

Pulmonary surfactant: phase behavior and function

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Pulmonary surfactant functions by first flowing rapidly into the alveolar air/water interface, but then resisting collapse from the surface when the adsorbed interfacial film is compressed during exhalation. Widely accepted models emphasize the importance of phase behavior in both processes. Recent studies show, however, that fluidity is a relatively minor determinant of adsorption and that solid films, which resist collapse, can form by kinetic processes unrelated to equilibrium phase behavior.

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Abbreviations

CLSE	calf lung surfactant extract
D	lateral diffusion coefficient
DOPC	dioleoyl phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
FRAP	fluorescence recovery after photobleaching
LE	liquid-expanded
π_e	maximum equilibrium surface pressure
SP-A, -B, -C	surfactant protein-A, -B, -C

Introduction

The function of pulmonary surfactant can be considered in terms of two transitions — one that must be accomplished quickly and a second that must be avoided. The first transition is the conversion of bilayer vesicles into an interfacial film. After synthesis and secretion by the type II pneumocytes, the mixture of approximately 80% phospholipid, 5–10% cholesterol-containing compounds and 5–10% protein acts as a surfactant, adsorbing to the air/water interface in the alveoli (Figure 1). During inflation of a fluid-filled or collapsed lung, whether at birth or when an aquatic mammal surfaces after a dive to great depth, the air/water interface expands enormously. The presence of a functional film during the first exhalation indicates that the surfactant film forms rapidly. The second transition, which must be avoided, is collapse from the interface. When compressed, monomolecular films exist at an air/water interface under equilibrium conditions only up to a maximum surface pressure (π_e), above which they collapse from the two-dimensional interface to form three-dimensional structures. (Surface pressure, defined as the extent to which a film reduces the surface tension of a clean interface, denotes the two-dimensional equivalent of pressure in three dimensions, or the force exerted by an interfacial film on its linear confining boundaries, with units of force/length [1].) Films can, however, exist at

higher surface pressures in metastable states if they are sufficiently rigid. When compressed by the shrinking alveolar surface area during exhalation, films of pulmonary surfactant reach and sustain surface pressures well above π_e in static lungs maintained at constant volume for at least tens of minutes [2,3]. This characteristic is essential for the ability of pulmonary surfactant to stabilize the small air spaces of the lungs. Existing models consider phase behavior an essential determinant both of adsorption, during which insertion of surfactant constituents into the interface raises the surface pressure to π_e , and of the metastability that allows compressed films to avoid collapse and reach surface pressures above π_e . In this review, we consider recent evidence concerning phase behavior in the precursor structures — bilayers for adsorption and monolayers for collapse — as well as in the transitions themselves. We emphasize in particular unanswered questions and findings that challenge long-standing views of the mechanisms by which pulmonary surfactant functions.

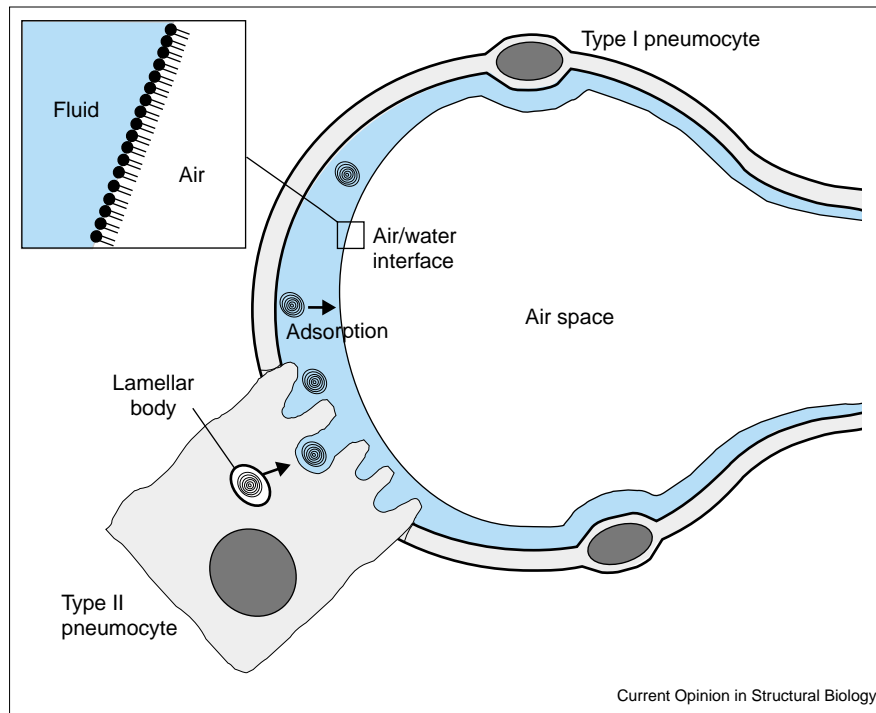
Phase behavior and function

The composition of the surfactant lipids and the phase behavior of simple lipid mixtures suggest three phases that might occur in the films present in the lungs. The disaturated compound dipalmitoyl phosphatidylcholine (DPPC) represents an unusually large fraction of lung surfactant relative to other biological phospholipids, approximately 30–45% by recent estimates [4], and its gel-to-liquid crystal melting transition of 41°C indicates the possibility of a solid phase at physiological temperatures. Along with phospholipids that have lower melting transitions, pulmonary surfactant also contains cholesterol, so there could be two fluid phases, ordered and disordered, distinguished in part by their cholesterol content [5–7]. For both bilayers and monolayers of pulmonary surfactant, the major questions include the following: how many phases are present, what are they and what is their physiological significance?

