Discrepancy between Phase Behavior of Lung Surfactant Phospholipids and the Classical Model of Surfactant Function

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ABSTRACT The studies reported here used fluorescence microscopy and Brewster angle microscopy to test the classical model of how pulmonary surfactant forms films that are metastable at high surface pressures in the lungs. The model predicts that the functional film is liquid-condensed (LC) and greatly enriched in dipalmitoyl phosphatidylcholine (DPPC). Both microscopic methods show that, in monolayers containing the complete set of phospholipids from calf surfactant, an expanded phase persists in coexistence with condensed domains at surface pressures approaching 70 mN/m. Constituents collapsed from the interface above 45 mN/m, but the relative area of the two phases changed little, and the LC phase never occupied more than 30% of the interface. Calculations based on these findings and on isotherms obtained on the continuous interface of a captive bubble estimated that collapse of other constituents increased the mol fraction of DPPC to no higher than 0.37. We conclude that monolayers containing the complete set of phospholipids achieve high surface pressures without forming a homogeneous LC film and with a mixed composition that falls far short of the nearly pure DPPC predicted previously. These findings contradict the classical model.

INTRODUCTION

Pulmonary surfactant forms thin films at the air–water interface in the alveoli of the lungs that have remarkable stability. When compressed during exhalation by the shrinking alveolar surface area, surfactant prevents collapse of the air spaces at small lung volumes by forming dense films with surface pressures approaching 70 mN/m that are maintained in static lungs for prolonged periods (Horie and Hildebrandt, 1971; Schürch et al., 1978). The ability to sustain such high surface pressures is characteristic of liquid-condensed (LC) films. When first compressed in vitro, however, surfactant more commonly shows the behavior of the liquid-expanded (LE) phase (Smith and Berg, 1980), collapsing rapidly from the air–water interface when it reaches the equilibrium spreading pressure of ~45 mN/m (Pison et al., 1996). These observations have led most investigators to accept that surfactant must undergo a process of compositional refinement before achieving stability at high surface pressures. Pulmonary surfactant contains a complex mixture of lipids and proteins, and, in single-component films over a wide range of temperatures up to physiological values, only dipalmitoyl phosphatidylcholine (DPPC), the most prevalent component, can form the LC phase. The main transition for the other phospholipids generally lies well below ambient temperatures (Kahn et al., 1995). The squeeze-out hypothesis proposes that refinement occurs above the equilibrium spreading pressure by selective exclusion of these more fluid constituents (Watkins, 1968; Clements, 1977; Bangham et al., 1979). More recent investigators have suggested that, instead of or in addition to squeeze-out, the refined composition results from selective adsorption of DPPC during formation of the film (Schürch et al., 1995). By whatever mechanism, the prevailing view, which we term the "classical model," contends that the functional film in the lung is LC (Bangham et al., 1979) and greatly enriched relative to the complete mixture in its fractional content of DPPC (Watkins, 1968; Clements, 1977; Bangham et al., 1979).

Given the extent of its wide-spread acceptance (Notter, 1984; Goerke and Clements, 1985; Van Golde et al., 1988; Hawgood, 1991; Keough, 1992; Pison et al., 1996; Veldhuizen and Haagsman, 2000), the classical model is supported by remarkably little experimental evidence. The model leads to specific hypotheses concerning both composition and phase behavior, but these have proven difficult to test. The small amount of material in an interfacial monolayer has hindered attempts using quantitative analysis to demonstrate the predicted enrichment in DPPC. Efforts to follow phase behavior using traditional analysis of surface pressure-area (π-A) isotherms have been complicated by the complexity of the surfactant mixture. The Gibbs phase rule, which constrains a phase transition for a single-component film to a constant surface pressure, places no such restriction on the behavior of multicomponent systems. Consequently, the absence of discontinuities in the π-A isotherms of pulmo-

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nary surfactant allows no conclusions concerning phase behavior. The most basic predictions of the classical model therefore remain largely untested.

Microscopic methods now provide direct access to phase behavior in films, including those with compositions as complex as pulmonary surfactant. Fluorescence microscopy and Brewster angle microscopy (BAM) both distinguish two phases that coexist over a broad range of surface pressures, at least up to 40 mN/m, in extracts of lung surfactant (Discher et al., 1996, 1999a; Nag et al., 1998). For the complete set of phospholipids purified from calf surfactant (purified phospholipid, PPL), prior analysis also shows that the more condensed phase contains almost pure DPPC (100 ± 4%), with the remaining constituents confined to the surrounding film (Discher et al., 1999b). The fractional area of each phase therefore also provides compositional information. The studies reported here monitor phase behavior in PPL monolayers during compression to very high surface pressures to test the prediction of the classical model that the film must be LC. We then use these data to estimate the extent to which the films become enriched in DPPC at these high pressures.

