

# Pressure-Dependent Changes in the Infrared C–H Vibrations of Monolayer Films at the Air/Water Interface Revealed by Two-Dimensional Infrared Correlation Spectroscopy

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Two-dimensional infrared correlation analysis (2D-IR) was applied to a set of surface pressure-dependent unpolarized IR spectra of a monolayer film of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at the air/water (A/W) interface. The experimentally measured asynchronous 2D-IR spectra were compared with synthetic spectra calculated by using an “overlapped peaks” model vs. a “frequency shifting” model. The results presented here show that when the experimentally observed monolayer IR spectra are acquired as a function of surface pressure, one model cannot be used exclusively for spectral interpretation. In this study, the monolayer IR spectra were divided into a low-pressure region subset (<11 mN/m) and a high-pressure region subset (>11 mN/m). When the monolayer IR spectra acquired as a function of surface pressure are analyzed by 2D correlation methods, the results strongly support the following conclusions: (1) the low-pressure subset, which encompasses both the liquid expanded (LE) and the liquid expanded/liquid condensed (LE/LC) regions of the DPPC monolayer isotherm, is best modeled by two overlapped peaks correlated with ordered and disordered conformational states of the monolayer film; and (2) the high-pressure subset, which reflects solely the liquid condensed (LC) phase of the monolayer isotherm, is best modeled by a single peak, which undergoes a minor frequency shift, and which may be primarily correlated with gradual packing of the liquid condensed structure. This interpretation of the 2D-IR correlation spectra is in agreement with the interpretation of sub-bands seen in polarized monolayer IR spectra previously reported by our laboratory.

Index Headings: Two-dimensional infrared; 2D-IR; Monolayer films; Pressure-dependent changes.

## INTRODUCTION

Since the development of the technique of external reflection Fourier transform infrared spectroscopy to study phospholipid films directly at the air/water (A/W) interface, a large number of studies have appeared in which IR spectroscopy has been used to study phospholipid monolayers as models of biomembrane interfaces (see, e.g., Refs. 1 and 2 for recent reviews). It has been shown that the IR reflectance method could identify vibrations due to the hydrocarbon acyl chains, carbonyl ester, and phosphate groups for these monolayer films, and that the external reflection method could differentiate between ordered and disordered conformations in phospholipid monolayer films at the air/water interface. In particular, the conformation-sensitive C–H stretching vibrations from the lipid hydrocarbon chains could be used to monitor the expanded-to-condensed thermodynamic phase

transition of the monolayer using infrared (IR) spectroscopy.

Previous experimental work has used both unpolarized and plane polarized (parallel,  $R_p$ , and perpendicular,  $R_s$ ) IR radiation to study monomolecular films at the A/W interface. Although polarized monolayer IR spectra are valuable in as much as the angle-dependent polarized reflectivities can be used in the calculation of monolayer molecular orientation,<sup>3,4</sup> both theoretical and experimental studies have shown that the use of polarized IR radiation at the A/W interface significantly degrades the final signal-to-noise ratio. In addition, the quality of the resulting spectra is highly dependent on the angle of incidence employed.<sup>5–7</sup>

Recent experiments in our laboratory have focused on the use of polarized IR external reflection-absorption spectroscopy to study biophysical monolayers. In experiments with well-defined single-component monolayers, we observed a band splitting in the methylene stretching vibrations that has not been previously described for IR monolayer spectra. The polarized monolayer spectra were able to distinguish individual, overlapping sub-bands within the methylene C–H vibrations that were correlated to ordered and disordered conformational states. Using the integrated intensities of these sub-bands, we have been able to semi-quantitatively track the formation of ordered and disordered conformational states of a monolayer film throughout its phase transition.<sup>8</sup>

Although these experiments demonstrate the power of polarized IR reflection spectroscopy for studying Langmuir monolayers, we are ultimately limited in our ability to make quantitative analyses of monolayer properties by the relatively poor signal-to-noise ratio in the polarized spectra. In order to enhance our ability to interpret IR monolayer spectra, we recently applied two-dimensional IR (2D-IR) correlation methods to study surface pressure-induced dynamical changes in monolayers at the A/W interface. In particular, we are interested in whether the application of 2D-IR methods to monolayer spectra collected by using unpolarized radiation can be used to confirm and further interpret the band splitting and spectral changes that we observe in the C–H region of polarized IR monolayer spectra.

Two-dimensional infrared can aid in the interpretation of complex spectra, especially spectra with the kind of broad, multiply overlapped peaks commonly encountered in condensed-phase vibrational spectroscopy.<sup>9,10</sup> In 2D-IR, the variations in the IR spectra obtained as a result of an external sample perturbation are mathematically

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cross-correlated to produce a two-dimensional correlation map. The resulting 2D-IR spectra are able to identify those vibrational modes which selectively respond (either in phase or out of phase) to the perturbation. Spectral resolution enhancement results when the perturbation affects the vibrational modes occurring at different wavelengths in a different manner.

