



Review

Recent advances in alveolar biology: Some new looks at the alveolar interface[☆]

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ABSTRACT

This article examines the manner in which some new methodologies and novel concepts have contributed to our understanding of how pulmonary surfactant reduces alveolar surface tension. Investigations utilizing small angle X-ray diffraction, inverted interface fluorescence microscopy, time of flight-secondary ion mass spectroscopy, atomic force microscopy, two-photon fluorescence microscopy and electrospray mass spectroscopy are highlighted and a new model of ventilation-induced acute lung injury described. This contribution attempts to emphasize how these new approaches have resulted in a fuller appreciation of events presumably occurring at the alveolar interface.

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Abbreviations: γ , surface tension; μ -domains, microdomains; π , surface pressure; AFM, atomic force microscopy; ALL, acute lung injury; ARDS, acute respiratory distress syndrome; β MCD, β -methyl cyclodextrin; BAL, bronchoalveolar lavage; BLES, bovine lipid extract surfactant; CBS, captive bubble surfactometer; DPPC, dipalmitoyl-phosphatidylcholine; DPPG, dipalmitoyl-phosphatidylglycerol; ESI-MS, electrospray ionization mass spectroscopy; ESR, electron spin resonance; FM, fluorescence microscopy; GP, generalized polarization; GUV, giant unilamellar vesicles; H_{II} , hexagonal II phase; KO, knock out; L_{α} , α -lamellar phase; L_{β} , β -lamellar phase; L_d , liquid disordered; L_o , liquid ordered; LA, large aggregates; LB, lamellar bodies; LE, liquid expanded; mLD, monolayer liquid disordered; mL0, monolayer liquid ordered; MS, mass spectroscopy; m/z , mass/charge; n-domains, nanodomains; NPS, natural pulmonary surfactant; NPSE, natural pulmonary surfactant extract; NPSL, natural pulmonary surfactant lipid; NPSP, natural pulmonary surfactant phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; SA, small aggregates; SAXD, small-angle X-ray diffraction; SP, surfactant protein; TC, tilted condensed; ToF-SIMS, time of flight-secondary ion mass spectroscopy; Vt, ventilation.

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1. Introduction

Although its major function is gaseous exchange, the lung also participates in host defence. The latter role arises from exposure of this organ's massive surface area, in the human situation equivalent to a tennis court, to large volumes of air. This article and the accompanying one seek to address recent advances related to both of these key pulmonary functions. The initial report focuses on new methodological and conceptual advances in investigating and understanding the role of pulmonary surfactant in promoting gaseous exchange by reducing surface tension (γ), thereby stabilizing the terminal air spaces. The accompanying article examines evolutionary and functional aspects of various alveolar proteins. These include the low molecular weight hydrophobic proteins, surfactant protein-B (SP-B) and SP-C, which are crucial for surfactant interfacial actions and may be involved in host defence as well. It concludes with discussions on surfactant collectins SP-A and SP-D, other collectins (e.g., the avian collectins), and antimicrobial peptides such as the defensins and cathelicidins.

Pulmonary surfactant is synthesized in alveolar type II cells and assembled and stored in organelles known as lamellar bodies (LB) (Fig. 1) (Possmayer et al., 1984; Possmayer, 2004). These organelles are subsequently secreted, both constitutively and under stimulation, into the alveolar hypophase. SP-A and SP-D are constitutively

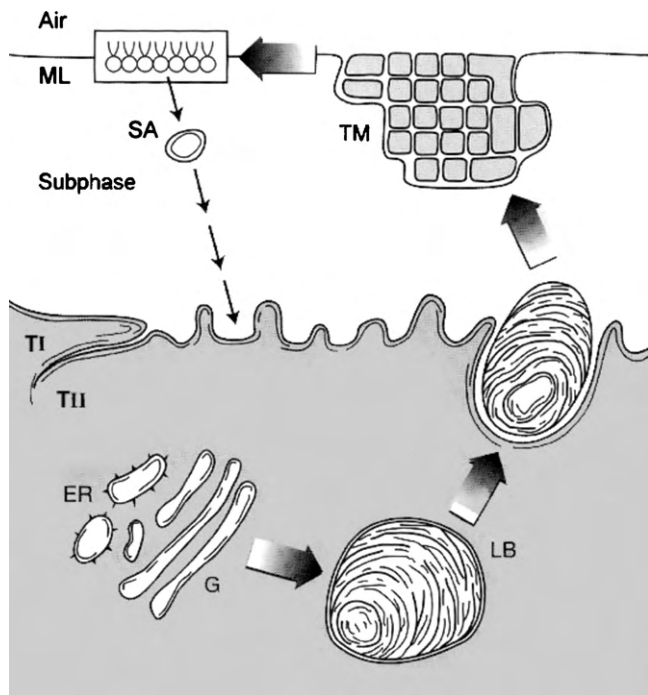


Fig. 1. Life cycle of pulmonary surfactant. Surfactant is synthesized in the endoplasmic reticulum (ER), processed through the Golgi (G) and assembled in lamellar bodies (LB). LB are secreted into the alveolar subphase, where they are converted to tubular myelin (TM). TM gives rise to the surface film/monolayer (ML). With repeated contraction, the film yields unilamellar small aggregates (SA), which are taken up by the type II cells for degradation and recycling. Modified from Possmayer et al. (1984) and Possmayer (2004).

secreted separate from LB, and SP-A reacts with secreted LB to form a specialized structure known as “tubular myelin”, likely the source of the surface film *in vivo*. With repeated compression:relaxation of the surface film, small, mainly unilamellar vesicles are formed which are partly taken up for degradation by alveolar macrophages, but primarily endocytosed by type II cells for catabolism by lysosomes and reutilization by LB. Surfactant proteins are also taken up by type II cells and recycled in part to LB. Surfactant obtained by bronchoalveolar lavage (BAL) can be centrifuged to generate a large aggregates fraction (LA), containing all of the alveolar surfactant constituents save the small (mainly unilamellar vesicles) aggregates fraction (SA). SA have the same phospholipid (PL) composition as LA but contain very little surfactant protein (Possmayer, 2004; Zuo et al., 2008c).

It is generally accepted that functional pulmonary surfactant must (1) adsorb rapidly to form a surface active film; (2) attain low γ during film compression, as occurs during expiration; and (3) adsorb or respread rapidly during film expansion, as occurs during inspiration (Possmayer, 2004; Perez-Gil and Keough, 1998; Perez-Gil, 2008; Rugonyi et al., 2008; Zuo et al., 2008c). Mechanistic insights into these important functional processes remain important goals for pulmonary surfactant research.

