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# The biophysical function of pulmonary surfactant

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ABSTRACT The type II pneumocytes of the lungs secrete a mixture of lipids and proteins that together acts as a surfactant. The material forms a thin film on the surface of the liquid layer that lines the alveolar air sacks. When compressed by the decreasing alveolar surface area during exhalation, the films reduce surface tension to exceptionally low levels. Pulmonary surfactant is essential for preserving the integrity of the barrier between alveolar air and capillary blood during normal breathing. This review focuses on the major biophysical processes by which endogenous pulmonary surfactant achieves its function and the mechanisms involved in those processes. Vesicles of pulmonary surfactant adsorb rapidly from the alveolar liquid to form the interfacial film. Interfacial insertion, which requires the hydrophobic surfactant protein SP-B, proceeds by a process analogous to the fusion of two vesicles. When compressed, the adsorbed film desorbs slowly. Constituents remain at the surface at high interfacial concentrations that reduce surface tensions well below equilibrium levels. We review the models proposed to explain how pulmonary surfactant achieves both the rapid adsorption and slow desorption characteristic of a functional film.

SIGNIFICANCE The surface tension of the liquid that lines the alveolar air sacks tends to deflate the lungs. By reducing that surface tension to exceptionally low levels, films of pulmonary surfactant allow the lungs to remain inflated. Normal breathing requires that function. Premature babies with deficient surfactant injure the barrier between alveolar air and capillary blood. Therapeutic surfactants have improved the survival of these infants. The mechanisms by which pulmonary surfactant achieves its function remain obscure. Understanding those mechanisms is essential for determining how abnormal surfactant contributes to other disorders and for the development of new therapeutic agents.

## INTRODUCTION

Pulmonary surfactant is the material secreted by the lungs that reduces alveolar surface tension. A thin layer of liquid lines the alveoli (1). The surface tension of that liquid tends to shrink the alveolar surface area and deflate the air space. The type II alveolar epithelial cells synthesize and secrete a mixture of lipids and proteins that together act as a surfactant. The constituents form a thin film at the surface of the liquid and reduce surface tension.

In addition to its biophysical function, the components of the secreted material have a role in host defense. Two of the four surfactant proteins are lectins that contribute to the phagocytosis of organisms (2). A third protein may promote lysosomal killing of bacteria (3). This review instead addresses the biophysical activity of endogenous pulmonary surfactant in minimizing alveolar surface tension.

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Our discussion emphasizes a fundamental characteristic of pulmonary surfactant. To function effectively in the lungs, pulmonary surfactant must insert into the air-water interface by a process that is effectively irreversible (4). The surfactant vesicles must adsorb within seconds to form the interfacial film (4,5). When compressed to high interfacial densities by the shrinking alveolar surface area during exhalation, the adsorbed film must desorb slowly. Constituents must remain at the surface so that interfacial density increases to the metastable values that reduce surface tension well below equilibrium levels. The dichotomy of rapid adsorption and slow desorption, pointed out long ago (4), remains largely unexplained.

### Background

#### *Function*

The importance of surface tension in the lungs was first demonstrated almost a century ago (6,7). The hydrostatic pressure required to maintain excised lungs at any particular volume is significantly lower when inflated with saline

rather than air (Fig. 1). Inflation with saline eliminates the air-liquid interface. In the absence of surface tension, the recoil forces, determined after full inflation of the lungs, that tend to deflate the air spaces decrease by roughly two-thirds (Fig. 1) (8).

The report on the importance of surface tension for pulmonary mechanics speculated that the lungs might benefit by producing a surfactant (6,7). Several decades later, observations on microscopic bubbles in tracheal aspirates provided the first direct evidence for the existence of a pulmonary surfactant (9). The persistence of any bubble requires a Laplace pressure, defined as a difference in hydrostatic pressure, $\Delta P$, across the air-water interface, which opposes the tendency of surface tension to shrink the interface. The equation of Young and Laplace for a spherical bubble, $\Delta P = 2\gamma/r$, indicates that for any given surface tension, $\gamma$, the magnitude of $\Delta P$ increases for bubbles with smaller radii, r. Larger $\Delta P$ drives faster diffusion of gas from the bubble into the surrounding medium. The persistence of very small bubbles suggested remarkably low surface tensions, implying the presence of a surfactant. Persistent bubbles might alternatively reflect slower gaseous diffusion rather than lower surface tensions (10). The original assessment, however, that the hypothetical surfactant virtually eliminates surface tension agrees with current estimates of alveolar values. At physiological temperatures, the surface tension of a clean air-water interface is 70 mN/m. A variety of different methods have arrived at the common conclusion that alveolar surface tensions reach ~1–2 mN/m (11).

The low surface tensions are essential for normal breathing. The first physiological change that must occur after birth is the shift of gas exchange from the placenta to the lungs. Babies born at a sufficiently early stage of gestation, before they have adequate amounts of pulmonary surfactant, have lungs that are anatomically normal at birth (12). Breathing progressively injures the thin barrier that separates capillary blood from alveolar air. During repeated opening of collapsed and flooded air spaces, an air-water interface traverses the pulmonary epithelium. The greater difference in hydrostatic pressure across an interface with increased surface tension disrupts cellular membranes (13,14). The babies develop the pulmonary edema and respiratory failure of the respiratory distress syndrome. After removal of pulmonary surfactant by repeated lavage, breathing produces the same injury in adult animals (15). The integrity of the lungs during ventilation requires low alveolar surface tensions.