When a band undergoes a frequency shift in the IR spectra, a distinctive pattern is observed in the corresponding 2D asynchronous plot. Since a frequency shift in the IR spectra can be caused by a simple shift in frequency of a single band or by the changes in relative intensities of two or more overlapped peaks, IR spectra have been simulated which correspond to both cases for an ideal system. Previous studies have shown that when a cross-correlation analysis is performed on each system, characteristic 2D-IR patterns are obtained in the asynchronous correlation plots.<sup>11–14</sup> These 2D-IR patterns are briefly described in this article.

In this current study we are able to show that these patterns can be used to determine the physical cause of the frequency shift of the methylene stretching vibrations in the monolayer IR spectra for a phospholipid at the A/W interface. When monolayer IR spectra are collected along the entire surface pressure–molecular area isotherm, one model cannot be used exclusively for spectral interpretation; however, the spectra can be divided into two subsets in which one model or the other can be used to determine the physical cause of the frequency shift observed in each subset. In our study we have divided the IR spectra into a low-pressure region subset and a high-pressure region subset. The spectral results strongly support the following conclusions: (1) the low-pressure subset, which encompasses both the liquid expanded (LE) and the liquid expanded/liquid condensed (LE/LC) regions of the DPPC monolayer isotherm, is best modeled by two overlapped peaks correlated with ordered and disordered conformational states of the monolayer film; and (2) the high-pressure subset, which reflects solely the liquid condensed (LC) phase of the monolayer isotherm, is best modeled by a single peak, which undergoes a minor frequency shift, and which may be correlated with gradual packing of the liquid condensed structure. Furthermore, this interpretation of the 2D-IR correlation spectra is in agreement with the observation of sub-bands seen in polarized monolayer IR spectra previously reported by our laboratory.

## MATERIALS AND METHODS

**Surface Chemistry.** The phospholipid used in these experiments [1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)] was obtained from Avanti Polar Lipids (Alabaster, AL) at 99+% stated purity and used without further purification. Subphase water was obtained from a Barnstead (Dubuque, LA) ROPure/Nanopure reverse osmosis/deionization system and had a nominal resistivity of 18.3 M $\Omega$  cm and a pH of approximately 5.6.

Sample solutions of DPPC were prepared by dissolving the lipid in a chloroform solution (Baker, HPLC grade) at a concentration of approximately 1.0 mg/mL. The lipid sample was spread onto a Nima 601M Series Langmuir film balance (Nima, Coventry, England) and

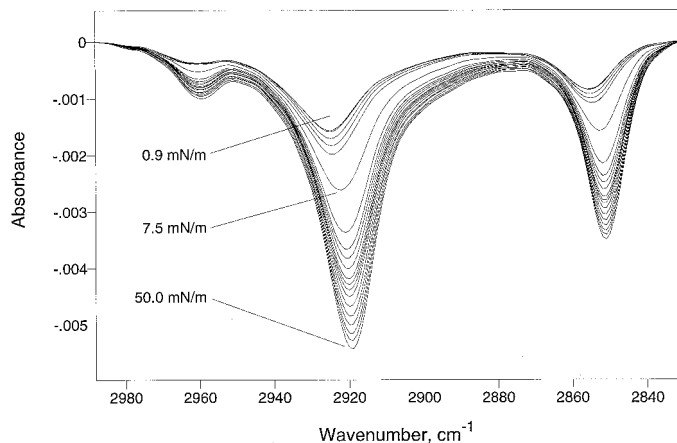


FIG. 1. Infrared external reflection-absorption spectra of DPPC monolayer films at the air/water interface. Reflection spectra were collected as a function of monolayer surface pressure and have not been normalized. Monolayer IR spectra were collected at the following surface pressures: 0.9, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, 12.0, 13.5, 15.0, 16.5, 18.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, and 50.0 mN/m.

allowed to settle for at least 10–15 min before compression began.

**Fourier Transform Infrared External Reflectance Measurements.** The external reflection-absorbance spectra were collected with a Perkin Elmer Spectrum 2000 Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer, Norwalk, CT). The optical interface of the IR spectrometer to the Langmuir film balance was designed in our laboratory. The IR beam coming from the spectrometer's external beam port reflected off a 60°, gold-coated, off-axis parabolic mirror (Janos Technology Inc., Townshend, VT), onto the surface of the Nima film balance, resulting in an incidence angle of 30° to the surface normal. The IR beam reflected off the subphase, sampling the film, and was recollimated by a second parabolic mirror that directed it onto the focusing mirror of a LN<sub>2</sub>-cooled narrow-band 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.

