A modified squeeze-out mechanism for generating high surface pressures with pulmonary surfactant

Eleonora Keating a,⁎, Yi Y. Zuo g, Seyed M. Tadayyon b, Nils O. Petersen h, Fred Possmayer c,d, Ruud A.W. Veldhuizen a,c,e

a Lawson Health Research Institute, The University of Western Ontario, London, Ontario, Canada
b Department of Chemistry, The University of Western Ontario, London, Ontario, Canada
c Department of Obstetrics & Gynaecology, The University of Western Ontario, London, Ontario, Canada
d Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada
e Department of Medicine, The University of Western Ontario, London, Ontario, Canada
f Department of Physiology & Pharmacology, The University of Western Ontario, London, Ontario, Canada
g Department of Mechanical Engineering, University of Hawaii at Manoa, Honolulu, HI, USA
h Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

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The exact mechanism by which pulmonary surfactant films reach the very low surface tensions required to stabilize the alveoli at end expiration remains uncertain. We utilized the nanoscale sensitivity of atomic force microscopy (AFM) to examine phospholipid (PL) phase transition and multilayer formation for two Langmuir–Blodgett (LB) systems: a simple 3 PL surfactant-like mixture and the more complex bovine lipid extract surfactant (BLES). AFM height images demonstrated that both systems develop two types of liquid condensed (LC) domains (micro- and nano-sized) within a liquid expanded phase (LE). The 3 PL mixture failed to form significant multilayers at high surface pressure (π) while BLES forms an extensive network of multilayer structures containing up to three bilayers. A close examination of the progression of multilayer formation reveals that multilayers start to form at the edge of the solid-like LC domains and also in the fluid-like LE phase. We used the elemental analysis capability of time-of-flight secondary ion mass spectrometry (ToF-SIMS) to show that multilayer structures are enriched in unsaturated PLs while the saturated PLs are concentrated in the remaining interfacial monolayer. This supports a modified squeeze-out model where film compression results in the hydrophobic surfactant protein-dependent formation of unsaturated PL-rich multilayers which remain functionally associated with a monolayer enriched in disaturated PL species. This allows the surface film to attain low surface tensions during compression and maintain values near equilibrium during expansion.

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1. Introduction

Considerable evidence indicates that lung surfactant is essential for normal lung function [1–5]. For example, premature delivery can result in inadequate surfactant levels, leading to neonatal respiratory distress syndrome (RDS). This condition can be reversed with exogenous surfactant supplementation. Additionally, a number of pulmonary (and some non-pulmonary) insults can result in acute lung injury (ALI) or the more serious analogous condition, acute respiratory distress syndrome (ARDS), with concomitant alterations in PS composition which contribute to pulmonary dysfunction. Here also, timely administration of exogenous surfactant can result in improved lung function, although not necessarily decreased mortality [6,7].

Pulmonary surfactant (PS) is secreted by alveolar type II epithelial cells as lamellar bodies that evolve into tubular myelin and adsorb to the air-liquid interface as a molecular film [1,8]. The composition of PS is relatively conserved among mammalian species, i.e. ~80% phospholipids (PL), 5–10% neutral lipids (primarily cholesterol) and 5–10% proteins [9–11]. The major phospholipid component is phosphatidylcholine (PC) accounting for ~80% of the total PL. In most cases, ~30–60% of the PC is dipalmitoylphosphatidylcholine (DPPC). Four surfactant-associated proteins are present: SP-A and SP-D are hydrophilic proteins that play a role in transport and storage of PS and in host defense [12], and SP-B and SP-C which are hydrophobic proteins that play a role in biophysical properties of PS [13].

The beneficial effects of PS on lung function primarily arise from three related physicochemical properties. First, surfactant adsorbs rapidly to the equilibrium surface pressure (π) of ~45 mN/m, which is equivalent to a surface tension of ~25 mN/m. This reduction in surface tension facilitates alveolar surface expansion, thus reducing the
work of breathing. Secondly, due to the ability of surfactant films to support \( \pi \) of ~70 mN/m during lateral compression (i.e. surface area reduction), surfactant stabilizes the alveoli at end-expiration, thereby limiting pulmonary edema [2]. Third, during inspiration, when the surface area increases, PS maintains surface tensions near equilibrium, either by resspreading or fresh adsorption.