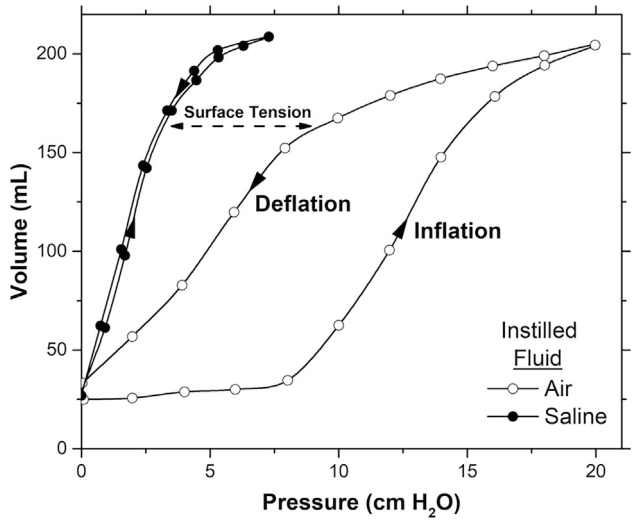


FIGURE 1 Dependence of pulmonary mechanics on the fluid instilled. Fully deaerated excised feline lungs at ambient temperatures were slowly inflated with either air or saline to the airway-pressures indicated, and then deflated (8). The horizontal dashed line indicates the effect of surface tension on the pulmonary mechanics of fully inflated lungs.

### *Composition*

Lavaging the lungs recovers phospholipid particles that can be separated by size (16) or density (17). The lavaged large and small particles separated by differential sedimentation contain roughly equal amounts of phospholipids that have the same composition (18). The large particles, which resemble the multilamellar vesicles synthesized and secreted by the type II pneumocytes, also contain the four surfactant proteins. The large particles replicate the effect of endogenous pulmonary surfactant on pulmonary mechanics (19). The small particles, which contain the same lipids but lack the proteins, are surface inactive (17,18). The large particles represent our best approximation of endogenous pulmonary surfactant.

The four surfactant proteins divide naturally into two categories according to their solubility in polar and nonpolar solvents. The two lectins, SP-A and SP-D, are water-soluble. Removal of these proteins during purification of pulmonary surfactant by extraction into nonpolar solvents has no significant effect on pulmonary mechanics (19). SP-A and SP-D have little role in surface activity. SP-B and SP-C, although both cationic, are sufficiently hydrophobic to extract with the surfactant lipids (20). Extracts of pulmonary surfactant, which are devoid of SP-A and SP-D, fully replicate the effects of endogenous surfactant on pulmonary mechanics (19).

The function of SP-C remains unknown. Patients and experimental animals that lack the protein develop interstitial fibrosis (21), but that process develops well after birth. The fibrosis may result from the toxicity of the mal-folded preprotein for SP-C rather than from a deficiency of its normal function (22). Evidence that SP-C has a major role in surface activity is lacking (23).

In contrast, SP-B is immediately essential for surfactant function (24). Babies and experimental animals with levels of the protein below a threshold value suffer the same immediate consequences as from the absence of

complete surfactant (24). In vitro experiments commonly study the native mixture of the two hydrophobic proteins, particularly since the matrix for their separation by gel permeation chromatography (25) became unavailable. The existing evidence argues that SP-B is responsible for their combined interfacial effects (23).

The lipids in pulmonary surfactant and other biological lipids are distinct. Phospholipids, which constitute approximately 92% (mol:mol) of extracted surfactant, contain an unusually large fraction of compounds with fully saturated fatty acids (26-28). Dipalmitoyl phosphatidylcholine (DPPC), which represents 35%–40% (mol:mol) of the surfactant phospholipids (26,27), has received considerable attention because of its temperature dependence. At physiological temperatures, DPPC remains just below its main structural transition and forms solid monolayers (29,30). The evolutionary selection of this compound may also have involved the absence of acyl double bonds and presumed resistance to oxidation. Sphingomyelins also have high melting temperatures, but the sphingosine group is unsaturated. Sphingomyelin is essentially absent from pulmonary surfactant (31).

Roughly 10% of the phospholipids are anionic (27). Those compounds are generally phosphatidylinositols in the fetus, changing to phosphatidylglycerols at some point around the time of birth. The composition of the alkyl chains with the two headgroups is considerably different (27). The two anionic phospholipids, however, apparently have equivalent biophysical function (32,33).

Pulmonary surfactant also contains ∼5%–10% (mol:mol phospholipid) cholesterol. In sufficient amounts, that compound prevents films of pulmonary surfactant and simple mimics from reducing surface tension to low levels (34-36). At physiological levels, however, the steroid has minimal functional effect (37,38).

## Adsorption

### *Fundamentals*

The interfacial insertion of pulmonary surfactant differs from the process for standard molecular surfactants, such as soaps and single-chain detergents. For those classic surfactants, the surface concentration, $\Gamma$, achieved by adsorption to any equilibrium surface tension, $\gamma$, depends on the monomeric concentration in the subphase, c, according to the Gibbs equation, $\Gamma = -c/RT \cdot \delta\gamma/\delta c$ (39). At and above a critical aggregate concentration (cac), analogous to the critical micelle concentration of soaps, phospholipids aggregate. Higher total concentrations produce no further increase in monomeric concentration and no further change in adsorption. This equilibration between the adsorbed monolayer and the aggregated structure defines the equilibrium spreading tension, which for phospholipids is ∼25 mN/m (40), reasonably independent of temperature.

