

## Infrared spectroscopy of aqueous biophysical monolayers

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### Abstract

We have used infrared external reflectance spectroscopy at the air/water interface to study: (1) polarized infrared (IR) monolayer spectroscopy at different incoming angles of incidence, and (2) ternary mixed model systems (i.e. two lipids and a peptide) that mimic the composition of pulmonary lung surfactant. Using polarized IR radiation, we have observed a splitting of the symmetric and antisymmetric methylene stretching vibrations in the spectra of long-chain hydrocarbon amphiphiles that has been previously unreported for unpolarized monolayer spectra. The splitting of the C–H bands results in sub-bands identifiable with ordered and disordered chain conformations. The splitting of these bands qualitatively tracks the fractional composition of ordered/disordered states upon monolayer compression. In a separate series of experiments, we have obtained the IR external reflectance spectra of monomolecular films of model mixtures relevant to pulmonary surfactant physiology. Monolayers were composed of 2:1 DPPC- $d_{62}$ :DOPG containing 0, 1 or 2 wt.% of the hydrophobic surfactant proteins SP-B and SP-C (SP-B + C). The  $\text{CH}_2$  antisymmetric and symmetric stretching bands ( $\sim 2920$  and  $2852\text{ cm}^{-1}$ ) along with the analogous  $\text{CD}_2$  stretching bands ( $\sim 2194$  and  $2089\text{ cm}^{-1}$ ) were analyzed, and band heights, integrated intensities and peak frequency positions were plotted as a function of measured surface pressure. These data indicate that the presence of surfactant protein appears the disorder the acyl chains of the DPPC- $d_{62}$  component in the film, opposite of what is seen for the protiated component, DOPG. Data from these model mixtures indicate that the surfactant protein interacts differently with each of the lipid components. © 1998 Elsevier Science S.A. All rights reserved

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### 1. Introduction

In 1985, Dluhy and Cornell published the first paper that reported that infrared (IR) external reflectance spectroscopy can be used to obtain spectra of monomolecular films on aqueous substrates [1]. Since that time, several research groups worldwide have adopted this approach for the study of monomolecular films [2–6]. While the use of external reflectance IR spectroscopy is a relatively new approach to the study of Langmuir monolayers, there already is a growing body of literature reporting the results of spectroscopic studies of a variety of these monomolecular films. Indeed, over the last few years, the work in this field has been reviewed in separate articles [7,8]. The majority of the IR spectroscopic work to date has been in conformational analysis and characterization of phase transitions in model monolayers [9,10], but there have also been several studies on naturally isolated pulmonary surfactant [11], cation

interactions with monolayer head groups [12,13], soluble ionic surfactants [14] and peptide monolayer films [15]. This paper reports on some recent work from our laboratory on two additional aspects of IR spectroscopy of Langmuir monolayers: polarized IR spectroscopy at different incoming angles of incidence, and the use of reflection IR spectroscopy to study ternary mixed model systems (i.e. two lipids and a peptide) that mimic the composition of pulmonary lung surfactant.

Recent experiments in our laboratory have focused on using polarized infrared external reflectance spectroscopy to study monomolecular films. Spectra were obtained at varying surface pressures, angles of incidence, and polarization settings (either parallel ( $R_p$ ) or perpendicular ( $R_s$ ) to the surface normal) at the air/water interface. Previous studies have shown that the reflectance of polarized radiation at the air/water (A/W) interface is highly dependent on the angle of incidence employed [16]. Calculations using the Fresnel reflectance formulae have previously been made of the reflectance of light vs. angle of incidence for both  $R_s$  and  $R_p$  polarizations at the air/water interface [17–19]. These

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calculations indicate that the absolute reflectance of the radiation at  $R_p$  polarization is greater at a 30-degree angle of incidence than at a 60-degree angle; however, the opposite is true for  $R_s$  polarized radiation. Thus, orientational as well as structural information of a monolayer film at the air/water interface may be obtained using polarized infrared external reflectance spectroscopy at varying angles of incidence.

