Neutral Lipids Induce Critical Behavior in Interfacial Monolayers of Pulmonary Surfactant†

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ABSTRACT: We have shown previously that lateral compression of pulmonary surfactant monolayers initially induces separation of two phases but that these remix when the films become more dense (*1*). In the studies reported here, we used fluorescence microscopy to examine the role of the different surfactant constituents in the remixing of the separated phases. Subfractions containing only the purified phospholipids (PPL), the surfactant proteins and phospholipids (SP&PL), and the neutral and phospholipids (N&PL) were obtained by chromatographic separation of the components in extracted calf surfactant (calf lung surfactant extract, CLSE). Compression of the different monolayers produced nonfluorescent domains that emerged for temperatures between 20 and 41 °C at similar surface pressures 6−8 mN/m higher than values observed for dipalmitoyl phosphatidylcholine (DPPC), the most prevalent component of pulmonary surfactant. Comparison of the different preparations showed that the neutral lipid increased the total nonfluorescent area at surface pressures up to 25 mN/m but dispersed that total area among a larger number of smaller domains. The surfactant proteins also produced smaller domains, but they had the opposite effect of decreasing the total nonfluorescent area. Only the neutral lipids caused remixing. In images from static monolayers, the domains for N&PL dropped from a maximum of 26 ± 3% of the interface at 25 mN/m to 4 ± 2% at 30 mN/m, similar to the previously reported behavior for CLSE. During continuous compression through a narrow range of pressure and molecular area, in N&PL, CLSE, and mixtures of PPL with 10% cholesterol, domains became highly distorted immediately prior to remixing. The characteristic transition in shape and abrupt termination of phase coexistence indicate that the remixing caused by the neutral lipids occurs at or close to a critical point.

The phase behavior of biological phospholipid films and membranes may be important in at least two respects. First, the different physical characteristics of the different phases can alter processes such as lateral diffusion. Second, phase separation can maintain components in distinct compartments and provide a means of organizing biological processes (*2*). Both of these issues may be important for the function of pulmonary surfactant. This complex mix of lipids and proteins coats the thin liquid layer that lines the lung's alveolar air spaces. When compressed by decreasing alveolar surface areas during normal exhalation, the surfactant film achieves very high densities, well above equilibrium values. Measurements in situ show that the surfactant films lower the surface tension of the air−liquid interface to very low levels (*3*) and in doing so stabilize the alveoli. Only films with the physical characteristics of a highly ordered phase seem likely to have sufficient rigidity to withstand compression to such high densities without collapse from the interface. The most prevalent component of surfactant, dipalmitoyl phosphatidylcholine (DPPC),[1] is capable of achieving the stable liquid condensed (LC) phase. When compressed in vitro, single component films of DPPC reach the high surface pressures implied by the measurements in situ. DPPC, however, represents only 30% (mol:mol) of the pulmonary surfactant lipids (*4*). Surfactant films therefore have long been thought to undergo a process of refinement in which components other than DPPC are eliminated from the interface before surface pressure can reach high values (*5*). Phase separation, in which DPPC segregates into LC compartments of the film that are significantly less susceptible to collapse from the interface than the liquid-expanded

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[1] Abbreviations: π−*A*, surface pressure−area; CLSE, calf lung surfactant extract; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSC, differential scanning calorimetry; HSC, 10 mM HEPES pH 7.0, 150 mM NaCl, and 1.5 mM $CaCl_2$; LC, liquid condensed; LE, liquid expanded; N&PL, neutral and phospholipids; PC, phosphatidylcholine; PPL, purified phospholipids; Rh-DPPE, rhodamine−dipalmitoyl phosphatidylethanolamine; SD, standard deviation; SP-B, surfactant protein B; SP-C, surfactant protein C; SP&PL, surfactant proteins and phospholipids.

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(LE) regions containing the other components, provides an appealing model of how such refinement might occur (*6*).

We have shown previously that phase separation does take place in monolayers of extracted calf surfactant (calf lung surfactant extract, CLSE) (*1*). Microscopic studies demonstrated the emergence and initial growth of discrete domains that had visual features characteristic of LC DPPC. On further compression, however, the area of the domains reached a maximum value and then abruptly declined. At the higher surface pressures most important for function in the lung, pulmonary surfactant formed a nearly homogeneous film. Nag and co-workers recently reported similar results with extracted porcine surfactant (*7*). In the studies presented here, we address the mechanism by which remixing of the monolayer occurs. We initially consider the compositional dependence of this phenomenon. Pulmonary surfactant includes approximately 1% (w:w) of the very hydrophobic surfactant proteins SP-B and SP-C and 5−10% (mol:mol) neutral lipid, almost all of which is cholesterol, in addition to the complex mixture of phospholipids. By removing the proteins and/or the neutral lipids, we have established which constituents are responsible for the behavior of CLSE. We also have observed the films more carefully during remixing. Our prior studies recorded images only from static films at predetermined surface pressures. During continuous compression, we show here that the condensed domains remix not by a progressive decrease in their size but rather by a sudden dissolution of the two-dimensional boundary between the two phases. The shape of the domains also becomes dramatically distorted just prior to the remixing. The abrupt termination of phase separation preceded by the characteristic transition in the shape of the domains demonstrate that remixing occurs at or very close to a critical point in the phase diagram.

EXPERIMENTAL PROCEDURES

Materials. Extracted calf surfactant (calf lung surfactant extract, CLSE), prepared as described previously (*8*), was obtained from Dr. Edmund Egan of ONY, Inc. (Amherst, NY), and Dr. Robert Notter of the University of Rochester. CLSE was fractionated to exclude specific components using column chromatography by a slight modification of a previously published protocol (*9*). Gel permeation chromatography separates the proteins, phospholipids, and neutral lipids into distinct peaks (*10*). Pooling selected fractions provides preparations that contain only the purified phospholipids (PPL), the neutral and phospholipids (N&PL), and the surfactant proteins and phospholipids (SP&PL). Each of these preparations then contains the complete set of surfactant phospholipids with or without the surfactant proteins and/or neutral lipids. Although in the original protocol (*9*) the protein and phospholipid peaks overlapped, a longer column achieved complete separation on a single pass for the materials studied here. Preparations were eluted from the LH-20 matrix (LKB-Pharmacia, Piscataway, NJ) with a solvent of acidified chloroform−methanol (0.1 N HCl−$CHCl_3$−CH_3-OH; 1:9:9 v:v:v) (*10*, *11*), followed by extraction of the constituents into chloroform (*12*). Samples of SP&PL suffered variable losses of proteins and were supplemented with protein purified separately to obtain the protein/phospholipid ratio found for CLSE (*9*). Experiments reported here that compared different preparations were performed simultaneously with the previously reported studies on CLSE (*1*) and used material derived from that common batch of surfactant. Experiments that monitored the shape of domains during remixing used the same material for CLSE but preparations derived from a different batch for PPL and N&PL. Results with different batches were qualitatively similar, although some quantities, such as the surface pressure at which the separated phases remixed, did vary by 5−7 mN/m. Prior analysis showed very little change in the composition of the phospholipids (*4*). The content of cholesterol does vary, and this seems the most likely explanation for the variation.

