is released. When a new TO bubble was formed, the new clean TO/W interface had a γ of 32 mN/m (γ of a clean interface), and changes in area did not deviate γ from 32 mN/m.

The Π/area relationship of EPC at a TO/water interface showed that as the bubble area decreased, Π increased at an increasing rate, as shown in Figure 7. The compressions, expansions, and oscillations all follow the same isotherms, which showed reversibility in the entire range of Π examined with virtually no hysteresis. The slope of the isotherm gradually increased without any breaks or abrupt changes. Since the Π/area isotherm was reversible and had no abrupt changes in the slope, it indicated there was no collapse of the monolayer and, thus, no phase transitions. The Π/area relationship after

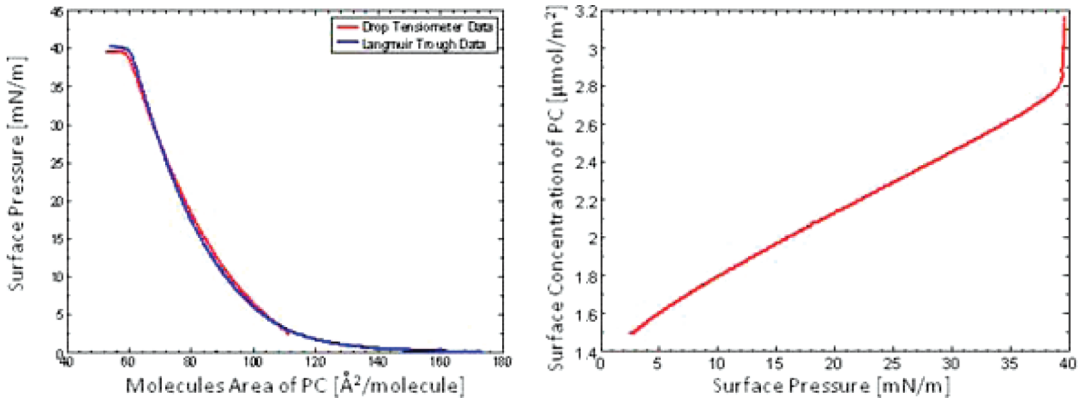

Figure 8. By overlaying the collapse isotherms of EPC at an A/W interface from a Langmuir trough (Figure 2) and from the bubble tensiometer using eq 4 (Figure 5), it is clear that they have very similar slopes (A). By converting area per molecule to concentration, the relationship between Π and Γ at the surface of an air bubble is shown (B).

washing out the lipid was independent of both the initial bulk lipid concentration and the waiting time before the washout. For example, if a lower concentration of lipid was added, γ fell more slowly but reached the same equilibrium value and behaved similarly upon compression and expansion after a washout. Alternatively, if the buffer was washed out at an earlier time, the Π/area relationship was the same as other isotherms (data not shown).

Discussion

At the A/W interface, EPC monolayers on a Langmuir trough (Figure 2A) and on a bubble (Figure 4) show similar characteristics. When the area per molecule is larger than 70 Å^2, the monolayer follows the same isotherm when compressed and reexpanded, showing it is reversible and has the characteristics of a liquid or liquid-expanded monolayer.[5] Both techniques show a clear collapse point at a Π equal to ~39 mN/m. At this point, the fatty acid chains of EPC are pressed tightly together, and upon further compression, they collapse irreversibly into a new bulk phase. Below the Π_C, according to eq 3, there is 1 degree of freedom [$C = 2$ (water and EPC), $P^B = 1$ (liquid water), and $P^S = 1$ (2-dimensional liquid surface)]. Thus, only one intrinsic variable, either Γ of EPC or Π, is needed to describe the system. Beyond the collapse point, there is one maximally compressed surface phase and a second bulk phase, which reduces the degrees of freedom to zero ($P^S = 1$, $P^B = 2$, $C = 2$). Thus, for an EPC monolayer, the collapse point is defined by only one possible area per molecule and one Π.