Bilayers

Two proposed mechanisms explain the adsorption of surfactant vesicles in terms of phase behavior. The first focuses on the fluidity of the bilayer and on the high content of DPPC, which by itself would occur as the solid gel phase at physiological temperatures. Gel phase bilayers adsorb slowly [8*] and the ability of surfactant constituents other than DPPC to accelerate adsorption was originally attributed to their ability to produce more fluid structures [9]. Recent studies confirm the proposed effect of the surfactant lipids other than DPPC on fluidity [10]. Measurements of fluorescence recovery after photobleaching (FRAP) for lipid probes show that lateral diffusion, which is inversely related to viscosity, is much faster in bilayers of extracted calf surfactant (calf lung

Figure 1



Schematic of pulmonary surfactant in an alveolus of the lung. Type II pneumocytes synthesize the surfactant constituents and package them into the concentric bilayers of lamellar bodies. After secretion, the bilayers unravel and adsorb to the air/water interface (see inset). During exhalation, the decreasing alveolar surface area compresses the interfacial film. Points of controversy simplified by this diagram include the following: at least part of the interfacial film consists of a multilayer [29,44], the lateral extent of which is uncertain; although continuous [67], the aqueous layer may at some points be too thin to be considered liquid [68]; and although strong physiological evidence either for or against such a process is lacking, respreading of collapsed material excluded from the interface by overcompression could contribute during re-expansion to the maintenance of a functional film [69].

surfactant extract, CLSE) than in gel phase DPPC and only slightly slower than in liquid-crystalline bilayers of dioleoyl phosphatidylcholine (DOPC) (Figure 2). Diffusion, however, correlates poorly with adsorption. CLSE adsorbs much more rapidly than DOPC, despite its slower diffusion. Adsorption of fluid vesicles is faster than for gel phase phospholipids [8*,11*], but that effect alone fails to explain the effects of the constituents other than DPPC in the adsorption of pulmonary surfactant.

The second proposed mechanism suggests that more rapid adsorption results not from the general disorder of a fluid phase but from localized defects in lipid packing at interfacial boundaries [11*]. Just as a phase boundary may represent a focus of instability that predisposes vesicles to fuse with other vesicles [12], so it might promote fusion with an air/water interface or pre-existing monolayer. Adsorption of single-component vesicles, for example, in some studies [11*] but not in others [8*], is fastest near the main phase transition. Several reports indicate that phase separation can exist in surfactant vesicles. Calorimetric experiments on extracted surfactant with [13] and without [14,15*] cholesterol demonstrate phase coexistence that extends over a broad range of temperatures, but ends at or just below 37°C. FRAP [10] and two-photon fluorescence microscopy [15*] provide further evidence for the coexistence and indicate that the two phases are solid and fluid. The termination of solid/fluid phase separation, however, at temperatures that have no major changes in adsorption rates (Figure 2) [16*] argues against any major role for interfacial boundaries in promoting adsorption.

Surfactant constituents accelerate adsorption not only when located in the vesicles, but also when present exclusively in a pre-existing monolayer. Both the hydrophobic proteins SP-B and SP-C [17], which represent only 1.5% (w:w) of surfactant but have the most dramatic effect on adsorption, and the phospholipids other than DPPC [18] have this ability to promote adsorption from both locations. For the phospholipids, the effect when confined to the monolayer or to the vesicle is equivalent. These observations suggest that an intermediate structure, equally accessible from monolayer and vesicle, determines the rate of adsorption. Analogy to bilayer fusion suggests that the intermediate structures might be highly curved, similar to lipids in the hexagonal-II (H_{II}) phase [19]. Phospholipid mixtures that form the H_{II} phase do adsorb rapidly [20]. Although unconfirmed, the facilitation of adsorption by the hydrophobic surfactant proteins via the formation of highly curved intermediates remains the most promising mechanism proposed to account for their effect. Whether catalysis by the surfactant proteins of the reverse process explains their tendency to encourage collapse during compression of films containing some simple phospholipid mixtures [20–24] also remains unknown.

Monolayers

The most impressive characteristic of pulmonary surfactant is the prolonged metastability of the interfacial films *in situ* at surface pressures well above π_c [2,3], which, for fluid monolayers at physiological temperatures, occurs at approximately 46 mN/m [8*]. This resistance to collapse indicates structures with the rigidity of a solid film. What we term

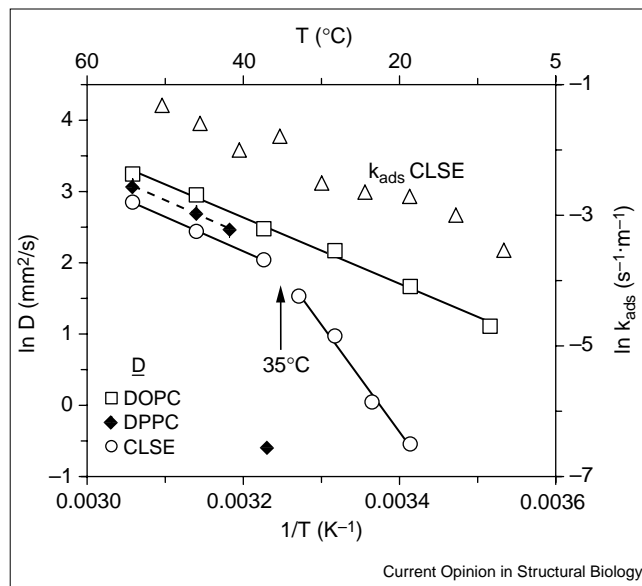
the 'classical model' contends that the functional film must therefore exist in a condensed phase (highly ordered with aligned acyl groups, whether tilted or untilted [25]) rather than in a fluid liquid-expanded (LE) phase. Because DPPC is the only constituent that can form a condensed phase at physiological temperatures, the model also holds that the film *in situ*, relative to freshly secreted surfactant, must be greatly enriched in DPPC [26–28].