2. Surfactant adsorption to form a surface active film

Recent studies by Stephen Hall's group have focused on the initial events in surfactant film formation. Hall proposes a model whereby surfactant constituents adsorb by way of a stalk or pore that bridges the gap between the vesicular bilayer and the air interface (Rugonyi et al., 2008; Chavarha et al., *in press*). Such fusion stalks can be considered analogous to the structures proposed for membrane fusion between cellular organelles, between cells or between coated viruses and cells. A model for an intermediate

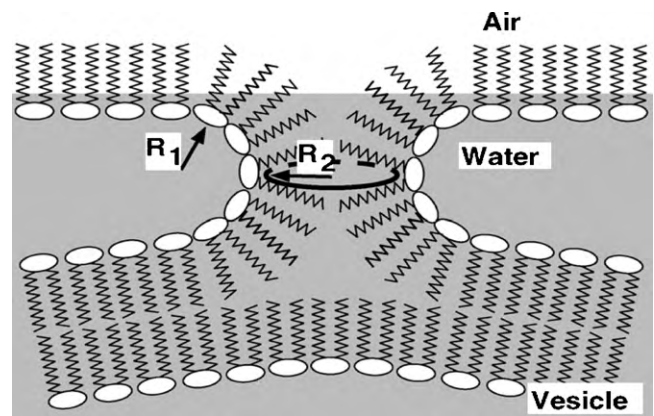


Fig. 2. Hypothetical kinetic intermediate in vesicular adsorption. R1 is the radius of curvature between the surface and the vesicle. R2, which approximates the length of a PL, is the radius of the stalk (Rugonyi et al., 2008).

structure would suggest that initially PL would flow from the outer leaflet of the underlying bilayer onto the surface (Fig. 2) (Rugonyi et al., 2008). Whether PL from both leaflets of the bilayer can readily flow onto the surface is unknown. Formation of this kinetic intermediate would determine the initial rate of adsorption (Rugonyi et al., 2008).

Accumulated evidence demonstrates that adsorption of surfactant PL is markedly enhanced by the low molecular weight surfactant proteins SP-B and/or SP-C. These proteins, to a much greater extent than unsaturated lipids, augment adsorption of disaturated PL. The discovery of these proteins marked an important advance in understanding surfactant function (Possmayer et al., 1984; Takahashi and Fujiwara, 1986; Yu and Possmayer, 1986; Possmayer, 1988; Wang et al., 1996). SP-B and SP-C likely facilitate formation and stabilization of the proposed adsorption stalks (Perez-Gil, 2008; Rugonyi et al., 2008; Zuo et al., 2008c).

Note the sharp curvature, marked R1 in Fig. 2, of the leaflet joining the underlying vesicle to the surface monolayer. The hydrophobic surfactant proteins could promote formation of the stalk by reducing the energy required to bend the lipid leaflets into this curved structure. Other factors could affect adsorption according to how they would influence the bending of a leaflet to form such a structure (Biswas et al., 2007; Perkins et al., 1996; Walters et al., 2000; Yu et al., 1984). So far, however, all evidence that the proteins might affect bending of the leaflets has been indirect. That problem may reflect the lipids with which the proteins have been studied. Phosphatidylcholines (PC), and mixtures of lipids dominated by those compounds, form bilayers. The reason for this is that glycerolipids like PC have a cylindrical shape, in which the hydrophobic and hydrophilic ends of the molecule have similar cross-sectional areas. This allows these molecules to pack readily into lamellar bilayers such that the hydrophobic fatty acyl groups are excluded from water (Hafez and Cullis, 2001). With lamellar bilayers, any tendency of a leaflet to curve is cancelled by the presence of the oppositely oriented, paired leaflet (Seddon and Templer, 1993). Demonstration of how the surfactant proteins affect the bending of leaflets could be facilitated by employing lipids that themselves can form curved structures.

The phosphatidylethanolamines (PE) provide such a system. At low temperatures PE molecules also possess a more or less cylindrical shape and form lamellar phases. However, as temperature is increased the fatty acids become more mobile and occupy a larger volume. As this occurs, due to the relatively small head-groups, PE adopts a conical shape which can no longer fit into bilayers while excluding the acyl groups from water. This causes PE molecules to adopt non-lamellar phase structures. To investi-

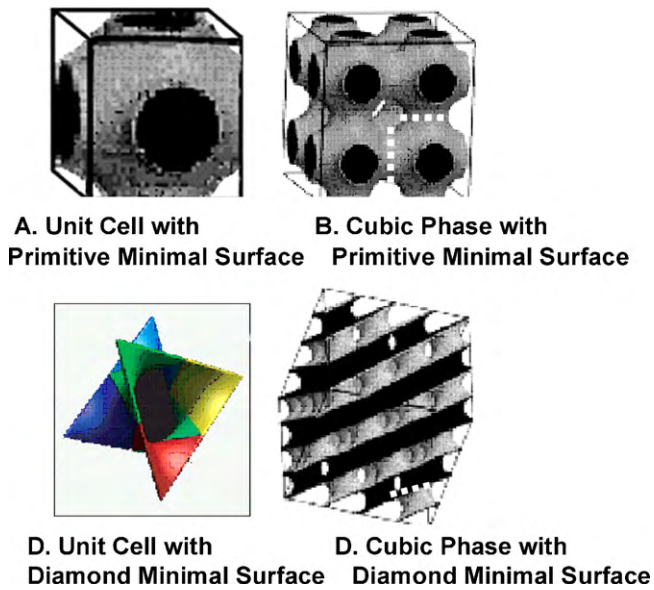


Fig. 3. Cubic phases generated with POPE in the presence of SP-B plus SP-C. (A) Depicts a single cubic unit cell with a primitive minimal surface. Note there are inside and outside compartments. (B) Illustrates some bicontinuous cubic phase composed of 8 unit cells with a continuous primitive minimal surface. A single cubic unit cell is segregated by the dotted lines towards the lower right. (C) Depicts a single cubic unit cell with diamond minimal surface and (D) illustrates some bicontinuous cubic phase with diamond minimal surface. A single cell is isolated by the slanted white dotted line towards the lower right. Modified from Marrink and Tieleman (2001).

gate whether the hydrophobic surfactant proteins affect membrane curvature, Hall's group combined mixtures of SP-B and SP-C with 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) and used small angle X-ray diffraction (SAXD) to examine the resulting structures (Chavarha et al., *in press*).