MATERIALS AND METHODS

Materials

DPPC was obtained from Avanti Polar Phospholipids (Alabaster, AL); *N*-(lissamine rhodamine-B sulfonyl)-dipalmitoyl phosphatidylethanolamine (Rh-DPPE) was purchased from Molecular Probes (Eugene, OR). Both were used without further purification or characterization. The purified phospholipids (PPL) were separated from extracts of calf surfactant (calf lung surfactant extract, CLSE) provided by Dr. Edmund Egan (ONY, Inc., Amherst, NY). Calf surfactant is obtained from bronchoalveolar lavage fluid by first removing cells using low-speed centrifugation and then pelleting the large surfactant aggregates from the cell-free supernatant (Notter et al., 1983). The hydrophobic constituents are then extracted into chloroform (Bligh and Dyer, 1959). Column chromatography separates the phospholipids from the other constituents to provide PPL (Hall et al., 1994).

The subphase for all experiments contained 10 mM Hepes pH 7.0, 150 mM NaCl, and 1.5 mM $CaCl_2$ (HSC) using ingredients purchased from Sigma. Water for these studies was first distilled and then purified by a multicartridge system (Barnstead, Dubuque, IA), and had a resistivity greater than 17 MΩ/cm. All glassware was acid-cleaned. All solvents were at least reagent-grade and contained no surface active stabilizing agents.

Methods

Phospholipid concentrations were determined by measuring the phosphate content of extracted material (Ames, 1966).

Isotherms

Microscopic measurements on interfacial films used a Langmuir trough (Labcon, Darlington, UK) with a computer-controlled barrier consisting of a continuous Teflon ribbon inserted vertically through the air–liquid interface (Tabak and Notter, 1977). Teflon blocks placed in the trough reduced the final surface area and increased the fractional compression during experiments with the fluorescence microscope. Experiments were performed at an ambient temperature of 23°C and were repeated with different solutions of PPL originating from different preparations of CLSE. Monolayers were deposited on the clean air–buffer interface from chloroform solutions before allowing approximately 30 min for evaporation of the solvent. Films were spread to an initial surface pressure of 7–16 mN/m to allow access to molecular areas sufficiently small to achieve surface pressures approaching 70 mN/m at the end of compression. With larger initial molecular areas at which surface pressure was undetectable, films had comparable phase behavior but could not reach surface pressures above 50–55 mN/m. Molecular area of the overspread films were normalized to values obtained for monolayers spread beyond lift-off. Compression occurred continuously at ~3 $Å^2$/(molecule·min).

Isotherms used to calculate compositional changes were obtained on a captive bubble (Schürch et al., 1989; Putz et al., 1994). The continuous interface of the bubble eliminates confining barriers and the possibility of leakage that complicates interpretation of changes in area on other devices at the high surface pressures of particular interest for pulmonary surfactant. Spread films of PPL or DPPC, formed using previously described methods (Crane et al., 1999; Crane and Hall, 2001), were compressed at <3 $Å^2$/(molecule·min) by increasing the hydrostatic pressure applied to the subphase and shrinking the size of the bubble. The difficulty of spreading small volumes at the bubble's surface introduced some uncertainty in the molecular content of the film, and molecular areas were therefore normalized for the PPL isotherms to values obtained on the Langmuir trough with films spread beyond lift-off. Average curves were obtained from multiple experiments by taking the mean of surface area and surface pressure, both of which are dependent variables with the captive bubble, at specific fractions of the initial volume.

Fluorescence microscopy

Fluorescence images were obtained using a Nikon epifluorescence microscope equipped with a 100× extra-long-working-distance objective and a C2400 SIT camera (Hamamatsu Corp., Hamamatsu City, Japan). Images of films containing ≤1% (mol:mol) of Rh-DPPE were captured directly to computer (Quadra 650, Apple, Cupertino, CA) via a frame grabber (LG-3, Scion Corp., Frederick, MD) and analyzed using the program Image, developed at the National Institutes of Health and available from the public domain on the internet at http://rsb.info.nih.gov/nih-image. To avoid selection bias, images were captured at a rate of approximately 1–2 images/min strictly according to time. The fractional area occupied by the nonfluorescent domains was calculated by counting pixels. A background image of a homogeneously fluorescent film was first subtracted from the experimental images to correct for inhomogeneities of illumination. In the resulting difference image, pixels with grayscale below a threshold value that distinguished between the two phases were then counted. Intensely fluorescent spots that appeared first at ~45 mN/m occurred outside the nonfluorescent domains and were included in the area of the fluorescent phase.

Brewster angle microscopy

BAM provided images of films containing no added fluorescent probes (Hénon and Meunier, 1991; Hönig and Möbius, 1991). The previously described home-built instrument (Frey et al., 1996; Schief et al., 2000a) uses a p-polarized laser beam of wavelength 532 nm and a power of approximately 90 mW (Coherent DPSS 532-100) to illuminate the interface within the ribbon barrier at $\theta \approx 53.1°$. A lens system focused an image of the interface through a polarizing analyzer onto a CCD camera for capture by computer. We have shown previously that grayscale values, which are linearly proportional to the reflectance of

interfacial structures to p-polarized light, are reproducible between experiments to within ±5 out of 256 units (Discher et al., 1999b).