It has been suggested that the mechanism by which PS operates to attain low tensions is through a squeeze-out mechanism where the more fluid PL components are selectively removed from the surface monolayer as \( \pi \) is raised during expiration [1,2,4,5,14,15]. With mixed monolayers at low \( \pi \), an ordered, liquid condensed (LC) phase co-exists with a disordered, liquid expanded (LE) phase [5,16–21]. Dipalmitoylated, gel phase PLs have been shown to exist primarily in the LC phase at physiological temperatures, while unsaturated, fluid phase PLs and proteins have been detected exclusively in the LE phase [2,4,14,19,20]. LC/LE phase separation could facilitate the squeeze-out of fluid components from the monolayer to form vesicles lost to the subphase. The squeeze-out mechanism requires that lipids can be selectively removed from the monolayer, but without a molecular description of how this could happen, and as a consequence it did not address the ability of surfactant films to respread during dynamic cycling.

The ability of surfactant to respread as surface area increases was first explained by the discovery of the surface-associated surfactant reservoir by Schürch and associates [22,23]. Captive bubble experiments using a subphase washout approach demonstrated that adsorbed films contained excess material corresponding to 3 to 5 compressed monolayers [22]. Evidence for this reservoir was further provided through fluorescence, atomic force microscopy (AFM) and electron microscopic studies with model and later, modified natural surfactants [22–29]. Extensive evidence suggests that surfactant proteins SP-B and SP-C are necessary for the reversible formation of multilayer structures from which respreading occurs upon film expansion [30–35].

Although the role of specific PS lipids and proteins and their possible interactions have been extensively investigated, the exact mechanism by which pulmonary surfactant attains low tensions remains uncertain. A logical extension of the original squeeze-out model would suggest that selective squeeze-out of the more fluid PL could form subsurface multilayers that remain functionally attached to a surfactant monolayer highly enriched in DPPC. There is evidence to counter the need for squeeze-out that arose from Langmuir balance and gold-coated mica was used as the substrate in AFM experiments and gold-coated mica was used as the substrate in ToF-SIMS experiments. Gold-coating was achieved by inserting freshly cleaved 1 x 1 cm² pieces of mica into a Hummer VI sputtering system (Technics EM, Springfield, VA) under reduced pressure at 100 mTorr. The Au was sputtered onto the substrate for 10 min at a plate current of 10 mA. Deposited films were imaged within 2 h of deposition.

2. Materials and methods

2.1. Materials

Phospholipids and deuterated phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL, USA). All were received as powders and were dissolved in chloroform at a concentration of 1 mg/mL prior to experiments. BLES, a clinical surfactant prepared from natural bovine surfactant, was a generous gift from the manufacturer (BLES Biochemicals, London, Ontario, Canada) and was extracted before use using a modified Bligh and Dyer technique [35].

2.2. Langmuir–Blodgett (LB) film preparation

A Kibron \( \mu \)Trough SE (Helsinki, Finland) was used to prepare the LB films. The Langmuir balance was equipped with a continuous PTFE ribbon to minimize film leakage. The trough contains ~90 mL subphase and has a working area of ~125 cm². All samples were dissolved in chloroform and were spread drop-wise on model and later, modified natural surfactants [22–29]. Extensive evidence suggests that surfactant proteins SP-B and SP-C are necessary for the reversible formation of multilayer structures from which respreading occurs upon film expansion [30–35].

Although the role of specific PS lipids and proteins and their possible interactions have been extensively investigated, the exact mechanism by which pulmonary surfactant attains low tensions remains uncertain. A logical extension of the original squeeze-out model would suggest that selective squeeze-out of the more fluid PL could form subsurface multilayers that remain functionally attached to a surfactant monolayer highly enriched in DPPC. There is evidence to counter the need for squeeze-out that arose from Langmuir balance fluorescence studies, for example by Hall’s group, which demonstrated that films of the PL fraction from bovine surfactant at \( \pi \) ~70 mN/m (surface tension ~0 mN/m) were primarily composed of fluorescent probe-labeled LE phase surrounding the (bulky) probe-excluding LC phase and has a working area of ~70 mN/m during lateral compression (i.e. surface area reduction), surfactant stabilizes the alveoli at end-expiration, thereby limiting pulmonary edema [2]. Third, during inspiration, when the surface area increases, PS maintains surface tensions near equilibrium, either by resspreading or fresh adsorption.