In addition to the polarized IR experiments, we have also used reflection IR spectroscopy at the A/W interface to study model mixtures that mimic the composition of pulmonary lung surfactant. First proposed by von Neergaard in 1929 [20], pulmonary surfactant is a substance composed of lipids and proteins that lines the lungs preventing alveolar collapse during respiration and providing a first lung defense against airborne infectious agents and irritants. A deficiency of pulmonary surfactant is the chief cause of respiratory distress syndrome (RDS) found in preterm infants [21] and is implicated in the related adult respiratory distress syndrome (ARDS) [22]. Surfactant replacement therapies are currently employed, and the development of more effective synthetic, exogenous surfactants is dependent upon gaining a better understanding of how the individual surfactant components interact and function on a molecular level.

Surfactant is secreted from type II epithelial cells and is composed of 90% lipid and 10% protein by weight. More than 80% of the lipid is phospholipid [23] with the most abundant single lipid being 1,2-dipalmitoyl-*sn*-3-phosphocholine (DPPC). Phosphoglycerols are the next largest phospholipid class comprising as much as 5% to 10% of the phospholipid population. Also found in mammalian pulmonary surfactants are four proteins specific to the lung: SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are low molecular weight (8.7 and 4.2 kDa, respectively) [24] hydrophobic proteins believed to help maintain low surface tensions in the film and to promote efficient spreading of the phospholipid components.

Monomolecular films of phospholipids at the A/W interface of a Langmuir film balance have long been used by researchers as model systems for studying pulmonary surfactant. Clements [25] studied the surface properties of monolayer films of surfactant extracts on a Langmuir film balance, and attributed the stabilizing function of the surfactant to a lowering of the surface tension at the air/alveolar interface to values less than 10 dynes/cm. To be effective, a model surfactant must be able to withstand high surface pressures (low surface tensions) during film compression and to re-spread quickly during film expansion. Films of DPPC alone will fulfill this first criterion, but do not re-spread quickly enough to function as effective exogenous surfactants in vivo. It is believed that unsaturated lipids, inorganic ions, and surfactant proteins SP-B and SP-C present in natural surfactant promote re-spreading of the DPPC component [26]. The present work describes recent external reflectance Fourier transform IR spectroscopic data col-

lected at the air/water interface which were used to investigate the effect these surfactant proteins have on binary phospholipid films of DPPC- $d_{62}$  with DOPG.

## 2. Experimental

Infrared external reflection-absorbance monolayer spectra at the A/W interface were acquired using a Perkin-Elmer (Norwalk, CT) 2000 Fourier transform infrared spectrometer. The IR beam was brought outside of the instrument using the spectrometer's external beam port and focused on the surface of a Langmuir film balance using gold-coated, off-axis parabolic mirrors (Janos, Townshend, VT) machined at either 30 or 60 degrees (which resulted in a angle of incidence at the surface of 60 and 30 degrees relative to the surface normal, respectively). A wire grid polarizer (IGP225, Cambridge) was placed in the beam path to collect polarized spectra; this polarizer was placed on a rotatable mount that allowed the polarization direction to be changed and the polarizer to be placed in and out of the external IR beam without a change in environmental conditions. Spectra were collected at 4  $\text{cm}^{-1}$  resolution. At a 30-degree angle of incidence 1024 scans were co-added for both  $R_p$  and  $R_s$  polarizations. At a 60-degree angle of incidence, 1024 scans were co-added for the  $R_s$  polarization, while 2048 scans were co-added for the  $R_p$  to obtain an acceptable signal-to-noise ratio; interferograms were apodized using the Beer-Norton (strong) function. The external IR reflectance setup was enclosed in a custom built chamber that allowed the humidity to be controlled. Spectral manipulations performed on the data such as baseline correction and curve fitting were performed using the Grams/32 software package (Galactic, Salem, NH). Vibrational frequencies were calculated using a center-of-gravity algorithm [27].