A single-beam spectrum of a pure water subphase was used as the background spectrum. The subphase temperature was held constant at 20 ± 1 °C by flowing thermostatted water through the hollow body of the trough. The temperature in the enclosed chamber was approximately 22 °C with a relative humidity of approximately 65%. The lipid sample in CHCl<sub>3</sub> solutions was spread via syringe onto the trough surface, where it was allowed to equilibrate for a period of 15 min before data collection began. The monolayer film was compressed intermittently and spectra collected over a range of surface pressures from 0.9 mN/m to a maximum of 50.0 mN/m. The specific surface pressures used are reported in the caption to Fig. 1. The surface number densities corresponding to each surface pressure have been previously reported.<sup>15</sup>

External reflection-absorbance spectra were collected with 1024 scans at 8 cm<sup>-1</sup> resolution, apodized with a Norton–Beer (medium) function, and were Fourier transformed with one level of zero filling. All monolayer IR spectra are presented as reflection-absorbance spectra;

i.e.,  $A = -\log(R/R_0)$ , where  $R$  is the IR reflectivity of the monolayer-covered surface, and  $R_0$  is the IR reflectivity of the bare water subphase background. The reflectance IR spectra used in the analyses presented here were baseline corrected with the use of the Grams/32 (Galactic Industries, Salem, NH) program. Adjustments for changes in surface density (i.e., intensity normalization) were also performed with Grams/32.

**Calculation of 2D-IR Correlation Spectra.** The 2D-IR synchronous and asynchronous spectra were calculated with the use of the Array Basic program "KG2D" written for Grams/32 and generously provided by Professor Yukihiro Ozaki of Kwansei-Gakuin University, Japan. This program uses Noda's most recent mathematical formalism, which replaces Fourier transforms with Hilbert transforms.<sup>16</sup> The 2D-IR correlation maps were calculated from the surface pressure-resolved IR spectra shown in Fig. 1. The average spectrum was subtracted from each spectrum in the set prior to the cross-correlation analysis. The synchronous spectrum,  $\Phi(\nu_1, \nu_2)$ , and the asynchronous spectrum,  $\Psi(\nu_1, \nu_2)$ , were then calculated by using Eqs. 1 and 2.

$$\Phi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n y(\nu_1, P_j) \cdot y(\nu_2, P_j) \quad (1)$$

$$\Psi(\nu_1, \nu_2) = \frac{1}{n-1} \sum_{j=1}^n y(\nu_1, P_j) \cdot \sum_{k=1}^n M_{jk} \cdot y(\nu_2, P_k) \quad (2)$$

In Eqs. 1 and 2,  $\nu_1$  and  $\nu_2$  represent two independent frequencies,  $P$  represents surface pressure,  $n$  represents the number of spectra used in the calculation, and  $M_{jk}$  is the Hilbert transform matrix, which is defined in Eq. 3.

$$M_{jk} = \begin{cases} 0 & \text{if } j = k \\ 1/\pi(k-j) & \text{otherwise} \end{cases} \quad (3)$$

To reduce the effect of noise in the experimental 2D-IR spectra, we removed correlation intensities less than 2% of the full, as previously described.<sup>12</sup> In the 2D spectra presented here, the contour levels are evenly spaced from 0 to the maximum value for positive correlations and from 0 to the minimum value for negative correlations.

## RESULTS AND DISCUSSION

The surface pressure-dependent conformational state of a monolayer film of DPPC at the air/water interface was studied by using infrared external-reflection spectroscopy and two-dimensional infrared correlation analysis. In a previous study, the IR spectra of the DPPC monolayer were collected with the use of polarized IR radiation, and a band splitting was observed in both the antisymmetric ( $\nu_a$ ) and symmetric ( $\nu_s$ ) methylene ( $\text{CH}_2$ ) stretching modes that was not observed with unpolarized radiation.<sup>8</sup> This band splitting was interpreted as being due to the presence of co-existing ordered and disordered conformational states (*trans* and *gauche*); however, definitive identification of conformational sub-bands is problematic due to the low signal-to-noise inherent in the polarized IR spectra.

**Experimental Data.** To further investigate the source of the observed band splitting and other spectral changes observed in the C-H region, we applied 2D-IR correlation analysis to a set of unpolarized IR spectra of the DPPC

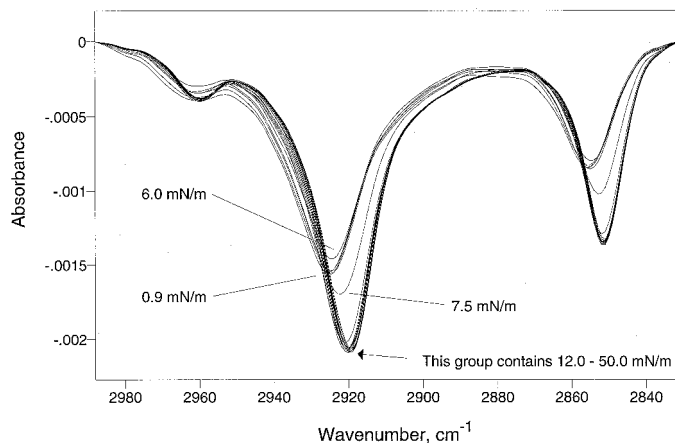


FIG. 2. Infrared external reflection-absorption spectra of DPPC monolayer films at the air/water interface normalized for changes in surface density as the trough area decreases. Specific surface pressures at which each spectra were obtained are given in the caption to Fig. 1.