The observation that homogeneous LC monolayers are not required to achieve high \( \pi \) emphasized the need for alternate explanations for the presence of multilayers. For example, it could be that as the multilayers expand under the surface it becomes several bilayers (plus a monolayer) thick. Thus, the multilayers could act as a monolayer-supporting scaffold or skeleton which increases mechanical stability even though all these layers contain both fluid and non-fluid PL [2,14,15,22–24,36–41].

The current study was designed to test the hypothesis that as PS films compress during expiration, a sorting mechanism exists that removes mainly unsaturated PL from the interfacial film to form multilayer stacks which remain attached to the film by surfactant-associated proteins. This sorting process would lead to an interfacial film highly enriched in disaturated PL which can support \( \pi \) of ~70 mN/m during lateral compression. Monolayer sorting would not be required for a multilayer-dependent increase in film stability. We took advantage of the nanoscale sensitivity of AFM to investigate the progression of multilayer formation and the lateral organization of the remaining interfacial monolayer. We then utilized the elemental analysis capabilities of ToF-SIMS to compare the molecular composition of the multilayer structures with the remaining monolayer regions. Two systems were employed: a 3 PL mixture containing DPPC, POPC and POPG, the most abundant saturated and unsaturated PLs in pulmonary surfactant; and BLES, an exogenous clinical surfactant containing only hydrophobic components of bovine natural surfactant. Our results are consistent with monolayer enrichment with non-fluid disaturated PL.

2.4. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging

ToF-SIMS images were collected using an ION-TOF ToF-SIMS IV (ION-TOF, Muenster, Germany) equipped with a bismuth liquid ion source at ACSES, University of Alberta or Surface Science Western, University of Western Ontario. The primary analysis beam was 25 keV Bi²⁺ operated in burst alignment mode with 10 kHz repetition rate and a pulse width of 100 ns. The target current was ~0.2 pA and the spot size was estimated to be ~300 nm. Mass analysis was performed via a single stage reflectron ToF analyzer at 2 keV with 10 keV post acceleration. The mass range was 0–800 amu (atomic mass unit) with unit mass resolution. Negative secondary ion images were acquired from regions of interest, typically with a 50 x 50 µm².
primary beam raster size. A 256 × 256 pixel image resolution was chosen, given the beam spot size and the raster area used in this work. Each image is normalized in intensity according to the minimum and maximum counts in a single pixel; these values are then mapped to a 256 increment, thermal color scale. The thermal gradient is chosen such that brighter areas in an image correspond to areas of increased secondary ion yield. In some cases the ToF-SIMS images were processed to enhance contrast by using a smoothing function available as part of the ION-TOF software.

In order to determine the lateral location of a specific component in a mixture by ToF-SIMS, an ion specific to that component must be selected. Three options were available to achieve this. The first was to image the intact molecular mass ions. For PLs, these high molecular mass ions give low secondary ion yield and therefore low contrast in images. Images with low SI yield were therefore subjected to a smoothing function, available as part of the ToF-SIMS software, to improve contrast. A second option was utilized in ToF-SIMS imaging of the 3 PL mix, where deuterated analogs of the PLs were used. More specifically, the mix consisted of DPPC that contains 4 deuteriums in the headgroup, POPG with 31 deuteriums in the palmitic acid tail and POPC. Previous studies have shown that deuterated PLs have a similar fragmentation pattern and phase behavior as the non-deuterated versions [42]. A third option, commonly used in ToF-SIMS experiments, is to spike a sample with trace amounts of a deuterated analog of the component of interest [43]. This option was used to determine the location of various PLs in BLES. Each experiment was repeated three times and at least five AFM and ToF-SIMS images were obtained for each sample.

3. Results

3.1. Phospholipid mixture

Fig. 1A shows a representative compression isotherm of a 50:30:20 DPPC:POPC:POPG film compressed to a π of 50 mN/m. As the film is compressed, the π increases steadily until it reaches ~45 mN/m where a slight kink is observed, followed by an increase to 50 mN/m. LB films deposited at π of 30 and 50 mN/m were imaged by AFM and characteristic images are shown in Fig. 1B–E. Fig. 1B shows a monolayer where domain formation is clear. A higher magnification of such a film (Fig. 1C) reveals that two types of LC domains form. There are large domains with an average diameter of 5 μm which we designated as LC micro-domains, as well as smaller domains with average diameter of 100 nm which we designated as LC nano-domains.