1,2-Dipalmitoyl-*sn*-3-phosphoglycerol (DPPG), 1,2-dioleoyl-*sn*-3-phosphoglycerol (DOPG), DPPC, and 1,2-dipalmitoyl- $[d_{62}]$ -*sn*-3-phosphocholine (acyl chain perdeuterated DPPC- $d_{62}$ ) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used as received. A mixture of the hydrophobic surfactant proteins B and C (SP-B + C) was generously provided by Dr. Robert H. Notter (Department of Pediatrics, University of Rochester Medical School). Subphase  $\text{H}_2\text{O}$  was obtained in-lab from a Barnstead (Dubuque, IA) ROPure/Nanopure reverse osmosis/deionization system having a nominal resistivity of 18  $\text{M}\Omega\text{-cm}$ .

Stock solutions of DPPC, DPPC- $d_{62}$  (~1 mg/ml in  $\text{CHCl}_3$ ), DPPG and DOPG (~1 mg/ml in 4:1  $\text{CHCl}_3$ :MeOH) were prepared and concentrations verified by inorganic phosphorus assay [28]. Solutions of 2:1 DPPC- $d_{62}$ :DPPG and 2:1 DPPC- $d_{62}$ :DOPG containing 0, 1, or 2 wt.% SP-B + C were prepared by mixing the appropriate amounts of the above lipid stock solutions and a 0.18 mg/ml stock solution of SP-B + C in 1:1  $\text{CHCl}_3$ :MeOH. The subphase for the DPPC monolayer experiments was deionized  $\text{H}_2\text{O}$

(18 M $\Omega$ -cm, pH 5.6); the subphase used for all surfactant experiments was 150 mM NaCl in deionized H<sub>2</sub>O (18 M $\Omega$ -cm, pH 5.6).

Surface–pressure isotherms and monolayer compression were accomplished using a Nima (Coventry, UK) 601M Langmuir film balance. The subphase was first cleaned by aspiration and a single beam spectrum was collected for use as a background. 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 environment chamber around the trough was typically  $24^\circ\text{C}$  and the relative humidity remained fairly constant at 70%. Typically, 5–10  $\mu\text{l}$  of sample were spread via syringe onto the trough surface. 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  $\sim 5 \text{ mN m}^{-1}$  to a maximum of 45–65  $\text{mN m}^{-1}$  depending on the nature of the film.

### 3. Results and discussion

#### 3.1. Polarized IR monolayer spectra

Infrared external reflection–absorption spectra at the air/water interface were acquired for DPPC monolayers at three

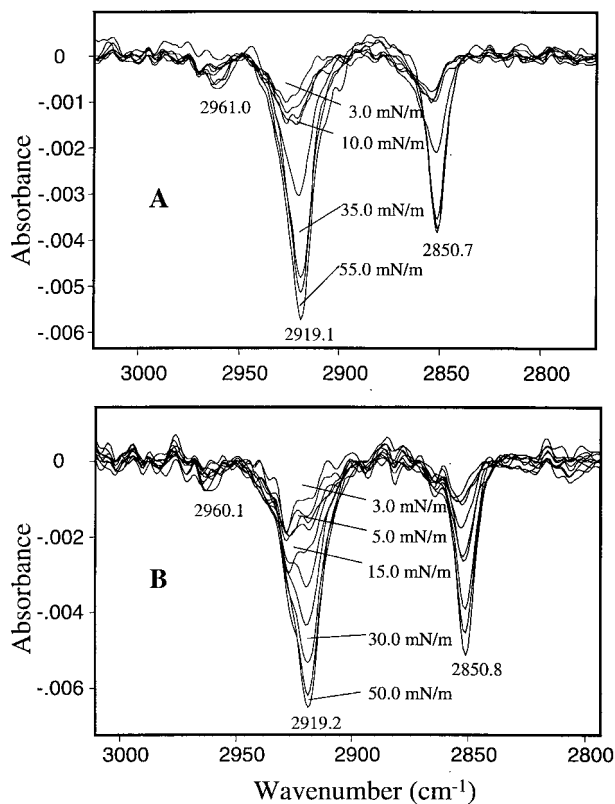


Fig. 1. IR external reflection–absorbance spectra at the air/water interface collected as a function of surface pressure for DPPC at a 30-degree angle of incidence. (A) Perpendicular ( $R_s$ ) polarized spectra. (B) Parallel ( $R_p$ ) polarized spectra.