The solubility of phospholipids is quite low. The cac is consequently also low, $\lesssim 10^{-10}$ M (41). The concentration of the surfactant phospholipids in the liquid that lines the alveolus is unknown but estimated to be substantially higher, as much as ∼100 mM (42). Concentrations that are easily measured, let alone physiologically relevant, are well above the cac. These considerations indicate that pulmonary surfactant dispersed in aqueous media exists almost exclusively as vesicles rather than individual monomers.

Pulmonary surfactant can insert into the air-water interface whenever surface tension exceeds the equilibrium spreading level. Expansion of the alveolar surface, and of the surfactant film, during a baby's first breath and subsequent inhalations can elevate surface tension sufficiently. Pulmonary surfactant then inserts into the interface as vesicles rather than individual molecules. Surface tension can fall during this process in discrete increments, consistent with interfacial insertion by packets of constituents rather than individual components (43). Comparison of monolayers artificially spread in volatile solvents with adsorbed films formed by surfactant vesicles leads to the same conclusion. Both films contain microscopically evident coexisting phases (44,45). The relative areas of those phases in the two films are equivalent. That observation suggests that adsorption, like spreading, delivers the complete contents of a vesicle to the interface (45). Microscopic studies have also directly visualized the interfacial insertion of surfactant vesicles into the air-water interface (46,47).

Adsorption of surfactant vesicles should involve distinct sequential steps (48,49). The vesicles must first diffuse to the interface and then closely approach the surface, followed by insertion of constituents into the interface, and then spreading from the point of insertion across the surface (Fig. 2). Experimental evidence argues that the first and last steps are relatively unimportant. Vesicles with similar dimensions but different compositions should diffuse at similar rates. Differences in diffusion cannot explain the different rates at which vesicles with and without the hydrophobic proteins reduce surface tension (Fig. 3). Insertion of vesicles into preexisting films instead provides insights concerning the importance of spreading. The composition of the preexisting monolayers should determine their viscosity and the ability of inserted material to spread. The failure of compositional differences in the preexisting films to affect the lowering of surface tensions provides indirect evidence against the importance of spreading (50).

Changes in composition do affect the other stages of adsorption. When vesicles first begin to reduce surface tension below the level for the clean interface, the anionic phospholipids, but not the hydrophobic proteins, significantly increase the rate at which surface tension falls (50). This finding suggests that an energetic barrier, which is at least partially electrostatic, limits the close approach of vesicles to the interface. The limitation to the close approach of two fusing vesicles by hydration forces (51) is well known.

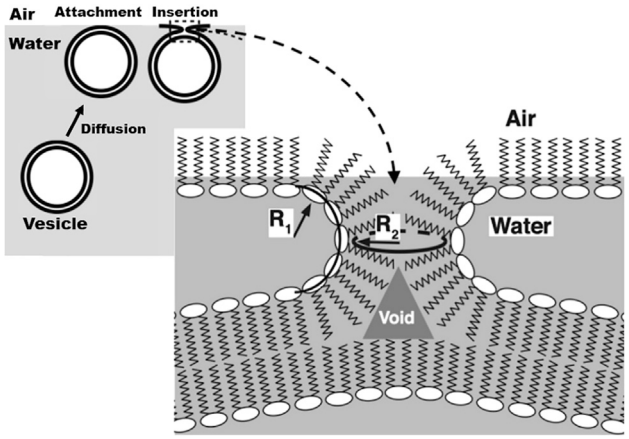


FIGURE 2 Hypothetical model for the adsorption by phospholipid vesicles to an air-water interface. Adsorption occurs by the following sequential stages: diffusion to the interface; attachment to the surface; insertion of constituents into the interface by a process involving a stalk connecting the vesicle with the surface; and subsequent spreading across the interface. The two principal radii of curvature, $R_1$ and $R_2$, for the stalk determine the principal curvatures (c) according to $c \equiv 1/R$. The two curvatures have opposite signs, $c_1 < 0$ and $c_2 > 0$. The dark "Void" would be unfilled by phospholipid leaflets with a constant thickness. Modified from (50,52).

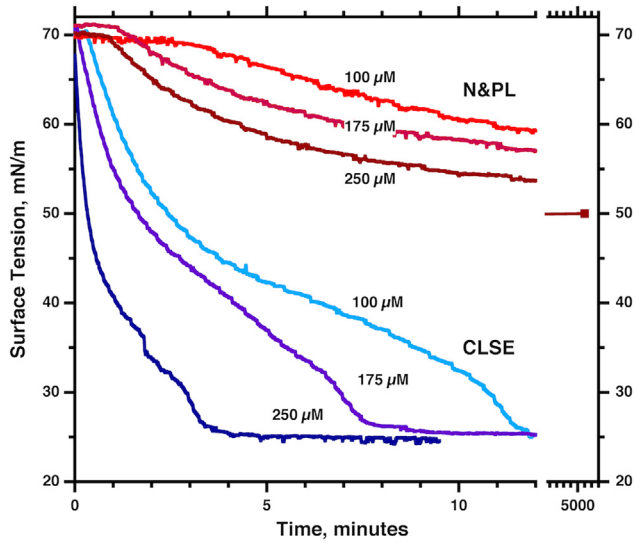


FIGURE 3 Effect of the hydrophobic surfactant proteins on adsorption of the surfactant lipids (52). Curves give the surface tensions achieved at times following the injection of samples into the subphase below an air/water interface. Calf lung surfactant extract (CLSE) contains the complete set of hydrophobic constituents in pulmonary surfactant obtained from calves. Nonpolar and phospholipids (N&PL) contains the full complement of surfactant lipids, obtained by removing the proteins from CLSE. Concentrations refer to phospholipids. To see this figure in color, go online.