A striking change in film topography was observed as the π was increased from 30 to 50 mN/m (Fig. 1D). A higher magnification of such LB film (Fig. 1E) reveals the presence of many irregularly shaped LC domains connected to a continuous network of LC phase with LE regions within. Sub-micron structures, seen as white spots in Fig. 1E, are also present. Many of these are located on the edges of the irregular LC domains (with a few within these domains). Section analysis gives a height difference in the range of 3.5–4 nm for these white structures, which corresponds to one fluid PL bilayer [44]. It may be that these protrusions act as precursors of bilayer vesicles which are lost from the film during compression. Also, we observe a height difference of ~0.8 nm between the continuous network and the lower areas, which is in close agreement with height difference between PL molecules in LC and LE phases [20,45].

Next, we took advantage of the imaging capabilities of ToF-SIMS to obtain a chemical map and therefore determine the lateral location of each component in this 3 PL mixture. M184 (C₈H₁₅NPO₄⁻, phosphocholine) (Fig. 2A) and M188 (C₉H₁₆D₃NPO₄⁻, d₄-phosphocholine) (Fig. 2B), both detected in positive ion mode, were used to unambiguously determine the location of POPC and d₄-DPPC, respectively. Fig. 2C shows the location of M281 (C₁₈H₃₃O₂⁻, oleate), which results from the fragmentation of both POPC and d₃₁-POPG, as detected in negative ion mode from a different area of the same film. Fig. 2A–C shows that at a π of 50 mN/m regions exist in the film which are enriched in the solid phase PL, d₄-DPPC (Fig. 2B), and lack the fluid phase PLs, POPC and d₃₁-POPG (Fig. 2A, C). These regions range in diameter from 2–5 μm.

Fig. 2D shows an AFM image obtained by scanning an LB film of the same 3 PL mix which was deposited on mica at the same time as the LB film was deposited on gold-coated mica for ToF-SIMS imaging. The AFM image shows domains which are similar in size and density as the regions seen in the ToF-SIMS images. Section analysis of this AFM image reveals a height difference of ~0.8 nm which is indicative of LC phase separation. The bilayer regions seen as occasional small white spots in Fig. 1D and E and Fig. 2E are too small to be resolved by ToF-SIMS and therefore their composition remains undefined.

3.2. Bovine lipid extract surfactant, BLES

BLES was spread on a Langmuir balance and compressed to different π where LB deposits were taken. Fig. 3A shows a representative π—a area isotherm of BLES. As the film is compressed the π increases rapidly until ~42 mN/m where a rising plateau is observed to ~48 mN/m followed by a rapid increase. Deposits were taken on mica at 5 different π: 30, 40, 44, 48 and 50 mN/m. These films were imaged by AFM and representative images are shown in Fig. 3. At a π of 30 mN/m, LC micro-domains are present ranging in diameter from 2 to 10 μm as well as nano-domains with an average diameter of ~100 nm. As π is increased to 40 mN/m, just before the start of the plateau, more LC nano-domains are present as well as somewhat irregularly shaped LC micro-domains. At the beginning of the plateau, π of 44 mN/m, multilayer structures are detected at the edges of LC micro-domains as well as in the network formed by LC micro-domains and the LE phase. As π is increased to 48 mN/m, the density of multilayer structures is increased and LC micro-domains are no longer clearly observed. Further compression of the film to 50 mN/m causes the multilayer structures to increase in size.

To study the progression of BLES multilayer formation, height analysis of AFM images was performed for LB films deposited at π of 44, 48 and 50 mN/m as shown in Fig. 4. At π of 44 mN/m, height differences of ~0.9 nm are detected which indicates phase separation. White spots with height differences of up to 12 nm are also detected which indicate that multilayer structures made up of 3 bilayers are present. At a π of 48 mN/m, LC domains are not evident but many structures made up of one or more bilayers are detected. At the highest π of 50 mN/m examined, two slightly different surface topographies were observed. This difference may be related to small variations in the length of the plateau. With some samples (Fig. 4E), nearly the entire surface was covered by multilayer structures. With these, up to 3 bilayer structures are detected which have grown in diameter compared to lower π. This growth tends to occur mainly at the base bilayer closest to the original monolayer. With other samples (Fig. 5F, G) extensive multilayer structures formed, however, small, approximately circular depressions or wells were detected which apparently correspond to the bare, uncovered monolayer. These wells can best be observed in the enlarged image (Fig. 5G). Height analysis revealed that the wells were ~4 nm deep, which corresponds to a single bilayer (results not shown).