Estimation of compositions

We used an iterative calculation to estimate the composition of the film and of the expanded phase according to ϕ_c, the measured fractional area of the condensed phase. In accordance with our experimental results, the calculation assumes that the domains contain only DPPC, that below 46 mN/m the number of molecules in the film remains constant, and that, at higher surface pressures, constituents are excluded out of the interfacial plane only from the expanded phase. The ratio r between the molecular areas for the expanded and condensed phases at 46 mN/m,

$$r = \frac{\bar{A}_e}{\bar{A}_c}$$

is assumed to remain constant during compression to higher pressures. This simplification allows calculation of the number of molecules in the expanded phase. Allowing r to vary produced significantly different results only if it reached unlikely values well above the ratio at lift-off.

The calculation follows n_e, n_c, and n, the number of molecules in the expanded and condensed phases and in the film, respectively, during sequential changes in the film's area by an amount ΔA. For the m^{th} iteration,

$$A^m = A^{m-1} + \Delta A$$

where $\Delta A < 0$ for compression. The surface pressure π^m at A^m is obtained from the original isotherm for PPL, and $\bar{A}_c(\pi^m)$, the molecular area of the condensed phase, is provided by the isotherm for DPPC. The number of molecules in the condensed phase is obtained from

$$n_c^m = \frac{A_c^m}{\bar{A}_c^m} = \frac{\phi_c \cdot A^m}{\bar{A}_c^m}.$$

Below 46 mN/m, n^m is constant, equal to n^0, the initial number of molecules spread, and

$$n_e^m = n^0 - n_c^m.$$

At higher pressures, collapse changes the content of the film for each iteration by an amount Δn^m,

$$\Delta n^m \equiv n^m - n^{m-1}$$

which can be obtained from the value of n^m at each iteration according to the sequence

$$\bar{A}_e^m = r \cdot \bar{A}_c^m,$$

$$n_e^m = \frac{A_e^m}{\bar{A}_e} = \frac{(1 - \phi_c) \cdot A^m}{\bar{A}_e},$$

and

$$n^m = n_c^m + n_e^m.$$

Constituents other than DPPC, designated (j), are confined to the expanded phase,

$$n(j) = n_e(j)$$

for $n(j)$ and $n_e(j)$ molecules of j in the film and in the expanded phase, respectively. Because collapse occurs only from the expanded phase,

$$\Delta n^m = \Delta n_e^m \quad \text{and} \quad \Delta n^m(j) = \Delta n_e^m(j).$$

For X and X_e, the mol fractions of the film and of the expanded phase,

$$X^m(j) = \frac{n^m(j)}{n^m} = \frac{n^{m-1}(j) + \Delta n^m(j)}{n^m} = \frac{n_e^{m-1}(j) + \Delta n^m \cdot X_e^{m-1}(j)}{n^m}.$$

and

$$X_e^m(j) = \frac{n_e^m(j)}{n_e^m} = \frac{n_e^{m-1}(j) + \Delta n_e^m(j)}{n_e^m} = \frac{n_e^{m-1}(j) + \Delta n^m \cdot X_e^{m-1}(j)}{n_e^m}.$$

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Finally,

$$X^m(\text{DPPC}) = 1 - X^m(j) \quad \text{and} \quad X_e^m(\text{DPPC}) = 1 - X_e^m(j).$$