At lower surface tensions, when vesicles insert into a partially occupied interface, the hydrophobic proteins produce a characteristic and unexpected behavior. Surface tension decreases during adsorption at rates that initially slow, as expected for vesicles inserting into a progressively crowded interface (Fig. 3) (52,53). With vesicles containing only the surfactant lipids, the fall in surface tension stalls well short of the equilibrium spreading tension (Fig. 3). With the proteins present, however, below an intermediate surface tension of ~30–40 mN/m, surface tension decreases at an accelerating rate (Fig. 3). The slope of the adsorption isotherm steepens. The hydrophobic proteins, and specifically SP-B, are crucial for the interfacial insertion of the surfactant constituents to reach the equilibrium spreading value.

SP-B achieves that function by a process other than simply destabilizing the vesicular bilayer. The hydrophobic proteins promote adsorption whether restricted to the adsorbing vesicle or to a preexisting monolayer at the interface (54,55). The proteins do not simply disrupt the structure of the bilayer. They instead stabilize a rate-limiting structure that is accessible from both the vesicle and the interface.

### *Stalk model*

The interfacial insertion by vesicles of pulmonary surfactant faces the same energetic problem as the fusion of two bilayers facilitated by other peptides with amphipathic helices. Constituents must reconfigure to insert into an adjacent structure without encountering the prohibitive hydrophobic interactions caused by exposure of nonpolar constituents to an aqueous environment. There is a significant difference between interfacial insertion and vesicular fusion. The reduction in interfacial energy achieved by lowering surface tension provides a major driving force for adsorption that is absent for fusion. The greater motivation suggests that specific energetically unfavorable intermediate structures are more likely to be transiently present during adsorption than fusion.

An early model proposed a possible structural intermediate in the pathway that would generate the fusion of two vesicles (56). The outer leaflets of each bilayer would form a stalk that bridges the gap between the two vesicles. A comparable stalk provides a possible structure for the rate-limiting intermediate in adsorption (Fig. 3) (50). The model has generated hypotheses concerning how SP-B promotes that process.

The stalk features prominent curvature. Forming that structure is likely to involve an energy of bending the lipids from their spontaneous curvature, $c_0$, adopted in the absence of applied force (57), to the configuration of the intermediate structure. The energy per unit area, $f_b$, of bending a thin sheet is given by $f_b = \kappa/2 \times [(c_1 + c_2) - c_0]^2 + \kappa_G c_1 c_2$, where $\kappa$ and $\kappa_G$ are the moduli of simple and saddle-splay bending (57). For simple bending, the energy depends on the deviation of the net curvature $(c_1 + c_2)$ from the spontaneous value and the rigidity of the lipids, indicated by $\kappa$. The proteins could lower that energy by reducing the modulus of bending. That possibility seems unlikely. Although the proteins soften phospholipid bilayers, their dose-response differs substantially from their effect on the kinetics of adsorption (58). The poor correlation argues that the

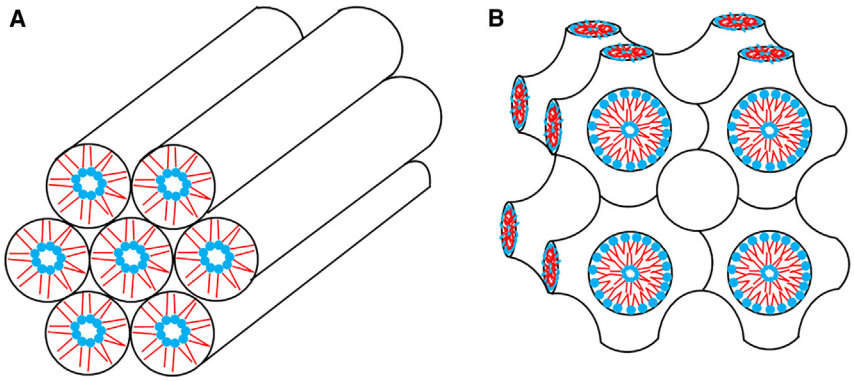


FIGURE 4 Relevant phospholipid polymorphisms. *A*. Inverse hexagonal ($H_{II}$) phase. Cylindrical monolayers contain an aqueous core and stack into a hexagonal array. *B*. Bicontinuous cubic ($Q_{II}$) phase. Continuous bilayers of phospholipids separate two aqueous compartments. The bilayers at all points have Gaussian curvature. The midpoint of the bilayer follows a periodic minimal surface, at which the two principal curvatures are equal in magnitude and opposite in sign, so that the surface lacks net curvature. The illustrated structure has the primitive surface of the $Im\bar{3}m$ space group. Phospholipids form structures with three of the 17 known periodic minimal surfaces (103). To see this figure in color, go online.

proteins promote adsorption by a mechanism other than reducing the lipids' rigidity.

A shift by the proteins of the spontaneous curvature toward the configuration of the rate-limiting structures seems more likely. The analysis requires measurements with lipids unrelated to pulmonary surfactant. The spontaneous curvature of individual lipid leaflets is unavailable from symmetric bilayers. The curvatures of the oppositely oriented leaflets cancel. The cylindrical monolayers of the inverse hexagonal ($H_{II}$) phase (Fig. 4) avoid that problem and allow expression of the spontaneous curvature. Phosphatidylethanolamine (59,60) and gramicidin-A (55) promote negative curvature, in which the hydrophilic face of the phospholipid leaflet is concave. Both compounds induce faster adsorption (55,59,60). Lysophosphatidylcholine, which forms positively curved micelles, has the opposite effect of inhibiting adsorption (61). These results suggest that negative curvature is an important feature of an intermediate structure that limits the rate of interfacial insertion by phospholipid vesicles.