ToF-SIMS was used to study the composition of BLES LB films at π of 50 mN/m. Representative positive and negative ion images of the samples where wells were detected are shown in Fig. 5. The top row shows the raw ToF-SIMS SI images and the second row was obtained by subjecting the top row images to a smoothing function to increase contrast and better visualize the distribution of various components of BLES. M104 and M735 were imaged in positive ion mode corresponding to choline (C₈H₁₄NO₃⁻) and DPPC-H⁺,
respectively. Although M104 is not specific to any one PC component in BLES, it clearly shows the presence of small, roughly circular regions at high \( \pi \). The image of M735 unambiguously shows the distribution of DPPC predominantly in these same circular regions. The fragments imaged in negative ion mode are: M255 (\( \text{C}_{16}\text{H}_{31}\text{O}_{2}^- \), palmitate), M281 (\( \text{C}_{18}\text{H}_{33}\text{O}_{2}^- \), oleate) and M721 (DPPG\(^-\)). The image of palmitate is not specific to one component in BLES but is known to show contrast between LC and LE regions because of concentration and difference in ease of fragmentation and ionization. It was included to show the presence of circular regions which are similar in size to those obtained in positive ion mode. Oleate is specific to the unsaturated PL components of BLES, such as POPC and POPG. Our ToF-SIMS data shows that oleate is mainly excluded from the circular regions. The image of DPPG\(^-\) shows this saturated PL is located almost exclusively in these circular regions, which contain high levels of palmitate and have frequencies of occurrence on the surface similar to those with DPPC. Note that the multilayer areas would also have monolayer present so some DPPC and DPPG would be expected.

AFM images were also obtained for such a BLES LB film deposited at \( \pi \) of 50 mN/m (Fig. 5F, G). These AFM images show the presence of small circular regions (see arrows) similar in size and density to the circular regions seen in ToF-SIMS images. These regions are \( \approx 4 \) nm lower than the surrounding phase. We interpret these regions as monolayer depressions or wells enriched in saturated PLs which are surrounded by higher multilayer structures.

In order to obtain more accurate images of the location of the fluid, unsaturated components of the films, BLES samples spiked with 10 mol\% d31-POPG or 10 mol\% d31-POPC were separately

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**Fig. 1.** A surface pressure — area isotherm is shown in A for a 50:30:20 DPPC:POPC:POPG film compressed to \( \pi \) of 50 mN/m. LB films were deposited on mica at \( \pi \) of 30 mN/m (B, C) and 50 mN/m (D, E) and imaged by AFM. C and E are high magnification images collected from the regions highlighted by black boxes in B and D, respectively. B and D are \( 50 \times 50 \) \( \mu \text{m} \) and C, E are \( 5 \times 5 \) \( \mu \text{m} \)².
compressed to \( \pi = 50 \) mN/m, deposited by LB technique and imaged by ToF-SIMS. Representative ToF-SIMS images are shown in Fig. 6. M2 was imaged in negative ion mode, corresponding to deuterium, and it unambiguously shows the distribution of d31-POPG (Fig. 6A) and d31-POPC (Fig. 6F). These data indicate that both unsaturated PL are highly excluded from the circular well regions. Images of M721 (on the same area as M2) and M735 (on different areas) were also obtained for both films corresponding to DPPG\(^-\) and DPPC-H\(^+\), respectively. These images demonstrate that both saturated PLs are concentrated inside the wells. AFM was used to obtain topography images of a BLES sample spiked with d31-POPG (Fig. 6D, E). Height analysis of AFM images further confirms the presence of multilayer structures (seen as light brown and white structures) as well as lower round depressions which we interpret as monolayer regions enriched in saturated PL.

4. Discussion

The apparent importance of monolayer-multilayer transitions for pulmonary surfactant function prompted a detailed examination of the progression of multilayer stack formation. The PL mixture containing the major molecular species present in surfactant but lacking SP-B or SP-C failed to form abundant multilayer structures during compression to high \( \pi \). These films consist mainly of a monolayer where LC and LE coexist plus a few small bilayer protrusions. In contrast, BLES films, which contain SP-B and SP-C, compressed to high \( \pi \) form extensive multilayer structures composed of up to three bilayers. During monolayer-multilayer transitions, unsaturated PL are mainly detected in multilayer structures by ToF-SIMS leaving apparent indentations or wells representing the interfacial monolayer enriched in disaturated PL like DPPC and DPPG. These data are consistent with a modified squeeze-out mechanism for attaining high \( \pi \).