Two mechanisms have been proposed to explain the change in composition required to produce such a film. First, DPPC could adsorb selectively to the interface [29] or, second, other constituents could be selectively excluded [26–28]. A basis for selective adsorption has not been specified. Selection based on thermodynamics during adsorption seems unlikely. The best available data suggest that all liquid-crystalline phospholipids share a common π_c that is higher than for compounds in the gel phase [8*]. According to these results, DPPC alone at physiological temperatures would be unable to adsorb to surface pressures achieved by the other surfactant phospholipids. The preferential adsorption of DPPC could instead be kinetic. Although recent studies suggest that solid/fluid phase separation terminates below physiological temperatures [10,14,15*], if fluid/fluid coexistence persists, sorting of constituents between the two phases might provide regions enriched in DPPC, which might in turn have distinct behavior. If surfactant constituents, however, adsorb as complete vesicles [30], even phase-separated bilayers would insert into the interface without a change in composition.

The limited available experimental data that directly address the composition of the adsorbed film also argue against selective adsorption. Fluorescence microscopy detects coexisting phases in surfactant films labeled with a fluorescent probe [31,32]. Studies with spread films of known composition suggest that the nonfluorescent phase contains mostly DPPC with some cholesterol (*vide infra*). Nonfluorescent regions are also evident during adsorption of extracted porcine surfactant, but their total area at any surface pressure is no larger than for spread films containing the complete set of constituents [32]. If the nonfluorescent phases in the adsorbed and spread films have the same compositions, then their similar molecular proportions, indicated by their relative areas, argue that the compositions of the two films should also be similar. These results suggest that the different constituents of surfactant vesicles adsorb equally.

A mechanism that would enrich the film in DPPC by selective exclusion, or 'squeeze-out', of constituents from the interface is more evident. Separation at the interface of DPPC from the other constituents would produce solid/fluid coexistence. Rapid collapse of the fluid phase at π_c would remove constituents other than DPPC, leaving the functional film of condensed DPPC proposed by the classical model [27,28]. For the phospholipids alone, with either the complete set from surfactant [33] or

Figure 2



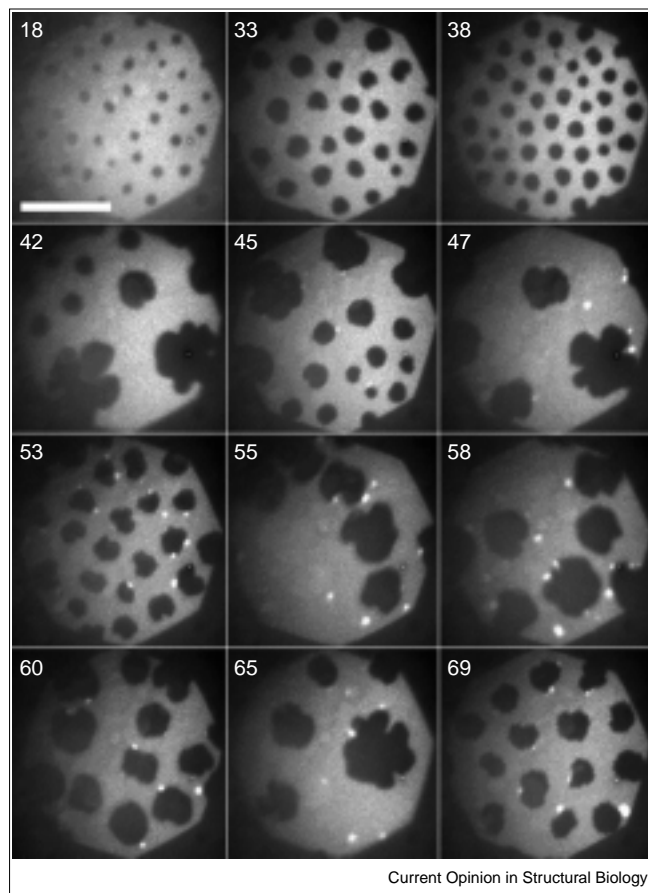
Temperature dependence of lipid diffusion in surfactant-related bilayers. Diffusion coefficients (D) of 0.5% (mol:mol) (7-nitrobenzoxa-2,3-diazol-4-yl)-dimyristoyl-phosphatidylethanolamine measured by FRAP, graphed as Arrhenius plots and fit by linear regression, provide a variable inversely related to viscosity. Diffusion in CLSE is slower than for DOPC at all temperatures. The apparent activation energy, obtained from the slope, shifts below 35°C for CLSE, consistent with the onset of gel/liquid crystal phase coexistence. Adsorption rates (k_{ads}) of CLSE [16*], indicated by unconnected open triangles, show no comparable change over the same range of temperature. (Modified from [10].)

simple model systems [34,35**], microscopic studies have demonstrated this solid/fluid coexistence. Both fluorescence microscopy and Brewster angle microscopy distinguish isolated domains from a surrounding continuous phase. The domains have the irregular shape, invariant optical thickness during compression and anisotropy expected of a condensed phase [33]. Direct localized compositional information is not yet available from secondary ion mass spectrometry [36], but the discontinuous domains grow in proportion to added DPPC and that growth extends to the mol fraction of pure DPPC. This behavior provides indirect evidence that the condensed phase contains almost exclusively DPPC [33].

The following observations, however, challenge selective exclusion based on phase separation:

1. The extent of the condensed phase at π_c can be quite limited. For extracted surfactant, the onset of phase separation detected by fluorescence microscopy over a broad range of temperatures lags behind the surface pressure at which coexistence begins for DPPC by approximately 6–10 mN/m [31]. At 37°C, the more ordered nonfluorescent phase in the surfactant films appears just below 45 mN/m and occupies only 4–6% of the interface when collapse from the fluorescent LE phase begins.

Figure 3



Fluorescence micrographs of monolayers containing the complete set of phospholipids in calf surfactant during continuous compression. Labels indicate surface pressures (mN/m). The inclusion of 1% (mol:mol) rhodamine-DPPE distinguishes three phases: the nonfluorescent condensed domain, which contains essentially pure DPPC [33]; the fluorescent LE phase in the monolayer; and the intensely fluorescent collapsed phase that appears above 45 mN/m and that, in separate experiments, is apparent by light-scattering microscopy, indicating three-dimensional structures. The LE phase persists during compression at $3 \text{ \AA}^2/(\text{molecule}\cdot\text{min})$ and 23°C to surface pressures well above π_c , contrary to the classical model. Scale bar represents $50 \mu\text{m}$. (Modified from [40].)