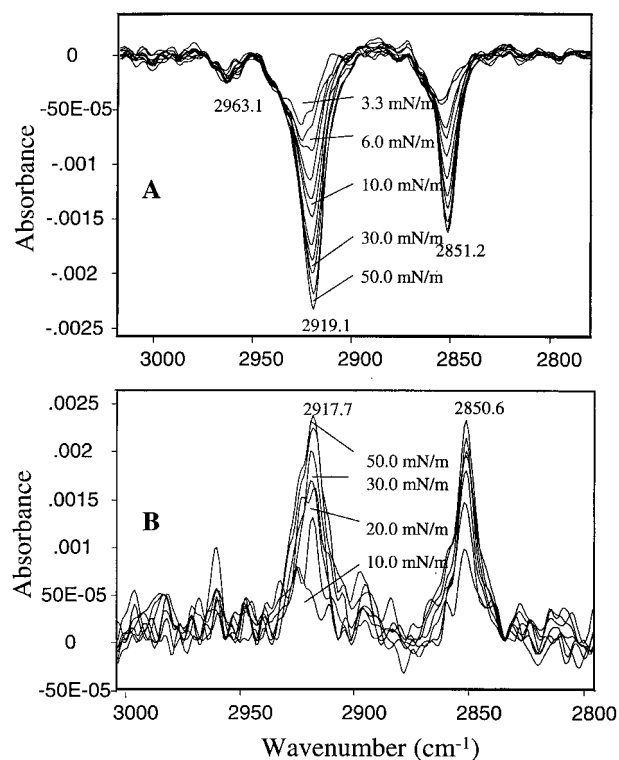


Fig. 2. IR external reflection–absorbance spectra at the air/water interface collected as a function of surface pressure for DPPC at a 60-degree angle of incidence. (A) Perpendicular ( $R_s$ ) polarized spectra. (B) Parallel ( $R_p$ ) polarized spectra.

different states of polarization of the incoming radiation: (1) without polarization, (2) with perpendicular ( $R_s$ ) polarization, and (3) with parallel ( $R_p$ ) polarization. Each series of spectra collected at a specific polarization setting were also collected at two different (30- and 60-degree) angles of incidence. Finally, each set of spectra was also collected at increasing surface pressures in order to monitor the thermodynamic state of the monolayer. These different types of monolayer spectra are illustrated in Figs. 1 and 2. Fig. 1 presents the methyl and methylene stretching bands for DPPC in the C–H stretching region between 3000–2800  $\text{cm}^{-1}$  with  $R_s$  and  $R_p$  polarization at a 30-degree angle of incidence. Fig. 2 illustrates reflection–absorbance spectra for DPPC in the C–H stretching region with  $R_s$  and  $R_p$  polarization at a 60-degree angle of incidence.

The polarized reflection–absorbance spectra for the methylene symmetric and antisymmetric stretching bands for DPPC at a 30-degree angle of incidence (Fig. 1) appear quite different from the unpolarized spectra (see e.g. [7]). In both the  $R_s$  and  $R_p$  polarized spectra, a splitting of the methylene antisymmetric stretching band at 2920  $\text{cm}^{-1}$  is observed. The splitting is most prominent in the  $R_p$  polarization. We have also observed such splitting in the  $R_s$  and  $R_p$  polarized spectra of the antisymmetric methyl stretching vibration ( $\sim 2960 \text{ cm}^{-1}$ ), although it is difficult to determine the trends in this band due to their lower intensities.

The polarized reflection–absorbance spectra for the

methylene symmetric and antisymmetric stretching bands at the 60-degree angle of incidence (Fig. 2) also appear quite different from the unpolarized spectra. For the perpendicu-

lar ( $R_s$ ) polarized spectra, a splitting of the C–H bands is observed at low surface pressures; the two bands appear to merge at intermediate surface pressures, and then the overall