More specifically, Langmuir compression of both the PL mixture and BLES generated relatively rapid increases in \( \pi \) to ~42 mN/m, slightly below equilibrium, where a kink occurred leading to increased compressibility, particularly with BLES. With BLES, a further inflection in the rising plateau occurred at ~50 mN/m. Previous studies have shown that further compression leads to high \( \pi \) near 70 mN/m [24,41,46]. AFM examination revealed both samples possessed similar overall structural characteristics at \( \pi \sim 30-40 \) mN/m, including LC micro and nano-domains. These observations are consistent with earlier studies on PL mixtures and natural surfactant extracts [18–21,24,41,47,48]. However, as equilibrium \( \pi \) was approached, only a few bilayer structures were noted with the PL film while numerous bilayer and multilayer stacks were observed with BLES. Since the literature indicates that significant multilayer formation has been observed only with samples containing SP-B and/or SP-C [31–34,41,49], we surmise that compositional differences, particularly the presence of the hydrophobic surfactant proteins in BLES, are responsible for the observed differences.

Monolayer-multilayer transitions were further examined by AFM of LB deposits from the beginning, middle and end of the plateau region. Interestingly, at \( \pi = 44 \) mN/m, many multilayer structures developed at the edges of the LC micro-domains, although many were noted within the LE phase. The few bilayer structures observed with the simpler PL mix also tended to be located at the edges of domains (Fig. 1D, E). Protrusion initiation at micro-domain edges has also been noted with mouse surfactant extracts [50]. This distribution could reflect film instability related to a mismatch in acyl heights at the edges of LC domains. Some of the multilayers found in the LE phase may initiate at the edges of the small LC nano-domains but it was not possible to confirm this by inspection. It should be evident that, if the more fluid components of the film were selectively directed into multilayers, the remaining monolayer would be enriched in more stable, gel components. Evidence for this concept was provided by a combination of AFM for structural height differences and ToF-SIMS for chemical composition. The AFM images obtained at \( \pi = 50 \) mN/m demonstrate an almost uniform mottled surface, but one which in some cases had small, almost circular indentations or wells located ~4 nm (i.e., one bilayer) below the resulting surface. ToF-SIMS

![Fig. 2. 50:30:20 d4-DPPC:POPC:d31-POPG films were compressed to \( \pi = 50 \) mN/m and deposited on gold-coated mica (A–C) and mica (D, E) by Langmuir–Blodgett technique. LB films were imaged by ToF-SIMS (A–C) and AFM (D, E). M184 (A) and M188 (B) collected in positive ion mode and M281 (C) in negative ion mode are shown. A and B are collected from the same area, C on a different area of the same film. A–D are 50×50 μm² and E is a high magnification image of the area highlighted by the black box in D.](image-url)
examination revealed surface regions containing areas highly enriched in oleate-containing (i.e. unsaturated) PL species and small circular regions, similar in size and frequency to those detected as wells by AFM, that were highly enriched in the disaturated species DPPC and DPPG. These data provide strong evidence indicating that monolayer regions become enriched in the disaturated PLs DPPC and DPPG, while unsaturated oleate-containing PL, such as POPC and POPG, were primarily present in the multilayer leaflets (Fig. 7).

Of the hypotheses proposed for surface tension reduction by surfactant to facilitate alveolar expansion during inspiration and stabilize the terminal air spaces, preventing edema and alveolar collapse during expiration, the modified squeeze-out:multilayer-generated stability models predominate [1,15,16,22,23,31,33,34,46,51]. Our study contributes two novel experimental findings to this literature. First, formation of PL bilayer protrusions can occur at the edges of the LC phase subsequently leading to the formation of multilayers. These observations are consistent with previous studies showing the formation of such protrusions with films of DPPC:DPPG containing SP-B or SP-C. Such films resemble natural and modified natural surfactant (e.g., extracts) in exhibiting plateaus near equilibrium and in having good respreading properties but differ in that such model films normally do not attain high Π near 70 mN/m [25–29,31,52]. These observations may appear surprising for films composed of gel phase PL.