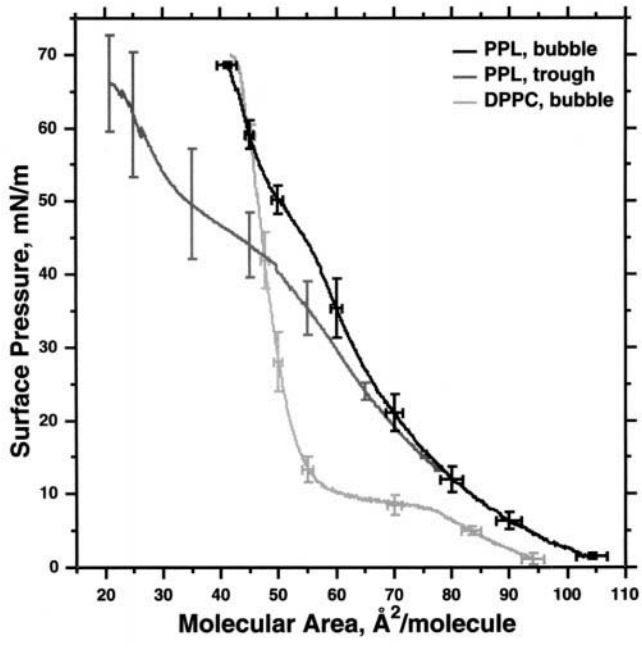

FIGURE 1 π-A isotherm for monolayers of PPL. Lipids in chloroform solution were deposited on a Langmuir trough or a captive bubble at 23°C on a subphase of HSC and then compressed at the constant rate of ~3 Å²/(molecule·min). Experiments with PPL on both the trough and the bubble used overspread films with initial areas below the point of lift-off, and molecular areas were normalized to values obtained at 17 mN/m with films spread to initial areas of 149 Å²/molecule on the Langmuir trough. Curves are averages, with SD shown only at selected points for clarity of presentation. $n = 5$ for DPPC, 5 for PPL on the bubble, and 10 for PPL on the trough.

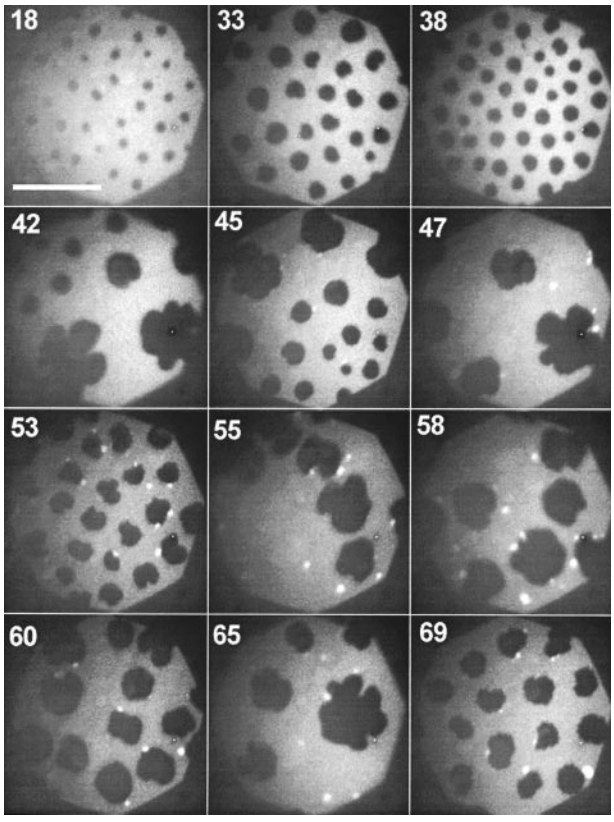

FIGURE 2 Microscopic images of PPL monolayer at different surface pressures. Numerical labels indicate surface pressures in mN/m. Images were obtained during continuous compression at 3 Å^2/(molecule·min) using fluorescence microscopy with a 100× objective and monolayers containing 1% (mol:mol) Rh-DPPE. Scale bar represents 50 μm.

The calculations used averaged isotherms obtained on the captive bubble for both PPL and DPPC, values of ϕ_c calculated from a polynomial fitted to the measured fractional areas, and an initial mol fraction of DPPC in the complete phospholipid mixture of 0.33 (Kahn et al., 1995).

RESULTS

In contrast to complete surfactant extracts (Discher et al., 1996), monolayers of PPL reached surface pressures approaching 70 mN/m when compressed on a Langmuir trough and sustained these values for many minutes after compression ceased (Fig. 1). At mid compression, the isotherm showed a significant decrease in slope, or increase in compressibility, beginning at ~40 mN/m before the curve again turned upward. Over the course of multiple experiments on the Langmuir trough, we found the shape of the isotherm and the area to be much more variable above 40 mN/m than at lower pressures (Fig. 1). Isotherms obtained on the continuous interface of a captive bubble, which eliminates the possibility of escape along confining barriers, showed the temporary increase in compressibility at ~46 mN/m but much less variation at higher pressures (Fig. 1). We presumed therefore that on the Langmuir trough, creep along the confining barrier contributed to some of the decrease in area at high surface pressures (Goerke and Gonzales, 1981). The shoulder on the bubble isotherm, however, must be explained by some other process, such as collapse of material from the interface. Judging from the isotherm alone, the film showed behavior at least consistent with the enrichment in DPPC predicted by the classical model, with the monolayer sustaining high surface pressure only after exclusion of some components.

In previous studies, fluorescence microscopy below 45 mN/m distinguished a condensed phase in PPL that contained almost exclusively DPPC (Discher et al., 1999b). The Rh-DPPC partitioned preferentially into the expanded phase, producing nonfluorescent condensed domains surrounded by the fluorescent expanded film. Our current experiments demonstrated similar behavior, with the appearance of nonfluorescent domains at approximately 8 mN/m that increased in size during further compression (Fig. 2). Domains tended to group and fuse during progression across the collapse plateau, leading to greater heterogeneity among different microscopic fields (Fig. 2). To eliminate selection bias, images were recorded at regular time intervals while convection moved different regions of the film below the microscope. The coexisting fluorescent and nonfluorescent phases persisted in all experiments to the highest surface pressures obtained at the end of compression (Fig. 2).

