

Effect of Hydrophobic Surfactant Proteins SP-B and SP-C on Binary Phospholipid Monolayers

II. Infrared External Reflectance-Absorption Spectroscopy

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ABSTRACT In situ external reflection infrared spectroscopy at the air-water interface was used to study the influence on phospholipid structure of an endogenous mixture of the two hydrophobic surfactant proteins, SP-B and SP-C, which are thought to play pivotal roles in the adsorption and function of pulmonary surfactant. Mixtures studied were 1:1, 2:1, and 7:1 (mol:mol) DPPC-d₆₂:DPPG, and 7:1 DPPC-d₆₂:DOPG, alone and in the presence of 0.5–10 wt % mixed SP-B/C purified chromatographically from calf lung surfactant extract. Perdeuteration of DPPC produced a shift in vibrational frequencies so that it could be differentiated spectroscopically from the phosphoglycerol component in the surface monolayer. CH₂ antisymmetric and symmetric stretching bands (~2920 and 2852 cm⁻¹) along with the analogous CD₂ stretching bands (~2194 and 2089 cm⁻¹) were analyzed, and band heights and peak wavenumber positions were assessed as a function of monolayer surface pressure. Small, near-physiological contents of 1–2 wt % SP-B/C typically produced the maximum observed spectroscopic effects, which were abolished at high protein contents of 10 wt %. Analysis of CH₂ and CD₂ stretching bands and C-H/C-D band height ratios indicated that SP-B/C affected PC and PG lipids differently within the surface monolayer. SP-B/C had preferential interactions with DPPG in 1:1, 2:1, and 7:1 DPPC-d₆₂:DPPG films that increased its acyl chain order. SP-B/C also interacted specifically with DOPG in 7:1 DPPC-d₆₂:DOPG monolayers, but in this case an increase in CH₂ band heights and peak wavenumber positions indicated a further disordering of the already fluid DOPG acyl chains. CD₂ band height and peak wavenumber analysis indicated that SP-B/C had no significant effect on the structure of DPPC-d₆₂ chains in 7:1 films with DPPG or DOPG, and had only a slight tendency to increase the acyl chain order in 1:1 films of DPPC-d₆₂:DPPG. SP-B/C had no significant effect on DPPC-d₆₂ structure in films with DOPG. Infrared results also indicated that interactions involving SP-B/C and lipids led to exclusion of PC and PG lipids from the compressed interfacial monolayer, in agreement with our previous report on the phase morphology of lipid monolayers containing 1 wt % SP-B/C.

INTRODUCTION

Pulmonary surfactant is a highly specialized substance found in all mammalian lungs that is known to promote lung expansion on inspiration and to prevent lung collapse upon expiration. Surfactant is necessary for normal breathing, and the lack of surfactant in the underdeveloped lungs of premature infants is the root cause of Respiratory Distress Syndrome (Avery and Mead, 1959). Disruption of surfactant activity is also implicated as contributing to the pathophysiology of clinical acute lung injury and Acute Respiratory Distress Syndrome (Pison et al., 1989; Lewis and Jobe, 1993; Notter and Wang, 1997).

Lung surfactant composition varies relatively little across a range of animal species, differing most significantly dur-

ing development or when lung disease or injury is present (Notter, 2000). Lavaged mammalian surfactant contains ~85% phospholipid, 7–10% protein, and 4–8% neutral lipid (Creuwels et al., 1997; Notter, 2000). The most abundant phospholipid class is phosphocholine, comprising ~80% of the phospholipid fraction. The single disaturated phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) accounts for about one-third or slightly more of total lung surfactant phospholipid (Hunt et al., 1991; Kahn et al., 1995; Holm et al., 1996). Anionic phospholipids including phosphatidylglycerols make up ~10–15% of lung surfactant phospholipids. Lung surfactant also contains four apoproteins, three of which are active in biophysical function: SP-A, SP-B, and SP-C. The largest of these, SP-A, is a relatively hydrophilic glycoprotein that also participates in surfactant biology and host defense (Crouch, 1998; Mason et al., 1998). SP-B and SP-C are small hydrophobic proteins known to have extensive interactions with phospholipids to enhance their adsorption and dynamic film behavior (Hawgood and Schiffer, 1991; Johansson et al., 1994b; Creuwels et al., 1997; Notter, 2000). It is the effect of these hydrophobic surfactant proteins on lipid structure in surface films that is probed in the current study.

From the time that lung surfactant extracts were first studied at the surface of a Langmuir-Wilhelmy film balance (Clements, 1957), researchers have used insoluble,

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Abbreviations used: A/W, air-water; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. DPPC-d₆₂, acyl chain perdeuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; LE, LC, liquid expanded, liquid condensed monolayer phase; PC, PG, phosphocholine, phosphoglycerol; SP-B/C, column isolated bovine hydrophobic surfactant proteins SP-B and -C; IRRAS, infrared external reflection-absorption spectroscopy.

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monomolecular films spread at the A/W interface as models for pulmonary surfactant (Notter, 1984; Notter, 2000). Surface balance techniques have been used to study the monolayer properties of the hydrophobic proteins SP-B and SP-C and their mixtures with lipids at the A/W interface (Oosterlaken-Dijksterhuis et al., 1991a; Taneva and Keough, 1994c; Taneva and Keough, 1994b; Taneva and Keough, 1994a; Wang et al., 1995; Wang et al., 1996). In addition, microscopic techniques such as electron (Tchoreloff et al., 1991), Brewster angle (Discher et al., 1996; Lipp et al., 1997b; Discher et al., 1999), fluorescence (Krüger et al., 1999; Ding et al., 2001; Lipp et al., 1996; Nag et al., 1996a; Nag et al., 1997; Takamoto et al., 2001), and near-field (Kramer et al., 2000), as well as scanning probe methods (Panaiotov et al., 1996; von Nahmen et al., 1997; Krol et al., 2000a) have been used to study surfactant preparations and model monomolecular films relevant for pulmonary surfactant. Whereas these microscopic techniques provide important biophysical information, they cannot give the same detailed molecular-level information about lipid-protein interactions that can be obtained using vibrational spectroscopic methods.

The use of external reflection Fourier transform infrared spectroscopy to study the structure of monomolecular films directly at the A/W interface was originally developed in the mid-1980s and progress in this field has recently been reviewed (Mendelsohn et al., 1995a; Dluhy, 2000). It has been shown that external reflectance infrared allows for the detection of the CH₂ stretching vibrations of the lipid acyl chains that can be used to monitor, in situ, the phase transitions that take place in monolayer films. In addition, the amide I vibration can be observed in pure or highly enriched lipid-protein films, and protein structural information can be obtained. This reflection IR technique has been applied to the study of monolayer films of extracted lung preparations (Dluhy et al., 1989) and more recently, to investigate the roles that SP-B and SP-C play in the function of lung surfactant (Pastrana-Rios et al., 1995; Gericke et al., 1997; Flach et al., 1999).

In the present investigation, ternary mixtures were constructed containing two phospholipids and column-isolated bovine hydrophobic surfactant proteins (SP-B/C); these mixtures were then studied in interfacial films using in situ external reflectance IR absorption spectroscopy. Phospholipids examined were DPPC plus either 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) or 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG). The acyl chains of the DPPC component were selectively deuterated, resulting in a shift in the vibrational frequencies to lower wavenumber values for DPPC. This enabled the effects of the surfactant protein on each of the phospholipid components to be monitored individually. SP-B/C were incorporated into phospholipid films at 0.5–10% by weight, a range extending above and below the physiologically relevant content of 1–2 wt % hydrophobic proteins in alveolar surfactant. Because

protein concentrations were too low to directly detect the protein amide bonds spectroscopically, the focus of study was on the influence of SP-B/C on the molecular behavior of phospholipids and on preferential lipid-protein associations in the surface film.

MATERIALS AND METHODS

Materials

The synthetic phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and 1,2-dipalmitoyl-d₆₂-*sn*-glycero-3-phosphocholine (DPPC-d₆₂) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used as received. ACS grade NaCl and HPLC grade methanol and chloroform were obtained from J. T. Baker. Subphase H₂O was obtained in-lab from a Barnstead (Dubuque, IA) ROPure/NANOpure reverse osmosis/deionization system having a nominal resistivity of 18.3 MΩ cm.