The Langmuir trough experiment showed that at the Π_C, each molecule occupies 58.9 Å^2. Because there are zero degrees of freedom at the collapse point, the area per molecule on the bubble surface at the collapse point should also be 58.9 Å^2. Using the Π_C as a reference point, the area per molecule on the bubble surface (and Γ) at other Πs can be estimated by multiplying the bubble area (in millimeters squared) at any Π by the area per molecule at collapse then dividing by the area of the bubble at the Π_C,

$$\frac{A(\Pi)_D[\mathrm{mm}^2] \times \mathrm{APM(collapse)}_T[\text{Å}^2/\text{molecule}]}{A(\mathrm{collapse})_D[\mathrm{mm}^2]} = \mathrm{APM}(\Pi)_D[\text{Å}^2/\text{molecule}] \quad (4)$$

where A is the bubble area, and APM is the area per molecule. The subscripts indicate the apparatus (D for drop tensiometer; T for Langmuir trough). The result of this conversion is shown in Figure 8A. Since the area of the surface and the area per molecule is now known, the number of EPC molecules on the surface and, thus, Γ can easily be calculated, which is shown in Figure 8B. The results from the drop tensiometer and the Langmuir trough yield almost identical isotherms, which is further evidence that the same phase changes and lipid interactions are occurring in both experiments. In the range of Π = 5−35 mN/m, the relationship between Π and Γ is approximately linear.

The mixed TO/EPC interface has two distinct changes in the slope (Figure 5). When Π was equal to 45 mN/m and area per molecule was equal to 100 Å^2, there was an abrupt reduction in the slope, and the isotherm became irreversible. This point is analogous to the collapse point for the pure EPC monolayer. Notably, both the Π_C and area per EPC molecule are larger than the pure EPC monolayer. This is possibly due to TO remaining in the monolayer at collapse acting as a spacer between EPC molecules, which reduces steric and charge−charge repulsions between the EPC headgroup.

In past experiments, our group has tried to estimate Γ of TO in EPC/TO emulsions.[26−28] An emulsion of EPC and TO was subjected to centrifugation, which broke the emulsion, releasing a core phase, which floated, and a "surface phase" which sunk. The core phase contained only TO. The surface phase was present as bilayers and initially was composed of ~6% TO/94% EPC. However, repeated resuspension and recentrifugation reduced the TO composition to ~4%. This was assumed to be the surface composition of the emulsion.[26] Further, incorporation of ^{13}C-labeled TO into EPC SUVs by cosonication of EPC/^{13}C TO mixtures showed the TO located both in the core phase and in the surface phase. A maximum of ~3% of the surface phase is composed of TO, which confirms the presence of TO in EPC monolayers at the surface of an emulsion.[28] In the Langmuir trough experiment with EPC/TO mixtures, the area per EPC molecule at high Π is higher than pure EPC monolayers, indicating that there is some TO in the monolayer, even at high Π before collapse.

From this study, the composition of the monolayer at the collapse point cannot be directly calculated. A spread monolayer of 17% TO (ratio 0.2:1) has an envelope point at ~33 mN/m and collapses at a higher Π and area than an EPC monolayer, indicating that the monolayer at collapse must have less than 17% TO. Presumably, if the envelope Π and collapse Π are the same at a certain mole fraction, that mole fraction will be equal to the composition of a mixed monolayer at the collapse