CLSE contains approximately 90% phospholipid, 8% neutral lipid, almost all of which is free cholesterol, and 1.5% (w:w) of the mixed proteins SP-B and SP-C (*9*). The phospholipids are 82% (mol:mol) phosphatidylcholine (PC), 6% phosphatidylglycerol, 3% phosphatidylinositol, 3% phosphatidylethanolamine, and 2% sphingomyelin (*9*). The following four major ester-linked compounds constitute 85% of the PCs: DPPC, 41%; palmitoyl−palmitoleoyl (16:0−16:1) PC, 18%; palmitoyl−oleoyl (16:0−18:1) PC, 14%; palmitoyl−myristoyl (16:0−14:0) PC, 13% (*4*). The remaining 15% of the PCs consists of a complex mixture of compounds, each of which constitutes less than a few percent of the PCs.

DPPC and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further analysis or purification. Rhodamine−DPPE (Rh−DPPE) labeled at the headgroup ((*N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) was purchased from either Avanti Polar Lipids (Alabaster, AL) or Molecular Probes (Eugene, OR) and used without further purification.

Reverse-osmosis grade water for these studies was obtained from purification systems purchased either from Millipore (Bedford, MA) or Barnstead (Dubuque, IA) and had resistivity of approximately 18 MΩ·cm. All glassware was acid-cleaned. All solvents were at least reagent-grade and contained no surface active stabilizing agents.

Methods. A. Biochemical Assays. Phospholipid concentrations were determined by measuring the phosphate content (*13*) of measured aliquots. Protein assays used the amido black method of Kaplan and Pedersen (*14*), with bovine serum albumin as a standard. Cholesterol (free and esterified) was measured by reduction with ferrous sulfate (*15*).

B. Compression Isotherms. Surface pressure (π)−area (A) isotherms of interfacial monolayers were measured for PPL, SP&PL, and N&PL on a commercially available trough (KSV-3000, KSV Instruments, Helsinki, Finland). Monolayers were compressed at a rate of 1.0 $Å^2$/(phospholipid/min). Water pumped through the base of the trough regulated subphase temperature. Monolayers were created by spreading stock solutions in chloroform at the air−liquid interface. A 10-min waiting period before beginning compression allowed for evaporation of the spreading solvent. π-A curves were selected from a group of three reproducible isotherms in which deviations in molecular area and surface pressure between different experiments were less than 2 $Å^2$/phospholipid and 0.4 mN/m, respectively. Molecular areas were expressed in terms only of phospholipid for reasons of simplicity and accuracy, with no attempt to correct for the presence of neutral lipid or protein. All experiments used a subphase of 10 mM HEPES pH 7.0, 150 mM NaCl, and 1.5 mM $CaCl_2$ (HSC).

C. Epifluorescence Microscopy. Epifluorescence microscopy monitored phase separation during compression of the different monolayers. All studies used no more than 1% (mole/mole surfactant phospholipid) Rh−DPPE. Samples were spread from chloroform to an initial molecular area of 150 Å^2/phospholipid. The films were compressed at 2.8 Å^2/(phospholipid/min).

Experiments used two different instruments. Measurements on static films containing the surfactant preparations used a previously described home-built Wilhelmy balance (*16*) and a Zeiss-ACM microscope (*17*) with a 50× objective. Images were obtained from static films unless noted otherwise. The fluorescent images were recorded by Hamamatsu C2400 SIT camera either to VHS videotape for later analysis or directly to computer (Quadra 650, Apple, Inc., Cupertino, CA, with LG-3 frame grabber, Scion Corp, Frederick, MD). A C-shaped Teflon mask placed directly in the trough and extending through the interface minimized flow and movement of the monolayer (*17*, *18*). Images obtained inside and outside the mask at frequent intervals ensured that the film remained comparable in both locations.

A second apparatus was used for the experiments with DPPC and for the images reported from the remixing for N&PL and cholesterol:PPL mixtures. The instrument consists of a Nikon epifluorescence microscope focused on the surface of a Langmuir trough with a ribbon barrier. The microscope uses infinity optics and a 100× super long working distance objective assembled on a custom-built stand. The ribbon barrier (Labcon, Darlington, U.K.) consists of a constant-perimeter continuous vertical Teflon tape. The confined section of the interface varies linearly in area by reconfiguration of the ribbon (*19*). The maximum area is 532 cm^2, and the subphase volume is approximately 1 L. A computer uses the graphical user interface LabView (National Instruments, Austin, TX) to control movement of the ribbon barrier as well as to measure surface area and surface pressure via a Wilhelmy plate. The C-shaped Teflon mask, SIT camera, frame grabber, and computer used for recording images were as described above for the other instrument.

D. Image Analysis. The total area, number, and individual size of the nonfluorescent domains in epifluorescence images of the monolayers were analyzed using the program Image, developed at the U.S. National Institutes of Health and available from the public domain on the Internet at http://rsb.info.gov/nih-image/. The marked contrast between dark domains and the surrounding fluorescent film facilitated counting and allowed direct measurements of the size of all domains in any given microscopic field based on digitally assigned pixel gray scale values. Each data point represents analysis of a minimum of three images recorded from different regions of the monolayer for each of four independent experiments. No efforts to quantitate nonfluorescent area were made on images that were uniformly fluorescent by inspection.