Rapid collapse of the surrounding fluid phase would require the exclusion of $>90\%$ of the interfacial monolayer before the film would contain only the condensed phase that could sustain surface pressures above π_c . Physiological measurements indicate no requirement for such a drastic reduction in area.

2. Phase coexistence is not always present at π_c . At $20\text{--}23^\circ\text{C}$ for films containing the neutral lipids as well as the phospholipids from calf surfactant, with or without the surfactant proteins, the initially separated phases remix before reaching π_c [31,37]. The homogeneous films provide no basis for selective exclusion. Physiological measurements show no correlation between pulmonary mechanics and phase separation at π_c .

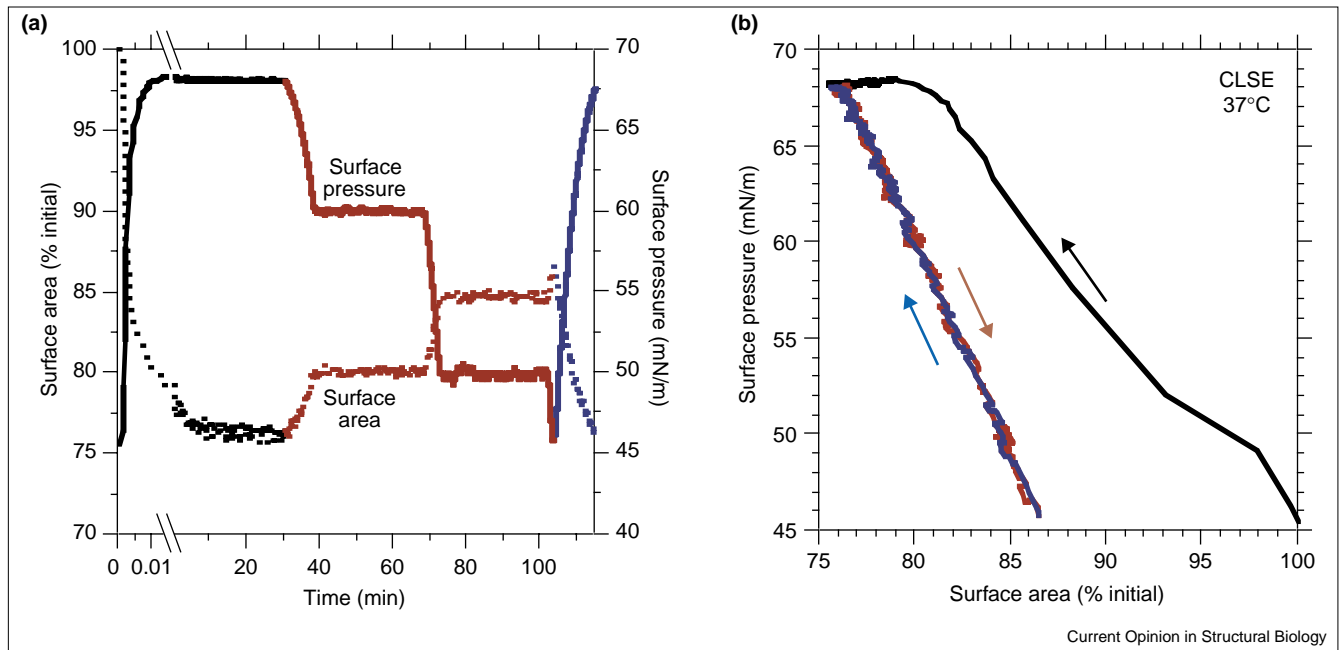
3. The more ordered phase may, in fact, be fluid rather than solid. In surfactant preparations that contain cholesterol, the nonfluorescent domains are generally circular, rather than having the irregular shapes seen in films containing the phospholipids alone [31,32,37]. In one study, the decrease in nonfluorescent area during compression occurs abruptly when the separated phases merge immediately following a transition of the domains to highly irregular shapes [37]. This behavior is consistent with remixing at a critical point [37], as seen with other mixtures of phospholipids containing disaturated compounds and cholesterol [6,38]. Although not tested directly, both the circular domains and the shape transition suggest that cholesterol changes the coexistence from solid/fluid for the phospholipids alone to fluid/fluid, analogous to the liquid-ordered and liquid-disordered phases seen in DPPC/cholesterol bilayers [5]. Whether such fluid phases have the differential stability required for selective exclusion remains unknown.

4. The differential stability of solid and fluid phases in phospholipid films is less than expected. For films composed of single-chain surfactants that exist as liquids in the bulk phase, collapse at π_c is “essentially instantaneous” [39]. Although fluid phospholipids have a bulk phase that is liquid-crystalline, rather than liquid, they were assumed to behave similarly. For both the complete set of surfactant phospholipids [40] and some binary mixtures [35], microscopic images show that, in fact, compression to surface pressures well above π_c occurs with minimal change in the ratio of the two phases (Figure 3).

All of these observations argue against selective exclusion of constituents other than DPPC.

Films that transiently reach surface pressures well above π_c during dynamic compression, but nonetheless remain fluid, with significant rates of collapse, would fail to replicate the prolonged metastability characteristic of surfactant films in static lungs. Recent studies show, however, that fluid monolayers can transform to solid films, defined by their slow flow into collapsed structures, by a process that need not reflect equilibrium phase behavior. Compression of fluid phospholipid monolayers at a rate sufficient to reach high surface pressures transforms the films from structures that collapse rapidly at π_c to forms that reproduce the behaviour observed in the lungs [41] (Figure 4). The transformation occurs not only for complete surfactant extracts but also for individual phosphatidylcholines [41,42]. The transformed films are kinetically trapped, in that when expanded slowly to π_c , at which they collapsed rapidly before transformation, they retain their resistance to collapse (Figure 4) [41,43]. When heated at π_c , the transformed films with individual phospholipids recover the ability to collapse, but without discontinuous changes in the area or slope of the heating isobar to suggest a standard phase transition [43]. Direct structural information is unavailable, but the behavior of fluid monolayers