Additional data suggest that the hydrophobic proteins facilitate interfacial insertion of surfactant vesicles by that mechanism. In $H_{II}$ structures, the proteins induce greater negative curvature (62). Several nuclear magnetic resonance studies show that SP-B or its functional fragments induce phospholipids to generate an isotropic signal (61,63–65), consistent with a tightly curved structure. The late acceleration of adsorption induced by the proteins also occurs with samples that contain only lipids. Preparations that include the phosphatidylethanolamines, which generate negative curvature, exhibit the same phenomenon. The late acceleration is apparently characteristic of samples that form negatively curved structure, including those induced by the hydrophobic proteins. This constellation of results suggests that the proteins, like other compounds that promote adsorption, achieve their effect by stabilizing negatively curved intermediate structures.

The hypothetical rate-limiting stalk has Gaussian curvature, given by the product of the two principal curvatures, $c_1c_2$. In the plane perpendicular to the interface, the curvature is negative, but in the plane parallel to the surface, the second principal curvature is positive (Fig. 2). The configuration of the individual lipid leaflets would resemble their structure in the inverse bicontinuous cubic ($Q_{II}$) phases (Fig. 4). The midpoint of the bilayer in those structures lies along a periodic minimal surface. The principal curvatures at all points on these surfaces are equal in magnitude and opposite in sign. Minimal surfaces lack net curvature. Each monolayer has its curvature defined instead at the pivotal plane, at which the molecular area remains constant during bending. Displacement of the pivotal plane from the minimal surface results in net negative curvature for each monolayer. To the extent that monolayers in the $Q_{II}$ phases and the hypothetical intermediate have similar configurations, factors that promote negative curvature should stabilize both structures. With appropriate phospholipids, the hydrophobic surfactant proteins induce formation of $Q_{II}$ phases (66,67).

The model in which SP-B promotes adsorption by inducing greater negative curvature does face a significant challenge. Factors can change spontaneous curvature by preferentially expanding one side of the lipid leaflet (68,69). To induce greater negative curvature, the protein should expand the hydrophobic face of the monolayer. The amino acid sequence of SP-B predicts a series of amphipathic helices (70). These structures should favor insertion in close proximity to the hydrophilic face of a phospholipid leaflet. Experimental evidence confirms that location (70,71). That position of the protein should induce positive curvature, opposite to the prediction of the model.

The proteins could stabilize the hypothetical stalk intermediate by processes other than reducing the energy of bending. Formation of that curved structure could also be limited by the disruption of chain packing. Any structure that includes paired sheets with uniform thickness that are not parallel must include unfilled space (72). In the stacked cylindrical monolayers of the $H_{II}$ phase, the acyl chains of the phospholipids fill that space by extending to different lengths. That variable extension disrupts optimal chain packing (72). Added alkanes can fill the potential void,

minimize the need for deviation of chains from their optimal length, and stabilize the $H_{II}$ phase at lower temperatures (72,73). The hypothetical stalk intermediate for adsorption would also involve disrupted chain packing (Fig. 2). SP-B might achieve its effect on adsorption by minimizing that disruption. To the best of our knowledge, that possibility remains untested.

Studies on vesicular fusion propose another possibility. Computational simulations (74) suggest that fusogenic proteins induce occasional flipping of an acyl group from the bilayer into the aqueous environment. Formation of a stalk that connects neighboring bilayers then follows promptly. The hydrophobic surfactant proteins might promote adsorption by altering the dynamic structure of the leaflets rather than changing their rigidity, spontaneous curvature, or chain packing.

## Metastability

### *Fundamentals*

Perhaps the most striking characteristic of pulmonary surfactant is the ability of the alveolar film to achieve and sustain remarkably low surface tensions. The equilibrium spreading tension is defined by the coexistence of a monolayer with its bulk phase (75). When compressed to surface tensions below that level, monomolecular films, including spread monolayers of pulmonary surfactant at physiological temperatures, reestablish equilibrium by collapse. Constituents leave the surface, reducing the interfacial density of the films and elevating surface tension (slowly compressed 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) in Fig. 5). For phospholipids, the equilibrium spreading tension is ~25 mN/m, with little variation among molecular species. Static films at lower surface tensions, such as the alveolar films, are metastable.

Phospholipid monolayers can collapse by either equilibrium or dynamic processes (Fig. 6). In both cases, the compressed monolayer folds from the interface to form a collapsed bilayer. Collapse involves regions of the film rather than individual constituents. Susceptibility to collapse therefore depends directly on regional structures and only indirectly on composition.

The equilibrium mechanism consists of the phase transition of the monomolecular film to form its bulk phase. This process is well known for single-chain surfactants (76), although with a subtle difference. Those compounds move from the monolayer to the bulk phase anywhere along the interface between them. Growth of the collapsed structure increases its perimeter, and collapse accelerates.

Phospholipid monolayers also collapse to form their bulk phase. The overcompressed monolayer folds from the interface to form an adjacent stack of bilayers (Fig. 6) (77–79). If compressed sufficiently, these structures can vesiculate, establishing the same equilibrium achieved by adsorbing vesicles (77). In contrast to the single-chain surfactants, the phospholipids flow into the collapsed structure through a confined focus (77). The kinetics of collapse for the two kinds of compounds are distinct (80).