POPE alone converted from β -lamellar (L_{β}) (gel) phase to α -lamellar (L_{α}) (fluid) phase (phases explained below) at $\sim 25^{\circ}\text{C}$, which in turn transitioned to the H_{II} phase above 70°C . In the presence of SP-B and SP-C, the lamellar phases were unaffected at low temperatures, but as temperature was increased with amounts of proteins as low as 0.03% (w:w), POPE formed two bicontinuous cubic phases (Fig. 3). With these structures, a continuous bilayer that separates two aqueous compartments adopts saddle-shaped curvature at all points. Because the two curvatures that define the saddle are opposite in size and equal in magnitude, the surfaces of these structures have no net curvature, defining them as minimal surfaces (Seddon and Templer, 1993). The two cubic phases observed have diamond and primitive minimal surfaces (Fig. 3). The ability of the surfactant hydrophobic proteins to alter the structural configuration of POPE leaflets supports a model whereby these proteins accelerate surfactant PL adsorption by promot-

ing saddle-shaped structures in the proposed adsorption:fusion intermediate.

Using another approach, Thomas Haller's group has examined the release of LB from alveolar type II cells and their interaction with the air-liquid interface (Haller et al., 2004). These investigators have developed an inverted interface fluorescence microscope combination (Bertocchi et al., 2005). With this apparatus, a small aqueous drop is suspended within the aperture plane by capillary action to create an essentially flat meniscus. Isolated LB are surprisingly stable, remaining intact in aqueous suspension for days. LB falling through the suspended drop can interact with the air-liquid interface by rapidly (i.e., s) adsorbing to form films (Fig. 4). However, as γ falls, increasing numbers of subsequently interacting LB tend to remain intact. This suggests the possibility that an activating transformation, which is more readily elicited at high γ , may be required for adsorption. Additionally, these observations confirm that surfactant adsorption does not require tubular myelin. The basis for the apparent structural stability of some LB inserted into the air-liquid interface remains unknown. However, it is evident that with sufficient LB and time, γ approaching the equilibrium value of $\sim 23\text{ mN/m}$ can be achieved (Bertocchi et al., 2005).

Appreciation of these experiments may be facilitated by a brief discussion of PL phase transitions (see Perez-Gil, 2008; Rugonyi et al., 2008; Zuo et al., 2008c for further review). PL bilayers exist in different lamellar phases depending on the polar head groups, the fatty acids and the temperature. For example, below 41°C dipalmitoyl-PC (DPPC) bilayers exist in the L_{β} or gel phase, in which both the fatty acyl chains and the arrangement of molecules are highly ordered. Above this temperature, known as the main transition temperature (T_m), both the fatty acyl chains and the molecular packing become disordered, leading to the L_{α} or fluid phase, where DPPC molecules can diffuse within the plane of their leaflet. PL phases are altered by the addition of cholesterol. Including cholesterol straightens the acyl chains and alters molecular packing, converting both L_{β} and L_{α} to an intermediate liquid ordered (L_o) phase. L_{α} phase is sometimes referred to as liquid disordered (L_d) phase, for example when cholesterol is present at concentrations too low to generate L_o phase. These phases have been defined with relatively simple model systems normally using one to three components (e.g. Mouritsen and Zuckermann, 2004; Veatch and Keller, 2005). For this reason, some investigators prefer to refer to liquid ordered-like and liquid disordered-like phases. The relative fluidity of these phases is $L_{\alpha}(L_d) > L_o > L_{\beta}$.

PL monolayers also form a series of phases that depends on the lipid, the acyl groups and the temperature. These monolayer phases can be considered analogous to the bilayer phases, but with the difference that monolayer transitions are highly influenced by PL surface density. PL surface density can be increased by reducing surface area, for example on a Langmuir-Willhelmy surface balance by increasing lateral pressure with the barrier. Surface pressure (π), which is the extent to which a film reduces the surface tension of

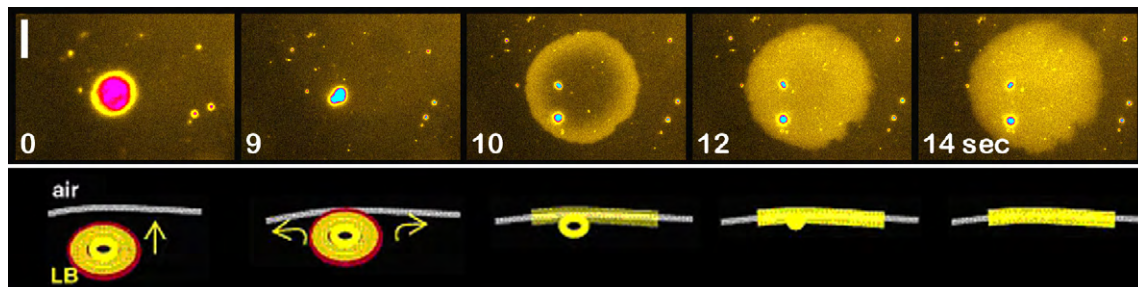


Fig. 4. Adsorption and spreading of a LB at the air-water interface. The upper series shows inverted interface microscopic images. The lower panels illustrate interpretation of the LB's behaviour. Modified from Haller et al. (2004) and Perez-Gil (2008).

the clean air–liquid interface, can be viewed as representing the applied force.

With bilayers, the effective lateral pressure is influenced by internal forces (corresponding to $\pi \sim 30$ mN/m under standard conditions) arising from the need to avoid excessive wetting of the hydrophobic fatty acids (Zwaal et al., 1976). With monolayers, where the acyl groups extend into (hydrophobic) air, there is no such limitation. As a result, it is possible to have very dilute PL monolayers where the lipids are in a gaseous phase. In this state they do not significantly affect π (or obviously γ). Increasing π , for example by reducing surface area on a surface balance, leads to generation of the fluid liquid expanded (LE) phase. With PL below the bilayer T_m , further reductions in surface area generate the tilted condensed (TC) phase. The TC phase may persist to γ approaching 0 mN/m ($\pi \sim 70$ mN/m at 37 °C), or, as has been suggested recently, it may convert to an untilted condensed phase (Perez-Gil, 2008).