Figure 4



Monomolecular films of CLSE manipulated on a captive bubble at 37°C. After initial compression at 112% initial area/s to 68 mN/m, the metastability of the film was demonstrated by holding the bubble's volume constant at the maximum surface pressure (black curves) and, after expansion at <5%/min, at 60 and 50 mN/m (red curves). After a final expansion to 45 mN/m, the persistent metastability of the film was demonstrated by recompression (blue curves) at the same slow rate to

68 mN/m. The same data are plotted as (a) surface area (dotted curves, left axis) and surface pressure (solid curves, right axis) as functions of time; (b) surface pressure as a function of surface area, with arrows indicating the temporal sequence of measurements. CLSE films compressed at <5%/min without the initial rapid compression sustain surface pressures no higher than 46 mN/m. (Modified from [41••].)

supercompressed to form films that resist collapse is analogous to that of three-dimensional liquids supercooled towards a glass transition [42]. The metastable films would then represent amorphous solids created by a kinetic process, rather than highly ordered solids that reflect equilibrium phase behavior.

For films containing complete surfactant extracts, a major change in composition, consistent with the classical model, during the transformation by rapid compression is unlikely. The individual phospholipids show that metastability of a film, long assumed to reflect a high content of DPPC [22], provides no information on composition. The small change in area during transformation of the surfactant extracts is inconsistent with any major enrichment in DPPC (Figure 4) [41••]. The results generally contradict the basic tenet of the classical model that only a film of DPPC in a condensed phase can replicate the behavior of surfactant films *in situ*.

Which of the two films — the condensed phase of DPPC or the amorphous structure with other components — actually exists in the lungs remains unknown. Both models have difficulty explaining how the functional films form. The problems of the classical model have been discussed above. For the amorphous multicomponent film, formation requires that the rate of compression must exceed the

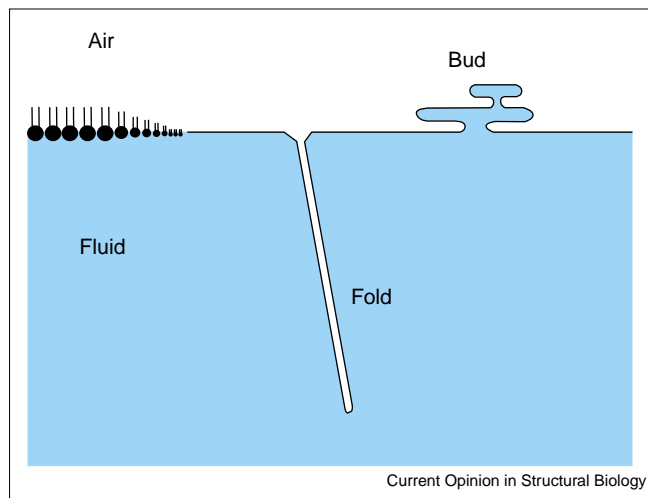
rate of collapse so that the film will reach high surface pressure. Although the rate required for extracted surfactant lies below the level of roughly 4%/s expected during normal tidal breathing [41••], much slower deflation has no deleterious effects on the pulmonary mechanics of excised lungs. Whether additional layers of surfactant adjacent to the interface [29,44], formed during adsorption or collapse [45,46•] *in vivo* but absent by design *in vitro*, would slow collapse [24] and lower the threshold rate required to reach high surface pressure is an intriguing but untested possibility.

For comparison of films *in vitro* and *in situ*, the melting temperature should provide a characteristic that could distinguish which film is present in the lungs. DPPC melts sharply at distinct temperatures [47]. The amorphous films with multiple components will presumably have different behavior. Unfortunately, physiological measurements of the temperatures at which films melt *in situ* have yielded varied results [27,48] and any useful comparison with studies *in vitro* must await resolution of those differences.

Collapsed phase

Microscopic studies are now documenting the detailed structures formed during the process of collapse that must be avoided in the lungs. Simple model systems indicate two forms in particular: bilayer folds from the

Figure 5



Schematic diagram of two microscopic structures that can form during collapse of phospholipid monolayers compressed above π_e . Variation of the focal plane indicates that the folds can extend hundreds of micrometers into the subphase [35**]. Buds grow into bilayer disks with lateral dimensions of approximately 2–3 μm that lie immediately adjacent to the interface and that can stack into multiple layers. (Placement of the buds above the monolayer is based on unpublished data suggesting that they are inaccessible to aqueous fluorescence quenchers in the subphase.) Studies with complete extracts of surfactant have so far detected only buds.

monolayer that can extend hundreds of micrometers into the subphase [35**,49,50] and buds [35**,51] that can grow into stacked bilayer disks immediately adjacent to the monolayer [23,52,53] (Figure 5). Studies with surfactant extracts or preparations derived directly from surfactant so far have found only the budded structure [31–33,37]. For both forms, the collapsed material reversibly reinserts into the expanding surface, suggesting lamellae that are continuous with the interfacial monolayer [23,35**,49,52,54]. The phospholipid films therefore collapse by flow of the complete monolayer into the third dimension, rather than by a standard phase transition in which constituents diffuse across an interfacial boundary [39]. If the relative rates of collapse and compression determine access to the high surface pressures at which fluid films become solid, then such subtleties of collapse may be critical to understanding how pulmonary surfactant functions in the lungs.

Conclusions

Despite half a century of investigation, some of the most fundamental aspects of pulmonary surfactant's function remain poorly understood. Most existing models have emphasized phase behavior in explaining the rapid adsorption of surfactant vesicles and the stability of the surfactant film. Recent advances, however, suggest that these models require re-evaluation.

(In the interests of space, this review has emphasized the hydrophobic constituents of native pulmonary surfactant.