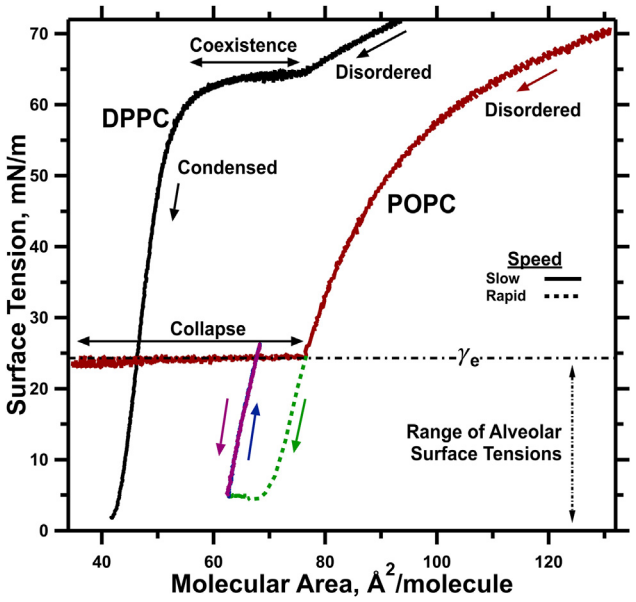


FIGURE 5 Phase behavior of compressed phospholipid monolayers. Films were produced by spreading solutions in immiscible, volatile solvents on the surface of captive bubbles. 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) forms a disordered monolayer. During slow compression (1.1 $h^{-1}$) at ambient temperatures (red curve), constituents collapse from the interface at the equilibrium spreading tension ($\gamma_e$), and surface tension falls no further. Initially disordered films of dipalmitoyl phosphatidylcholine (DPPC) undergo a phase transition to a condensed phase that resists collapse (*black*) and readily reduces surface tension to alveolar levels. POPC also reaches low surface tension if compressed faster than the films can collapse (*green*). At sufficiently low surface tensions, the films transform. They become resistant to collapse and undergo slow cyclic expansion to the $\gamma_e$ (*blue*) and recompression (*magenta*) without evidence of collapse. Modified from (104). To see this figure in color, go online.

Overcompressed monolayers can alternatively collapse by the mechanical process of buckling. This form of collapse occurs at variable surface tensions below the equilibrium spreading level (81), consistent with a system not at equilibrium. The buckled structures form bilayers that extend great distances (μm) into the subphase (Fig. 6) (82). Analysis routinely considers this form of collapse in terms of a sheet compressed to the point of mechanical failure. The nonzero surface tension, however, indicates that the collapsing film remains under tension (83). That observation may require reconsideration of the process involved in collapse by buckling.

### *Models*

Films with different compositions, proposed as models of pulmonary surfactant, collapse by one or the other of these two processes (Fig. 6) (77,82). Monolayers closest to the composition of pulmonary surfactant favor the phase

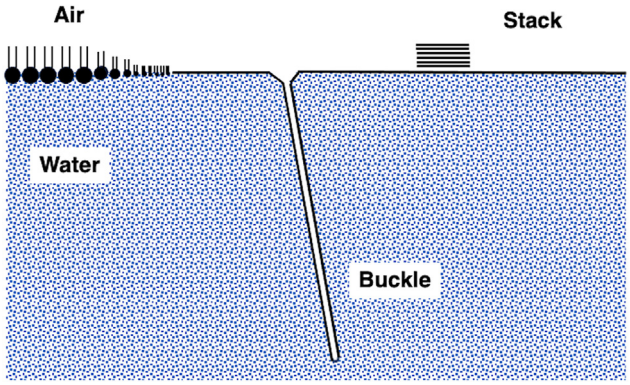


FIGURE 6 Mechanisms of collapse. Compressed phospholipid monolayers may collapse either by flowing at the equilibrium spreading pressure into stacks of the bulk smectic phase adjacent to the interface or by buckling from the interface at lower surface tensions to form bilayers that extend great distances into the subphase. Modified from (105). To see this figure in color, go online.

transition (77). The important question for pulmonary surfactant, however, is not how the films collapse but rather how the alveolar film avoids collapse to achieve very low surface tensions. The characteristics of films that resist collapse by either mechanism are similar. Solid films, defined by their inability to flow, fail to undergo the phase transition to form the bulk, collapsed structure at the equilibrium spreading tension (DPPC in Fig. 5). More rigid films are less prone to buckling. The resistance to collapse indicates that the alveolar film has the characteristics of a solid.

Experiments with bubbles suggest that surfactant films may achieve that solid characteristic during adsorption. Films formed on the continuous surface of bubbles can be competent to resist collapse and achieve low surface tensions from the beginning of the first intended compression (9,42,84–86). This observation contradicts several models of how the alveolar film becomes metastable. Those models require some structural change during the initial portion of the first compression before the film becomes competent to resist collapse.

The results with bubbles are subject to a significant reservation. Gas dissolving from a bubble into the surrounding medium can shrink its interfacial area. That change can subject the films to the compression that the models predict converts them to a solid structure (87). This possibility could reasonably explain the stable low surface tensions suggested for Pattle's uncompressed bubbles in tracheal aspirates (9). Such an unintended compression seems less likely for films on captive bubbles under continuous observation. The apparent ability of adsorbed films to resist collapse from the beginning of the first compression is a significant constraint on the validity of the different models.