monolayer. Figure 1 illustrates the unpolarized external reflectance IR spectra of a DPPC monolayer film in the C-H stretching region between 2988 and 2830  $\text{cm}^{-1}$ . In the acquisition of these spectra, the monolayer was applied to the surface of the film balance, allowed to equilibrate, and then compressed step-wise over a range of surface pressures from 0.9 mN/m to 50.0 mN/m with spectra collected during the interval between each successive compression. Clearly evident in the spectra are the antisymmetric ( $\nu_a$ )  $\text{CH}_2$  stretching band ( $\sim 2920 \text{ cm}^{-1}$ ), the symmetric ( $\nu_s$ )  $\text{CH}_2$  stretching band ( $\sim 2850 \text{ cm}^{-1}$ ), and the antisymmetric  $\text{CH}_3$  stretching band ( $\sim 2960 \text{ cm}^{-1}$ ). The symmetric methyl vibration ( $\sim 2870 \text{ cm}^{-1}$ ) is barely apparent as a slight shoulder on the symmetric  $\text{CH}_2$  band. The C-H vibrations grow in intensity as the lipid surface density increases with increasing surface pressure and the lipid molecules become more ordered. Also observed in these spectra as the monolayer surface pressure increases is the shift of the IR peak maxima frequencies to lower wavenumbers. Specifically, the  $\nu_a$   $\text{CH}_2$  band maximum shifts from  $\sim 2925$  to  $2919 \text{ cm}^{-1}$  while the  $\nu_s$   $\text{CH}_2$  band maximum shifts from  $\sim 2855$  to  $2851 \text{ cm}^{-1}$ . The shift of the  $\text{CH}_2$  peak frequency to lower wavenumbers has long been used to distinguish hydrocarbon order in a variety of alkane systems.<sup>17</sup> The magnitude values of the wavenumber shifts for the  $\text{CH}_2$  modes seen in Fig. 1 are consistent with previously reported values for monolayer systems and have been used to provide a direct, although qualitative, measure of acyl chain order in monolayers at the A/W interface.<sup>1,2</sup>

Figure 2 shows the IR spectra of the DPPC monolayer after normalization to account for changing surface coverage due to monolayer compression. This normalization process refers to the adjustment of monolayer IR spectral intensities for changes in surface density as the trough area available to the monomolecular film intermittently decreases.

Several distinct changes are apparent in the spectral features for the  $\nu_a$  and  $\nu_s$   $\text{CH}_2$  stretching vibrations in the normalized monolayer IR spectra in Fig. 2 that are not as readily observed in the non-normalized spectra. Specifically, a significant increase in IR intensity occurs from  $\sim 7 \text{ mN/m}$  to  $\sim 11 \text{ mN/m}$ . This change occurs just after

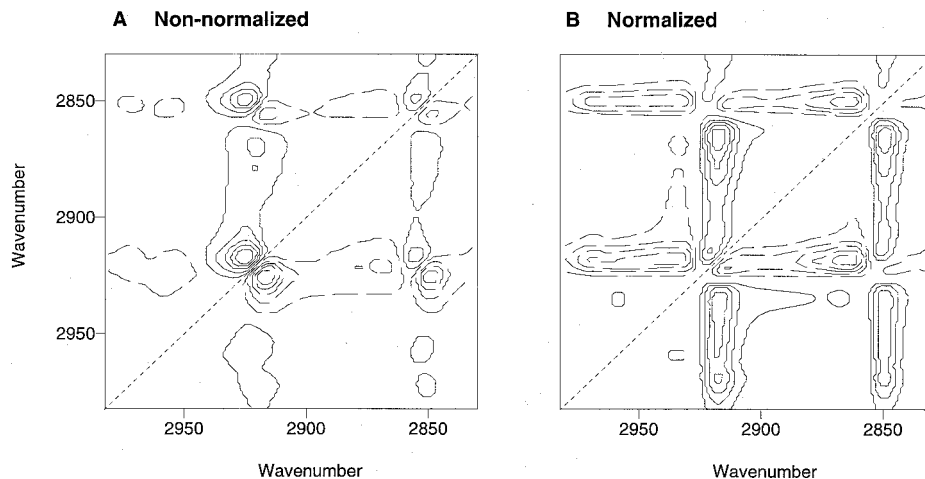


FIG. 3. Experimentally measured 2D-IR asynchronous correlation maps for the low-pressure region subset of spectra collected between 0 and 11 mN/m of the DPPC monolayer at the A/W interface. Solid lines indicate regions of positive correlation intensity, while dashed lines indicate regions of negative correlation intensity. (A) Correlations calculated for non-normalized spectra. (B) Correlations calculated for normalized spectra.