Isolation and purification of surfactant hydrophobic protein fraction

Mixed hydrophobic surfactant proteins, SP-B/C, were obtained from calf lung surfactant extract (CLSE). Whole lung surfactant was obtained by repeated saline lavage of lungs from freshly killed calves followed by centrifugation (250 × *g* for 10 min) to remove cells and debris. The resulting supernatant was centrifuged at a higher speed (12,500 × *g* for 30 min) to pellet large surface-active aggregates as described previously (Notter et al., 1983; Whitsett et al., 1986; Notter and Shapiro, 1987). The whole surfactant pellets were then resuspended, and the hydrophobic constituents extracted in CHCl₃:MeOH (Bligh and Dyer, 1959) to obtain CLSE. Separation of SP-B/C from lipids in CLSE was performed by gel permeation column chromatography through an LH-20 column (Pharmacia-LKB Biotechnology, Piscataway, NJ) using 2:1 CHCl₃:MeOH as the elution solvent (Hall et al., 1994). Two column passes were required to completely eliminate lipids, and the final pooled protein fraction contained no phospholipids as measured by phosphorus assay (Ames, 1966). Purity was assessed by SDS-PAGE and by N-terminal amino acid sequencing. The latter technique indicated that the mixed protein preparation contained only sequences specific for SP-B and SP-C. The total content of SP-B/C in CLSE was 1.5% based on the Folin phenol reagent assay (Lowry et al., 1951) modified by the addition of 15% SDS to allow for quantitative protein determination in the presence of lipid. ELISA testing based on an antibody to bovine SP-B (rabbit-derived) indicated an SP-B content of ~0.9 wt % in CLSE (Notter et al., 2002). Our previous results have shown that the column-purified SP-B/C mixture used here can be combined with purified calf lung surfactant lipids to give overall surface and physiological activities very similar to CLSE (Hall et al., 1994; Wang et al., 1995), indicating that the purified proteins represent the closest physiological analogy feasible for such material. The final mixed SP-B/C isolate was dissolved in 1:1 CHCl₃:MeOH at a concentration of 0.18 mg/ml and stored at low temperature until use.

Preparation of samples

Stock solutions of DPPC-d₆₂ (~1 mg/ml in CHCl₃), DPPG and DOPG (~1 mg/ml in 4:1 CHCl₃:MeOH) were prepared and concentrations verified by inorganic phosphorus assay (Chen et al., 1956). Solutions of the desired DPPC-d₆₂:DPPG and DPPC-d₆₂:DOPG mole ratios (1:1, 2:1, or 7:1) containing between 0 and 10 wt % SP-B/C were prepared by mixing the appropriate amounts of the above phospholipid stock solutions and the 0.18 mg/ml stock solution of SP-B/C in 1:1 CHCl₃:MeOH. The subphase used for all experiments was 150 mM NaCl in deionized H₂O (pH 5.6).

FTIR external reflectance measurements

Infrared external reflection-absorbance spectra of monolayers at the A/W interface were collected using a PerkinElmer Spectrum 2000 FTIR spectrometer equipped with an external sample beam. A 60°, gold-coated, off-axis parabolic mirror (Janos Technology, Townshend, VT) reflected the beam coming from the spectrometer onto the surface of a Nima 601M film balance (Coventry, England) at an incidence angle of 30° to the surface normal. The beam reflects off the subphase, sampling the film, and a second parabolic mirror collects the beam and directs it into a collection mirror and then onto the sensing chip of a liquid N₂-cooled HgCdTe detector. The film balance, optical components, and detector are housed in a sealed, Plexiglas chamber that allows humidity control of the local trough environment, thus improving water vapor background subtraction.

The subphase was first cleaned by aspiration and a single beam spectrum was collected for use as the IR background spectrum. The subphase temperature was held constant at $22 \pm 1^\circ\text{C}$ by flowing thermostatted water through the hollow body of the trough. The temperature in the enclosed chamber was typically 24°C and the relative humidity remained fairly constant at 70%. Typically 5–10 μl of sample was spread via syringe onto the trough surface resulting in an initial surface pressure of 3–6 mN/m. The film was allowed to equilibrate for a period of 30 min and then was compressed intermittently and spectra collected over a range of surface pressures from ~ 5 mN/m to a maximum of 45–65 mN/m depending on the nature of the film.

Parameters for IR spectra collection included 1024 coadded scans at 4 cm^{-1} resolution. Interferograms were apodized using a Norton-Beer (strong) function, and Fourier transformed with one level of zero filling. Monolayer IR spectra are presented as IRRAS spectra, i.e., $-\log(R/R_0)$, where R is IR reflectivity of the monolayer surface and R_0 is the IR reflectivity of the bare water subphase background. The reflectance IR spectra were baseline corrected before determination of peak positions and band intensities using the GRAMS/32 (Thermo Galactic, Salem, NH) software package. Otherwise, the spectra have not been smoothed or further processed. Vibrational wavenumber peak positions and band heights were calculated using a five-point center-of-gravity algorithm (Cameron et al., 1982), written in our laboratory for the Grams/32 environment.

RESULTS

IRRAS spectra of lipid-protein monolayer films

Infrared external reflectance-absorption spectra obtained at the A/W interface for a monolayer film of 7:1 DPPC-d₆₂:DPPG plus 5 wt % SP-B/C are shown in Fig. 1. IR spectra were acquired while the monolayer was held at specific surface pressure values from 6.0 mN/m to 60.0 mN/m during stepwise compression. The overlaid data in Fig. 1 are monolayer IRRAS absorbance spectra that show the C-H stretching region (Fig. 1 A, 3000–2800 cm^{-1}) and analogous C-D stretching region (Fig. 1 B, 2250–2050 cm^{-1}) at various surface pressures. Clearly evident in the Fig. 1 A spectra of the C-H stretching region are the CH₂ antisymmetric stretching band at 2920 cm^{-1} , the CH₂ symmetric stretching bands at 2850 cm^{-1} , and the CH₃ antisymmetric stretching bands at 2960 cm^{-1} , which is seen as a shoulder on the antisymmetric CH₂ band (Snyder et al., 1982). Similarly, in Fig. 1 B, the spectra of the C-D stretching region show the CD₂ antisymmetric stretching band at 2194 cm^{-1} , the CD₂ symmetric stretching bands at 2090 cm^{-1} , and the CD₃ antisymmetric stretching bands at 2218 cm^{-1} (Bunow and Levin, 1977).

As seen in Fig. 1, both C-H and C-D vibrational bands grow in intensity as the surface pressure increases and the surface density of the lipid molecules increases. As the average surface area per molecule is reduced, hydrophobic interactions predominate and the lipids begin to adopt a more ordered configuration. Thus, inhomogeneous line broadening is reduced, the peaks become sharper, and the peak maxima are shifted to lower frequency values (see, e.g., Mendelsohn et al., 1995b or Dluhy, 2000). The measured peak wavenumber position therefore reflects the intramolecular conformation of the phospholipid acyl chains, whereby a higher wavenumber value is indicative of a disordered chain, whereas a lower wavenumber value reflects an ordered, *trans* segment of the hydrocarbon tails (Snyder et al., 1978). This shift in peak position is seen most dramatically for the CH₂ antisymmetric stretching band contributed by the DPPG lipid component of the DPPG:DPPC-d₆₂ binary mixture in Fig. 1 A.

Deuteration of the DPPC acyl chains produces a shift in the vibrational bands to lower wavenumber values, thereby allowing the two phospholipid species to be individually identified in monolayer IRRAS spectra (Dluhy et al., 1985; Cameron and Dluhy, 1986). As seen in Fig. 1, substitution of deuterium for hydrogen in the lipid acyl chains results in a wavenumber shift of $\sqrt{2}$ for the perdeuterated C-D bands, as predicted by the harmonic oscillator model. Therefore, the new C-D vibrations are located in the spectral range 2000–2300 cm^{-1} , a spectral region free from other vibrational modes and easily observed spectroscopically using IRRAS.

The intensity and wavenumber values of the peaks provide information not only concerning chain conformations, but also about the number density of the individual phospholipid components present at the film surface (Rana et al., 1993b). Isotopic substitution not only causes wavenumber shifts in IR bands, but also influences their integrated intensities (Pinchas and Laulicht, 1971). We have previously empirically determined that the ratio of integrated intensities of the C-H to C-D vibrations for a 1:1 mol:mol DPPC-d₆₂:DPPG monolayer film was 0.95 (Rana et al., 1993a). Assuming a linear relationship between mole fraction and integrated intensity, one would therefore expect the band height ratio of C-H to C-D bands in a 7:1 mol:mol DPPC-d₆₂:DPPG film to be ~ 0.24 . Instead, calculations based on the spectra in Fig. 1 indicate that the apparent band height ratio for this film (a 7:1 DPPC-d₆₂:DPPG monolayer with 5 wt % SP-B/C added) is ~ 0.6 . The difference seen in these band height ratios reflects a preferential interaction of SP-B/C with DPPG in the binary lipid film, as explained further below.