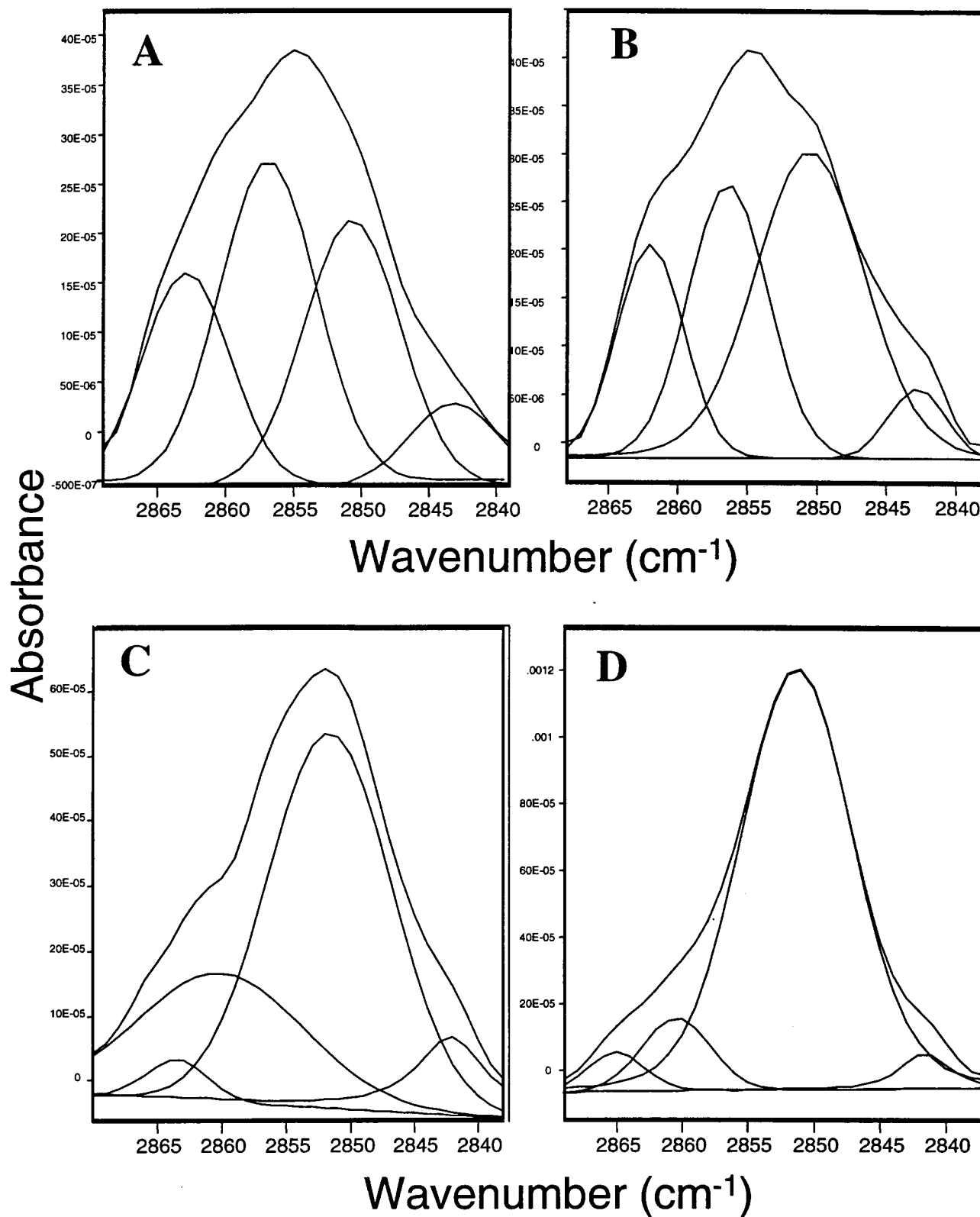


Fig. 3. Curve-fitting results for the 2850  $\text{cm}^{-1}$  band of DPPC as a function of increasing surface pressure. Spectra used for curve fitting were collected unpolarized at a 60-degree incident angle. (A) 3.0  $\text{mN m}^{-2}$ ; (B) 5.0  $\text{mN m}^{-2}$ ; (C) 10.0  $\text{mN m}^{-2}$ ; (D) 30  $\text{mN m}^{-2}$ .