*Classical model.* The classical model was proposed early after the function and phospholipid composition of pulmonary surfactant were defined. The model contends that the functional alveolar film has the structure of monolayers formed by DPPC below that compound's main transition temperature (4). DPPC is the most prevalent constituent of pulmonary surfactant from most animals (28). It is the only major component that at 37°C forms bilayers in the $L_\beta$ or $L_\beta$' phase and monolayers in which the acyl tails occupy a crystalline lattice. Compressed films of DPPC avoid formation of the bulk phase at the equilibrium spreading tension (Fig. 5) and do not buckle until they reach extremely low surface tensions (88). Over the range of metastability from 0 to 25 mN/m, they readily undergo reexpansion and slow recompression without evidence of collapse. They fully replicate the performance of the alveolar film.

DPPC represents ∼35%–40% (mol:mol) of the phospholipids in pulmonary surfactant from most animals (28). Microscopic methods at ambient laboratory temperatures show that spread monolayers (44) and adsorbed films (45) of pulmonary surfactant form coexisting phases (Fig. 7). In films containing only the complete set of surfactant phospholipids, without cholesterol or the surfactant proteins, compositional analysis indicates that domains of the discontinuous phase (Fig. 7, isolated domains) contain essentially pure DPPC (89). Measurements of surface potential suggest that with the complete set of lipids, including cholesterol, the steroid partitions into the discontinuous domains (90). At physiological levels of cholesterol, compressed films of DPPC-cholesterol collapse slowly (38). Selective collapse of the continuous phase (Fig. 7, film surrounding the domains), containing the phospholipids other than DPPC, would leave a film of cholesterol-DPPC. That film should mimic the resistance to collapse of the alveolar film (37,38).

Experiments at 37°C have produced conflicting structural results, some of which seriously challenge the classical model. In contrast to the single-component films of DPPC, the phase rule for multicomponent monolayers of pulmonary surfactant allows coexistence to extend over a broad range of surface tensions (44). As expected, the appearance of coexisting phases in films of pulmonary surfactant required compression to lower surface tensions than for DPPC. At 37°C, the discontinuous domains emerge in monolayers of pulmonary surfactant only after compression to surface tensions just above the equilibrium spreading tension. When collapse first becomes possible, the phase rich in DPPC occupies ∼5% of the interface (44). Elimination of the film surrounding these domains by selective collapse would require a nonphysiological 95% compression.

These results, obtained with fluorescence microscopy (44), differ from findings with atomic force microscopy (AFM) (91). Both microscopic methods detect the domains observed by fluorescence, ∼2 μm in diameter (Fig. 7). AFM also found a set of much smaller domains, with diameters of ∼50 nm (91). The smaller domains have the thickness of the condensed phase in films of DPPC. The mechanism by which domains with two distinctly different sizes would

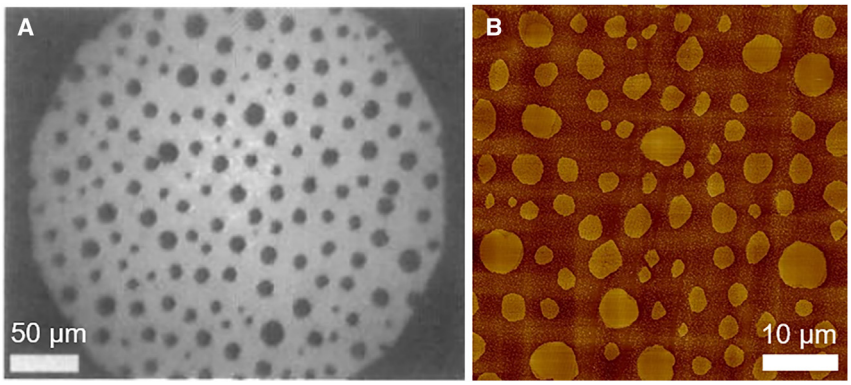


FIGURE 7 Coexisting phases in monolayers of extracted calf surfactant. Micrographs depict monolayers of calf lung surfactant extract (CLSE) compressed to a surface pressure of 30 mN/m at ambient temperatures. *A*. Fluorescence microscopy (from (44)). *B*. Atomic force microscopy (from (106)). To see this figure in color, go online.

form is unclear. Under equilibrium conditions, the combination of interfacial tension between the two-dimensional coexisting phases and the difference in their dipole densities determines the size of the discontinuous domains (92). This consideration provides the basis for forming domains of one radius rather than two. The total area of the thicker regions, which reaches ∼70% of the interface, is nonetheless more consistent than the results by fluorescence with the predictions of the classical model.

Opposing explanations for the difference between the results favor each set of findings. The resolution of fluorescence microscopy could simply be inadequate to detect the smaller domains. The transfer of the films from the air-water interface to a solid support, required for AFM but not for fluorescence microscopy, could instead artifactually induce the smaller domains. Which set of results more accurately reflects the structure of the alveolar film remains uncertain.

The initial version of the classical model would exclude constituents during some initial stage of compression before the films resist collapse. This prediction fits poorly with the results that suggest that the adsorbed film can sustain low surface tensions from the beginning of the first compression. An additional variant of the classical model proposes formation of the functional film by a different mechanism. Rather than selective exclusion of constituents, the film that resists collapse would result from selective interfacial insertion of DPPC. That process would be distinct from selective adsorption. The available evidence indicates that vesicles insert their full complement of constituents to form the initial monolayer (45). The compositional shift would occur by exchange between the interfacial monolayer and material in the subphase.

The model of selective insertion could explain the dependence of the compressed film on the subphase concentration. Several studies have found that higher concentrations of pulmonary surfactant in the subphase improve the performance of adsorbed films during the first compression (42,84–86). The model, however, lacks a physical motivation. Phospholipids share a common equilibrium spreading tension. An obvious force that would drive the selective exchange is missing.