This approach ignores interesting recent work concerning components for therapeutic surfactants [55–60] and also the effects of the collectin SP-A [61–64]. Although the omission of SP-A, which constitutes most of the protein in surfactant lavaged from the lungs, is controversial, we feel that the limited biophysical effects of its removal, either by extraction [65] or by genetic manipulation [66], argue that the surface activity of pulmonary surfactant results primarily from its hydrophobic constituents.)

Update

Recent studies show that the shear viscosity of lipid monolayers that have coexisting phases increases abruptly when the fraction of condensed area reaches a critical value [70*]. This phenomenon, observed for solid/fluid coexistence and interpreted in terms of a two-dimensional colloidal suspension, could also occur at the percolation threshold for coexisting fluid phases, which may more accurately reflect behavior in surfactant films. The importance of such findings for the stability of surfactant films is uncertain. Collapse may depend only on localized fluidity and therefore only on the extent of the phase with the lowest viscosity. If collapse occurs, however, by flow of continuous lamellae into the third dimension, then an increase in shear viscosity might produce slower collapse. Rather than the condensed monolayer required by the classical model, a metastable surfactant film might then require only a certain proportion of the coexisting phases. Because these studies deal with model systems, however, they do not address the poor correlation discussed above between phase behavior and monolayer metastability for the complete biological mixture.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
 - of outstanding interest
1. Gaines GL Jr: *Insoluble Monolayers at Liquid-Gas Interfaces*. New York: Wiley (Interscience); 1966.
 2. Horie T, Hildebrandt J: **Dynamic compliance, limit cycles, and static equilibria of excised cat lung**. *J Appl Physiol* 1971, 31:423-430.
 3. Schürch S, Goerke J, Clements JA: **Direct determination of volume- and time-dependence of alveolar surface tension in excised lungs**. *Proc Natl Acad Sci USA* 1978, 75:3417-3421.
 4. Postle AD, Heeley EL, Wilton DC: **A comparison of the molecular species compositions of mammalian lung surfactant phospholipids**. *Comp Biochem Physiol A Mol Integr Physiol* 2001, 129:65-73.
 5. Brown DA, London E: **Structure and function of sphingolipid- and cholesterol-rich membrane rafts**. *J Biol Chem* 2000, 275:17221-17224.
 6. Hagen JP, McConnell HM: **Liquid-liquid immiscibility in lipid monolayers**. *Biochim Biophys Acta* 1997, 1329:7-11.
 7. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, Gratton E: **Lipid rafts reconstituted in model membranes**. *Biophys J* 2001, 80:1417-1428.