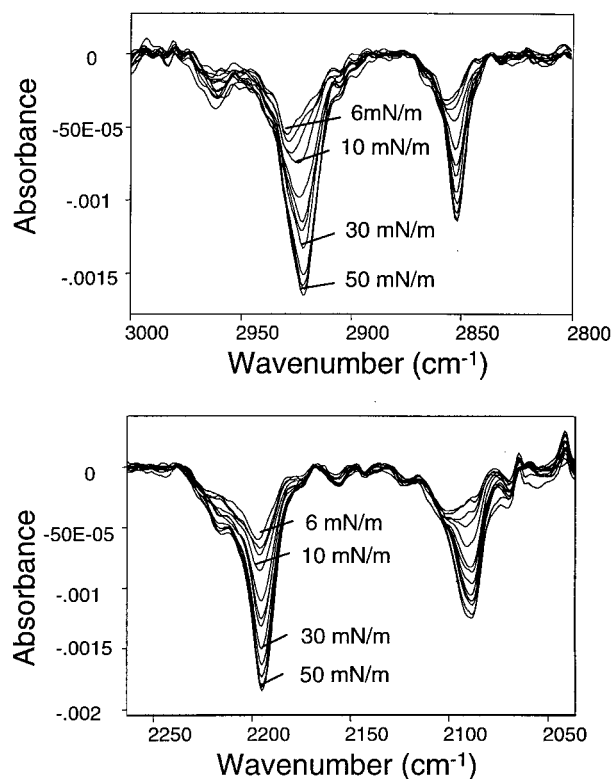


Fig. 4. IR external reflection-absorbance spectra of a 2:1 DPPC- $d_{62}$ :DPPG binary monolayer film collected at various surface pressures. The C-H (top) and C-D (bottom) stretching regions are illustrated.

vibrational band appears to shift to lower wavenumber. Similar behavior is observed in the parallel ( $R_p$ ) polarized spectra collected at a 30-degree angle of incidence. However, the spectra collected at the  $R_p$  polarization at a 60-degree angle of incidence are qualitatively different from the spectra collected at the 30-degree incident angle. The methylene stretching bands have reversed intensity and are now positive rather than negative. This band intensity reversal has been theoretically predicted and previously observed [16,29]. The DPPC antisymmetric stretching band for the  $R_p$  polarization at a 60-degree angle of incidence also appears to exhibit splitting characteristic of the spectra at a 30-degree angle of incidence. However, the signal-to-noise level is much lower for the  $R_p$  polarization at a 60-degree angle of incidence due to the fact that the absolute reflectivity of the  $R_p$  polarization is very near zero at angles close to the Brewster angle [16]. It is for these reasons that it is difficult to determine the degree of splitting in the antisymmetric band observed with  $R_p$  polarization at 60 degrees.

We have correlated the polarized splitting of the methylene antisymmetric stretching band at low surface pressures with the presence of two different conformational structures, ordered (solid) and disordered (liquid) phases. Curve fitting of the methylene symmetric stretching band results in the ability to determine the amount of either liquid or solid phase lipid as a function of surface pressure. Representative curve-fitting results obtained at varying surface pressures at 60-degree incident angle are presented in Fig.

3. These data show a liquid (disordered) phase band at  $\sim 2859\text{ cm}^{-1}$  and a solid (ordered) phase band at  $\sim 2852\text{ cm}^{-1}$ . At low surface pressures these bands are approximately of equal intensity, and their presence results in the splitting observed in the methylene vibrations. As the surface pressure of the lipid monolayer is increased, the intensity of the liquid-phase band decreases while the solid-phase band increases. This increase in intensity for the solid-phase band corresponds to a shift to lower wavenumber for the methylene stretching bands. At high surface pressures the band due to the liquid phase virtually (but not entirely) disappears as the intensity of the solid band predominates.