Effect of SP-B/C proteins on DPPC-d₆₂:DPPG monolayers

In Fig. 2, the CH₂ antisymmetric stretching vibration wavenumber and band heights are plotted as a function of measured surface pressure for monomolecular films of 7:1

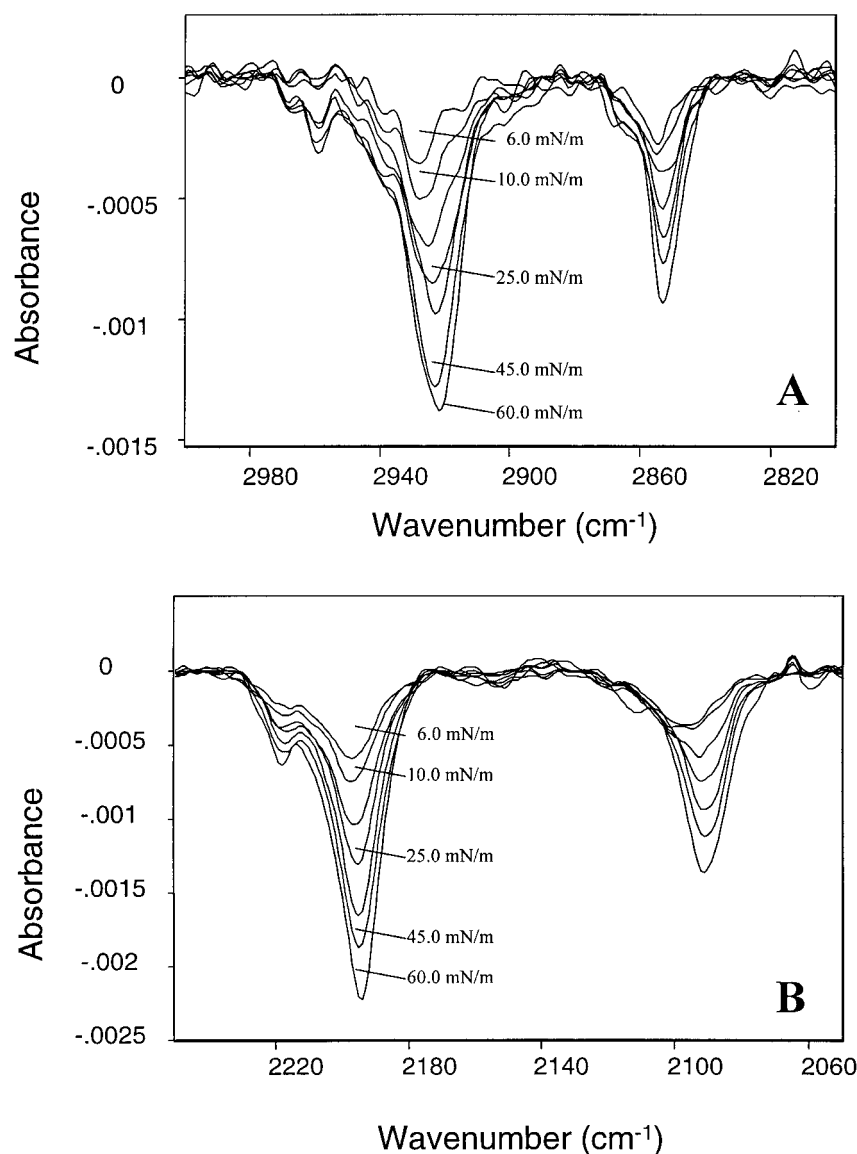


FIGURE 1 IR external reflection-absorption spectra of a 7:1 DPPC- d_{62} :DPPG + 5 wt % SP-B/C monolayer film collected at surface pressures from 6.0 to 60.0 mN/m. (A) CH₂ stretching region between 3000–2800 cm⁻¹. (B) CD₂ stretching region between 2250–2050 cm⁻¹.

DPPC- d_{62} :DPPG with and without added SP-B/C. These plots provide information about the structure of the DPPG component in the film. The data in Fig. 2 were obtained from the CH₂ antisymmetric band at ~ 2920 cm⁻¹, which was used in analysis because of its larger intensity and higher signal-to-noise ratio compared to the corresponding symmetric bands. This allowed molecular behavior to be probed in the film at low surface pressures where average molecular areas are high and peak intensities are inherently weak. It also proved crucial for the analysis of films containing a low percentage of protiated lipid (i.e., the monolayer contains only $\sim 12\%$ PG in 7:1 DPPC- d_{62} :DPPG and DPPC- d_{62} :DOPG films) and consequently exhibited C-H bands that were extremely weak. Due to the very low band intensities on which these analyses are based, there is a fair amount of scatter in some of the data sets. Lines are thus

drawn through the individual points in Fig. 2 and subsequent figures to help point out trends. These trend lines are for illustrative purposes only, and are not intended to be a rigorous mathematical model of the relationship among the given variables. In addition, representative error bars illustrating the level of uncertainty for a specific point within a data set are included (error bars for each point are not included out of concern for legibility). The center-of-gravity algorithm used here to process IRRAS spectra defined wavenumber peak positions to better than ± 0.01 cm⁻¹ precision (Cameron et al., 1982). The observed scatter is thus due solely to inherent data fluctuations in the monolayer sample. For the IRRAS data here, typical levels of uncertainty were ~ 1.0 cm⁻¹ in peak positions and 10% in peak heights for strong bands in the IR spectra of repetitive data sets.

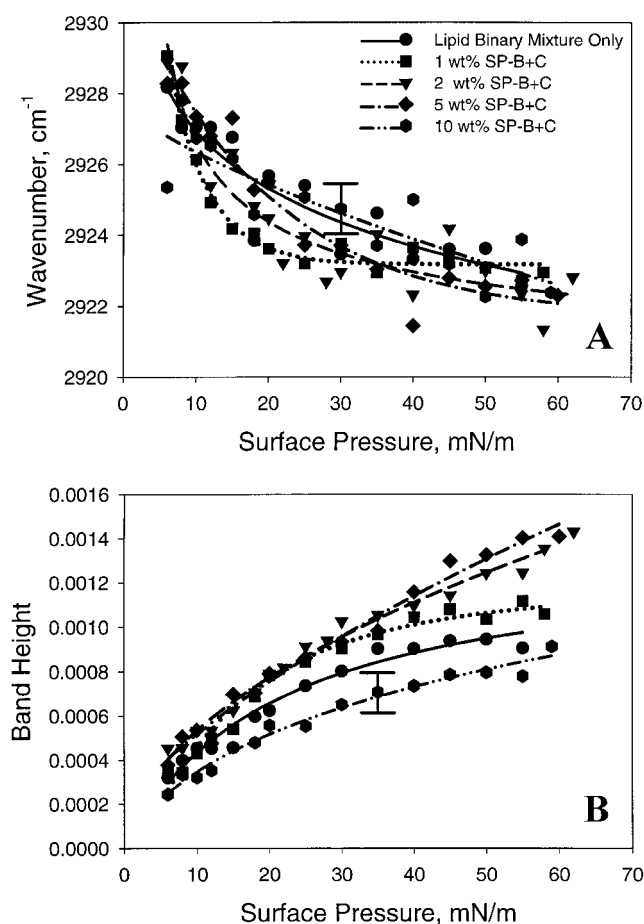


FIGURE 2 IR external reflection-absorption C-H spectral band parameters for 7:1 DPPC- d_{62} :DPPG binary monolayer films with and without SP-B/C added. C-H band parameters reflect changes in structure for the DPPG component in the monolayer. Variations in wavenumber (*A*) and band height (*B*) for the CH_2 antisymmetric stretching band are shown plotted as a function of surface pressure. See key in *A* for symbols.

For the model mixtures shown in Fig. 2 *A*, the wavenumber values observed for the CH_2 antisymmetric stretch decreased by as much as 6.1 cm^{-1} as surface pressure increased, reflecting a more condensed, ordered state for the DPPG component in the monolayer. A close study of the data reveals that there are differences in the extent of this ordering that depend upon the amount of SP-B/C incorporated into the film, with the largest effect seen upon addition of the smallest amount of protein. The greater shift in wavenumber values between 10 mN/m and 30 mN/m in films of 7:1 DPPC- d_{62} :DPPG plus 1 wt % SP-B/C indicates the greatest extent of acyl chain ordering. Wavenumber values also decreased relative to the lipids alone as the amount of protein was increased to 2 and then 5 wt %, but not as much as for the 1 wt % mixture. Further increase in the amount of surfactant protein to 10 wt % resulted in wavenumber shifts for the DPPG component that were indistinguishable from the pure binary lipid mixture itself. A

consideration of the uncertainties associated with these data indicate that only the monolayers containing 1 and 2 wt % of SP-B/C protein in Fig. 2 produced wavenumber shifts statistically different from the lipid-only monolayer.

Measurement of the height of the CH_2 asymmetric vibration in these lipid-protein monolayers also reflects the influence of the SP-B/C proteins on lipid structure (Fig. 2 *B*). All of the model mixtures in Fig. 2 *B* show an increase in band height with an increase in the measured surface pressure; this increased band height results from both an increase in surface density of molecules sampled by the infrared beam as well as increased ordering and reorientation of the DPPG acyl chains. Fig. 2 *B* shows a significant difference in the band height versus surface pressure curves for the different model mixtures. At any given surface pressure, an incremental increase in band height occurred for SP-B/C contents of 1 wt %, 2 wt %, and 5 wt % in the mixed monolayer. In contrast, addition of 10 wt % SP-B/C to the monolayer resulted in band height values that were lower than those seen for the pure lipid film alone. Hence, small amounts of surfactant protein appear to interact with the DPPG in the monolayer, causing an increase in acyl chain ordering/reorientation, but higher amounts of SP-B/C (10 wt %) actually caused a reduction in the perturbation to both wavenumber shift and band height.