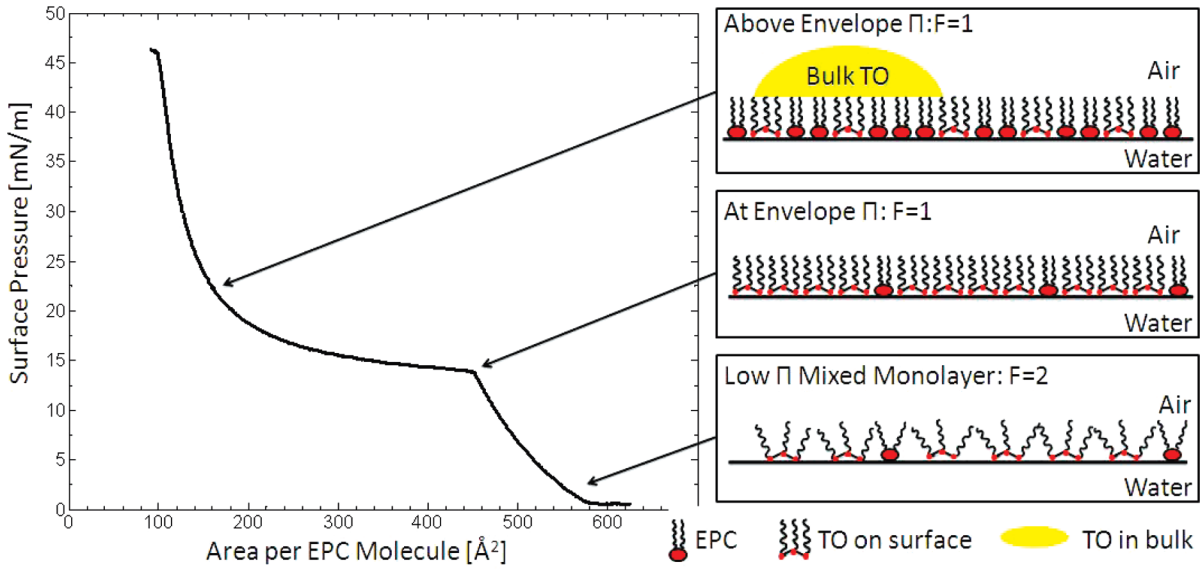

Figure 9. A model of a mixed TO/EPC (ratio of 3.5:1) monolayer on a Langmuir trough at an A/W interface, which is being isothermally compressed, illustrates change in the monolayer as a function of Π and Γ (from Figure 5). Below the envelope point, the lipids form a homogeneous mixed monolayer (lower right panel). The fatty acid chains are in an expanded liquid state. As the surface density increased, the chains were pushed closer together. At the envelope point, the chains of the EPC and TO are more aligned with one another but still liquid (center right panel). Any further compression causes the TO to be expelled from the monolayer and create a new bulk hydrophobic phase outside of the monolayer (top right panel). As Π increased, more TO was ejected, and the TO/EPC ratio of the monolayer was decreased. The red area of the molecules indicates a hydrophilic region on the molecule and the squiggly lines indicate the fatty acid chains. Above 45 mN/m, the collapse point, an EPC-rich phase also separates out of the surface. At this point, there are three bulk phases and one surface phase, which reduces the degrees of freedom to zero ($F = 3 - 3 - 1 + 1$). Upon further compression, the Π remains constant.

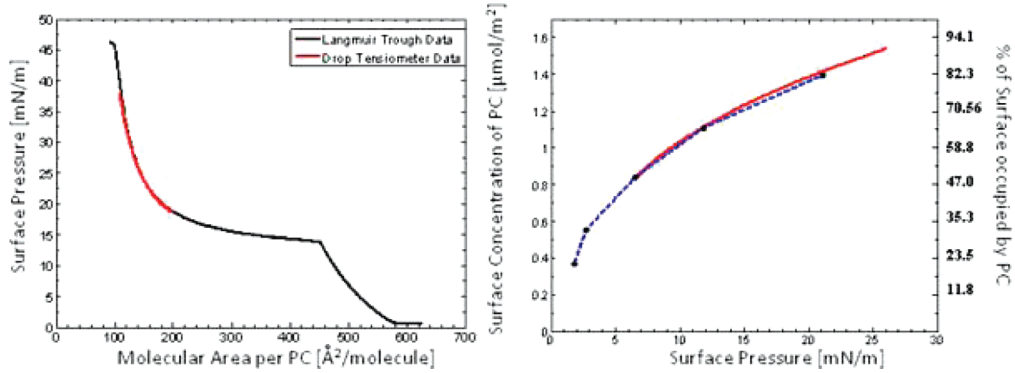

Figure 10. (A) To calculate the concentration of EPC at the surface of a TO bubble, a mixed TO/EPC compression isotherm at a ratio of 3.5:1 (Figure 5, black) can be overlaid with the compression isotherm of EPC on a TO/W interface (Figure 7, shown in red). To account for the fact that the mixed TO/EPC monolayer was compressed at an A/W interface, 12.1 mN/m was added to the value of the TO bubble Π because that is the collapse Π of pure TO (Figure 2B). (B) By converting from area per molecule to Γ (surface concentration of EPC), the relationship between Π and Γ is established (red). At the envelope point, the composition of the monolayer is known, so the percentage of the molecules at the surface at these points is also known (black dots). Thus, as the Π on the TO drop increases from 2 to 26 mN/m, the mole percent of EPC is enriched from ~25 to ~90%, and the amount of TO in the surface decreases in a reciprocal manner.

point. An exponential fit of the data ($x_{TO} = 2.23e^{-0.077\Pi}$, $R^2 = 0.962$) predicts that an envelope Π of 45 mN/m will occur at an initial spreading mole fraction of TO of 0.0697; that is, ~7%. This shows that in a compressed monolayer of EPC and TO, there is TO in the surface and indicates that on the surface of an emulsion, there is potentially more than 3−6% TO found by more disruptive techniques.[26−28]