### 3.2. IR spectroscopy of ternary surfactant mixtures

External reflectance spectra of the methylene stretching region ( $3000\text{--}2800\text{ cm}^{-1}$ ) and the analogous  $\text{CD}_2$  stretching region ( $2250\text{--}2050\text{ cm}^{-1}$ ) for a monolayer of 2:1 DPPC- $d_{62}$ :DPPG compressed over a range of surface pressures is shown in Fig. 4. As the film is compressed, the average area per molecule is reduced, resulting in an increase in the peak intensity. As the pressure of the film increases, an ordering of the acyl chains occurs and the frequencies of the peak maxima are observed to shift to lower wavenumbers. The

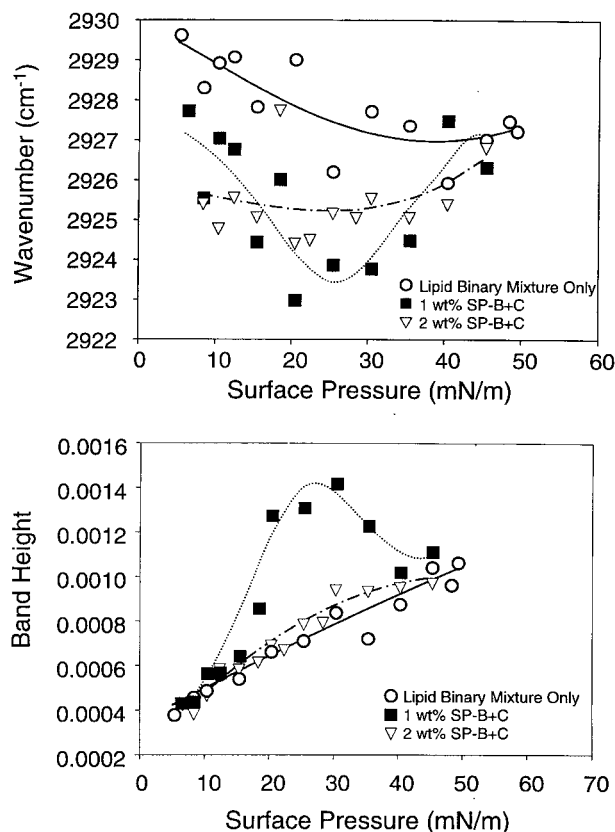


Fig. 5. Spectral band parameters of the DOPG component in a 2:1 DPPC- $d_{62}$ :DOPG binary monolayer film plotted as a function of surface pressure. (Top) Variation in measured frequency vs. surface pressure for the  $\text{CH}_2$  antisymmetric stretching vibration. (Bottom) Variation in band height vs. surface pressure for the  $\text{CH}_2$  antisymmetric stretching vibration.

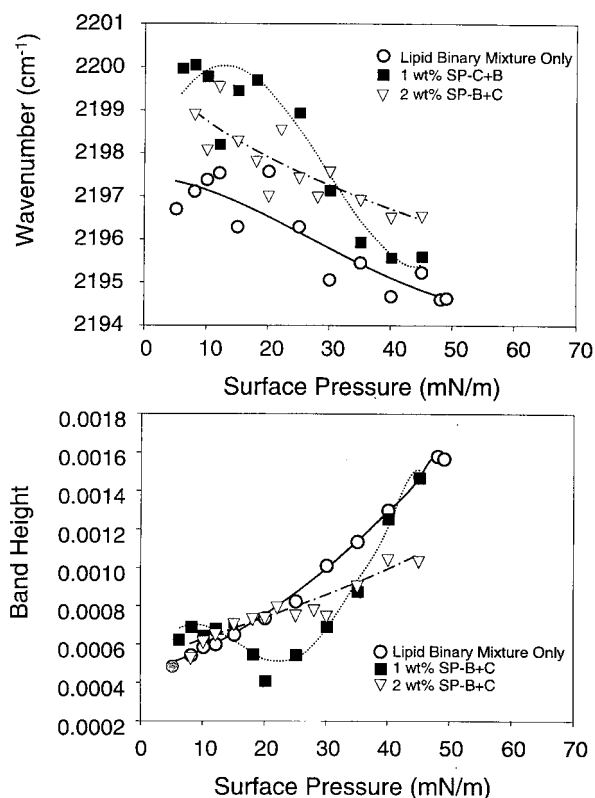


Fig. 6. Spectral band parameters of the DPPC- $d_{62}$  component in a 2:1 DPPC- $d_{62}$ :DOPG binary monolayer film plotted as a function of surface pressure. (Top) Variation in measured frequency vs. surface pressure for the CD<sub>2</sub> antisymmetric stretching vibration. (Bottom) Variation in band height vs. surface pressure for the CD<sub>2</sub> antisymmetric stretching vibration.