Fig. 3, *A* and *B*, are the same type of plots shown in Fig. 2, *A* and *B*, the difference being that the data are obtained from the CD_2 antisymmetric stretching band of the DPPC- d_{62} component for these same 7:1 DPPC- d_{62} :DPPG films. The CD_2 vibrational modes in Fig. 3 exhibited similar behavior to the CH_2 modes in Fig. 2: the wavenumber peak positions decreased and band heights grew larger as surface pressure increased. The overall magnitude of change in the band parameters of the perdeuterated DPPC was smaller than that of its protiated counterpart (4.4 cm^{-1} vs. 6.1 cm^{-1}). The smaller wavenumber shift for perdeuterated acyl chains relative to protiated chains is well known and is related to the thermodynamic properties of deuterated phospholipids (Dluhy et al., 1985). Most important in Fig. 3, however, was that unlike the CH_2 bands from the DPPG in this model mixture, the addition of differing amounts of SP-B/C had relatively little influence on the wavenumber or band height of the antisymmetric CD_2 stretching bands compared to the pure lipid mixture. The surface pressure-induced wavenumber shifts for all lipid-protein monolayers essentially overlapped (Fig. 3 *A*). Similarly, the measured band heights of the CD_2 antisymmetric vibration were only minimally affected by addition of SP-B/C at all of the protein concentrations studied (Fig. 3 *B*). These results indicate that no statistically significant preferential interaction involving wavenumber shifts or band height changes occurred between the SP-B/C proteins and the DPPC- d_{62} component of the 7:1 DPPC- d_{62} :DPPG binary mixture.

The differential effect of the SP-B/C surfactant proteins on each of the two lipid components in this 7:1 DPPC-

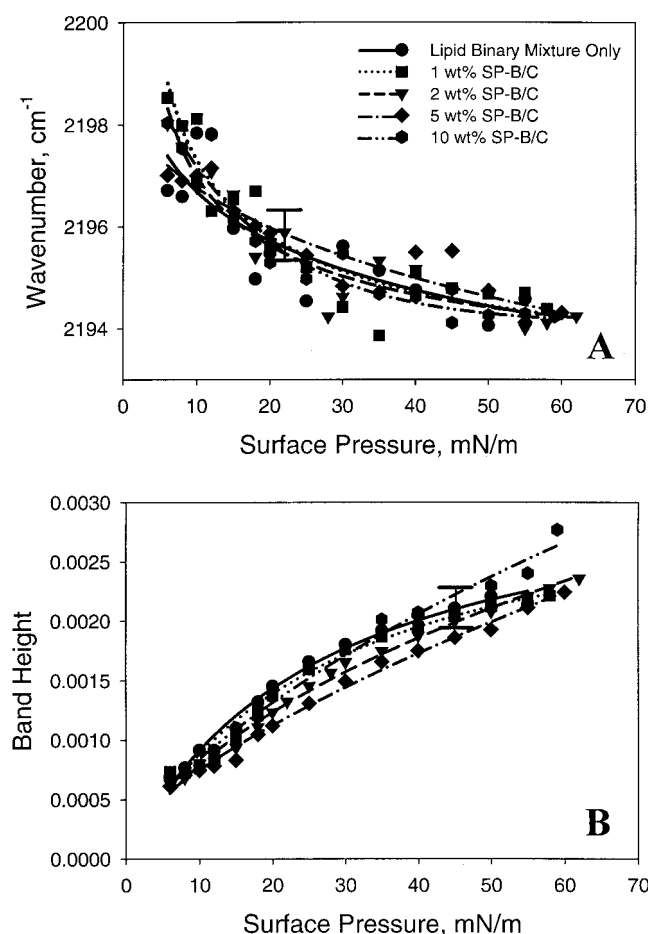


FIGURE 3 IR external reflection-absorption C-D spectral band parameters for a 7:1 DPPC-d₆₂:DPPG binary monolayer films with and without SP-B/C added. C-D band parameters reflect changes in structure for the DPPC-d₆₂ component in the monolayer. Variations in wavenumber (A) and band height (B) for the CD₂ antisymmetric stretching band are shown plotted as a function of surface pressure. See key in A for symbols.

d₆₂:DPPG model mixture can be seen quite clearly in Fig. 4. Here the ratios of the CH₂ versus CD₂ band heights for the antisymmetric CH₂ and CD₂ stretching bands are plotted as a function of surface pressure. The use of band height ratios to indicate specific interactions in lipid-protein systems has been prevalent in biophysical vibrational spectroscopy, particularly in Raman spectroscopy (Levin, 1984). Band height ratios correlate directly with interchain configuration, as perturbed by protein interactions. With increasing surface pressure, the band height ratio in Fig. 4 remained essentially constant within the 7:1 DPPC-d₆₂:DPPG film, consistent with a proportional increase in the vibrational intensities of the CH₂ and CD₂ bands with compression. However, the band height ratio at any given surface pressure changed as the content of SP-B/C was increased in the lipid-protein monolayers. Films containing 1, 2, and 5 wt % surfactant protein had a significant increase in the C-H/C-D band height ratio at fixed surface pressure, indicating a specific protein

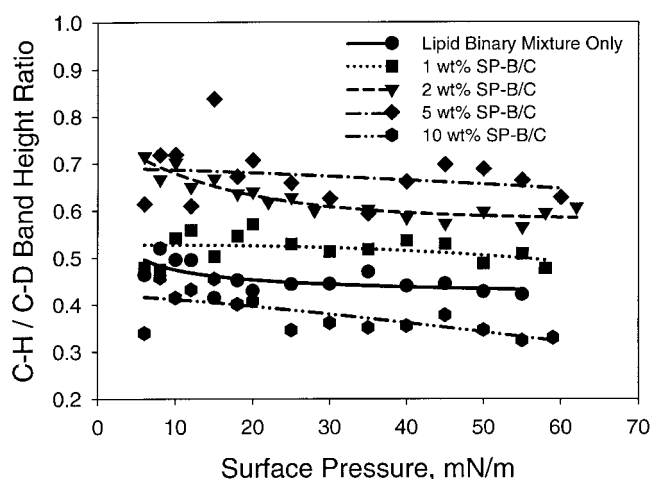


FIGURE 4 IR external reflection-absorption spectral band parameters for a 7:1 DPPC-d₆₂:DPPG binary monolayer films with and without SP-B/C added. Variation in band height ratio for the CH₂/CD₂ antisymmetric stretching bands is shown plotted as a function of surface pressure. Changes in C-H/C-D band height ratio indicate relative changes in the DPPG versus DPPC-d₆₂ components. See key in A for symbols.

interaction with the C-H monolayer component (i.e., the DPPG). These results are consistent with the SP-B/C proteins ordering the DPPG component but not significantly affecting the DPPC-d₆₂ component in 7:1 DPPC-d₆₂:DPPG films, as referred to earlier in Figs. 2 and 3. The fact that 5 wt % protein had a substantial effect on band heights is consistent with the unexpectedly high C-H/C-D intensity ratio noted earlier for the spectra in Fig. 1.

The band height ratio changes in Fig. 4 indicate a significant preferential effect of the (1–5 wt %) SP-B/C proteins with DPPG versus DPPC in the monolayer. However, when the protein content in the film reached 10 wt %, C-H/C-D ratios actually dropped to values below those of the pure lipid mixture alone. Similar behavior to that in Fig. 4 for CH₂/CD₂ band height ratios was also found for the symmetric C-H versus C-D stretching bands (data not shown). It is also worth noting that there is a very small tendency toward a negative slope in the trend lines of band height ratio versus surface pressure when SP-B/C proteins are present in the lipid monolayers. This trend is related to the loss of monolayer material upon compression, and is discussed in more detail later.

In addition to 7:1 mol:mol DPPC-d₆₂:DPPG monolayer films, the influence of SP-B/C proteins on lipid structure was also determined for monolayer films of 1:1 and 2:1 mol:mol DPPC-d₆₂:DPPG. Fig. 5, A and B, show plots of the wavenumber shifts as a function of surface pressure for the C-H and C-D components in 1:1 DPPC-d₆₂:DPPG films. As with the 7:1 films in earlier figures, a marked decrease in the CH₂ antisymmetric peak frequency values was found in the presence of 1 and 2 wt % SP-B/C. At low surface pressures for the DPPG component of the 1:1 monolayer (Fig. 5 A), the

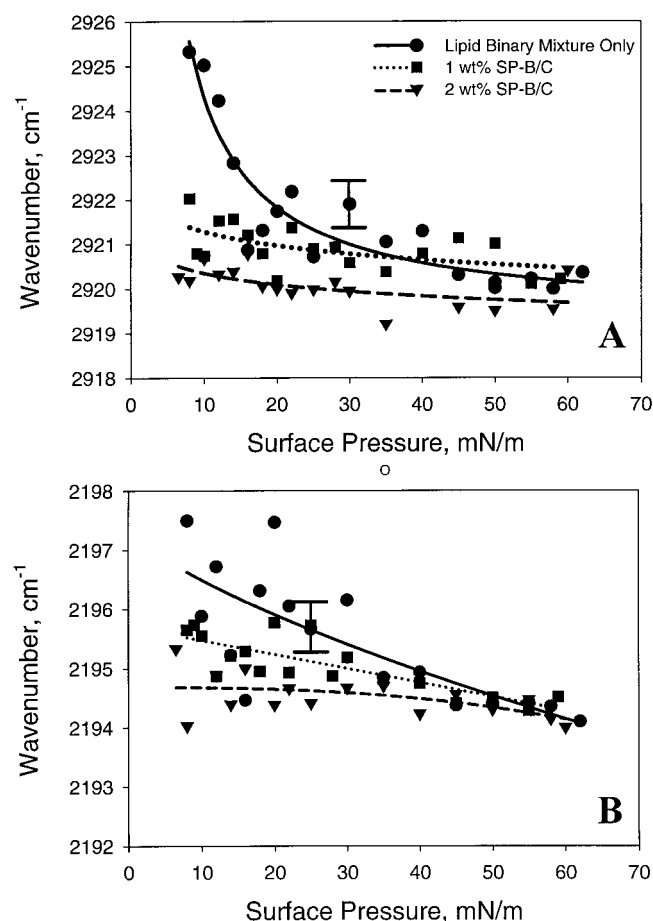


FIGURE 5 IR external reflection-absorption C-H and C-D spectral band parameters for 1:1 DPPC-d₆₂:DPPG binary monolayer films with and without SP-B/C added. C-H band parameters reflect changes in structure for the DPPG component in the monolayer whereas C-D band parameters reflect changes in structure for the DPPC-d₆₂ component. Variation in wavenumber for the CH₂ (A) and CD₂ (B) antisymmetric stretching bands is shown plotted as a function of surface pressure. See key in A for symbols.

drop in wavenumber of ~ 3 cm⁻¹ upon addition of 1 wt % protein and ~ 5 cm⁻¹ upon addition of 2 wt % protein is consistent with a statistically significant increase in the ordering of the DPPG acyl chains. For the DPPC-d₆₂ component of the 1:1 monolayer, the shift in the CD₂ antisymmetric band peak position to lower values (Fig. 5 B) indicates that the addition of SP-B/C caused a small but statistically significant increase in ordering of the DPPC-d₆₂ chains, whereas these proteins had no significant effect on the deuterated bands in the 7:1 monolayer (Fig. 3 A).