the main transition plateau (LE/LC phase transition region) in the surface pressure–molecular area isotherm of the DPPC monolayer and is primarily attributed to the large change in molecular reorientation occurring in this region. Also, in the surface pressure regime greater than  $\sim 11$  mN/m a very minor frequency shift with a minimal amount of IR intensity fluctuation is observed from  $\sim 2920.5$  to  $\sim 2919$   $\text{cm}^{-1}$  for the  $\nu_a$   $\text{CH}_2$  band and from  $\sim 2852$  to  $\sim 2851$  for the  $\nu_s$   $\text{CH}_2$  band. This small frequency shift is also attributed to hydrocarbon conformational changes, since small changes in Fermi resonance interactions, which may explain the shift in the  $\nu_a$   $\text{CH}_2$  band, do not explain the concurrent shift in the  $\nu_s$   $\text{CH}_2$  band.

The experimentally measured 2D-IR asynchronous correlation maps for the DPPC monolayer at the A/W interface are shown in Figs. 3 and 4. For the purposes of this study, we performed cross-correlation analyses on both the non-normalized and the normalized IR spectra. In addition, we have divided the monolayer data set into two distinct regions for analysis: a low-pressure region below  $\sim 11$  mN/m that corresponds to the liquid expand-

ed (LE) and liquid expanded/liquid condensed (LE/LC) isotherm phases and a high-pressure region greater than  $\sim 11$  mN/m that corresponds to the liquid condensed (LC) phase of the DPPC monolayer.

Figure 3A shows the results of the 2D-IR analysis for the non-normalized DPPC monolayer spectra in the low-pressure region below  $\sim 11$  mN/m. Since no information is obtained from the synchronous plots which can help determine the physical source of a frequency shift in IR spectra,<sup>11–14</sup> only the asynchronous plots are presented here. The most dominant cross peaks appear at 2925, 2916, 2855, and 2849  $\text{cm}^{-1}$  and are easily assigned to the  $\nu_a$  and  $\nu_s$   $\text{CH}_2$  stretching modes. Figure 3 shows that most of the information contained in the low-pressure region is obtained from the *non-normalized* spectra (Fig. 3A). The low-pressure normalized spectra are dominated by noise and baseline fluctuations (Fig. 3B).

For the high-pressure region above  $\sim 11$  mN/m the most significant 2D-IR asynchronous cross peaks are located at approximately 2922, 2915, 2852, and 2949  $\text{cm}^{-1}$  (see Fig. 4B). These peaks are also easily assigned to the  $\nu_a$  and  $\nu_s$   $\text{CH}_2$  stretching modes. For the normalized DPPC mono-

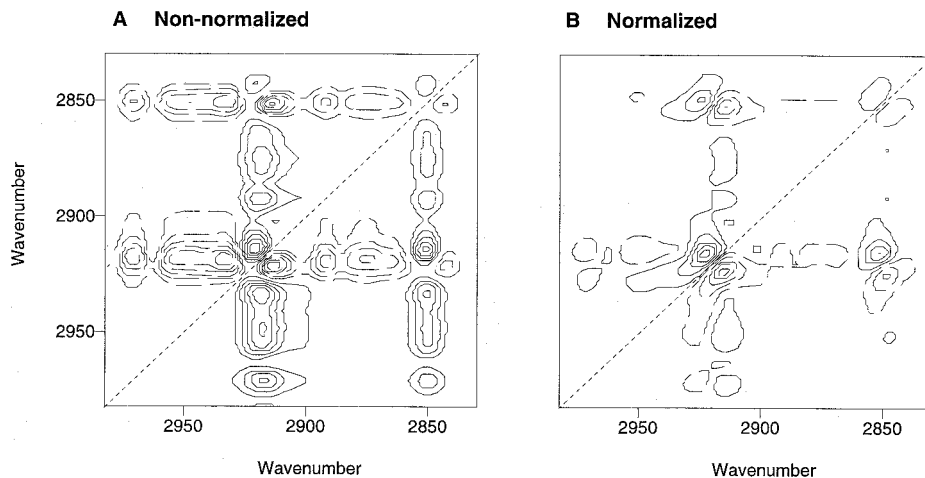


FIG. 4. Experimentally measured 2D-IR asynchronous correlation maps for the high-pressure region subset of spectra collected between 12 and 50 mN/m of the DPPC monolayer at the A/W interface. Solid lines indicate regions of positive correlation intensity, while dashed lines indicate regions of negative correlation intensity. (A) Correlations calculated for non-normalized spectra. (B) Correlations calculated for normalized spectra.