Beginning at ~45 mN/m, images also showed small, intensely fluorescent spots outside or at the edges of the

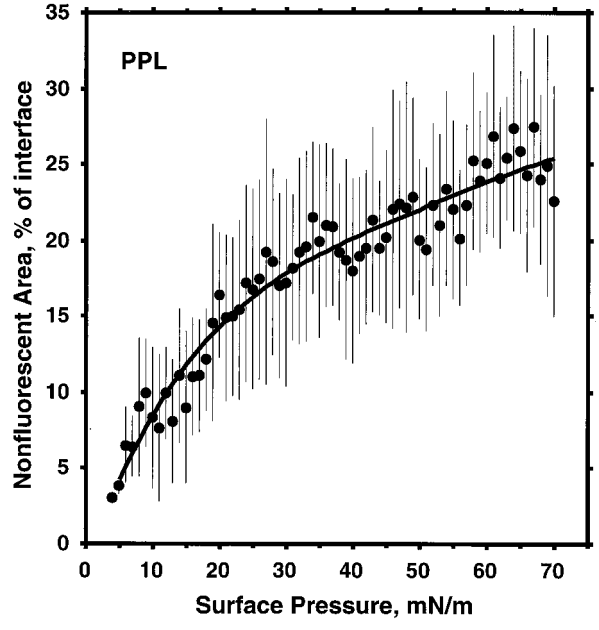

FIGURE 3 Variation of nonfluorescent area with surface pressure for PPL monolayers. The nonfluorescent area from multiple images obtained during continuous compression at 3 Å^2/(molecule·min) was boxcar averaged over ten experiments at 1 mN/m intervals to provide mean ± SD Solid line gives the best fit to a fourth-order polynomial.

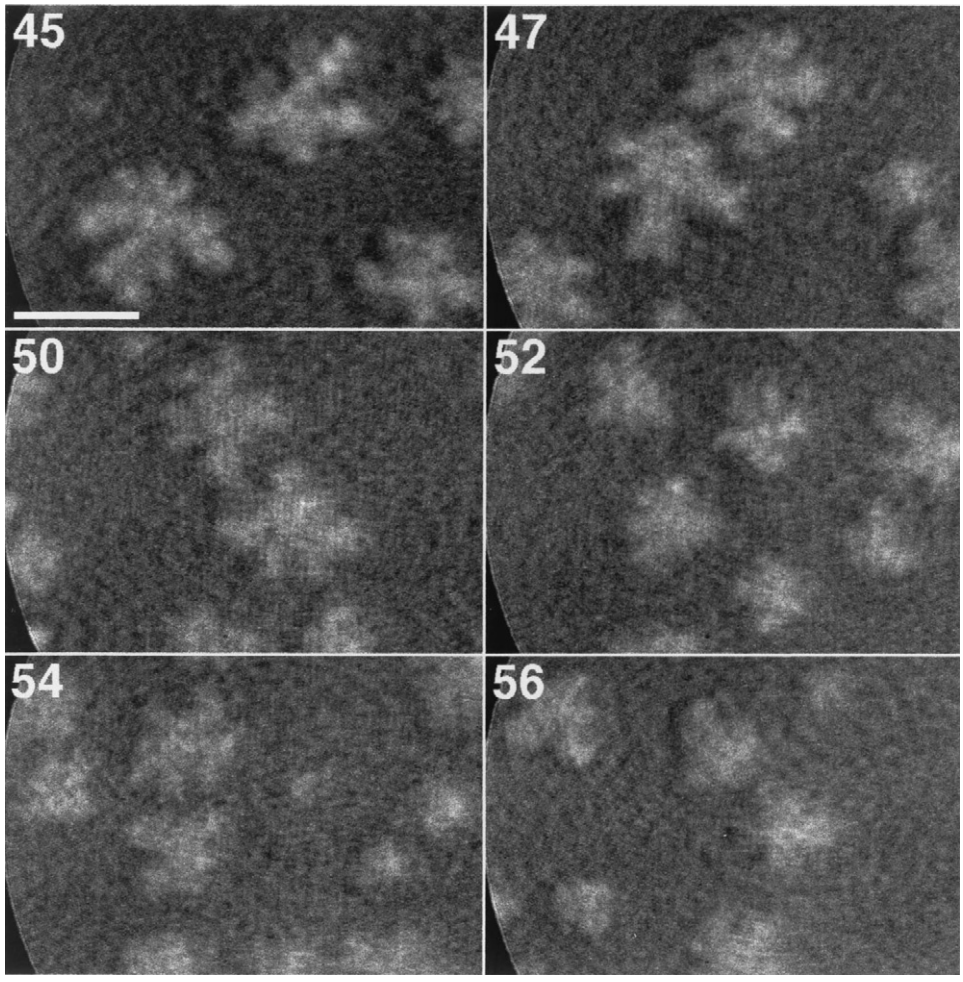

FIGURE 4 Brewster angle microscopy of PPL monolayers at high surface pressures. Solutions of PPL in chloroform were spread on a subphase of HSC at room temperature and compressed at 1.4 Å^2/(molecule·min). Images are labeled with the surface pressure (mN/m) at which they were obtained during continuous compression. Contrast in all images has been enhanced identically to preserve the relative grayscale. Scale bar is 50 μm. Micrographs at lower surface pressures have been published (Discher et al., 1999b).