The density of domains was calculated as the number per image divided by the area of an image. Average densities were again obtained from at least three images for each of four experiments. The software provided a count of the number and individual area of all domains in each image analyzed. The density of domains and their distribution among the different sizes were calculated from these data. Histograms for the size of individual domains were constructed using boxcar intervals in area adjusted for the range of sizes observed for each sample. Domains for which the true area was unknown because they were partially interrupted by the aperture of the microscope were not included. The normalized frequency for the different areas was obtained by dividing the number of domains in any specific interval by the total number analyzed.

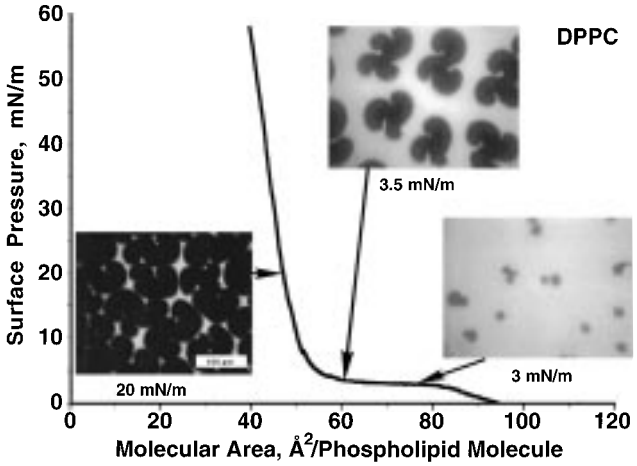

FIGURE 1: Surface pressure−area isotherm with epifluorescence micrographs of DPPC monolayers. Chloroform solutions containing 1% (mol/mol) Rh−DPPE were spread at 20 °C on HSC. Images were obtained from static films following compression at 2.8 Å^2/(phospholipid/min) to the desired pressure. Representative images are given at the specified surface pressures. Scale bar is 100 μm. Isotherms were obtained in separate experiments for DPPC without the addition of Rh−DPPE during compression at 1 Å^2/(phospholipid/min).

The nonfluorescent fraction of the monolayer was calculated as the sum of the area occupied by the individual domains observed in an image expressed as the percentage of its total area.

Data are expressed as mean ±SD.

RESULTS

These studies use preparations of surfactant constituents to determine the role of different components in the previously demonstrated remixing of separated phases in CLSE monolayers (*1*). Column chromatography separated CLSE into distinct peaks containing the surfactant proteins, the phospholipids, and the neutral lipids. Collection of appropriate fractions provided preparations containing the purified phospholipids (PPL) alone, the surfactant proteins and phospholipids (SP&PL), or the neutral and phospholipid (N&PL) (*9*). Comparison of films containing the different preparations to monolayers of DPPC and of CLSE then established the roles of the surfactant proteins, the neutral lipids, and the phospholipids other than DPPC.

The $\pi-A$ isotherms for the different preparations were similar to each other and to the curve for CLSE (*1*). The isotherm for CLSE is a continuous smooth curve (*1*) that shows no discontinuity suggestive of the plateau observed for DPPC (Figure 1) corresponding to the LE-to-LC phase transition. The isotherms for PPL (Figure 2), SP&PL (Figure 3), and N&PL (Figure 4) all showed similarly smooth isotherms without evidence of any discontinuity below 40 mN/m. Surface pressure up to this point decreased by at most 1−2 mN/m when compression stopped, indicating the stability of the monolayers.

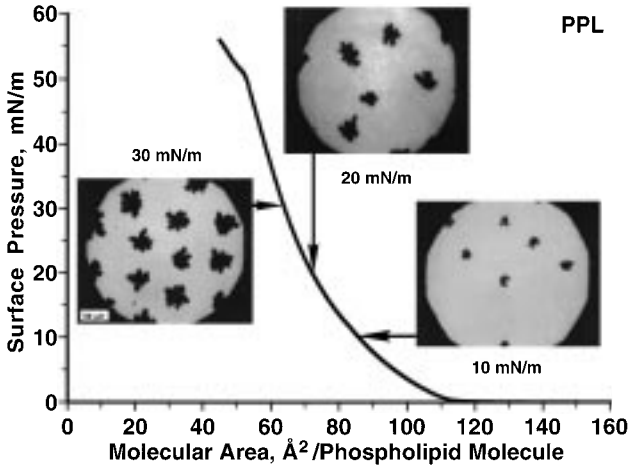

FIGURE 2: Images and isotherms for compression of PPL monolayers. Conditions are identical to those for Figure 1, except that the scale bar represents 50 μm and the isotherm was obtained in a separate experiment without added Rh−DPPE during compression at 1 Å^2/(phospholipid/min).

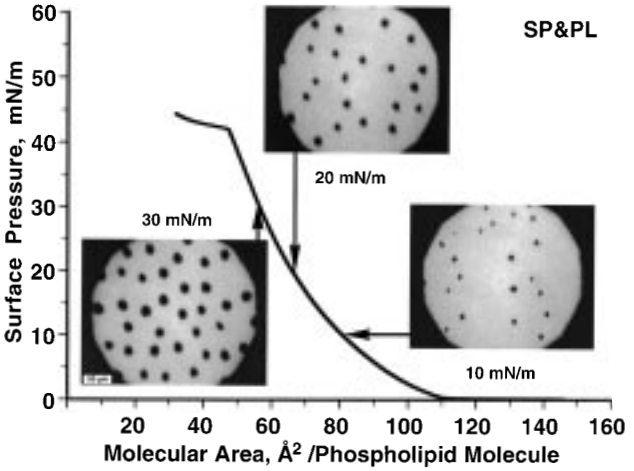

FIGURE 3: Images and isotherms for compression of SP&PL monolayers. Conditions are identical to those for Figure 2.