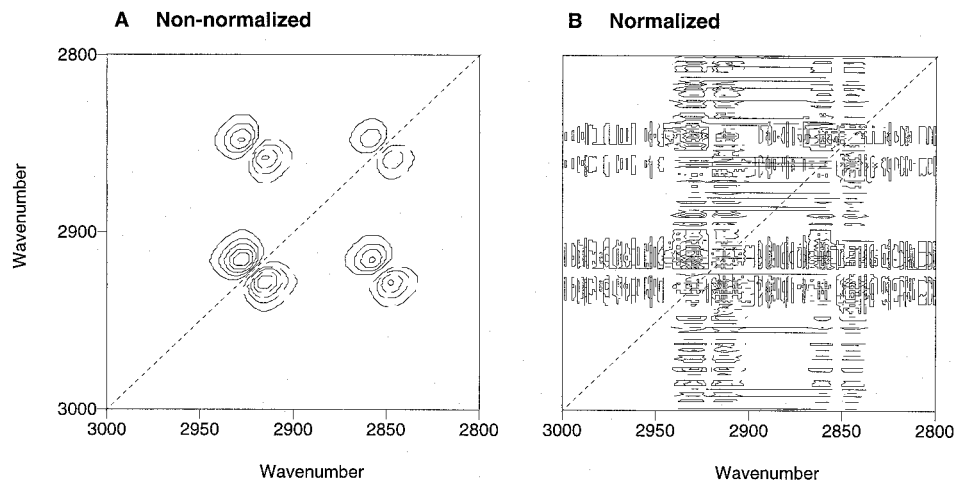


FIG. 5. Computer simulated 2D-IR asynchronous correlation maps for the “overlapped peaks” model in which two closely related peaks representing ordered and disordered frequencies change relative intensity but do not shift frequency. Details of the conditions of this model are given in Ref. 13. (A) Correlations calculated for non-normalized spectra. (B) Correlations calculated for normalized spectra.

layer spectrum, a cross peak quartet and curved elongations along the diagonal are observed that correspond to a peak shift range of  $\sim 2920$  to  $\sim 2919$   $\text{cm}^{-1}$  for the  $\nu_a$   $\text{CH}_2$  band and  $\sim 2952$  to  $\sim 2951$   $\text{cm}^{-1}$  for the  $\nu_s$   $\text{CH}_2$  band (Fig. 4B). However, in contrast to the low-pressure region, a more distinctive pattern in the high-pressure region is observed in the *normalized* asynchronous plot rather than the non-normalized asynchronous plot (compare Figs. 4A and 4B). The non-normalized asynchronous plot for the high-pressure region above 11 mN/m is dominated by noise and baseline fluctuations (see Fig. 4A).

#### Two-Dimensional Infrared Spectral Simulations.

Since frequency shifts and band splitting in 2D-IR spectra may be due to several causes, computer simulations were undertaken to help elucidate the exact cause of the observed splitting in the low-pressure region DPPC 2D asynchronous spectrum. For the present study, synthetic monolayer IR spectra were calculated for two limiting cases. The first limiting case was an “overlapped peaks” model in which an overall vibrational band was calculated as the sum of two individual sub-bands whose frequencies remained constant but whose relative intensities

changed through the simulated monolayer transition. A total of 11 synthetic spectra were used to calculate this model. The second limiting case was a “frequency shifting” model in which a single band undergoes a simple frequency shift. Nine synthetic spectra were generated to represent this model. A detailed description of these models is given elsewhere.<sup>13</sup>

The results of these computer simulations are presented in Figs. 5 and 6. For the “overlapped peaks” model, the simulated 2D asynchronous spectrum resulting from two overlapped peaks shows a correlation intensity cross peak doublet, one positive and one negative with no elongation along the diagonal (Fig. 5). In contrast, the computer simulations showed that a simple frequency shift (as simulated in the “frequency shifting” model) could be distinguished in the simulated 2D asynchronous spectrum by the presence of a cross peak quartet, two with positive correlation intensities, and two with negative (Fig. 6). In addition, a curved elongation of these cross peaks along the diagonal was associated with this frequency shift. Similar results have been seen in previous 2D-IR simulations.<sup>11,12</sup>