nonfluorescent domains (Fig. 2). The number of these spots and, to a lesser extent, their individual size grew during compression beyond 45 mN/m, but their total area never exceeded 3–6% of the interface. Prior experiments in our laboratories using simultaneous fluorescence and light scattering microscopies (Schief et al., 2000a,b) with films of CLSE and binary mixtures of DPPC-dihydrocholesterol have shown that similar intensely fluorescent spots scatter significantly more light than the edges of two-dimensional gas bubbles or LC domains in monolayers (data not shown). The intensely fluorescent regions in the PPL films therefore have greater thickness than the surrounding film, consistent with three-dimensional structures formed by collapse of the two-dimensional monolayer.

The marked contrast between the fluorescent and nonfluorescent phases allowed measurement of their relative areas (Fig. 3). The nonfluorescent domains grew over the course of compression. When compared over the same range of surface pressures $\leq$40 mN/m, they occupied fractional areas comparable to values reported previously (Discher et al., 1999b). At 45 mN/m, our current measurements obtained a nonfluorescent area averaged over 10 experiments of 20.2 $\pm$ 5.7% of the interface. The rate at which the domains grew during compression, however, slowed at higher pressures. For some individual experiments, the relative areas of the two phases changed little above 45 mN/m. At 65 mN/m, the nonfluorescent area averaged over the 10 experiments occupied 25.8 $\pm$ 5.3% of the interface. The progression toward a uniformly LC film predicted by the classical model of surfactant function failed to occur.

BAM demonstrated that the persistence of phase coexistence to high surface pressures was unrelated to the presence of the extraneous probe (Fig. 4). Bright domains, with greater optical thickness than the surrounding film, were similar in size and shape in the BAM images to the nonfluorescent phase in the fluorescence micrographs when compared at the same surface pressures. Contrast between the two phases in the BAM images decreased at the end of compression, but even at the highest pressures, the two phases remained evident.

BAM also showed that a number of properties characteristic of each phase below 45 mN/m persisted at higher pressures. We have previously used the dependence of grayscale on the angle of an analyzing polarizer in the

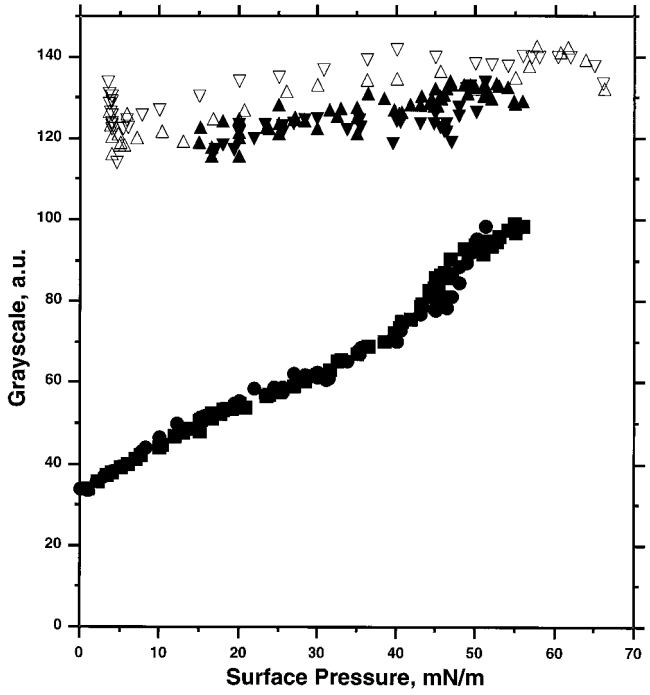

FIGURE 5 Variation of grayscale in Brewster angle micrographs of PPL monolayers during compression. Measurements give grayscale values from two experiments for domains (*filled triangles*) and the surrounding film (*filled squares* and *circles*) in monolayers of PPL, and for monolayers of DPPC in the LC phase (*open triangles*). Each measurement is the mean of a Gaussian fit to a grayscale histogram compiled from 2–5 images at a particular pressure in a particular experiment. The grayscale of the plain air–buffer interface, which effectively corresponds to zero reflectivity, was 17.5. Values below 45 mN/m have been published previously (Discher et al., 1999b).