Due to kinks arising from double bonds, unsaturated PL do not pack readily and consequently possess low bilayer T_m . As a result, unsaturated PL monolayers do not form TC phase at physiological or even room temperatures. Increasing atmospheric pressure increases order with L_α phase bilayers by limiting acyl mobility. In a similar vein, with PL monolayers increasing π can reverse acyl chain melting slightly above the bilayer T_m , resulting in formation of TC phase (Yan et al., 2007).

As in the case of bilayers, cholesterol has profound effects on monolayer phase transition. Incorporating cholesterol into TC phase generates the more fluid monolayer liquid ordered (mLO) phase. Two mLO phases have been identified (McConnell and Vrljic, 2003). With low cholesterol, mLO α phase is created while mLO β phase is generated at higher sterol levels. With the ternary system, dioleoyl-PC:DPPC:cholesterol, the shift from the α -region to the β -region occurs at ~ 15 –25 wt%. This is influenced by the dioleoyl-PC:DPPC ratio and by π (Stottrup et al., 2005; Stottrup and Keller, 2006). Adding cholesterol also converts LE to mLO phase. As in the case of bilayers, these phases were defined using simple model systems. The relative fluidities of these phases are: LE > mLO > TC.

Returning to the Haller LB experiments, phase separation events occurring as LB adsorb can be followed using phase selective fluorescent dyes and by reflection microscopy. The adsorbed monolayers are formed as complex films, apparently containing LE, mLO and TC regions. In addition, there is evidence for multilayer formation, presumably arising from the multilaminations of the LB. Previous studies have shown that spontaneously adsorbing DPPC:SP-C vesicles form gaseous, LE and TC phases (Nag et al., 1996). However, the present experiments further stress that the lateral and spatial phase organizations of LB films are quite distinct from those observed with more commonly utilized surfactants, such as for example the clinical surfactant Curosurf (a modified natural pulmonary surfactant). These other surfactants adsorb much more slowly and form considerably less complex surface films. These observations also serve to emphasize the sometimes disregarded differences between natural and clinical surfactants. These, in turn, arise from their distinct lipid and protein compositions.

In related studies, interactions between isolated rat type II cells and the air–liquid interface were investigated. Type II cells responded to proximity to the air–liquid interface by a graded Ca^{++} response, which results in elevated LB secretion. These observations suggest the possibility that air–liquid interfacial forces are detected as a potential “threat” to cell stability. The cells respond by generating Ca^{++} gradients which act as a stimulus to release LB and therefore regulate γ near the cells. Note that these γ forces act on both type II cells and, as indicated earlier, the LB. This implies that the physical environment in the vicinity of the cells can initiate a complex biological response that, in turn, modulates the physical environment of the cells via a feedback loop.

3. Chemical and structural analysis of surfactant surface films

Classically, it has long been considered that mammalian lungs at low lung volumes are stabilized by the presence of a monolayer at the alveolar surface which is highly enriched in the disaturated PC, DPPC (Watkins, 1968; Clements, 1977; Bangham et al., 1979). It has further been proposed that such DPPC-enriched monolayers arise through selective squeeze-out during monolayer compression of the unsaturated PL (which melt to fluid phases at temperatures below 37 °C) (Bangham et al., 1984; Perez-Gil and Keough, 1998; Veldhuizen et al., 1998; Possmayer, 2001, 2004; Perez-Gil, 2008; Rugonyi et al., 2008; Zuo et al., 2008c). Consistent with these proposals, compression of spread monolayers of organic extracts of natural surfactants containing (bulky) fluorescent probes reveals the appearance of dark probe-excluding microdomains (μ -domains), the number and intensity of which increase as π is elevated (Discher et al., 1996; Nag et al., 1998). The DPPC-enriched monolayer classical model and the squeeze-out hypothesis would predict that the total area covered by these μ -domains should increase until the surface monolayer becomes essentially free of fluorescent fluid regions as γ decreases to near zero. In practice, however, increasing π leads to the disappearance of these presumably DPPC-enriched μ -domains (Discher et al., 1996; Nag et al., 1998). In fact at 37 °C, probe-excluding domains can barely be observed. Additional evidence contrary to the classical model and the squeeze-out hypothesis cannot be discussed in detail here, but to date we know of no compelling evidence supporting the classical model or the squeeze-out mechanism with natural or extract surfactants (Yu and Possmayer, 2001; Pikhova et al., 2002; Yan et al., 2007; Rugonyi et al., 2008; Zuo et al., 2008c).

The classical model makes distinct predictions concerning the chemical composition of the surface film. Understanding events occurring at the alveolar interface would benefit from chemical analysis of such films. Time of flight-secondary ion mass spectroscopy (ToF-SIMS) focuses a potent ion beam on the surface of a sample so as to generate ionized and non-ionized fragments. Ionized fragments are subsequently analyzed by mass spectroscopy (MS). The high energy beam is rastered across the sample in such a manner as to yield over 5 million measurements per view, providing spatially localized, detailed compositional information. Nils Petersen's group has applied ToF-SIMS to surfactant films transferred to gold-covered mica to generate precise chemical information on the μ m scale. Application of this approach to surfactant films transferred at various π confirmed that, as expected, μ -domains appearing identical in size and number to those observed by fluorescence microscopy (FM) and atomic force microscopy (AFM; see later) are composed of DPPC and dipalmitoyl-PG (DPPG) (Keating et al., 2007). The analytical precision of this approach can be markedly enhanced through spiking the samples with compounds of interest labelled with stable isotopes. This approach has been used in attempts to localize surfactant proteins. Discrimination can also be elevated through computer-based processing techniques (Biesinger et al., 2006).