However, they are consistent with the previously described location of SP-B and SP-C in the LE phase[2,4,14,15] and the ability of these proteins to initially increase collapse rate of surfactant films near equilibrium [25,31] but then result in stiffening allowing the films to attain high Π [24,41,46,53]. The second novel observation is the demonstration that the multilayer regions are highly enriched in oleate-containing LE fluid phase PL, implying that the remaining monolayer was enriched in the gel phase PL, DPPC and DPPG. This latter suggestion was directly confirmed by ToF-SIMS imaging of the remaining circular areas of the monolayer available for analyses. Infrared reflection-absorption spectroscopic analyses of the multilayers arising from DPPC:DPPG:cholesterol:SP-C indicate that they are enriched in DPPG [29]. The present study extends those results by directly demonstrating unsaturated PL enrichment of multilayers with increased DPPC and DPPG in the remaining monolayer.

Incorporating our findings with earlier observations on natural, organic extract, and model pulmonary surfactants provides an updated model of the manner by which surfactant films maintain Π between ~40 and ~70 mN/m during compression:expansion cycling. Considerable evidence supports the requirement for the hydrophobic surfactant proteins in enhancing surfactant adsorption [31–34,49]. Furthermore, the fusogenic properties of these proteins suggest that these peptides perform this function through formation of
“adsorption/fusion pores or hemifusion stalks” \[3,5,49\]. Existence of fusion pores or stalks is supported by computer simulation studies employing PL:SP-B \[28\]. Such fusion pores or stalks could contribute to multilayer formation. The observations are consistent with a model in which multilayer formation originates at nucleation sites corresponding to SP-B and/or SP-C-based fusion pores within the LE phase. Lateral compression of the film would result in preferential flow of unsaturated LE phase PL via the fusion pores into the multilayers. The acyl mismatch indicated by the height differences between LC:LE micro-domains and nano-domains could contribute to the initiation of protrusion formation. The latter structures then act as nucleation sites for bilayers and multilayers. Furthermore, preferential flow of fluid components would be promoted by exclusion of the relatively large gel phase PL-containing micro-domains and nano-domains from migrating through fusion pores. This would result in progressive depletion of monolayer fluid components until a percolation threshold was achieved where \(\pi\) increases dramatically toward 70 mN/m. This concept is supported by recent studies by Zhang et al. \[41,46\] who used AFM to compare multilayer formation with a number of clinical surfactants. In particular, examination of Survanta, a bovine surfactant-based preparation supplemented with DPPC, by this group and Alonso et al. \[48\], revealed large LC plates surrounded by protrusions initiating in LE regions. Such pores would not only act as nucleation sites for protrusion formation, allowing surface PL to flow into the multilayers during film compression, but also for PL to be reincorporated into the monolayer during film expansion. Reversible multilayer formation is critical because hydrophobic protein-dependent PL respreading abrogates the requirement for replacing significant amounts of monolayer during each compression:expansion cycle.

It should be noted that the modified squeeze-out model is based on Langmuir observations employing spread surfactant films. However, this model can also provide insight into the mechanism of surface tension reduction related to surfactant adsorption as occurs in vivo. In this situation multilayers are during surfactant adsorption. In contrast to the Langmuir observations described here, adsorbed multilayers would arise from excess bilayer material which did not manage to enter the overlying monolayer before equilibrium \(\pi\) was attained \[2,15,22–24,30\]. Consequently, following the scenario described above, PL entering the monolayer through adsorption pores would logically be enriched in LE phase fluid species while the remaining multilayers would contain LC gel phase PL such as DPPC. Although
sufficient DPPC could remain in the LE phase to allow the adsorbed monolayer to attain high $\pi$ during compression, it seems counterproductive for the lung to form a predominantly fluid monolayer in order to generate DPPC enriched films. A potential explanation arises from the ability of SP-B and SP-C to promote PL structural modification through hemifusion, as evidenced by lipid mixing with vesicles. This indicates that SP-B and SP-C catalyze hemifusion with the air:liquid interface [11,15,54]. The flow of lipids from the outer leaflet of the vesicle to the surface apparently results in instability because, as previously documented, surfactant adsorption spontaneously proceeds to equilibrium. Furthermore, the resulting monolayers contain both fluid LE and gel LC regions [5,54,55]. This supports the suggestion that surfactant adsorption occurs in “packets” [23]. It also highlights a fundamental difference between the creation of surfactant reservoirs during adsorption compared to monolayer compression due to involvement of the air:water interface with the former situation [15]. SP-B and/or SP-C accelerate PL adsorption whether present at the surface or in the adsorbing vesicles [56]. This supports the concept of fusion pores. In either case, adsorption ceases once equilibrium is attained even though excess material remains functionally associated with the monolayer [29,30,57]. Although the mechanism is unknown, the excess surfactant material clearly contributes to decreased compressibility of the monolayer. Films adsorbed from dilute suspensions of BLES attain low surface tensions with ~30% surface area reduction from equilibrium while films generated with concentrated BLES or with SP-A require initial compressions of 20% or less [15,58]. Consideration of these observations along with the fact that BLES contains