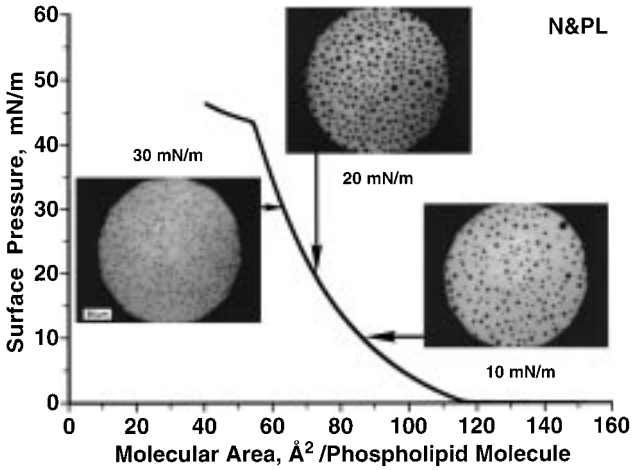

FIGURE 4: Images and isotherms for compression of N&PL monolayers. Conditions are identical to those for Figure 2.

We used fluorescence microscopy to image directly phase behavior within the monolayer during compression on a Wilhelmy balance. Images of static films at 5 mN/m increments in surface pressures detected the formation of nonfluorescent domains in each of the fractions (Figures 2−4).

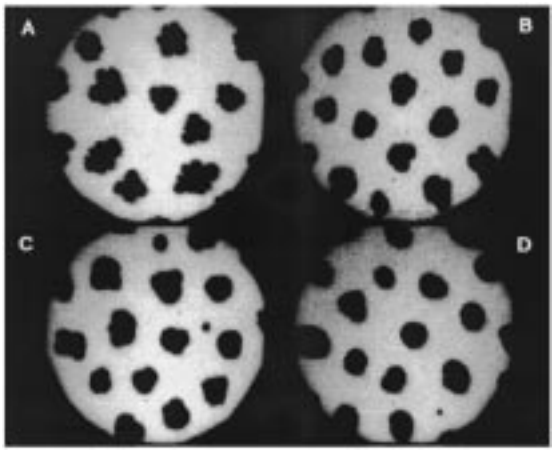

FIGURE 5: Variation of the shape of domains in PPL with time at 25 mN/m. Films of PPL containing 1% (mol/mol) Rh−DPPE were spread to an initial area of 150 Å^2/phospholipid and compressed at 2.8 Å^2/(phospholipid/min) to 25 mN/m. Images of the static films were then recorded at the following number of hours after cessation of compression: A, 0; B, 2.5; C, 3.5; D, 6.5.

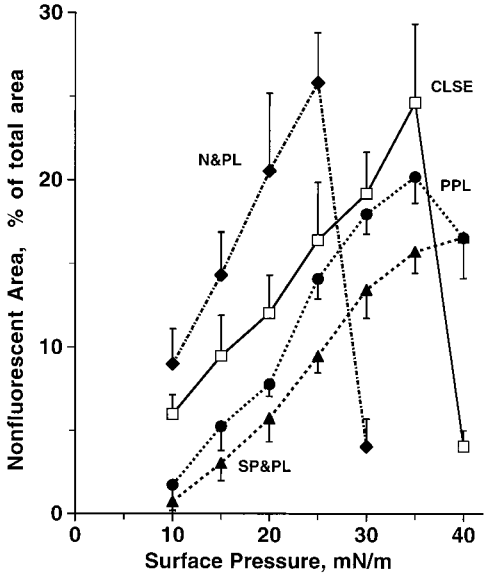

FIGURE 6: Variation of total area of condensed domains with surface pressure for the different preparations of surfactant components. Total area of the domains is expressed as the fraction of the total image area analyzed. At least three images were analyzed at each surface pressure for each of four experiments. (See Methods for details). Values are mean ± SD.

Domains emerged between 5 and 10 mN/m and then increased in size over a broad range of surface pressures for all preparations. The domains for PPL initially had shapes substantially different from the circular forms in CLSE (Figure 2). These, however, represented nonequilibrium structures. In films of PPL held at fixed surface pressures over a period of hours, the domains gradually changed to more regular shapes (Figure 5). Many domains adopted the characteristic kidney bean configuration seen in freshly compressed monolayers of pure DPPC (*20*). The domains for both N&PL and SP&PL were circular (Figures 3 and 4, respectively).

Measurements of the nonfluorescent area allowed quantitative comparison of the domains among the different preparations (Figure 6). The total nonfluorescent areas for N&PL and SP&PL varied in opposite directions relative to

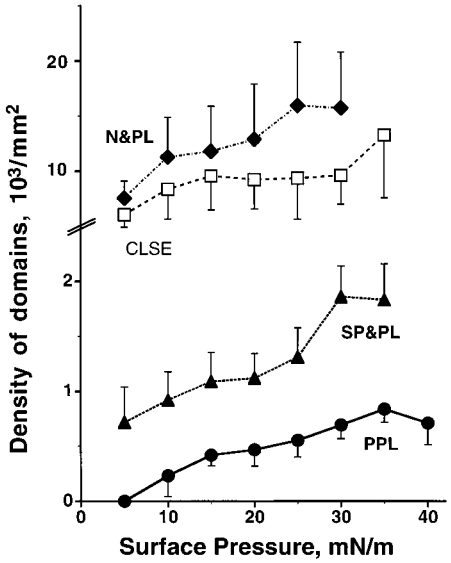

FIGURE 7: Density per area of domains in different preparations of surfactant components. The preparations were spread and compressed to specific surface pressures. Images were recorded from static films at the pressures indicated. The number of domains was counted from three images at each surface pressure for each preparation in four experiments. Values are mean ± SD.

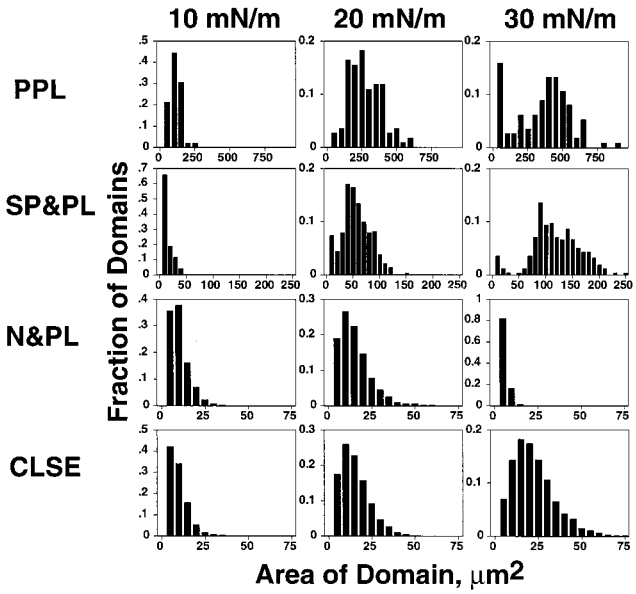

FIGURE 8: Size distribution of domains in monolayers of surfactant constituents. Results are expressed as the fraction of domains analyzed that occurred within specific ranges of size. The different preparations produced different maximum sizes and required the following different box car intervals in area for this analysis: 5 μm^2 for CLSE and N&PL; 10 μm^2 for SP&PL; 50 μm^2 for PPL. Results at each surface pressure were averaged from at least three images for each of four experiments.