values for both the CH<sub>2</sub> (2920 and 2852 cm<sup>-1</sup>) and CD<sub>2</sub> (2194 and 2089 cm<sup>-1</sup>) antisymmetric and symmetric stretching bands at a surface pressure of 50 mN m<sup>-1</sup> indicate that both phospholipid species in the binary film exist in a relatively ordered state. Similar spectra are seen for a 2:1 DPPC- $d_{62}$ :DOPG mixture.

Shown in Fig. 5 (top and bottom) are the antisymmetric CH<sub>2</sub> stretching vibration frequency and band height plotted against measured surface pressure for 2:1 DPPC- $d_{62}$ :DOPG with and without surfactant protein added. Antisymmetric bands were chosen for analysis due to the higher signal to noise ratio than the corresponding symmetric bands. This was crucial for the low surface pressures where average molecular areas are high and peak intensities are inherently weak. Band height data was chosen over integrated intensity data due to the lesser influence by the partially overlapping CH<sub>3</sub>/CD<sub>3</sub> stretching bands. Data were collected for surface pressures from 5 to 50 mN m<sup>-1</sup> (where the film begins to collapse due to the thermodynamic properties of the DOPG component). The lipid only film shows a steady lowering of the frequency value as the surface pressure increases and the DOPG acyl chains become more ordered (more all-*trans* configuration). This ordering, along with a decrease in average molecular area causes the band height to increase with pressure as is seen in Fig. 5 (bottom).

Adding surfactant protein appears to affect the C–H and

C–D components of the film differently. The addition of 1 wt.% protein shifts the antisymmetric CH<sub>2</sub> stretching peak to lower wavenumber values at all surface pressures, but with maximum displacements of 3–5 cm<sup>-1</sup> occurring in the range from 15–35 mN m<sup>-1</sup>. This effect is also clearly seen in Fig. 5 (bottom) as a marked increase in the band height for pressures in the 15–35 mN m<sup>-1</sup> range. The addition of 2 wt.% SP-(B + C) appears to also lower the frequency of the DOPG antisymmetric stretching band, but the magnitude of the decrease is constant over the entire range of pressures, as no maximum lowering is observed in either the wavenumber or band height plots. Overall, the addition of both 1 and 2 wt.% protein acts to order the DOPG lipid acyl chains.

Addition of surfactant protein has the opposite effect on the deuterated lipid, DPPC- $d_{62}$ , as seen in Fig. 6. The addition of both 1 and 2 wt.% SP-(B + C) causes approximately a 2 cm<sup>-1</sup> increase in peak frequency over the entire range of surface pressures. The protein is also observed to shift the band heights to lower values than those seen for the lipid binary mixture only at pressures higher than 20 mN m<sup>-1</sup>. A small dip in the band height data occurs at pressures from 15–30 mN m<sup>-1</sup> with the addition of 1 wt.% protein, paralleling the trend seen in the DOPG data. The presence of surfactant protein appears the disorder the acyl chains of the DPPC- $d_{62}$  component in the film, opposite to what was seen for the protiated component, DOPG.

Due to the presence of unsaturated sites in the acyl chains of DOPG, the thermodynamic properties of DPPC- $d_{62}$ :DOPG monomolecular films are very different than those of films of DPPC- $d_{62}$ :DPPG. Films of 2:1 DPPC- $d_{62}$ :DPPG are more stable at high surface pressures, and were compressed to values near 70 mN m<sup>-1</sup> before collapse. The exact nature of the plots of frequency and band height versus surface pressure (data not shown) were not the same for the DPPG mixture as they were for the DOPG, but the overall trends upon addition of surfactant protein were alike, where a similar ordering of the C–H component and slight disordering of the C–D component occurred.

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