Insights obtained through FM and ToF-SIMS have been greatly expanded through application of AFM. With AFM, a very fine probe, which detects the electron clouds from molecules on the sample surface, provides topographical discrimination in the x - y dimensions at the sub- μ m range and in the z - (up:down) dimension at the sub-nanometer (nm) range. Examination of transferred spread films of BLES (bovine lipid extract surfactant), a clinical surfactant used to treat neonatal respiratory distress in Canada, on a Langmuir–Wilhelmy surface balance by Yi Zuo and co-workers revealed that as π increases, μ -domains appear and then begin to disappear (Fig. 5). As the μ -domains disappear, they are apparently replaced by nanodomains (n-domains) of the same height

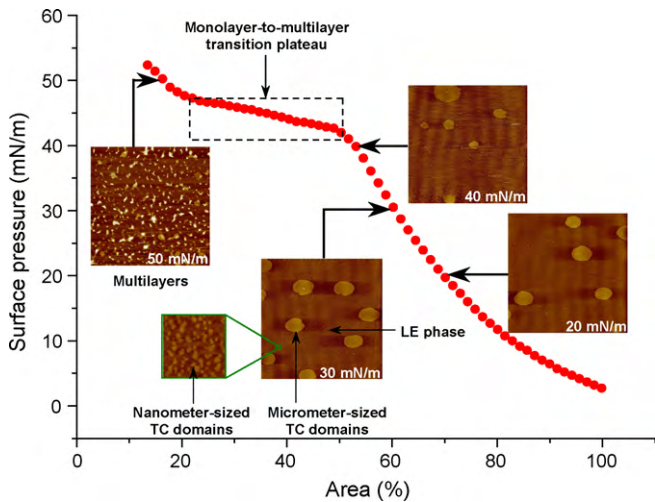


Fig. 5. AFM of BLES samples transferred at increasing π , showing the formation of μ -domains, n-domains, and multilayers. Modified from Zuo et al. (2008c).

which are too small to be detected by FM or ToF-SIMS (Zuo et al., 2008a,b,c). Similar overall conclusions arose from studies on DPPC:DPPG films (Cruz et al., 2004).

Above π of about 45, which approximates equilibrium γ , a rising plateau is observed above which π increases to the values thought necessary to protect the terminal air spaces (Perez-Gil and Keough, 1998; Veldhuizen et al., 1998; Piknova et al., 2002; Keough, 2003; Rugonyi et al., 2008; Zuo et al., 2008a,c). Within and above this rising plateau, the surface film obtains a mottled appearance due to the presence of multilayers. These multilayers could act as a surface-associated surfactant reservoir, functionally associated with the overlying monolayer. This provides a mechanism for film preservation through reversible collapse (Schürch et al., 1995; Qanbar et al., 1996; Amrein et al., 1997; Malcharek et al., 2005; Yu and Possmayer, 2001; see Schürch et al., 1998, 2001; Possmayer et al., 2001; Possmayer, 2004; Perez-Gil, 2008; Zuo et al., 2008c for review). Such surfactant reservoirs can be generated during adsorption or during film compression (Possmayer, 2004). Multilayer formation can be promoted by either SP-B or SP-C, and by SP-A in the presence of SP-B.

An important remaining question is the manner in which surfactant monolayers containing 50% or more unsaturated, fluid PL attain γ near zero during film compression. Several mechanisms have been proposed:

- (1) The n-domains created by compression and through fragmentation of μ -domains could contribute composite or alloy-like properties which distribute the load on the monolayer more evenly, thereby strengthening the film (Perez-Gil and Keough, 1998; Keough, 2003; Zuo et al., 2008a,c). However, it is difficult to discern n-domains on surface monolayers in the presence of the protruding multilayers, thus limiting evidence for this mechanism.
- (2) The more fluid unsaturated PL may be selectively infused into the forming multilayers resulting in disaturated PL enrichment of the remaining interfacial monolayer. As elaborated in the symposium, Petersen's group has obtained some evidence for this proposal. Fig. 6 shows that combining ToF-SIMS and AFM studies of BLES films at high π shows deep "wells" which appear composed exclusively of DPPC and DPPG, consistent with modified squeeze-out. However, the possibility remains that these "wells" merely represent μ -domains which have not broken up to form n-domains and it has not been possible to obtain analyses of more than very small parts of the underlying monolayer.
- (3) Hall's group has discovered that compressing surfactant extract films and even monolayers containing unsaturated PL, such as POPC, faster than they can collapse (rates $\geq 50\%$ area reduction/s) leads to films that can support π near 70 mN/m (γ near zero) (Crane and Hall, 2001; Smith et al., 2003; see Piknova et al., 2002; Keough, 2003; Perez-Gil, 2008; Rugonyi et al., 2008; Zuo et al., 2008c for review). Such films are metastable, i.e., below equilibrium, but persist at low γ for long periods (*h*). The authors suggest these persistently metastable films may arise through an amorphous, non-crystalline structure. Whether such supercompressed or "jammed" films, or for that matter DPPC-enriched monolayers, occur *in vivo* at the alveolar interface is still not clear.

4. Phase transitions in pulmonary surfactant bilayers

In order to obtain further insights into pulmonary surfactant phase segregation, Jorge Bernardino de la Serna, Luis Bagatolli

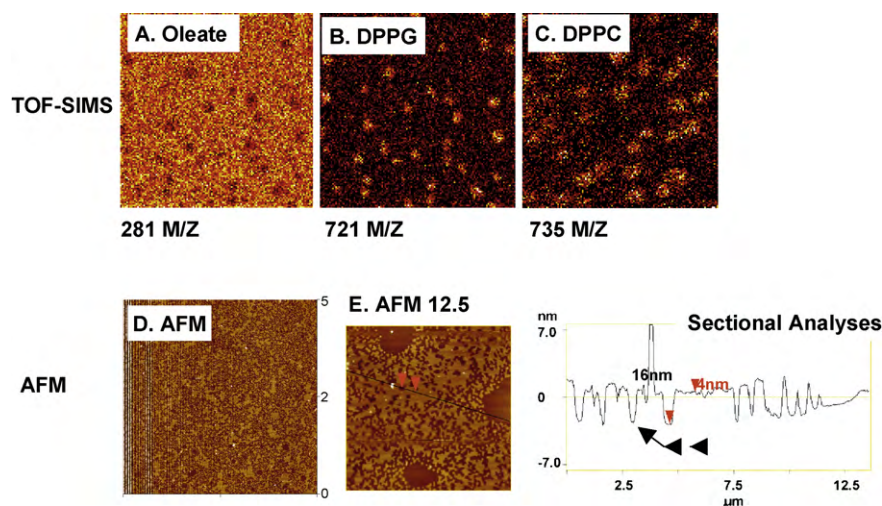


Fig. 6. ToF-SIMS and AFM of BLES multilayers at $\pi = 50$ mN/m. ToF-SIMS ($50 \mu\text{m} \times 50 \mu\text{m}$ each) shows oleate-containing PL (A) are present throughout most of the sample, but DPPC (B) and DPPG (C) are present in small limited areas. These latter areas are suggested to correspond to the small regions observed in the corresponding AFM picture (D) and in the enlarged AFM view (E) ($12.5 \mu\text{m} \times 12.5 \mu\text{m}$). The "wells" indicated by the arrow in the AFM height sectional analysis (F) could be part of the underlying monolayer below the multilayers. This is consistent with a condensed monolayer being associated with the multilayer, which could support $\pi \sim 70$ mN/m (Petersen et al., 2009).