PPL (Figure 6). Domains in SP&PL occupied the smallest interfacial area, reaching only 16 ± 1% of the interface at 35 mN/m in contrast to 20 ± 2% for PPL. N&PL produced the largest nonfluorescent phase, reaching a maximum of 26 ± 3% of the interface at 25 mN/m for which the domains in PPL occupied only 14 ± 1%. The domains in CLSE occupied 25 ± 5% of the interface at 35 mN/m, with areas at lower pressures generally lying intermediate to SP&PL and N&PL and comparable to values for PPL (*1*). The neutral lipids therefore increased the total area in N&PL relative to PPL and in CLSE relative to SP&PL. The proteins had the opposite effect of decreasing the area for SP&PL relative to PPL and for CLSE relative to N&PL. The combined presence of both components in CLSE tended to counteract each other.

The domains in monolayers containing the surfactant subfractions underwent the remixing observed previously in CLSE (*1*) only for films of N&PL (Figure 6). The nonfluorescent area for this preparation abruptly declined between 25 and 30 mN/m from 26 ± 3% to 4 ± 2% of the interface. Images at higher pressures showed only the uniformly fluorescent monolayer. The magnitude of the abrupt decrease in nonfluorescent area for N&PL was similar to the change observed previously for CLSE, which dropped from 25 ± 5% of the interface at 35 mN/m to 4 ± 2% at 40 mN/m (*1*). The domains in the PPL films also passed through a maximum area at 35 mN/m, but the decrease over the subsequent 5 mN/m increase in surface pressure was only 3%, and the nonfluorescent phases remained distinct. The total area of the SP&PL domains increased during the entire compression and reached a maximum of 17 ± 1% at 40 mN/m. Remixing therefore occurred in both preparations, CLSE and N&PL, that contain the neutral lipids but not in the two from which these compounds had been removed.

The distribution of the nonfluorescent area into domains of different sizes also varied among the different preparations. PPL produced small numbers of large domains (Figures 7 and 8). In N&PL, the additional presence of the neutral lipids instead yielded a much larger number of much smaller domains. At 20 mN/m, for instance, the most frequent size for domains in PPL was 250 μm^2 but only 10 μm^2 for N&PL. The density of domains was (12.9 ± 5.0) × 10^3 domains/mm^2 for N&PL but only (0.5 ± 0.2) × 10^3/mm^2 for PPL. Domains in SP&PL were intermediate in size between PPL and N&PL. The most frequent size at 20 mN/m was 45 μm^2 for SP&PL, with a density of (1.1 ± 0.2) × 10^3 domains/mm^2. CLSE produced a distribution more similar to N&PL, with the most frequent size occurring at 10 μm^2 for 20 mN/m with a density of (9.2 ± 2.7) × 10^3 domains /mm^2. The additional presence of the neutral lipids and the proteins in the different preparations then both caused the distribution of the nonfluorescent area among more numerous domains, with the neutral lipids having the greater effect.

We also measured the surface pressure at which domains first emerged for the different preparations over the temperature range from 20 to 41 °C (Figure 9). For all three preparations, the nonfluorescent phase first appeared at a pressure higher than for pure DPPC. The phospholipids other than DPPC accounted for most of that change. Domains emerged in PPL at pressures 6−8 mN/m higher than DPPC over the full range of temperatures. The additional presence of the neutral lipids in N&PL and the proteins in SP&PL produced little further effect. Formation of domains in N&PL required significantly higher pressures than PPL only at 20 °C. The pressures for these subfractions of components were slightly lower than our previously established values for CLSE (*1*), especially above 36 °C. These differences, however, were generally small, and the pressure at which domains first emerged for all surfactant preparations paralleled the behavior for DPPC at pressures that were higher by 6−8 mN/m (Figure 9).

To characterize the process of remixing more thoroughly, we also conducted experiments on N&PL in which compres-

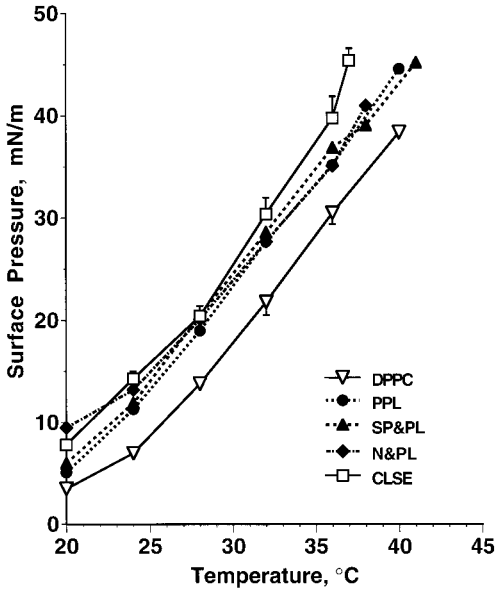

FIGURE 9: Temperature dependence of the surface pressure at which domains first emerged. Films of the different preparations spread at the temperatures indicated to 150 Å^2/phospholipid were then compressed continuously at 2.8 Å^2/(phospholipid/min). Values are mean ± SD; $n = 3$.