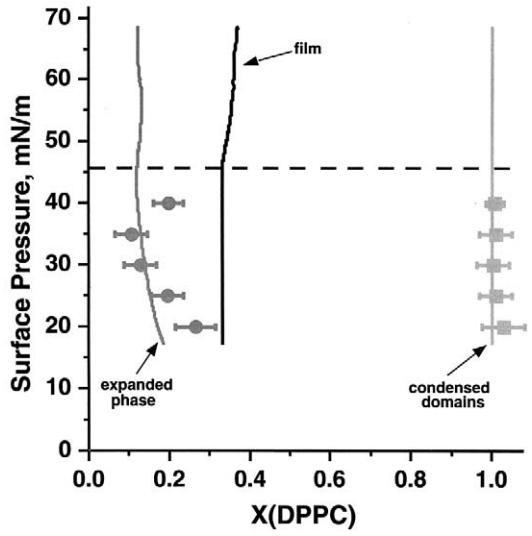

FIGURE 6 Estimated compositions in monolayers of PPL. Solid lines give X (DPPC), the mol fraction of DPPC, estimated for the complete film (*black line*) and for the expanded phase (*dark gray line*) with an iterative calculation, and assumed for the condensed domains (*light gray line*). In addition to domains that contain only DPPC, the iterative calculation assumes that no material is excluded from the interface below 46 mN/m (*horizontal dashed line*), that exclusion occurs only from the expanded phase at higher pressures, and that the ratio of molecular areas for the two phases remains constant above 46 mN/m. Symbols give the previously published mol fractions of DPPC (mean ± SD) for the condensed domains (*light gray squares*) and the surrounding film (*dark gray circles*) (Discher et al., 1999b).

reflected beam to show that, below 45 mN/m, the condensed domains in PPL are anisotropic, surrounded by an isotropic expanded phase (Discher et al., 1999b). The two phases retained these characteristics above 45 mN/m (data not shown). We also showed previously that the grayscale in BAM images provides a reproducible quantitative measurement of the reflected intensity that can be used to compare specific regions within the same or different monolayers (Discher et al., 1999b). Below 45 mN/m, the grayscale for the two phases had distinct behavior. Values for the condensed phase were similar to grayscales for the LC phase of DPPC both in magnitude and in lack of variation during compression. In contrast, values for the expanded phase in PPL were lower and showed a greater dependence on surface pressure (Discher et al., 1999b). Similarly distinct behavior for the two phases persisted above 45 mN/m (Fig. 5). The grayscale for the condensed domains was again relatively invariant (slope of 0.29 ± 0.03 a.u.·m/mN) during compression from 45 to 56 mN/m, and maintained a value (129 ± 4 a.u.) only slightly lower than for LC DPPC (137 ± 3 a.u.) over the same range (Fig. 5). For the expanded phase, the grayscales at the higher surface pressures were again lower and more variable during compression, although the slope of rising grayscale showed a distinct increase at ~40 mN/m from 1.0 ± 0.1 to 1.7 ± 0.2 a.u.·m/mN (Fig. 5). A limited number of observations with light scattering microscopy demonstrated a marked increase in the number of point scatterers detected in the expanded phase above 60 mN/m. Because diffuse scattering can alter the intensity observed by BAM (Schief et al., 2000a), grayscale no longer provided a reliable indicator of optical thickness, and no measurements were made above 60 mN/m. In the range between 45 and 60 mN/m, however, the characteristics of each phase remained well behaved relative to their properties at lower surface pressures.

The measured phase behavior provided the basis for estimating the variation during compression of composition in the expanded phase and in the film. Previously, we have shown that, over the range of surface pressure from 20 to 40 mN/m, the condensed phase in PPL contains essentially pure DPPC (100 ± 4%) (Discher et al., 1999b). If that composition persists to higher surface pressures, then the condensed phase should have the same metastability as films of DPPC, and material should collapse only from the surrounding film. Our model assumed no loss of constituents up to 46 mN/m, and exclusion only from the expanded

phase at higher pressures. An iterative calculation estimated the change of the molecular content for each phase during sequential changes in area, based on their molecular areas and the measured fractional areas (see Methods). Because we suspect that the films were incompletely confined on the Langmuir trough, these calculations used isotherms obtained on the captive bubble for both PPL and DPPC. After the onset of collapse at 46 mN/m, we used the simplification that the ratio of molecular areas in the two phases remained constant. The compositions predicted for the expanded phase below 46 mN/m were comparable to previously determined values (Discher et al., 1999b). The predicted mol fractions of DPPC increased above 46 mN/m, but only from 0.12 to 0.13 for the expanded phase and from 0.33 to 0.37 for the complete film (Fig. 6).