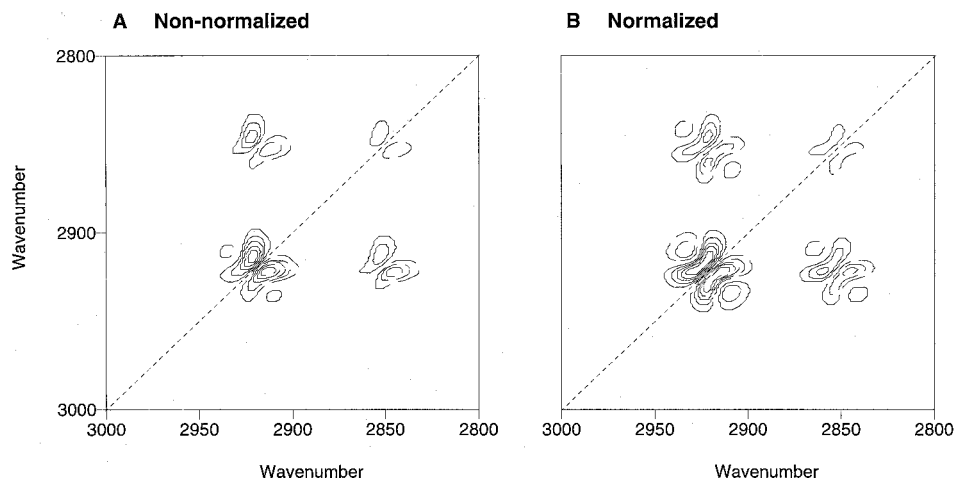


FIG. 6. Computer simulated 2D-IR asynchronous correlation maps for the “frequency shifting” model in which a single IR band undergoes a shift in frequency through the monolayer phase transition. Details of the conditions of this model are given in Ref. 13. Correlations calculated for non-normalized spectra. (B) Correlations calculated for normalized spectra.

In the present study, the results of the 2D-IR simulations depend upon whether normalized or non-normalized surface pressure-resolved simulated IR spectra are each used in a correlation analysis. The effect of intensity normalization on dynamic IR spectra has recently been described.<sup>18</sup> In the present case of monolayer IR spectra, as the mole fractions of the two components in the “overlapped peaks” model vary under an external perturbation the non-normalized spectral intensities for the two components vary out of phase with each other, and correlation cross peaks are observed in the asynchronous spectrum (Fig. 5A). However, if the spectral intensities are normalized, the spectral intensities vary anti-phase to each other, and no correlation cross peaks are observed in the corresponding asynchronous spectrum (Fig. 5B). Experimentally, as changes in spectral intensities approach this ideal anti-phase relationship, the cross peaks observed in the asynchronous plot diminish in correlation intensity; specifically, the noise and baseline fluctuations become more apparent as the correlation intensities associated with IR transitions decrease.<sup>12</sup>

For the “frequency shifting” model, cross-correlation peaks are observed in the 2D-IR asynchronous spectrum calculated from both the normalized IR spectra and the asynchronous spectrum calculated from the non-normalized IR spectra. Furthermore, the frequency shifting pattern is more defined in the asynchronous plot calculated by using the normalized data.<sup>11,13</sup>

**Comparison of Experimental and Simulated 2D-IR Spectra.** A comparison of the experimentally obtained 2D-IR cross-correlation maps for both normalized and non-normalized surface pressure-resolved spectra with the simulations performed for the two limiting case scenarios can be used to help elucidate the cause of the observed band splitting and frequency shifts that are observed in the original monolayer IR spectra.

Figure 3 presents the 2D-IR correlation analysis for the experimental IR monolayer spectra in the low-pressure region below  $\sim 11$  mN/m. The most dominant cross peaks appear at 2925, 2916, 2855, and 2849  $\text{cm}^{-1}$  and are easily assigned to the  $\nu_a$  and  $\nu_s$   $\text{CH}_2$  stretching modes. Since these cross peaks contain the highest correlation intensities, we will restrict our analysis to these peaks. When non-normalized monolayer spectra are used, Fig. 3A clearly shows  $\text{CH}_2$  bands both split into two components in the low-pressure region. The resulting 2D-IR asynchronous map closely resembles the non-normalized “overlapped peaks” model (compare Fig. 3A with Fig. 5A). The peak splitting corresponds to the peak maxima range of  $\sim 2925$   $\text{cm}^{-1}$  to  $\sim 2920$   $\text{cm}^{-1}$  for the  $\nu_a$   $\text{CH}_2$  band and  $\sim 2956$   $\text{cm}^{-1}$  to  $\sim 2952$   $\text{cm}^{-1}$  for the  $\nu_s$   $\text{CH}_2$  band in the IR spectra. While the non-normalized low-pressure monolayer spectra show characteristic band splitting, the *normalized* spectra are dominated by noise and baseline fluctuations. This feature is in agreement with the normalized “overlapped peaks” model (compare Fig. 3B with Fig. 5B).