8. Lee S, Kim DH, Needham D: **Equilibrium and dynamic interfacial tension measurements at microscopic interfaces using a micropipet technique. 2. Dynamics of phospholipid monolayer formation and equilibrium tensions at water-air interface.** *Langmuir* 2001, **17**:5544-5550.
- With a method that uses small volumes and therefore allows high concentrations and faster adsorption, the authors demonstrate the dependence of π_e for different phospholipids only on T/T_m , the temperature relative to T_m , the gel-to-liquid crystal transition temperature.
9. Notter RH: **Surface chemistry of pulmonary surfactant: the role of individual components.** In *Pulmonary Surfactant*. Edited by Robertson B, van Golde LMG, Batenburg JJ. Amsterdam: Elsevier; 1984:17-64.
10. Schram V, Clark HM, Hall SB: **SP-B and SP-C reduce lateral diffusion in pulmonary surfactant bilayers.** *Biophys J* 2002, **82**:2678.
11. Gugliotti M, Politi MJ: **The role of the gel \leftrightarrow liquid-crystalline phase transition in the lung surfactant cycle.** *Biophys Chem* 2001, **89**:243-251.
- An intriguing paper suggesting a direct correlation between interfacial boundaries and film formation. The adsorption of pure lipids is maximal near their main phase transition temperatures.
12. Leckband DE, Helm CA, Israelachvili J: **Role of calcium in the adhesion and fusion of bilayers.** *Biochemistry* 1993, **32**:1127-1140.
13. Dluhy RA, Reilly KE, Hunt RD, Mitchell ML, Mautone AJ, Mendelsohn R: **Infrared spectroscopic investigations of pulmonary surfactant. Surface film transitions at the air-water interface and bulk phase thermotropism.** *Biophys J* 1989, **56**:1173-1181.
14. Ebel H, Grabitz P, Heimburg T: **Enthalpy and volume changes in lipid membranes. I. The proportionality of heat and volume changes in the lipid melting transition and its implication for the elastic constants.** *J Phys Chem B* 2001, **105**:7353-7360.
15. Nag K, Pao J-S, Harbottle RR, Possmayer F, Petersen NO, Bagatolli LA: **Segregation of saturated chain lipids in pulmonary surfactant films and bilayers.** *Biophys J* 2002, **82**:2041-2051.
- Two-photon fluorescence microscopy of Laurdan in giant unilamellar vesicles made from cholesterol-depleted bovine surfactant reveals coexisting liquid-crystalline and gel phases between 20 and 37°C. Quantitative analysis of the Laurdan fluorescence indicates low energetic differences between the two phases, suggesting relatively similar compositions.
16. Schram V, Hall SB: **Thermodynamic effects of the hydrophobic surfactant proteins on the early adsorption of pulmonary surfactant.** *Biophys J* 2001, **81**:1536-1546.
- The rate-limiting barrier to adsorption that is reduced by the hydrophobic surfactant proteins is enthalpic rather than entropic, consistent with a rate-limiting intermediate that is highly curved.
17. Oosterlaken-Dijksterhuis MA, Haagsman HP, van Golde LMG, Demel RA: **Interaction of lipid vesicles with monomolecular layers containing lung surfactant proteins SP-B or SP-C.** *Biochemistry* 1991, **30**:8276-8281.
18. Walters RW, Jenq RR, Hall SB: **Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface.** *Biophys J* 2000, **78**:257-266.
19. Yu S-H, Harding PGR, Possmayer F: **Artificial pulmonary surfactant. Potential role for hexagonal H(II) phase in the formation of a surface-active monolayer.** *Biochim Biophys Acta* 1984, **776**:37-47.
20. Perkins WR, Dause RB, Parente RA, Minchey SR, Neuman KC, Gruner SM, Taraschi TF, Janoff AS: **Role of lipid polymorphism in pulmonary surfactant.** *Science* 1996, **273**:330-332.
21. Krüger P, Schalke M, Wang Z, Notter RH, Dluhy RA, Lösche M: **Effect of hydrophobic surfactant peptides SP-B and SP-C on binary phospholipid monolayers.** *Biophys J* 1999, **77**:903-914.
22. Veldhuizen EJA, Diemel RV, Putz G, van Golde LMG, Batenburg JJ, Haagsman HP: **Effect of the hydrophobic surfactant proteins on the surface activity of spread films in the captive bubble surfactometer.** *Chem Phys Lipids* 2001, **110**:47-55.
23. von Nahmen A, Post A, Galla HJ, Sieber M: **The phase behavior of lipid monolayers containing pulmonary surfactant protein C studied by fluorescence light microscopy.** *Eur Biophys J Biophys Lett* 1997, **26**:359-369.
24. Cruz A, Worthman LA, Serrano AG, Casals C, Keough KMW, Perez-Gil J: **Microstructure and dynamic surface properties of surfactant protein SP-B/dipalmitoylphosphatidylcholine interfacial films spread from lipid-protein bilayers.** *Eur Biophys J Biophys Lett* 2000, **29**:204-213.
25. Kaganer VM, Möhwald H, Dutta P: **Structure and phase transitions in Langmuir monolayers.** *Rev Mod Phys* 1999, **71**:779-819.
26. Watkins JC: **The surface properties of pure phospholipids in relation to those of lung extracts.** *Biochim Biophys Acta* 1968, **152**:293-306.
27. Clements JA: **Functions of the alveolar lining.** *Am Rev Respir Dis* 1977, **115**:67-71.
28. Bangham AD, Morley CJ, Phillips MC: **The physical properties of an effective lung surfactant.** *Biochim Biophys Acta* 1979, **573**:552-556.
29. Schürch S, Qanbar R, Bachofen H, Possmayer F: **The surface-associated surfactant reservoir in the alveolar lining.** *Biol Neonate* 1995, **67**(suppl 1):61-76.
30. Schürch S, Schürch D, Curstedt T, Robertson B: **Surface activity of lipid extract surfactant in relation to film area compression and collapse.** *J Appl Physiol* 1994, **77**:974-986.
31. Discher BM, Maloney KM, Schief WR Jr, Grainger DW, Vogel V, Hall SB: **Lateral phase separation in interfacial films of pulmonary surfactant.** *Biophys J* 1996, **71**:2583-2590.
32. Nag K, Perez-Gil J, Ruano ML, Worthman LA, Stewart J, Casals C, Keough KM: **Phase transitions in films of lung surfactant at the air-water interface.** *Biophys J* 1998, **74**:2983-2995.
33. Discher BM, Schief WR, Vogel V, Hall SB: **Phase separation in monolayers of pulmonary surfactant phospholipids at the air-water interface: composition and structure.** *Biophys J* 1999, **77**:2051-2061.
34. Nag K, Keough KMW: **Epifluorescence microscopic studies of monolayers containing mixtures of dioleoyl- and dipalmitoylphosphatidylcholines.** *Biophys J* 1993, **65**:1019-1026.
35. Gopal A, Lee KYC: **Morphology and collapse transitions in binary phospholipid monolayers.** *J Phys Chem B* 2001, **105**:10348-10354.
- Depending on the temperature of the experiment, the same phospholipid monolayer can collapse to form buckled folds or budded disks, which act like distinct equilibrium phases.
36. Bourdos N, Kollmer F, Benninghoven A, Ross M, Sieber M, Galla HJ: **Analysis of lung surfactant model systems with time-of-flight secondary ion mass spectrometry.** *Biophys J* 2000, **79**:357-369.
37. Discher BM, Maloney KM, Grainger DW, Sousa CA, Hall SB: **Neutral lipids induce critical behavior in interfacial monolayers of pulmonary surfactant.** *Biochemistry* 1999, **38**:374-383.
38. Keller SL, Pitcher WH, Huestis WH, McConnell HM: **Red blood cell lipids form immiscible liquids.** *Phys Rev Lett* 1998, **81**:5019-5022.
39. Smith RD, Berg JC: **The collapse of surfactant monolayers at the air-water interface.** *J Colloid Interface Sci* 1980, **74**:273-286.
40. Piknova B, Schief WR, Vogel V, Discher BM, Hall SB: **Discrepancy between phase behavior of lung surfactant phospholipids and the classical model of surfactant function.** *Biophys J* 2001, **81**:2172-2180.
- Monolayers containing the complete set of surfactant phospholipids achieve high surface pressures during continuous slow compression without significant changes in composition.
41. Crane JM, Hall SB: **Rapid compression transforms interfacial monolayers of pulmonary surfactant.** *Biophys J* 2001, **80**:1863-1872.
- Monolayers of surfactant extracts as well as LE dimyristoyl phosphatidylcholine (DMPC) transform into metastable films on a captive bubble if compressed faster than a threshold rate required to reach high surface pressures.
42. Hall SB, Smith EC, Crane JM, Laderas TG, Shapiro I: **Metastable supercompressed fluid monolayers at the air/water interface.** *Biophys J* 2002, **82**:162A.
43. Laderas TG, Smith EC, Crane J, Hall SB: **Persistent metastability of rapidly compressed monolayers at the air-water interface.** *Biophys J* 2002, **82**:152A.
44. Hills BA: *The Biology of Surfactant*. New York: Cambridge University Press; 1988:222-235.
45. Knebel D, Sieber M, Reichelt R, Galla HJ, Amrein M: **Scanning force microscopy at the air-water interface of an air bubble coated with pulmonary surfactant.** *Biophys J* 2002, **82**:474-480.