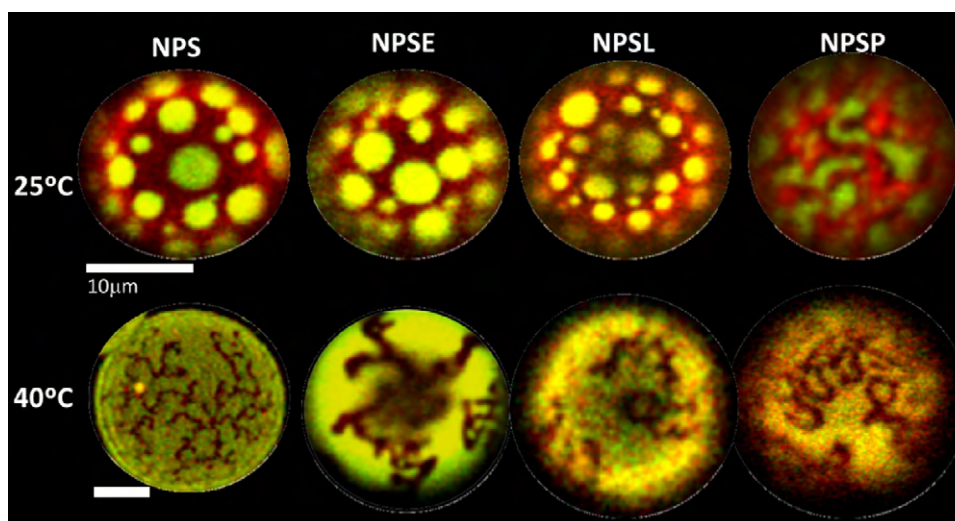


Fig. 7. Phase segregation below and above physiological temperature in GUV of natural porcine pulmonary surfactant (NPS) and fractions thereof. Scale bar indicates 10 μm for all images except NPS at 40 $^{\circ}\text{C}$, which has its own bar, also 10 μm (see text and Bernardino de la Serna et al., 2009 for further details).

and Jesus Perez-Gil applied two-photon excitation FM and other approaches to examine phase transitions in surfactant bilayers and monolayers (Bernardino de la Serna et al., 2004, 2009). These investigations examined natural (porcine) pulmonary surfactant (NPS), organic solvent extracts of NPS (NPSE), the isolated lipid fraction of NPSE (NPSL, i.e., no SP-B or SP-C), and the PL fraction from NPSL (NPSP, i.e., no cholesterol). Bilayers were microscopically examined as giant unilamellar vesicles (GUV) generated by using low field alternating electric current.

Differential scanning calorimetry studies revealed similar endothermic transitions for all of these surfactant preparations with heat absorption (acyl chain melting) initiating below 20 $^{\circ}\text{C}$, peaking near 32 $^{\circ}\text{C}$, and completing sharply just above 37 $^{\circ}\text{C}$. These results confirmed that PL acyl groups are primarily responsible for the thermotropic properties. Confocal FM imaging using fluorescent lipid probes (Fig. 7) revealed distinct phase separation within these bilayers at 25 $^{\circ}\text{C}$, consisting of μm -sized L_{α} -like fluid domains within an L_{α} -like background, except for the PL fraction (NPSP), where L_{α} fluid μ -domains were present within a L_{β} gel matrix. This difference reflects the absence of cholesterol in the NPSP fraction. Most of the μ -domains were abolished at 40 $^{\circ}\text{C}$ (Fig. 7).

Two-photon excitation FM investigations were used to monitor fluorescence emitted from the probe LAURDAN. LAURDAN is a very useful probe because it reports fluorescence information in both gel and fluid phase environments (Bagatolli, 2006). These data are expressed as a generalized polarization (GP) function varying from 1 to -1, where higher numbers reflect lower fluidity, whereas lower numbers indicate increasing fluidity, providing unique structural and dynamic information about membrane packing. LAURDAN GP measurements were consistent with the above GUV bilayer preparations possessing fluid L_{α} domains within a larger L_{α} phase. These authors prefer to report their results as coexisting liquid ordered-like and liquid disordered-like phases (similar but possibly not precisely identical to a coexistence of L_{α} and L_{β} (or L_{α}) phases) when cholesterol is present.

Surprisingly, application of electron spin resonance (ESR) using nitroxide-labelled probes along the fatty acyl groups failed to detect two different acyl group mobilities as temperature increased through the physiological range (Bernardino de la Serna et al., 2009). This suggests either that lipid chain mobility does not differ greatly between the fluid disordered-like and fluid ordered-like regions at 37 $^{\circ}\text{C}$ (FM) or that PL exchange between these two regions is very fast at this temperature (ESR). Although a single

mobile component was observed, samples devoid of surfactant proteins and/or cholesterol showed the fastest acyl group motion below 40 $^{\circ}\text{C}$. Similarly, line pulsed field gradient nuclear magnetic resonance revealed a single lateral diffusion component in these samples. The fastest translational diffusion coefficient of PL was observed in NPSP (i.e., no SP-B, SP-C or cholesterol), but this difference was evident only below 30 $^{\circ}\text{C}$. These results show that the particular lateral structures observed in pulmonary surfactant bilayers display different dynamic features than those resolved in PC: sphingomyelin: cholesterol “canonical raft” mixtures where liquid ordered-liquid disordered phase coexistence is also observed (Bagatolli, 2006). Further studies are necessary to fully understand these observations, particularly at physiological temperatures.

These approaches have been extended to pulmonary surfactant from SP-D deficient (SP-D KO) mice. Such mice have large alveolar surfactant pools arising from diminished uptake and catabolism (Hawgood et al., 2002; Ikegami et al., 2005). Interestingly, Bernardino de la Serna and Bagatolli found SP-D KO surfactant possessed a slightly higher disaturated PL content, but similar overall surface tension characteristics on a surface balance. SP-D KO surfactant yielded larger μ -domains than surfactant from wild type mice but domain area coverage was somewhat reduced. Two-photon excitation FM studies demonstrated alterations in the LAURDAN GP function, consistent with more gel-like, less fluid bilayers and with reduced PL miscibility with SP-D KO mice.