*Supercompressed fluid model.* The absence of films other than DPPC that could replicate the performance of the alveolar film has supported the classical model. That restriction is no longer valid. Fluid films of phospholipids transform if they achieve surface tensions well below the equilibrium spreading levels (rapidly compressed POPC in Fig. 4). If compressed slowly from high surface tensions, films of POPC (93), or of extracted calf surfactant (29), collapse readily at the equilibrium spreading tension of ∼25 mN/m (Fig. 4, red curve). When subjected to a pulsed compression to lower surface tensions (Fig. 4, green curve), the films collapse at rates that progressively increase, as expected, at surface tensions further from equilibrium. Those rates, however, reach a sharply defined maximum and then decline at lower surface tensions. The films become metastable and resist collapse (93).

That metastability persists during expansion of the films to the surface tensions at which they initially collapsed. After slow reexpansion of the films to the equilibrium spreading tension (Fig. 4, blue curve), they reduce surface tension to low values during slow recompression without evidence of collapse (Fig. 4, magenta curve) (93). Although the supercompressed fluid films by no means disprove the classical model, they show that films other than DPPC can replicate the performance of the alveolar film. Like the classical model, the supercompressed model requires an initial manipulation, in this case the pulsed compression, before the films can sustain low surface tensions. The model is incompatible with the data which suggest that adsorbed films are competent to resist collapse before compression begins.

*Multilamellar model.* Microscopic studies indicate that at least portions of the alveolar film are multilamellar (94). This observation has prompted speculation that the additional layers inhibit collapse of the interfacial monolayer (95). Multilayers, however, form during collapse (Fig. 5)

(77). The additional layers in the alveolus fail to distinguish whether they minimize collapse or result from that process.

Microscopic studies show that adsorption can form multilayered structures. Rather than the continuous multilayers seen in situ (94), however, these structures are isolated stacks of bilayers, surrounded by monomolecular film (96). That heterogeneity of thickness perhaps explains the different results with x-ray reflectivity. Those measurements, which average results across the footprint of the incident beam, detect only a monolayer (97). The isolated stacks fail to explain why the intervening monolayer resists collapse. Direct evidence that multilamellar structures, formed by whatever process, resist collapse is sparse.

*Model of interfacial curvature.* Fluorescence microscopy shows that the morphology of coexisting phases in interfacial monolayers can depend on the curvature of the surface (98). On static bubbles with progressively smaller radii, the nonfluorescent domains deviate from their circular shape. The discontinuous domains interconnect at a percolation threshold to form a continuous meshwork (98). Although not tested, such a continuous solid structure should resist collapse. The curvature of the alveolar surface would determine the stability of the alveolar film.

The model depends heavily on the alveolar anatomy. Histologic studies (99) have indicated that the alveolar air spaces are polyhedral, with surfaces that are planar rather than curved. Those methods, however, are notoriously subject to morphological distortion caused by dehydration of the stained tissue. Recent microtomographic studies using synchrotron-generated x-rays show that in the lungs of living mice, the alveolar air space is spherical (100). The alveolar air-water interface has the curvature required by the model.

This proposal has the advantage that it requires no initial manipulation to generate a film that sustains low surface tensions. The model, however, does require curvature well below the level at which surfactant films resist collapse. Quasi-static compression of surfactant films on captive bubbles with a diameter of ~5 mm, and curvature of roughly 0.4 $mm^{-1}$, readily reduce surface tension to low levels (42). The curvatures of the alveolar air space, ~25 $mm^{-1}$ (100), or of the bubbles that produce reconfigurations of the coexisting phases, ~10 $mm^{-1}$, appear unnecessary to yield a functional film.

### Respreading

Once the functional film forms, tidal breathing may require only infrequent replacement of interfacial surfactant. Inhalation and exhalation may well only sample the linear portion of the isotherm (Fig. 5, blue and magenta curves for POPC). Changes in area may be insufficient to produce either the low surface tensions at which the films collapse or the high tensions necessary for interfacial insertion (101). Deeper inhalations, however, such as sighs, occur regularly (102). Both interfacial insertion and collapse would then occur.

Material that has collapsed from the interface during overcompression can reenter the interface (36). In vitro experiments show that when surface tension exceeds the equilibrium spreading value, constituents that collapsed either by flow into adjacent stacks of bilayers or by buckling (Fig. 6) can respread into the interface. The two processes seem likely to occur by similar mechanisms involving a tightly curved structure. Either respreading or adsorption could maintain a saturated film at the equilibrium spreading tension during cyclic changes in area.

The relative importance of the two processes is difficult to distinguish. Physiological circumstances exist during which interfacial insertion can occur theoretically only by adsorption. During a baby's first inhalation, before the initial exhalation compresses the alveolar film, no collapsed material exists to respread. Constituents can only insert into the interface by adsorption, not respreading. No comparable circumstances exist during which only respreading is possible. The alveolar subphase always contains constituents that could insert into the expanding film by adsorption.

## CONCLUSIONS

Pulmonary surfactant has profound effects on multiple aspects of pulmonary physiology and pathophysiology and presents direct therapeutic opportunities. Surfactant vesicles insert their full complement of constituents into the air-water interface via a rate-limiting intermediate that is promoted by the hydrophobic protein, SP-B. Several models predict processes by which the adsorbed film forms a structure that sustains surface tensions well below equilibrium values. None fully explains all of the available experimental data. Although fundamental questions remain, previously unused interfacial methods are providing new information concerning the mechanisms by which pulmonary surfactant achieves its unusual and essential function.

## AUTHOR CONTRIBUTIONS

S.B.H. and Y.Y.Z. wrote the paper.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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