Band height ratios for the C-H versus C-D antisymmetric bands were calculated for the 1:1 DPPC-d₆₂:DPPG model mixtures in the presence and absence of surfactant protein; these ratios are presented in Fig. 6 A. Trends in the C-H versus C-D band height ratios for the 1:1 films were similar, but not identical, to those found for 7:1 DPPC-d₆₂:DPPG films. When 1 wt % protein was present in the 1:1 DPPC-

d₆₂:DPPG monolayer, there was a significant increase in the C-H/C-D ratio over the pressure range 10–25 mN/m (Fig. 6 A). However, when the protein content in the film was raised to 2 wt %, this effect was not present.

Band height ratios are plotted in Fig. 6 B as a function of surface pressure for 2:1 mol:mol DPPC-d₆₂:DPPG monolayers with 0, 1, and 2 wt % added SP-B/C. The results for the 2:1 DPPC-d₆₂:DPPG films are very similar to those of the 7:1 monolayers. At SP-B/C contents of both 1 and 2 wt %, the surfactant proteins interacted preferentially with the DPPG component causing the band height ratio to increase. Analysis of the antisymmetric CH₂ band parameters indicated a slight ordering of the DPPG acyl chains with increasing surface pressure in the 2:1 system based on an increase in band height and a decrease in peak frequency values in the presence of protein (data not shown). How-

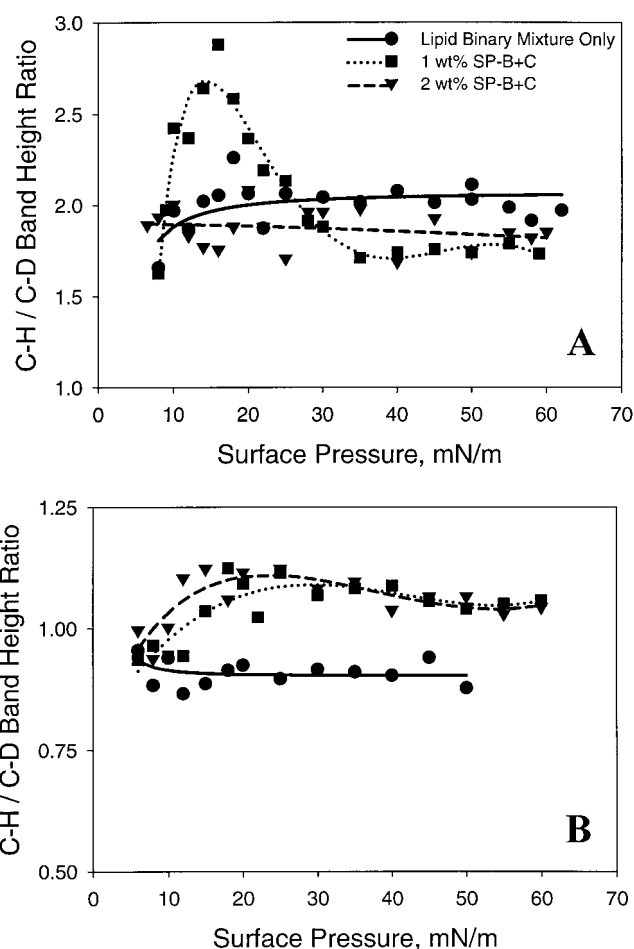


FIGURE 6 IR external reflection-absorption spectral band parameters for (A) 1:1 DPPC-d₆₂:DPPG and (B) 2:1 DPPC-d₆₂:DPPG binary monolayer films with and without SP-B/C added. Variation in band height ratio for the CH₂/CD₂ antisymmetric stretching bands is shown plotted as a function of surface pressure. Changes in C-H/C-D band height ratio indicate relative changes in the DPPG versus DPPC-d₆₂ components. See key in A for symbols.

ever, there did not appear to be any effect of SP-B/C on the DPPC-d₆₂ component, as no significant difference in the antisymmetric CD₂ band parameters (wavenumber or band height) was apparent in the presence of protein in the 2:1 DPPC-d₆₂:DPPG monolayer system.

In summary, the major differences in the data for the three different mole ratios of DPPC-d₆₂:DPPG appeared to be the interaction of the protein with the phosphoglycerol constituent. In all of the mixtures, SP-B/C acted to order the DPPG component in the film. In terms of the PC component, the effects of SP-B/C depended on film composition. When the content of DPPC-d₆₂ in the film was high (~88 mol % in the 7:1 mixtures and 66 mol % in the 2:1 mixture), SP-B/C had little effect on the PC component. When the proportion of DPPC-d₆₂ was lowered to 50 mol %, (in the 1:1 mixture), SP-B/C appeared to have only a slight ordering effect on this film component.

Effect of SP-B/C proteins on DPPC-d₆₂:DOPG monolayer films

A final set of studies examined films containing the unsaturated phosphoglycerol DOPG in place of the DPPG. Spectra were collected at various surface pressures for 7:1 DPPC-d₆₂:DOPG mixtures containing 0, 0.5, 1, and 2 wt % SP-B/C, and trends in the resulting CH₂ antisymmetric stretching band parameters are shown plotted in Fig. 7, *A* and *B*. The wavenumber values obtained at fixed surface pressures for 7:1 DPPC-d₆₂:DOPG films were on average much higher than those found for the corresponding films with DPPG. This is due to the inherent thermodynamic properties of the DOPG molecule. The acyl chains of DOPG pack together less efficiently because of the 9-*cis* oleoyl double bond, causing a shift in peak frequencies to higher values. However, the overall extent of ordering of the acyl chains does increase as surface pressure is increased just as in the case of films containing DPPG. Addition of SP-B/C is seen to affect the packing of the DOPG component, but in a different way than in the DPPG films. The presence of SP-B/C at 1 and 2 wt % in 7:1 DPPC-d₆₂:DOPG films increased CH₂ band peak wavenumber values (Fig. 7 *A*), and also led to small increases in band height (Fig. 7 *B*). When compared to the lipid binary monolayer alone, the wavenumber shifts for the 1 and 2 wt % monolayers showed a significant difference at all surface pressures. Low amounts of SP-B/C (0.5 wt %) increased peak wavenumber values only at surface pressures <20 mN/m, but gave the largest increase in band heights. These results suggest that SP-B/C specifically interacted with DOPG, thereby increasing the effective surface density of CH₂ groups in the IR sample beam. At the same time, this lipid-protein interaction caused further disorder in the acyl chain.

The effects of SP-B/C on the deuterated PC component in 7:1 DPPC-d₆₂:DOPG films are shown in Fig. 8, *A* and *B*. SP-

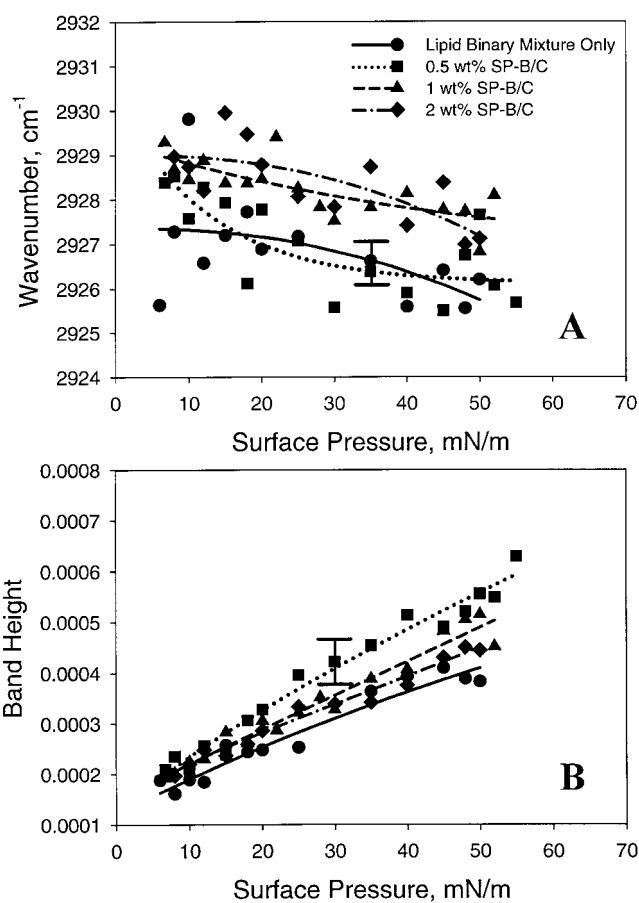


FIGURE 7 IR external reflection-absorption C-H spectral band parameters for a 7:1 DPPC-d₆₂:DOPG binary monolayer films with and without SP-B/C added. C-H band parameters reflect changes in structure for the DOPG component in the monolayer. Variations in wavenumber (*A*) and band height (*B*) for the CH₂ antisymmetric stretching band are shown plotted as a function of surface pressure. See key in *A* for symbols.