The mixed TO/EPC monolayers had different characteristics from that of the pure EPC monolayer. A model of the phase changes during an isothermal compression is shown in Figure 9. When Π is below the envelope Π (Figure 9, bottom right panel), there is one liquid surface phase, and $F = 2$. Thus, Γ and Π are independent of one another and depend on the starting ratio of lipids added to the surface as well as Π. For example, at Π = 10 mN/m, the area per EPC molecule estimated as if only EPC were in the surface varies between 156 and 475 Å^2 at the molar ratios shown (Figure 5). For Π and area per molecule (which is inversely proportional to Γ) to be independent, there must be at least 2 degrees of freedom. In this system, there are three components: water, EPC, and TO. Because EPC and TO are virtually insoluble in water, both lipids will separate from the water and occupy the surface phase at Π = 10 mN/m. There must also be a bulk water phase. According to eq 2, for F to be equal to 2, P^S must be equal to 1, meaning that there is only one homogeneous liquid surface phase, which is composed of TO and EPC.

Above the envelope point (Figure 9, middle right panel) at a specific surface Π, there is only one possible area per molecule. This is demonstrated at Π = 25 mN/m, where four different TO/EPC ratios all overlay with one another above their envelope point at an area of 140 Å^2 per molecule (Figure 5). This is equivalent to saying that only one intensive variable must be

specified (in this case, Π) to describe the system between the envelope and Π_C. The direct relationship between Π and Γ indicates only one degree of freedom. There are still three components in the system, so to reduce the degrees of freedom from 2 to 1, a new phase must be formed. Because TO is not miscible with water and is less surface-active than EPC, it should be pushed out of the mixed monolayer to form a new hydrophobic TO bulk phase outside of the monolayer. When pure TO was added to the surface above the envelope point, it formed a lens on the surface. Thus, the TO expelled from the monolayer above the envelope point probably forms a microlens outside of the monolayer. Because EPC is not soluble in either TO[26] or water, it will remain at the interface. Thus, the envelope point is the point at which the first TO molecule begins to be ejected from the monolayer At the envelope point, the composition of the monolayer is identical to that in the solution that was first spread on the surface. Above the envelope point, TO is progressively ejected from the interface into a bulk hydrophobic TO phase presumably present as microlens(es) (Figure 9, right upper panel).

At Πs greater than the envelope Π, the system studied on the Langmuir trough is composed of a liquid (bulk) TO and a bulk water phase with a mixed TO/EPC monolayer between them. This is analogous to bulk water and a TO bubble when EPC is deposited onto the TO surface, creating an EPC/TO monolayer, since the same phases coexist and there is 1 degree of freedom. Thus, Γ of EPC at a TO/W interface varies with Π and can be estimated, similar to the technique used to align the A/W interface isotherms (Figure 5). The Π_C of a pure TO monolayer is 12.1 mN/m (Figure 2B). Since there is always an excess of the bulk TO in the drop phase, by adding 12.1 mN/m to the value of Π of the bubble measurements and multiplying the bubble area by the area per molecule at a given Π between the collapse and envelope points and dividing by the bubble area at the same Π, the area per EPC molecule on the bubble surface can be estimated (eq 4), which is shown in Figure 10A. This can then be converted to Γ of EPC. At the envelope point, no molecules have left the monolayer, so the mean molecular area can be calculated. The fatty acid chains are aligned roughly parallel to one another and perpendicular to the interface. At this point, the choline headgroup will limit the area per molecule. Since the headgroup is unchanged, it is reasonable to assume that each EPC molecule occupies approximately the same area as a pure EPC monolayer at Π_C (Figures 2 and 8). Using this assumption, the area occupied by EPC molecules and percentage of the surface covered by EPC and TO can be calculated. The Γ and percentage of surface occupied by EPC is shown in Figure 10B.

EPC is deposited on the surface of an air or TO bubble by injecting SUVs into the bulk. It rapidly lowers γ and binds irreversibly to the interface. This is done without the addition of any other molecules, such as organic solvents or other lipids. After removing the EPC from the aqueous phase, the compression isotherm of the monolayers on the bubble behave identically to its characteristics on a Langmuir trough, which indicates that it is a monolayer and not multiple layers stacked on top of one another. By using the 2-dimensional phase rule to evaluate phase changes, Γ has been estimated. On the surface of a TO/EPC/W interface, there will be some exposed TO, even at high Π, which will be accessible to proteins or other molecules that could potentially interact with the surface. In the future, this technique can be used to investigate the properties of a mixed protein−phospholipid−TAG monolayer, which is more closely related to a true biological interfaces of cellular lipid droplets and lipoproteins than previous monolayer studies at the A/W or TO/W interface.

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