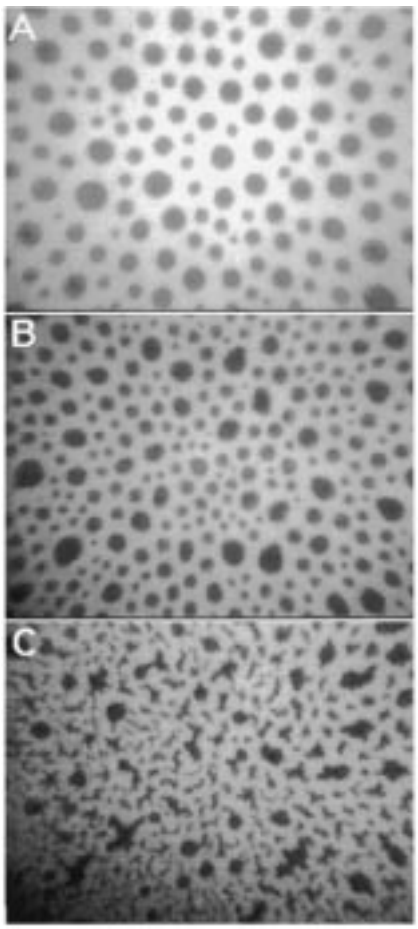

FIGURE 10: Fluorescence images of films containing N&PL. Films were spread in chloroform and compressed at 2.8 Å^2/(phospholipid/min) until microscopy suggested the beginning of the shape transition. Images were then recorded from static films at the surface pressures A, 30 mN/m, B, 31 mN/m, and C, 31 mN/m, with compression of the film from Figure 10B only to overcome the small decay in surface pressure after the cessation of compression.

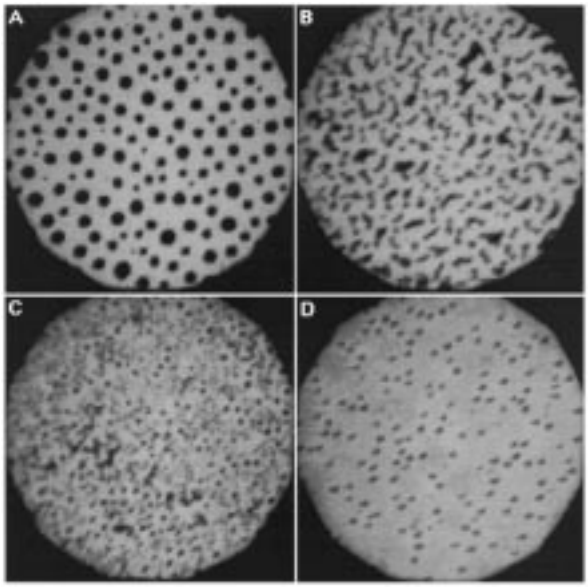

FIGURE 11: Remixing of separated phases in monolayers of CLSE. The film containing 1% (mol:mol) Rh−DPPE was spread in chloroform to 150 Å^2/phospholipid, compressed at 2.8 Å^2/(phospholipid/min) to 30 mN/m, and then held at constant area for 3 min before recording image A. The subsequent images were then recorded during continuous compression at 2.8 Å^2/(phospholipid/min) at the following intervals in time (and molecular area) after reaching 34 mN/m: B, 0 s (0 Å^2/phospholipid); C, 4 s (0.2 Å^2/phospholipid); D, 13 s (0.6 Å^2/phospholipid).

sion was halted according to the appearance of the film. Domains underwent a dramatic change in shape before dissipating into the surrounding monolayer (Figure 10). The original circular shape (Figure 10A) became first slightly elongated (Figure 10B) and then progressively distorted, adopting highly irregular profiles (Figure 10C), before the domains mixed with the surrounding film. These changes occurred during minimal compression with changes in surface pressure of only 1 mN/m. Material used in comparative studies described above differed from the preparation used in these experiments, and remixing occurred at a higher surface pressure, reflecting our general finding that this phenomenon occurs at slightly different points during compression for different lots of material. This distinctive transformation of domain shape, however, with minimal compression of the monolayer was a characteristic finding for all films in which remixing occurred, including for CLSE in experiments examining the complete mixture (Figure 11).

Addition of 10% (mol:mol) cholesterol to PPL converted its behavior to that of N&PL (Figure 12). Instead of the small number of large, irregularly shaped domains seen in films of PPL, the condensed phase in the cholesterol:PPL films instead was dispersed among numerous small circular domains (Figure 12). The separated phases remixed during continuous compression at approximately 30 mN/m. The domains underwent the same shape transition observed for N&PL prior to remixing, with circular forms again becoming progressively distorted until the condensed phase dissipated into the surrounding film (Figure 12). The configuration of the domains again changed during quite limited compression of the film. These results support the essential role of the cholesterol in the remixing of the separated phases.

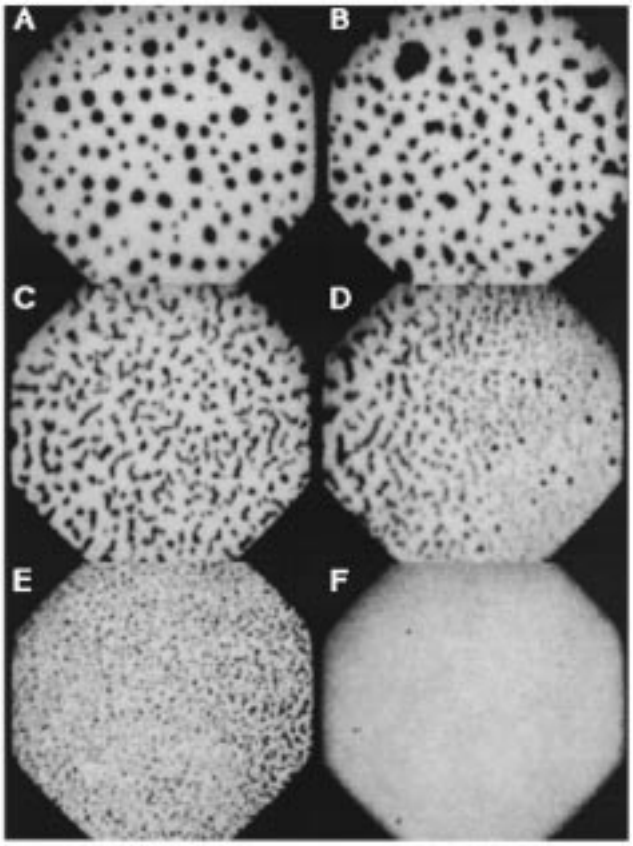

FIGURE 12: Fluorescence images of films containing mixtures of cholesterol:PPL (1:9, mol:mol). Images were recorded during continuous compression at 2.8 Å^2/(phospholipid/min) of films spread in chloroform to initial molecular areas of 125 Å^2/phospholipid. Images correspond to the following molecular areas (Å^2/phospholipid): A, 68.0; B, 64.3; C, 64.2; D, 63.8; E, 60.9; F, 58.0.