B/C appeared to have relatively little influence on the chain conformation of the DPPC-d₆₂ component, inasmuch as there was no statistically significant difference in the values of the CD₂ antisymmetric peak frequencies between the lipid-only and surfactant added films (Fig. 8 *A*). As was the case with the DOPG component, a large increase in measured band heights was found when SP-B/C is added. This may again be explained as occurring as a result of a general condensing effect of the surfactant proteins on the phospholipids in the films, although no specific structural change to the DPPC-d₆₂ component in 7:1 DPPC-d₆₂:DOPG films was noted in the presence of SP-B/C.

The data for 7:1 DPPC-d₆₂:DOPG monolayers indicate a specific interaction of SP-B/C with the phosphoglycerol component as found earlier with films containing DPPG. Fig. 9 shows substantial increases in the C-H/C-D band height ratios with the addition of 0.5–2 wt % SP-B/C in the 7:1 DPPC-d₆₂:DOPG film. The increases in C-H/C-D band height ratios are not as large as found in DPPG-containing

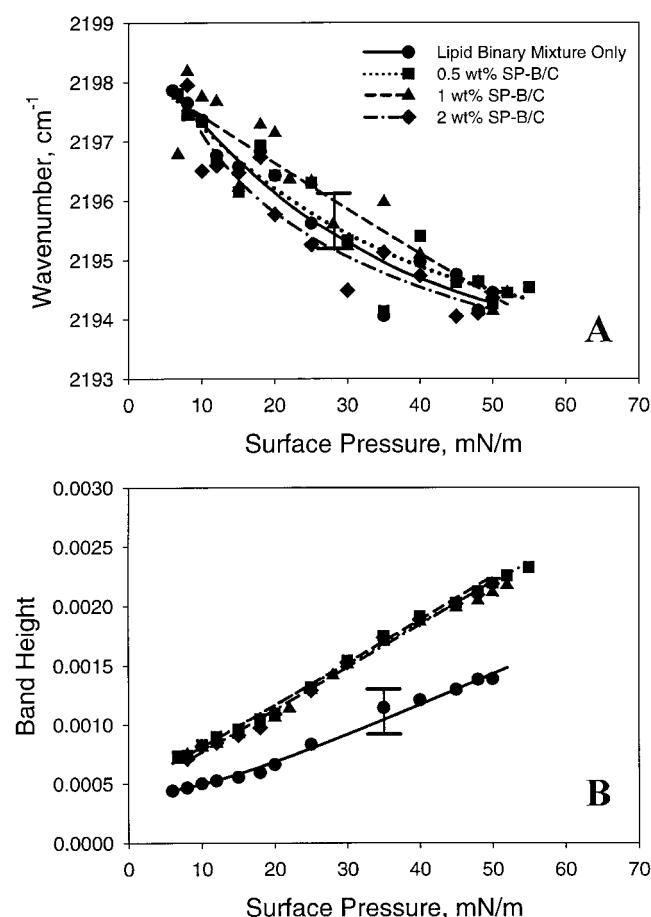


FIGURE 8 IR external reflection-absorption C-D spectral band parameters for a 7:1 DPPC- d_{62} :DOPG binary monolayer films with and without SP-B/C added. C-D band parameters reflect changes in structure for the DPPC- d_{62} component in the monolayer. Variations in wavenumber (A) and band height (B) for the CD_2 antisymmetric stretching band are shown plotted as a function of surface pressure. See key in A for symbols.

films in Fig. 4 (note the expanded scale in Fig. 9), but they represent a significant change compared to the lipid-only mixture. Again, negative slopes were found in the trend lines in all plots of band height ratio versus surface pressure in Fig. 9, although their magnitudes were lower than in Fig. 4 for films containing DPPG. As discussed in the next section, this negative slope likely results from the loss of DOPG from the film upon compression, as unsaturated lipid components have been inferred by *in situ* IR analysis to be squeezed out of films of DPPC- d_{62} (Pastrana-Rios et al., 1994).

DISCUSSION

Using *in situ* IR spectroscopy, we have investigated the influence of differing levels of the hydrophobic lung surfactant proteins SP-B/C on phospholipid structure in surface monolayers containing deuterated DPPC plus either DPPG or DOPG. The results indicate a specific interaction between the SP-B/C proteins and negatively charged anionic

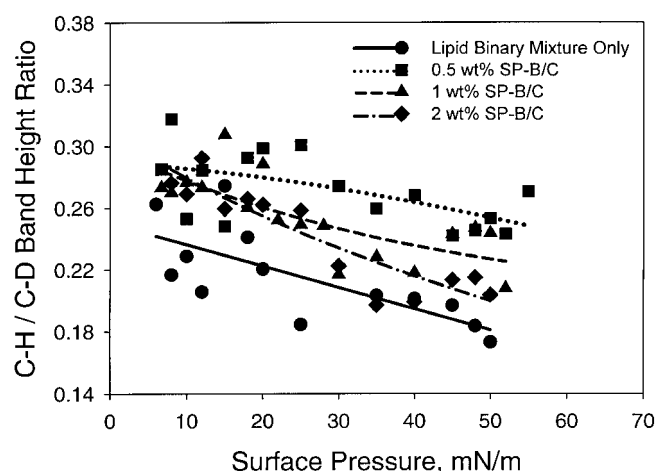


FIGURE 9 IR external reflection-absorption spectra band parameters for a 7:1 DPPC- d_{62} :DOPG binary monolayer films with and without SP-B/C added. Variation in band height ratio for the CH_2/CD_2 antisymmetric stretching bands is shown plotted as a function of surface pressure. Changes in C-H/C-D band height ratio indicate relative changes in the DPPG versus DPPC- d_{62} components. See key in A for symbols.

PG headgroups in the binary PC/PG mixture, leading to the exclusion of a lipid-protein complex from the monolayer. The addition of SP-B/C caused a disproportionate increase in the C-H band height, consistent with increased acyl chain order in DPPG. SP-B/C also interacted specifically with DOPG to increase its surface density and to further disorder acyl chains. SP-B/C had no significant effect on the structure of DPPC- d_{62} chains in 7:1 films with DPPG or DOPG, and had only a slight tendency to increase the acyl chain order in 1:1 films of DPPC- d_{62} :DPPG. Also, an important result of this work is that the effects of the SP-B/C surfactant proteins are highly concentration dependent, with the maximum effect occurring at 1–2 wt %, the physiologically relevant concentration. Indeed, higher amounts of protein were seen to abolish the measured spectroscopic changes, and to induce no discernable structural differences when compared to the lipid mixture alone. These IR spectroscopic results provide a structural complement to our previous optical microscopy images concerning the influence of the SP-B/C proteins on the phase morphology of PC/PG monolayers (Krüger et al., 1999).

Comments on the use of physiologically relevant amounts of protein

The current study examined the effects of a chromatographically purified mixture of SP-B/C that presumptively maintained the endogenous ratio of the two hydrophobic proteins. A number of prior studies have addressed the biophysical roles played by SP-B and SP-C in the function of pulmonary surfactant. Most of these studies, however, have not utilized the naturally occurring combination of both proteins. Moreover, it has not been uncommon in previous

work to study protein contents much higher than those encountered *in vivo*. The current work examined a range of protein contents, but with a focus on physiologically relevant levels (1–2 wt %) in investigating interactions with lipids in the surface monolayer. Using an endogenous mixture of SP-B/C at physiologically relevant contents in lipid mixtures has the advantage that it more closely mimics true lung surfactant composition, but at the cost of being unable to differentiate the effects of SP-B from those of SP-C. In addition, the use of low physiological levels of SP-B/C also makes it difficult or impossible to directly study protein vibrational bands.