The results presented by this group suggest that the presence of cholesterol can play an important role in regulating the structural and dynamic properties of pulmonary surfactant bilayers. However, the relationship between the structural/dynamic features observed in surfactant bilayer systems (occurring at several time and length scales) and surfactant function under physiological conditions remains open.

5. Pulmonary surfactant in the clinical setting

The development of electrospray ionization mass spectroscopy (ESI-MS) methods for analysis of lipid molecular species has revolutionized PL compositional investigations. Anthony Postle and his group have pioneered application of this approach to pulmonary surfactant (Postle and Hunt, 2009). With this technique, solvent droplets optimized toward containing single molecules of the sample are evaporated. The dried ionized particles are then separated by mass over charge (m/z) using mass spectroscopy in either pos-

itive or negative mode. Analysis can be conducted on direct mass distribution or by diagnostic scans using tandem MS/MS, where individual peaks are disrupted to ionized fragments via collision gases, for example the phosphocholine fragment (m/z , 184) for PC.

Applying ESI-MS analyses to BAL samples from control and asthmatic patients under basal conditions demonstrated similar total PC/PG ratios and molecular distribution for these PL (Heeley et al., 2000). Allergen challenge led to an increase in PC species containing linoleic acid (i.e., 16:0/18:2-PC, 18:0/18:2-PC, and 18:1/18:2-PC). These molecular species proved characteristic of plasma PC from the same patients, consistent with the altered PC composition arising from leakage of plasma lipoproteins into the alveolar space. Levels of 18:2-containing-PC species correlated directly with BAL total protein levels and indirectly with the γ lowering ability of the BAL samples. These observations strongly suggest that the PC composition alterations occurring after allergen challenge arise from serum leakage and this may also contribute to the functional changes.

Another study using ESI-MS revealed that the fractional concentration of 16:0/16:0-PC correlated with airway resistance and decreased relative 16:0/16:0-PC levels arose from elevations in cell membrane PL such as 16:0/18:1-PC and 18:0/20:4-PI in BAL from children with structural pulmonary abnormalities, asthma, lung infections or cystic fibrosis (Mander et al., 2002). Surfactant activity was suppressed in BAL from patients with infection or cystic fibrosis. Infection was accompanied by increased LA PL levels, possibly due to membrane components from inflammatory cells.

Traditional approaches to studying pulmonary surfactant PL synthesis rely on incorporation of radio-labelled substrates. Results were often limited to total disaturated PC, typically isolated after OsO_4 oxidation of unsaturated lipids. This procedure is not very robust because some monounsaturated lipid is also isolated (Chan et al., 1983). Its use tended to encourage equating surfactant with DPPC. It should be noted that erythrocytes and myelin also have significant levels of DPPC (Veldhuizen et al., 1998) and that the DPPC content of spleen is similar to or higher than that of surfactant.

Surfactant secretion has normally been evaluated using radio-labelled techniques usually employing isolated type II cells. While providing useful information, these approaches are limited in terms of molecular detail and are obviously not applicable to whole animal or clinical settings. Recent advances in MS and availability of PL metabolic precursors containing stable isotopes have made metabolic studies in experimental animals more practical and human clinical studies ethically feasible (Fig. 8). For example, human studies employing deuterium-labelled choline showed that, following a 6 h lag period, there was a 30 h linear increase of labelled PC in induced sputum samples. Fractional labelling of unsaturated PCs such as 16:0/18:1-PC was initially higher than for 16:0/16:0-PC at 12 h, but equalized at 48 h. These data suggest that de novo newly synthesized PC incorporates rapidly into LB and is secreted independently from 16:0/16:0-PC arising from the deacylation/reacylation pathway. Labelled choline was incorporated more rapidly into plasma PC, presumably as a result of the slower incorporation of this lipid into LBs prior to secretion and possibly time for migration through the bronchiolar system from the peripheral lung.

Additional studies are being conducted with animal models, in particular mice bearing completed and conditional gene KO mutations that do not produce functional SP-D, stearoyl-CoA dehydrogenase (produces oleoyl-CoA), or the ABCA3 PL transporter (transports PC into LB).

This report will close with discussion of an animal model of acute lung injury/acute respiratory distress syndrome (ALI/ARDS), an important clinical manifestation of an acute, life-threatening pulmonary injury. ALI/ARDS arises through a variety of insults, including direct pulmonary causes such as microbial infections,

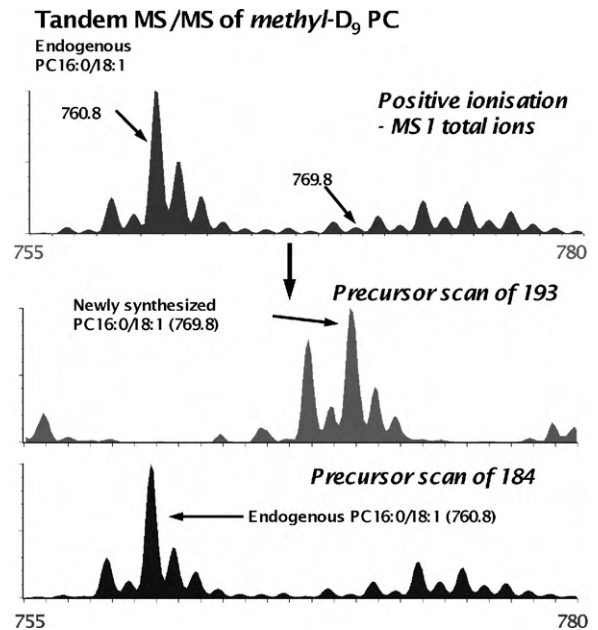


Fig. 8. Tandem MS/MS of endogenous and newly synthesized PC in surfactant isolated from D9-choline infused mice. The upper spectrum shows total positive mode PL. The center panel depicts newly synthesized (D9-labelled) PC while the lower spectrum gives endogenous (nonlabelled) PC (Postle et al., 2009).

(stomach) acid aspiration or near drowning, and indirect causes such as systemic infections, trauma, pancreatitis, or brain injury (Lewis and Veldhuizen, 2003; Bosma and Lewis, 2007). The final common mechanism for ALI/ARDS appears to involve leukocyte activation, resulting in inflammation which leads to surfactant inactivation. The leukocyte activation can induce a cytokine cascade with increased cellular activity beyond the lung. Consequently death due to ALI/ARDS often occurs as multi-organ failure initiated by the original pulmonary dysfunction.