A different situation exists in the high-pressure region above  $\sim 11$  mN/m. Figure 4 presents the 2D-IR correlation analysis for the experimental IR monolayer spectra in the high-pressure region. The most notable features in Fig. 4 are the quartet of cross peaks and curved elongations along the diagonal that are observed in the nor-

malized spectra. This pattern corresponds to the peak shift range of  $\sim 2920$   $\text{cm}^{-1}$  to  $\sim 2919$   $\text{cm}^{-1}$  for the  $\nu_a$   $\text{CH}_2$  band and  $\sim 2952$   $\text{cm}^{-1}$  to  $\sim 2951$   $\text{cm}^{-1}$  for the  $\nu_s$   $\text{CH}_2$  band in the IR spectra. These patterns compare well with the “frequency shifting” model in which a single band undergoes a simple shift in band frequency (compare Fig. 4B with Fig. 6B). In contrast with the low-pressure data, it is the *non-normalized* asynchronous correlation map that is dominated by noise and baseline fluctuations in the high-pressure monolayer data (see Fig. 4A).

While the simulations shown in Figs. 5 and 6 and the corresponding experimental spectra shown in Figs. 3 and 4 illustrate the correspondence of the 2D-IR theory and experimental monolayer IR spectra, care must be exercised in the interpretation of these 2D-IR correlation maps. In particular, problems with baseline fluctuations are very important in the 2D analysis of monolayer IR spectra. This effect has been described in detail by Czarnecki,<sup>12</sup> and the effect can be nicely illustrated in the 2D-IR asynchronous maps constructed from the low-pressure and high-pressure experimental monolayer spectra (see Figs. 3B and 4A).

Other groups have previously used 2D-IR to investigate the C–H region of macromolecules. Two studies, in particular, are relevant to the current situation of co-existing ordered and disordered conformational states in a monomolecular film. First, a 2D-IR study of temperature-induced changes in nylon 12 discovered a splitting in the C–H stretching modes in the 2D asynchronous spectrum.<sup>19</sup> In this case, the authors attributed the observed band splitting to the presence of crystalline and amorphous forms of the nylon polymer, an analogous situation to that encountered with co-existing ordered and disordered regions in monomolecular films. In the second instance, a splitting of the antisymmetric methylene and methyl stretching modes was reported in a 2D-IR study of poly( $\beta$ -hydroxybutyrate) using a sinusoidal strain perturbation and a dynamic IR linear dichroism method.<sup>20</sup> In this case, the splitting of both the  $\nu_a$  and  $\nu_s$   $\text{CH}_2$  vibrational modes was again seen in the 2D asynchronous correlation spectrum. The pattern of the  $\text{CH}_2$  splitting observed in the 2D asynchronous map in this study matches the pattern observed by us for the DPPC monolayer and the “overlapped peaks” model. As in the previous study, the splitting seen in the 2D spectrum was attributed to the presence of crystalline solid and noncrystalline disordered regions in poly( $\beta$ -hydroxybutyrate).

In contrast to these studies is a recent paper by Nabet et al., who performed a 2D-IR study on the temperature-dependent phase transitions in bulk DPPC multilayer dispersions in water, in which a change in sample temperature was used as the external perturbation.<sup>14</sup> These authors concluded that the frequency shift observed in the IR spectra is predominately caused by the simple frequency shift of a single peak, and that the frequency shift has a linear relationship with the isomeric ordering of the sample. Our current results for the ordered region of the DPPC monolayer agree with these authors’ results for the ordered region of the DPPC multilayer dispersion. However, our results for the expanded region of the monolayer isotherm indicate the presence of two overlapped peaks correlated with an ordered and a disordered phase in the monolayer. This pattern differs from the results obtained

by Nabet et al. for their high-temperature, liquid-crystalline phase of the DPPC multilayer dispersions, in which their data can best be explained by a one-phase, frequency-shifting model.

Recently a study of bulk phase, multilayer phospholipid phase transitions using FT-IR spectroscopy and singular value decomposition analysis was performed by Kota et al.<sup>21</sup> This group concluded that two separable contributions to the spectral intensity exist for the ordered and the disordered segments of the phospholipid acyl chain, and that the change in the relative intensity of these bands accounts for the frequency shift observed in the both the  $\nu_a$  and  $\nu_s$  CH<sub>2</sub> bands. The results of this study are in agreement with our interpretation of co-existing ordered and disordered phases in the LE/LC transition region of DPPC monolayer films.

## CONCLUSION

The 2D-IR asynchronous spectra of the low-pressure region of DPPC monolayer films at the A/W interface show that the liquid expanded-to-condensed LE/LC phase transition region is best modeled by two overlapped peaks in the C-H stretching region. The frequencies of these two sub-bands indicated that they may be correlated with ordered and disordered conformational states of the DPPC monolayer film, as previously described.<sup>8</sup> In contrast, the high-pressure region associated with the LC phase is best modeled by a single peak that undergoes a frequency shift. The frequency of this latter peak indicates an ordered monolayer film, and the minor frequency shift observed in the high-pressure regions is primarily correlated with a gradual packing of the liquid condensed structure. These results support the interpretation that the sub-bands observed in the polarized monolayer IR spectra and reported by our laboratory are correlated with ordered and disordered conformational states.

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