DISCUSSION

PPL provides a system with several advantages for testing the classical model of surfactant function, which contends that the interfacial film at high surface pressures is LC and substantially enriched in DPPC. In contrast to complete extracts of calf surfactant, compression of PPL monolayers readily achieves the range of high pressures at which the model can be tested. PPL contains the complete set of surfactant phospholipids (Hall et al., 1994) including the full complement of compounds that, in single-component monolayers, collapse readily above 45 mN/m. The classical model therefore makes the same prediction for PPL as for complete surfactant, that films should be unable to reach high surface pressures until their composition changes. The squeeze-out hypothesis suggests that the necessary refinement occurs when constituents other than DPPC collapse from the interface above 45 mN/m, and the π-A isotherm, particularly on the Langmuir trough, is at least superficially consistent with the specific predictions of that hypothesis. Surface pressure only achieves high values after a shoulder in the isotherm indicates the onset of collapse, during which the composition of the film might change. The phase behavior of PPL is also particularly helpful not only because the separated phases induced by compression are clearly evident in microscopic images, but also because they provide important compositional information. Condensed domains contain almost pure DPPC (mol fraction 1.00 $\pm$ 0.04), with the other constituents confined to the surrounding film (Discher et al., 1999b). PPL therefore provides a physiologically relevant system to study whether and how the composition of the phospholipids changes at high surface pressures.

The predicted conversion of the PPL film to a LC monolayer at high surface pressure does not occur. Both coexisting phases that we have demonstrated previously below 45 mN/m (Discher et al., 1999a,b) persist to pressures approaching 70 mN/m. The phase that surrounds the condensed domains has the same characteristics above 45 mN/m that we have demonstrated previously at lower pressures. In films containing a fluorescent lipid probe, the surrounding phase remains enriched in fluorophores at high pressures. In the absence of probe, BAM shows that the lower optical thickness and isotropy that were evident below 45 mN/m continues at higher pressures. The greater variation of optical thickness with surface pressure also extends over the full range of surface pressures, and suggests the greater compressibility of an LE phase. The phase that covers approximately three quarters of the interface at $\sim$70 mN/m therefore exhibits behavior characteristic of an LE phase. It is also continuous with regions of the film that, below 45 mN/m, have a larger molecular area than the condensed domains (Discher et al., 1999b), and, therefore, is LE by that criterion as well.

The one unexpected property of the expanded phase is its stability. This phase of mixed constituents in PPL is considerably less prone to collapse than fluid films containing single compounds (Smith and Berg, 1980). The difference may reflect the favorable entropy of mixing in the multicomponent phase, or the presence of some DPPC (Discher et al., 1999b) and of the second disaturated compound palmitoyl-myristoyl phosphatidylcholine (Kahn et al., 1995) that might increase the viscosity and slow collapse. Whatever the explanation, our results contradict a fundamental assertion of the classical model. The formation of a homogeneous film containing only a condensed phase represents a basic mechanism, whether explicit (Bangham et al., 1979) or implicit (Clements, 1977), by which previous investigators have explained the stability of the surfactant film in the lung. The persistence of the LE phase to high surface pressures requires at least significant revision of the classical model.

Our estimates of the composition at high surface pressure assume that constituents leave the film only from the expanded phase. If the domains continue to contain essentially pure DPPC, then collapse only from the surrounding film is likely. Our current methods, however, are inadequate to determine conclusively the source of excluded material within the film, and so this feature remains an assumption. BAM has inadequate lateral resolution to detect collapsed structures that were evident by fluorescence, and fluorescence microscopy would not detect collapse from domains that lack probe. Preliminary experiments with light scattering microscopy did detect three-dimensional structures within the expanded phase, but not within the domains or at their boundaries. Our working model therefore is that material is excluded into the bright fluorescent structures that lie above or below the monolayer only from the LE phase. An iterative calculation based on this view predicts that the mol fraction of DPPC, which is 0.33 in our original preparation (Kahn et al., 1995), reaches 0.37 in the film and 0.13 in the expanded phase. We consider these to be upper limits. The values would be lower if any material is in fact excluded from the condensed phase. The calculations assume

a fixed ratio of molecular areas, $\bar{A}_e/\bar{A}_c$, and if, in fact, the measured decline in the ratio below 46 mN/m continues at higher pressures, the amount of material excluded from the film would be less, so that the mol fractions of DPPC would again be lower. The minimal enrichment of DPPC does preserve that essential element of the squeeze-out hypothesis, but the resulting composition falls far short of the >90% DPPC suggested previously (Hildebran et al., 1979).

We conclude that the classical model of surfactant function requires serious reevaluation. The model contends that only an LC film could have the stability observed in the lung, that compounds could form the LC phase only at temperatures below their gel-to-liquid crystal transition temperatures (T_c), and that, because DPPC is the only major constituent that remains below T_c at physiological temperatures, the functional film must consist mostly of DPPC. Results reported elsewhere show that single-component monolayers containing phospholipids above T_c can, in fact, be transformed to structures with physiological stability (Crane and Hall, 2001). Our results here demonstrate that LE films of mixed components, over half of which exceed their T_c in our experiments, can also achieve and maintain high surface pressure.

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