ARDS often arises through a “two-hit” scenario, in which ventilation contributes to the second hit. The Veldhuizen/Lewis group has developed a ventilation-induced model of injured lungs in which rats are ventilated at 3.7-fold (high Vt) the normal non-injurious volumes (low Vt) (Maruscak et al., 2008; Vockeroth et al., 2010). Physiological indicators of lung function, such as oxygenation, were maintained for the 120 min of ventilation in the low Vt group. In high Vt animals lung function was maintained for the first 60 min but decreased rapidly thereafter. Surfactant samples obtained at 120 min were examined with the captive bubble surfactometer (CBS) introduced by Schürch et al. (1989). Crude and gradient-purified LA from low Vt and high Vt animals adsorbed at similar rates, which were more rapid than with organic-extracted LA. Addition of SP-A, which enhances adsorption and is removed by extraction, restored adsorption by low Vt but not high Vt surfactant, suggesting a fundamental functional difference.

Subjecting crude control LA to compression:expansion cycling of surface area resulted in an initial high minimum γ (γ_{\min}), which improved thereafter. Purified low Vt LA exhibited low initial γ_{\min} , consistent with gradient removal of inhibitory serum proteins (Cockshutt et al., 1990). Neither crude, purified nor extracted high Vt LA were able to lower γ to low values, despite prolonged cycling. Cholesterol content was elevated in high Vt surfactant. Incubation with β -methyl cyclodextrin (β MCD), which binds sterols, led to some improvement in γ_{\min} , such that the γ_{\min} values for extracted high Vt LA approached those for low Vt. Adding cholesterol restored the inhibition. This suggests that increasing surfactant cholesterol above physiological values is detrimental to pulmonary surfactant (Gunther et al., 1996; Keating et al., 2007; Gunasekara et al., 2008;

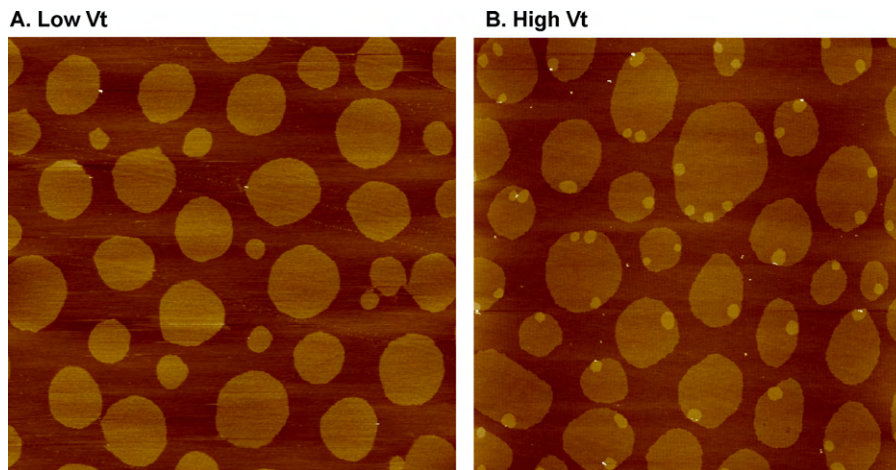


Fig. 9. AFM of natural pulmonary surfactant from low Vt (A) and high Vt (B) rats at $\pi = 30$ mN/m. Both samples show numerous μ -domains but μ -domains from high Vt surfactant show “domains-within-domains” (Veldhuizen et al., 2009).

Vockeroth et al., 2010). Despite cholesterol removal with β MCD, surface activity with Vt extracts was not optimal, possibly related to a reduction in immunoreactive SP-B.

To provide a structural correlate to the functional studies, AFM was performed. Preliminary data revealed that μ -domains from high Vt differed from low Vt surfactant in having small, higher domains within the normally observed mLO μ -domains at a surface pressure of 30 mN/m (Fig. 9). The nature of these “domains-within-domains”, and whether they contribute to the surfactant dysfunction, is unknown, but they can be generated in BLES by elevating cholesterol, and therefore apparently involve that compound.

6. Conclusion

This article has attempted to illustrate how recent advances in membranology have contributed to our understanding of surfactant function with a view toward encouraging other investigators to apply these techniques to their particular biological problems, whether or not related to surfactant. While the investigations described did not (as anticipated) resolve all previous difficulties, they did add considerable new detail and helped to refine a number of critical questions. SAXD investigations have provided evidence indicating that SP-B and/or SP-C may accelerate adsorption by promoting the formation of curved structures. While the inverted interface microscope extends our capacity to follow adsorption processes, this novel approach will require further application and possibly further development to contribute additional insights into LB adsorption.

Likewise, application of ToF-SIMS to chemical definition of spread films provides information about film organization. This approach has provided novel insights, such as the realization that surfactant dysfunction might reflect altered organization of a film rather than only, compositional changes. ToF-SIMS has been complemented by AFM. Nevertheless, these approaches, while admittedly helpful, serve to emphasize certain questions such as: (1) Do the μ -domains and n-domains contribute to film stability and multilayer formation? (2) Do μ -domains, n-domains, or multilayers stabilize compressed films at high π ? (3) What is the chemical nature of the monolayer, functionally attached to the multilayers, at π near 70 mN/m?

Investigations with two-photon excitation FM have shown that cholesterol has a major effect on phase segregation in bilayers. On the other hand, high cholesterol levels in the alveolus (as well as

in the blood) appear to be deleterious. The role of physiological and superphysiological levels of this sterol on monolayer phase transitions must be further studied. The influence of surfactant cholesterol on γ must be clarified. The reason why phase distributions in surfactant monolayers and bilayers appear to differ must be resolved. The nature of the “domains-within-domains” observed with high cholesterol needs to be determined. Whether reducing surfactant cholesterol to or below normal physiological levels will improve lung function in ALI/ARDS must still be investigated.

ESI-MS provides a simple, rapid and effective method for accurately determining the composition of PL molecular species with extremely small samples. This approach has provided important new information on surfactant composition from humans and experimental animals and this procedure has proven practical in the clinical setting. This technique, especially when applied to metabolic studies through the use of stable isotopes, holds great potential for addressing questions related to alterations in surfactant synthesis and turnover with animal models and clinical situations.

The past decade has proven rewarding in terms of surfactant research due in no small part to application of methodology outlined in this symposium. We submit that these approaches and other new imaginative methodologies hold even greater promise for the future.

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