DISCUSSION

These studies address the remixing of separated phases in interfacial films of pulmonary surfactant. We have found previously that compression of interfacial monolayers containing CLSE produced separation of a condensed phase from the surrounding film (*1*). The condensed domains increased in size during initial compression, but the two phases then abruptly remix. In the experiments reported here, we asked which components of the complex mixture that constitutes pulmonary surfactant contribute to the observed remixing. Our experimental design emphasizes preparations that contain complete sets of the different surfactant constituents. By comparing DPPC and CLSE with preparations from which specific components have been removed, we can determine the effect of the surfactant proteins, the neutral lipids, and the phospholipids other than DPPC on the behavior of complete surfactant. This approach preserves effects that might be missed in studies with simpler model systems.

Films containing PPL, SP&PL, and N&PL mixed with small amounts of fluorescent phospholipid all develop nonfluorescent domains during compression at the air−liquid interface (Figures 2−4). These domains have the same characteristics which suggested previously that the nonfluorescent phase in CLSE contained predominately DPPC. The exclusion of the fluorescent probe from the domains suggests that they are condensed relative to the surrounding film. The pressures at which the domains first emerge again closely parallel values for DPPC over a range of temperatures (Figure 9) for which no other surfactant constituents should form a condensed phase (*4*). The total area of the nonfluorescent phase in each case remains below or comparable to the values expected if all DPPC resided in LC domains. The fraction f_c of constituents located within condensed domains with molecular area $\tilde{A}_c$ that occupy the interfacial fraction ϕ_c of a film with average molecular area $\tilde{A}$ is given by (*21*)

$$f_c = \phi_c \frac{\tilde{A}}{\tilde{A}_c} \quad (1)$$

Using values from DPPC isotherms for $\tilde{A}_c$, this equation predicts that, at their maximum areas, the domains contain 27, 18, and 36% of the constituents in PPL, SP&PL, and N&PL, respectively. Prior quantitative analysis indicates that DPPC constitutes 33% of the phospholipids and 30% of the total lipids in CLSE (*4*). The estimated content of DPPC in the nonfluorescent domains remains therefore either below or quite close to the content of DPPC in CLSE, and so the area of the domains remains consistent with a composition of mostly DPPC. For the preparations containing the neutral lipids, the remixing discussed below does suggest that the domains contain material other than DPPC. Our results in general, however, suggest that, relative to the surrounding film, the domains in PPL, SP&PL, and N&PL, like those in CLSE, are more condensed and significantly enriched in DPPC.

The condensed phase remixes in both preparations, N&PL and CLSE, that contain the neutral lipids but in neither PPL nor SP&PL from which they are absent (Figure 6). The condensed phase in PPL containing only the phospholipids reaches a maximum total area and then decreases to a very limited extent at 40 mN/m.[2] Similar results have been reported previously for binary mixtures of DPPC and dioleoyl PC (*22*). This behavior, however, differs greatly from the much larger decrease observed for CLSE over a narrow range of surface pressure (*1*). Only N&PL produces a similarly abrupt drop in the nonfluorescent area. The domains for SP&PL increase in area continuously during compression. These results and the ability of cholesterol to convert the behavior of PPL to that of N&PL (Figure 12) show that the neutral lipids are responsible for the remixing of the separated phases.

Two characteristics of the remixing suggest that it occurs at or at least very close to a critical point in the phase diagram. A critical point is defined as the conditions at which separation of two coexisting phases in equilibrium terminates (*23*). The properties of two distinct phases become progressively similar in a system approaching a critical point until they merge. The separated phases become miscible with minimal change in conditions and without the discontinuity of state characteristic of a first-order transition (*24*, *25*). The abrupt remixing of the separated phases in pulmonary surfactant films then indicates critical behavior. The remixing

[2] In a limited number of experiments not reported in detail here, continuous compression of PPL monolayers to surface pressure above 60 mN/m failed to produce any further decrease in nonfluorescent area sufficiently large to be obvious by simple inspection or any transition in the shape of the domains.

does *not* occur by the gradual partitioning of constituents from one phase into the other. For a standard first-order process, the domains would shrink progressively while the surrounding phase enlarged until the domains become undetectably small. Instead the condensed phase in the surfactant films suddenly dissolves into the surrounding film at a specific point during compression. In some experiments, much smaller domains persisted beyond the abrupt dissolution of most of the nonfluorescent area. These residual domains could represent another nonfluorescent phase with a composition different from the original domains. It seems more likely that these regions instead simply reflect "critical slow down" (*26*, *27*). Close to a critical point, relaxation times in general become slow because the similar properties of the two phases reduce the driving force to equilibrium, and so the complete mixing of two miscible phases may simply require longer times. Most of the nonfluorescent phase, however, dissolves within a range of less than 2 mN/m. This behavior fits the definition of critical behavior.

The domains also exhibit a characteristic shape transition immediately prior to remixing. The shape of the domains is normally circular because that configuration produces the smallest interfacial boundary. The energy of interfacial tension, or line tension, is then also at a minimum. Near the critical point, however, the characteristics of the two phases become similar, and line tension approaches zero (*28*−*32*). The reduced interfacial energy then allows distorted shapes with much more prolonged boundaries. The shape transition observed for CLSE, N&PL, and cholesterol:PPL (Figures 11, 10, and 12, respectively) has been demonstrated previously with simple model systems to be highly characteristic of critical behavior (*30*−*35*) and provides further supportive evidence that the remixing in our films occurs in the proximity of a critical point.

Critical behavior has been demonstrated previously in binary lipid mixtures pertinent to the surfactant films. Appropriate mixtures of dimyristoyl phosphatidylcholine (DMPC) and cholesterol (*30*−*32*) and DPPC−cholesterol (*34*, *35*) exhibit the same characteristics of a critical point, although at pressures well below the values at which remixing occurs here. Surfactant preparations, however, that contain the complete set of phospholipids are far from binary mixtures. Two-thirds of the phospholipids are compounds other than DPPC, and these other constituents are likely to explain the difference in critical pressures. Considerably smaller amounts of phospholipid elevate the critical pressure of the DMPC−cholesterol mixture to the range of remixing in our experiments (*33*). Results with the simple model systems therefore predict a critical point in complex mixtures of phospholipids and cholesterol. To the best of our knowledge, our results with pulmonary surfactant represent the first demonstration of such behavior in a full biological system.