Fig. 5. Monolayers of BLES were spread and compressed to $\pi$ of 50 mN/m. LB films were deposited on gold-coated mica and imaged by ToF-SIMS. Positive (A, B) and negative (C–E) SI images are shown. Images A1–E1 were obtained by subjecting A–E to a smoothing function to improve contrast. BLES LB films were also deposited on mica at $\pi$ 50 mN/m and imaged by AFM (F, G). Images A and B were collected from the same area while images C–E were collected from a different area of the same film. Images A–F are 50×50 $\mu$m$^2$ and G is 13×13 $\mu$m$^2$. Figs. 5F, G were reproduced from conference summary [5].

Fig. 6. Monolayers of BLES spiked with 10 mol% d31-POPG (A–E) and 10 mol% d31-POPC (F–H) were spread, compressed to $\pi$ 50 mN/m, deposited on gold-coated mica and imaged by ToF-SIMS. Negative (A, B, F, G) and positive ion mode (C, H) SI images are shown. BLES +10 mol% d31-POPG LB films were also deposited on mica and imaged by AFM (D, E). Images A, B, F and G were collected from the same area while images C and H were collected from a different area of the same film. A–D, F–H are 50×50 $\mu$m$^2$ and E is 13×13 $\mu$m$^2$. 
\(-30\%\) DPPC and \(<50\%\) total disaturates [54] led to the suggestion of selective adsorption of DPPC into the monolayer [1-4,8,15,53,56]. In contrast to above, the present model would not suggest selective disaturated adsorption but rather implicates selective desorption during the first and subsequent compressions. This interpretation is consistent with the observation that film efficiency improves markedly with repeated compression. It should also be noted that a percolation threshold capable of attaining \(\pi\) near 70 mN/m would not require depletion of all fluid PL species.

It is acknowledged that this interpretation lacks a number of specific details and future studies are indicated. For example, the nature of the adsorption/fusion pores remains vague. Furthermore, the current experiments were conducted at room temperature. The observation that the multilayer protrusions can initiate at domain edges and the suggestion that these structures contribute to sorting of fluid versus non-fluid lipids serves to provide a rational explanation for the role of such domains. However, whether nano-domains or micro-domains are present in surfactant monolayers at 37 \(^\circ\)C has not been established. This would clearly depend on \(\pi\) [22,39]. In this regard LE/LC structures predominate at 37 \(^\circ\)C in giant unilamellar bilayer vesicles prepared from natural and organic extract bovine, porcine or mouse surfactants [50,53,59], although this does not necessarily mean they persist in monolayers at that temperature. Fluorescence microscopic examination of compressed Langmuir films of the PL fraction from calf lung surfactant demonstrated micro-domains up to 41 \(^\circ\)C [19]. Including the neutral fraction (or cholesterol) led to apparent PL remixing. This occurs because nano-domains are not detectable with fluorescence microscopy. More recently, AFM studies have shown that this apparent remixing results from the conversion of micro-domains to nano-domains [22], presumably due to reduced line tension [60].

Finally, it should also be mentioned that not all experimental evidence is consistent with the ability of surfactant to generate high \(\pi\) through a modified squeeze-out mechanism by which \(\pi\) fluid phase PL flow through adsorption/fusion pores into multilayer stacks. Hall and co-workers have demonstrated that very rapid compression rates (faster than 50\% surface area reduction/s) produce “jammed” phase monolayers capable of maintaining \(\pi\) \(-70\) mN/m for long periods despite being composed entirely of LE phase components [3,16]. Whether such rapid surface area reductions occur physiologically is not known. However, it is evident that this latter mechanism differs from the present model in that the hydrophobic proteins would be required only to enhance adsorption to form the surface monolayer. In other words, multilayer formation would not be necessary. Whether either mechanism occurs in the alveoli in vivo must still be established.

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