46. Knebel D, Sieber M, Reichelt R, Galla H-J, Amrein M: **Fluorescence light microscopy of pulmonary surfactant at the air-water interface of an air bubble of adjustable size.** *Biophys J* 2002, **83**:547-555.
In this paper and [45], the authors describe the direct observation of films on the surface of alveolar-sized bubbles. With adsorbed films, bilayers remain associated with the monolayer and act as a reservoir during surface expansion. Measurements of surface tension and surface area are not yet possible.
47. Crane JM, Putz G, Hall SB: **Persistence of phase coexistence in disaturated phosphatidylcholine monolayers at high surface pressures.** *Biophys J* 1999, **77**:3134-3143.
48. Inoue H, Inoue C, Hildebrandt J: **Temperature effects on lung mechanics in air- and liquid-filled rabbit lungs.** *J Appl Physiol* 1982, **53**:567-575.
49. Lipp MM, Lee KYC, Takamoto DY, Zasadzinski JA, Waring AJ: **Coexistence of buckled and flat monolayers.** *Phys Rev Lett* 1998, **81**:1650-1653.
50. Takamoto DY, Lipp MM, von Nahmen A, Lee KYC, Waring AJ, Zasadzinski JA: **Interaction of lung surfactant proteins with anionic phospholipids.** *Biophys J* 2001, **81**:153-169.
51. Schief WR, Touryan L, Hall SB, Vogel V: **Nanoscale topographic instabilities of a phospholipid monolayer.** *J Phys Chem B* 2000, **104**:7388-7393.
52. Amrein M, von Nahmen A, Sieber M: **A scanning force and fluorescence light microscopy study of the structure and function of a model pulmonary surfactant.** *Eur Biophys J Biophys Lett* 1997, **26**:349-357.
53. Kramer A, Wintergalen A, Sieber M, Galla HJ, Amrein M, Guckenberger R: **Distribution of the surfactant-associated protein C within a lung surfactant model film investigated by near-field optical microscopy.** *Biophys J* 2000, **78**:458-465.
54. Krol S, Ross M, Sieber M, Kunneke S, Galla HJ, Janshoff A: **Formation of three-dimensional protein-lipid aggregates in monolayer films induced by surfactant protein B.** *Biophys J* 2000, **79**:904-918.
55. Bringezu F, Ding JQ, Brezesinski G, Zasadzinski JA: **Changes in model lung surfactant monolayers induced by palmitic acid.** *Langmuir* 2001, **17**:4641-4648.
56. Ding J, Takamoto DY, von Nahmen A, Lipp MM, Lee KY, Waring AJ, Zasadzinski JA: **Effects of lung surfactant proteins, SP-B and SP-C, and palmitic acid on monolayer stability.** *Biophys J* 2001, **80**:2262-2272.
57. Lee KYC, Majewski J, Kuhl TL, Howes PB, Kjaer K, Lipp MM, Waring AJ, Zasadzinski JA, Smith GS: **Synchrotron X-ray study of lung surfactant-specific protein SP-B in lipid monolayers.** *Biophys J* 2001, **81**:572-585.
58. Lu KW, Taeusch HW, Robertson B, Goerke J, Clements JA: **Polyethylene glycol/surfactant mixtures improve lung function after HCl and endotoxin lung injuries.** *Am J Respir Crit Care Med* 2001, **164**:1531-1536.
59. Wu CW, Lee KYC, Barron AE: **Polypeptoids for biological mimicry of surfactant proteins: a novel exogenous lung surfactant replacement.** *Biophys J* 2001, **80**:2553A.
60. Lee KYC, Gopal A, von Nahmen A, Zasadzinski JA, Majewski J, Smith GS, Howes PB, Kjaer K: **Influence of palmitic acid and hexadecanol on the phase transition temperature and molecular packing of dipalmitoylphosphatidylcholine monolayers at the air-water interface.** *J Chem Phys* 2002, **116**:774-783.
61. Ruano MLF, Nag K, Casals C, Perez-Gil J, Keough KMW: **Interactions of pulmonary surfactant protein A with phospholipid monolayers change with pH.** *Biophys J* 1999, **77**:1469-1476.
62. Taneva SG, Keough KMW: **Differential effects of surfactant protein A on regional organization of phospholipid monolayers containing surfactant protein B or C.** *Biophys J* 2000, **79**:2010-2023.
63. Worthman LA, Nag K, Rich N, Ruano ML, Casals C, Perez-Gil J, Keough KM: **Pulmonary surfactant protein A interacts with gel-like regions in monolayers of pulmonary surfactant lipid extract.** *Biophys J* 2000, **79**:2657-2666.
64. Bi XH, Taneva S, Keough KMW, Mendelsohn R, Flach CR: **Thermal stability and DPPC/Ca²⁺ interactions of pulmonary surfactant SP-A from bulk-phase and monolayer IR spectroscopy.** *Biochemistry* 2001, **40**:13659-13669.
65. Hall SB, Venkataraman AR, Whitsett JA, Holm BA, Notter RH: **Importance of hydrophobic apoproteins as constituents of clinical exogenous surfactants.** *Am Rev Respir Dis* 1992, **145**:24-30.
66. Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, Stripp BR, Morris RE, Glasser SW *et al.*: **Altered surfactant function and structure in SP-A gene targeted mice.** *Proc Natl Acad Sci USA* 1996, **93**:9594-9599.
67. Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA: **Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung.** *J Appl Physiol* 1995, **79**:1615-1628.
68. Dorrington KL, Young JD: **Development of the concept of a liquid pulmonary alveolar lining layer.** *Br J Anaesth* 2001, **86**:614-617.
69. Notter RH, Tabak SA, Mavis RD: **Surface properties of binary mixtures of some pulmonary surfactant components.** *J Lipid Res* 1980, **21**:10-22.
70. Ding JQ, Warriner HE, Zasadzinski JA: **Viscosity of two-dimensional suspensions.** *Phys Rev Lett* 2002, **88**:168102.
Direct measurements of shear viscosity and phase behavior in monolayers containing simple lipid mixtures.