Some studies have examined the effects of physiologically relevant levels of surfactant proteins in lipid mixtures. Dluhy et al. (1989), used IR spectroscopy to study molecular behavior in films of bovine lung surfactant containing endogenous ratios of protein and lipid components. In addition, several recent investigations have used near-field and scanning probe techniques to visualize transferred monolayer films containing SP-B and SP-C at levels in the neighborhood of 2 wt % (Kramer et al., 2000; Krol et al., 2000b). However, other IR studies of SP-B and SP-C in lipid monolayers at the A/W interface or in bulk phase vesicles have incorporated these proteins at between 3 and 80 wt % (Pastrana-Rios et al., 1995; Gericke et al., 1997; Gordon et al., 2000). Similarly, optical microscopy studies of monolayers containing SP-C or synthetic peptides based on the truncated N-terminus of SP-B (i.e., SP-B₁₋₂₅) have used protein amounts that span a wide range between 2 and 28 wt % (Lipp et al., 1996; Nag et al., 1996a; Lipp et al., 1997a; Nag et al., 1997; Ding et al., 2001; Takamoto et al., 2001). One study (Perez-Gil et al., 1992) found that SP-C affected lipid monolayer domains in two different ways depending upon the concentration, and stated that a precise determination of the amounts of SP-B and SP-C present in surfactant may be critical. Other authors (Pastrana-Rios et al., 1995) have also commented on the use of above-normal amounts of protein in model systems. The influence of protein concentration on experimental results was particularly apparent in this study where trends in data were often reversed upon addition of higher amounts of SP-B/C (e.g., 10 wt %).

The use of high, nonphysiological levels of exogenous protein in surfactant biophysical studies is sometimes necessary due to the limitations of methodology or to specifically study protein monolayers. Nevertheless, sufficiently high protein concentrations inevitably lead to aggregation and lipid-protein interactions driven by non-physiological energetics. Our prior optical microscopy data indicate a distinct difference in monolayer morphology upon addition of higher amounts of protein (Krüger et al., 1999). From the data presented here on the anomalous effects of 10 wt % SP-B/C in films, it appears that a nonphysiological response occurs at smaller protein concentrations than previously reported. This fact must be considered when trying to draw conclusions concerning physiological re-

sponses from model systems containing artificially high protein levels.

Comments on data error and significance

One factor in interpreting our results on interactions between SP-B/C and phospholipids concerns the degree of variability or scatter present in the IR data. This scatter is due to the very small IR intensities inherent in the monolayer IRRAS experiment ($\sim 10^{-3}$ to 10^{-4} AU) as well as the very small amounts of protein used in these experiments, which caused concomitantly small shifts in the measured IR band parameters. Despite this expected data variability, the IR band parameters showed clear and consistent patterns indicating the relative effects of different amounts of SP-B/C protein on lipid molecular packing and interfacial order. In addition, specific interactions of these proteins with DPPG or DOPG relative to DPPC in the binary films are easily discernable.

Several methods of data analysis were employed to enhance interpretations. Representative error bars were utilized on standard wavenumber versus surface pressure or band intensity versus surface pressure plots to illustrate the typical uncertainty for a particular data set. Because wavenumber peak positions were calculated to better than $\pm 0.01 \text{ cm}^{-1}$ precision (see Methods), the scatter in these data represented inherent fluctuations in the monolayer. Our results indicated an uncertainty of $\sim 1.0 \text{ cm}^{-1}$ in peak positions and 10% in peak heights for the measured monolayer IRRAS spectra. Interpretations on lipid-protein interactions here were based on data that were statistically significant relative to monolayers of lipids alone, as noted in the Results section. A further refinement used band height ratio analyses in addition to absolute shifts in band parameters to enhance insights about interactions between specific film components and surfactant proteins. Band height ratios have been widely used in biophysical vibrational spectroscopy to indicate specific interactions in lipid-protein systems. Band height ratios correlate directly with interchain configuration, as perturbed by protein interactions (Levin, 1984). Therefore, C-H/C-D ratios reflect acyl chain conformation and configuration, similar to wavenumber shifts. Band height ratios were necessary in the present study because of the very small protein concentrations used and the very small shifts in the wavenumber and intensity parameters observed. Analysis based on band heights ratios in Figs. 4, 6, and 9 provided important added evidence for specific lipid-protein interactions to enhance interpretations based on wavenumber shifts and intensity differences observed in the other Figures.

Relationship of the current study to previous surfactant research

In terms of lipid components, the studies here utilized well-defined mixtures containing only two phospholipids: DPPC

plus either DPPG or DOPG. Studies on simple lipid mixtures such as these greatly facilitate the ability of spectroscopic assessments to discern molecular effects. At the same time, such studies clearly oversimplify the complex lipid composition of pulmonary surfactant. The results here show a dependence of apoprotein-induced effects on lipid composition even in simple binary mixtures. Endogenous surfactant contains multiple saturated and unsaturated phosphatidylcholines plus a broad distribution of fatty chain derivatives in the anionic phospholipid classes that are present (PG, phosphatidylinositol, and phosphatidylserine). It is very likely that specific molecular activities observed here for SP-B/C in binary lipid mixtures will be modified to some extent as a result of multicomponent associations and interactions in the biological material. Nonetheless, the findings here are relevant in elucidating the potential range of molecular actions of SP-B/C.

The ability of a naturally derived mixture of SP-B/C to interact preferentially with the net negatively charged headgroups of DPPG and DOPG was clearly demonstrated in the current IR results. In all of the lipid binary systems studied, the addition of SP-B/C caused a disproportionate increase in the band heights due to the phosphoglycerol component as seen in the CH_2/CD_2 band height ratio plots of Figs. 4, 6, and 9. This effect of the SP-B/C proteins on the phosphoglycerol headgroups appears analogous to the effect that divalent cations in the subphase have on PG (Williams et al., 1995). Differential interactions of SP-B/C with DPPC versus DPPG must reflect the influence of headgroup structure, inasmuch as the acyl chains of the lipids are identical in length and saturation. In films of 1:1, 2:1, and 7:1 DPPC- d_{62} :DPPG, the interaction of SP-B/C with DPPG headgroups was accompanied by an ordering of the lipid acyl chains indicated by a shift in peak frequencies to lower values. In DPPC- d_{62} :DOPG films, differential interactions of SP-B/C with the two lipids may reflect differences in both headgroups and chains, because the oleoyl acyl chains in DOPG are two carbon atoms longer than DPPC and contain a 9-*cis* unsaturated double bond. Addition of SP-B/C in 7:1 DPPC- d_{62} :DOPG films caused an interaction with the DOPG headgroup, but without the accompanying increase in acyl chain ordering. Instead, the DOPG acyl chains became increasingly disordered with the addition of the mixed surfactant proteins.

Our study could not separate the individual contributions of SP-B and SP-C in spectroscopic assessments, but both likely contributed to the molecular biophysical changes observed. The structural and molecular properties of SP-B and SP-C have been examined in a variety of studies (for reviews see Hawgood and Schiffer, 1991; Johansson et al., 1994a; Creuwels et al., 1997; Notter, 2000). SP-B has a highly conserved sequence yielding an amphipathic structure that is particularly suited to interact with phospholipid headgroups as well as chains. The fully processed form of human SP-B is a highly charged protein composed of 79 amino acids. Although many of these amino acids are

hydrophobic, SP-B also contains 10 basic Lys/Arg residues and two acidic Glu/Asp residues and has a net positive charge at neutral pH. The protein exists in both monomer and oligomeric forms, including a presumptively active homodimer in most species. Amphipathic α -helical segments are thought to locate SP-B relatively peripherally in phospholipid bilayers, rather than as a transmembrane (or transmonolayer) protein. Using polarized ATR Fourier transform IR spectroscopy, the hydrophobic domains of SP-B were reported to be associated with the phospholipid headgroups with the short α -helical portions located only slightly inside the bilayer (Vandenbussche et al., 1992). Fluorescence anisotropy was applied to the problem and also determined that SP-B was not a transmembrane protein, but was associated with the membrane surface (Baatz et al., 1990). Tryptophan fluorescence was used to locate SP-B-like peptides incorporated into phospholipid films and found them to be present mostly at the polar interfacial area of the lipid monolayers (Cochrane and Revak, 1991).

Experimental evidence also indicates the existence of a specific interaction of SP-B with anionic phospholipids. Fluorescence microscopy studies (Lipp et al., 1996) indicate that positively charged SP-B protein segments complex with the negatively charged lipid, palmitic acid. Incorporation of a charged peptide of SP-B into monolayer films of palmitic acid induced a change in the appearance of the isotherm whereas an uncharged mutant of the same synthetic SP-B peptide did not (Longo et al., 1993). It was therefore concluded that a specific charge interaction between the cationic peptide and the anionic lipid must be present. Our previous optical microscopy studies with SP-B/C proteins clearly implied that electrostatic interactions are critical for lung surfactant function (Krüger et al., 1999). At low subphase pH (~ 1.9), there was no change in the observed fluorescence phase morphology throughout the liquid expanded/liquid condensed (LE/LC) phase transition of model lipid mixtures regardless of whether the SP-B/C proteins were present in the monolayer. Only when the subphase pH was raised to 6.2 (which is a value above the pK of the PG headgroup), did the monolayer exhibit a distinct new protein-induced morphology. These data suggest that an electrostatic component is required for the formation of physiologically relevant complexes.