The distribution of the condensed phase among the domains in the different preparations provides further insight into the effect of the neutral lipids. The size of the domains reflects at least in part the effect of line tension. At equilibrium, the conflicting influences of line tension and electrostatic interactions determine the radius of the circular domains (*36*). Line tension tends to minimize the total interfacial perimeter by producing a smaller number of larger domains. In contrast, the electrostatic repulsion among the greater density of dipoles within the domains tends to disperse the condensed components into a larger number of smaller domains. In the surfactant preparations, the neutral lipids produce smaller domains (Figure 8). Whether or not the surfactant proteins are present, the neutral lipids increase the number and decrease the size of the nonfluorescent domains (Figures 7 and 8). The neutral lipids seem unlikely to produce this change strictly by electrostatic effects. The difference in dipole density between the two phases would in that case increase. The known tendency of cholesterol to condense LE phospholipids (*37*) suggests in fact that the neutral lipids should have the opposite effect. The smaller domains then suggest that cholesterol lowers line tension. This finding is consistent with previous reports that cholesterol lowers line tension in monolayers containing only cholesterol and DPPC (*38*). Because line tension must become zero at a critical point, this effect of the neutral lipids is consistent with their induction of critical behavior.

The neutral lipids must alter substantially the characteristics of at least one of the two separated phases to generate critical behavior. The properties of the two phases must become indistinguishable at the critical point, and so the addition of neutral lipid to PPL must produce a major change in at least one, if not both, of the separated phases. Direct observation shows that the neutral lipids increase the nonfluorescent area in the surfactant films (Figure 6). The total nonfluorescent area is greater for N&PL than for PPL and for CLSE than for SP&PL at all surface pressures up to the point of remixing. These findings suggest that at least some of the neutral lipid partitions into the domains. Prior studies, however, indicate that the maximum content of cholesterol in condensed DPPC should be limited. Small amounts of cholesterol added to films of DPPC elevate the surface pressure of the LE−LC transition and suggest that the LC phase can solubilize 2−5% cholesterol (*39*). If the only phospholipid in the domains were DPPC, then they should accommodate only a small fraction of the cholesterol present. DPPC represents 33% of the phospholipids in CLSE (*4*). Given a mixture with 5% cholesterol, the domains would solubilize at most 33% of the cholesterol in N&PL even if all DPPC occurred within the domains. It therefore seems likely that the condensed phase contains a limited amount of phospholipids other than DPPC that increases the solubility for cholesterol. An alternate possibility is that the domains represent a phase of mixed DPPC−cholesterol that is more expanded than LC DPPC and therefore occupies a larger fraction of the interface but which is still sufficiently dense that its solubility for the fluorescent probe is much less than in the surrounding film of mixed phospholipids. It of course remains quite possible that much of the cholesterol partitions into the expanded phase. The significant increase in nonfluorescent area, however, indicates that the presence of the neutral lipids at least changes the condensed phase.

The surfactant proteins have the opposite effect on the area of the condensed phase (Figure 6). These hydrophobic proteins decrease the condensed area at all pressures up to 35 mN/m independent of the presence of the neutral lipids. These results suggest that the proteins limit the tendency of the phospholipids to partition into condensed domains. Calculations of the fraction of constituents contained in the domains for monolayers with and without the proteins provide a quantitative estimate of the number of lipids interacting with each protein molecule. Equation 1 predicts that if the

constituents of domains have the average molecular area of pure DPPC at the same surface pressure, then the domains for SP&PL reach a maximum content of only 18% of the total constituents in the monolayer, down from 27% for PPL. The additional presence of the proteins in CLSE similarly reduces the maximum content of the domains from 36% for N&PL to 32%. The ratio of 9.8 μg of protein/μmol of phospholipid in these preparations (*9*) then indicates that 1 μg of protein prevents 10 nmol of phospholipid from entering the domains in the absence of neutral lipids or 6 nmol in their presence. A recently published assay for the hydrophobic surfactant proteins suggests the presence of equivalent amounts of SP-B and SP-C (w:w) in extracted surfactant (*40*). The average molecular weight resulting from this ratio indicates 36−60 phospholipid bound per protein. Similar figures have been published previously for the effect of these proteins on the phase transition in lipid bilayers. Differential scanning calorimetry (DSC) with mixtures of SP-B and DPPC showed that each protein removed 51 lipid molecules from the gel-to-liquid crystal phase transition (*41*). The value for SP-C was 35 phospholipids per protein. Our results and the findings with DSC suggest that, both in monolayers containing the full array of surfactant phospholipids and in bilayers of pure DPPC, the proteins prevented a comparable fraction of phospholipid from condensing to a more ordered state. The effect of surfactant proteins on the nonfluorescent area is possibly also related to the difference between N&PL and CLSE in the surface pressure of the critical point.

The pronounced effect of the neutral lipids raises the possibility that phase behavior is a physiologically regulated variable. Cholesterol constitutes most of the neutral lipid in surfactant, and levels of this compound vary in response to a number of physiological and pathophysiological factors. These include exercise (*42*), hyperventilation (*43*), the development of pulmonary fibrosis (*44*), and temperature for reptilian species whose body temperature can vary (*45*). Cholesterol levels appear to be tightly regulated, with alterations in breathing patterns for periods as brief as 15 min producing significant changes (*43*). The marked shifts in condensed area produced by the neutral lipids in our studies and the changes in the physical characteristics of the film that should result from these alterations suggest that phase behavior may be the important response to the changes in cholesterol levels.

In summary, these studies show that the neutral lipids cause remixing of the separated phases during compression of interfacial films of pulmonary surfactant. The large distortions of the domain's shape prior to their disappearance as well as the narrow range of compression during which the domains again become miscible with the surrounding film indicate that remixing occurs at or close to a critical point. Both the termination of phase separation and the presence of a critical point raise interesting issues concerning the physiological function of pulmonary surfactant films.

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