SP-C as well as SP-B also is known to interact significantly with lipids as a result of its structure and properties (Hawgood and Schiffer, 1991; Johansson et al., 1994a; Creuwels et al., 1997; Notter, 2000). Fully processed human SP-C consists of 35 amino acids, most of which are very hydrophobic. The sequence and overall extreme hydrophobicity of SP-C is highly conserved among animal species. Cysteine residues at positions 5 and 6 of SP-C in humans and many other species are linked by thioester bonds to palmitic acid moieties (see e.g., Curstedt et al., 1990). SP-C is primarily α -helical with a length able to span a phospholipid bilayer. Although the majority of its inter-

actions with lipids are hydrophobic, it does contain some hydrophilic residues including positively charged Arg/Lys residues at positions 11 and 12 in most species. Hydrophilic residues near the N-terminus of SP-C have the potential to interact with charged groups or ions in the plane of the phospholipid headgroups. A number of studies has been done on the effects of SP-C on the phospholipid components in monomolecular films. Fluorescence microscopy suggests that SP-C enhances the adsorption of DPPC to the monolayer surface from vesicles (Nag et al., 1996b), an effect thought to involve a perturbation of the packing arrangements of the phospholipid molecules. The incorporation of SP-C (and to a smaller extent SP-B) into monolayers reduces the size of condensed phases in these films (Perez-Gil et al., 1992; Nag et al., 1996a; Nag et al., 1997). As noted, the interaction of SP-C with monolayer phospholipids is theorized to be primarily hydrophobic in nature. Isotherm evidence was first used to predict that SP-C has its α -helical axis oriented parallel to the A/W interface (Oosterlaken-Dijksterhuis et al., 1991a). Recent dichroic FTIR investigations of SP-C in the liquid expanded monolayer phase of DPPC showed an orientation angle of the SP-C helices of $\sim 30^\circ$ from the surface, whereas at higher surface pressure, an orientation of the helix closer to the surface normal was reported (Gericke et al., 1997). Recent results from our laboratory (Krüger et al., 2002) have argued that SP-C acts analogously to a molecular machine, or loaded spring, in which the stored energy of the hydrophobic rigid α -helix is released upon reorientation around its flexible, more hydrophilic N-terminus, which is anchored at the A/W interface. If such a reorientation does in fact occur, it would appear from the present study to be associated with a decreased phospholipid packing density and lowering of band heights for the DPPC- d_{62} component, although still maintaining an ordered PC acyl chain conformation.

The current study did not attempt to address the functional importance to surface activity of the hydrophobic surfactant protein-induced effects found here in spectroscopic experiments. Both SP-B and SP-C are known to enhance the surface properties of phospholipids, but SP-B is more effective in doing so on either a weight or molar basis (Curstedt et al., 1987; Revak et al., 1988; Yu and Possmayer, 1988; Sarin et al., 1990; Oosterlaken-Dijksterhuis et al., 1991a; Oosterlaken-Dijksterhuis et al., 1991b; Seeger et al., 1992; Wang et al., 1996; Johansson et al., 1998). SP-B has been shown to be more active than SP-C in increasing both adsorption and dynamic surface tension lowering when combined with phospholipids. These activity findings correlate with the fact that SP-B has a fourfold higher capacity than SP-C on a weight basis for binding lipid vesicles to interfacial films (Oosterlaken-Dijksterhuis et al., 1991a; Oosterlaken-Dijksterhuis et al., 1991b). Mixtures of phospholipids with SP-B versus SP-C also have an improved ability to resist inhibition by serum albumin (Seeger et al., 1992; Wang et al., 1996). Physiological studies have shown that installed

exogenous surfactants containing lipids plus SP-B have higher activity in improving lung mechanics and function in animals relative to corresponding mixtures containing SP-C (Rider et al., 1993). Supplementation with SP-B or related synthetic peptides has also been found to improve the surface and physiological activity of the clinical exogenous surfactant Survanta, which contains SP-C but has only minimal levels of SP-B (Mizuno et al., 1995; Walther et al., 1997).

A previous study examined the adsorption, dynamic film behavior, and physiological activity of naturally derived lung surfactant mixtures with and without anionic phospholipids (Wang et al., 1997). Gel permeation chromatography was used to obtain the complete mix of zwitterionic and anionic calf lung surfactant phospholipids, as well as the subfraction containing only the zwitterionic components. These phospholipid mixtures were then studied for activity when combined with column-isolated SP-B/C. Depletion in anionic phospholipids led to only small decreases in adsorption and dynamic surface tension lowering ability, and no significant detriment in physiological activity in restoring pressure-volume mechanics in lavaged rat lungs. The relevance of these findings on complex multicomponent mixtures of lung surfactant phospholipids to the present studies on spread binary films of synthetic phospholipids is uncertain. Additional studies need to address the functional importance of specific molecular interactions between anionic phospholipids and SP-B/C in lung surfactant activity.

Implications for surface structure reorganization

The results of the present study not only addressed the effects of SP-B/C on molecular order within the surface film, but also provide insights about film refining or squeeze-out during cycling. This is the process whereby some components of pulmonary surfactant are ejected from the alveolar surface film during compression and reinserted back into it during expansion in the breathing cycle (Notter, 1984, 2000). IR spectroscopic evidence has shown that both unsaturated lipids (Pastrana-Rios et al., 1994) and SP-B (Pastrana-Rios et al., 1995) are excluded from monolayer films as the surface pressure is increased. More recently, it has been established using optical microscopy techniques that SP-C is incorporated in exclusion particles that adhere to the surface structure after transfer to solid substrates (Kramer et al., 2000). In this study, the expulsion of DOPG from the films can be inferred from the negative slopes seen in the band height ratio data plotted in Fig. 9. These data are consistent with the interpretation that mixed SP-B/C interacts strongly with the charged headgroup of the DOPG so that a lipid-protein complex is excluded from monomolecular films. This is also supported by the PC data in Fig. 8. The presence of SP-B/C did not affect peak wavenumber values but did significantly increase band heights for DPPC- d_{62} in the 7:1 DPPC- d_{62} :DOPG film. This increase in band height could potentially be attributed to the formation of three-

dimensional exclusion particles that begin with specific SP-B/C:PG interactions, but also incorporate a significant amount of PC lipid.

The ability of SP-B/C to influence squeeze-out from lipid-rich monolayers is also supported by our recent fluorescence and dark-field microscopy studies (Krüger et al., 1999). These studies have directly shown by dark-field microscopy that the addition of SP-B/C (or the synthetic equivalent of SP-C) induces the formation of a new monolayer phase of different morphology than either the LC or LE phases that form in pure lipid mixtures. Material is excluded from the lipid-protein surface film upon compression and forms three-dimensional, surface-associated structures of micron dimensions. Such exclusion bodies form with SP-B/C peptides at the physiological levels (1 wt %) used here, or in the presence of synthetic SP-C at higher (10 wt %) concentrations (Krüger et al., 2002).

Our current IR results and previous microscopic studies would be consistent with the following conceptual model. Anionic components such as phosphoglycerols in phospholipid films preferentially associate with SP-B and/or SP-C. The positively charged, amphiphatic structure of SP-B has multiple regions where such interactions could occur, whereas the SP-C peptide has the potential for association with anionic lipids in the vicinity of its N-terminus where several hydrophilic amino acid residues are present. Upon monolayer compression, the overall energy of the system rises continuously, leading to a driving force for surface film reorganization. Lipid molecules associated through electrostatic and/or hydrophobic contacts with SP-B and/or SP-C are lifted from the monolayer surface and inverted to form a localized bilayer structure, possibly serving as a nucleation site for additional material including PC lipids, thereby leading to the formation of larger-scale interfacial structures visible via dark-field microscopy (Krüger et al., 1999). The formation of subinterfacial domains of lipid-protein material ejected from the film is also possible. This model would be consistent with the IR wavenumber shifts and band height changes seen in the C-H region (e.g., Figs. 2 and 5) as preferential interaction of the SP-B/C proteins with the phosphoglycerol lipids. Also, the slope of the band height ratios (Figs. 4 and 9) can be interpreted as evidence for the formation of three-dimensional, surface-associated structures. A more detailed explanation of the energetics of how SP-C might individually interact with anionic phospholipids in surface films is presented elsewhere (Krüger et al., 2002).

CONCLUSIONS

The primary conclusions drawn from these in situ IR measurements on surface monolayers containing DPPC-d₆₂:DPPG or DPPC-d₆₂:DOPG plus chromatographically isolated bovine SP-B/C are as follows.

Bovine SP-B/C in the endogenous ratio had molecular interactions with both PC and PG components in binary lipid films to influence acyl chain order and lipid packing density during stepwise compression from surface pressures of 6 to 60 mN/m. Specific effects depended upon the lipid component, protein content, and monolayer composition. Of particular note was the observation that maximal spectroscopic effects were found for hydrophobic protein contents in the physiological range of 1–2 wt %, and these effects were abolished at artificially high protein levels (10 wt %).

Although SP-B/C interacted with both PC and PG in binary lipid films, analysis of C-H/C-D spectroscopic signals indicated preferential interactions with the anionic phospholipid component. The mixed proteins ordered the acyl chains of DPPG in 7:1, 2:1, and 1:1 monolayers of DPPC-d₆₂:DPPG, and disordered the acyl chains of DOPG in 7:1 films of DPPC-d₆₂:DOPG. SP-B/C had no significant effect on DPPC acyl chain structure in 7:1 DPPC-d₆₂:DPPG or DPPC-d₆₂:DOPG monolayers, with only slight ordering effects noted for the PC acyl chains in 1:1 DPPC-d₆₂:DPPG monolayers.

IR spectroscopic data were consistent with ejection of PC and PG lipids from the surface monolayer during compression to high surface pressures, consistent with previous near-field, fluorescence, and Brewster-angle microscopic studies indicating phase morphology changes and surface aggregate formation in compressed lung surfactant films.

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