Application of 2D IR correlation analysis to phase transitions in Langmuir monolayer films

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Abstract

The surface pressure-dependent conformational state of a monolayer film of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) at the air–water (A/W) interface was studied using infrared external-reflection spectroscopy and two-dimensional infrared (2D IR) correlation analysis. When the IR spectra of the DPPC monolayer was collected using polarized IR radiation, a band splitting was observed in both the antisymmetric ($v_a$) and symmetric ($v_s$) methylene CH$_2$ stretching modes that was not observed with unpolarized radiation. This band splitting was interpreted as being due to the presence of co-existing ordered and disordered conformational states, however, definitive identification of conformational sub-bands is problematic due to the low signal-to-noise inherent in the polarized IR spectra. To further investigate the spectral changes observed in the C–H region, 2D IR correlation analysis was applied to a set of pressure-dependent unpolarized IR spectra of the DPPC monolayer. When these unpolarized spectra were analyzed using 2D IR methods, the 2D asynchronous correlation spectrum of the DPPC monolayer clearly showed that cross peaks attributable to the $v_a$ and $v_s$ CH$_2$ bands both split into two components, in agreement with the polarized IR monolayer spectra. Since 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 DPPC 2D asynchronous spectrum. Synthetic monolayer IR spectra were calculated for two limiting cases. The first was a ‘frequency shifting’ model in which a single band underwent a simple frequency shift. The second 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. The results of the computer simulations indicated that a simple frequency shift could be distinguished in the 2D asynchronous spectrum by the presence of a quartet of cross peaks, two with positive correlation intensities, and two with negative. In addition, a curved elongation of these cross peaks along the diagonal was associated with this frequency shift. In contrast, the 2D asynchronous spectrum for two overlapped peaks resulted in a correlation intensity cross peak doublet, one positive and one negative with no elongation along the diagonal. The experimentally measured 2D IR asynchronous correlation spectrum for the DPPC monolayer closely resembled the computer-simulated spectra for the ‘overlapped peaks’ model. Therefore, the origin of the band splitting in the $v_a$ and $v_s$ CH$_2$ bands in the 2D asynchronous spectrum is due to overlapping sub-bands that represent the ordered and disordered conformational states of the monolayer. Furthermore, these results also support the
interpretation that the sub-bands observed in the polarized monolayer IR spectra are correlated with ordered and disordered monolayer states. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

1.1. IR spectroscopy at the air–water interface

In 1985 Dluhy and Cornell published the first paper which reported that IR reflection spectroscopy could be used to acquire spectra of monomolecular films on aqueous substrates [1]. Since this initial proof of applicability to monomolecular films at the air–water (A/W) interface, infrared external reflection–absorption spectroscopy has been utilized to study many different types of insoluble monolayers, and there is a growing body of literature in this field (see, [2,3]). Although the major application of this method has been in the conformational analysis and phase transitions of model monolayers, there also have been several groups that have applied IR spectroscopy to study monolayer headgroup interactions as well as polymer and peptide monolayers [4–8].

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 [9,10], 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 and is highly dependent on the angle of incidence employed [11–13].

Recent experiments in our laboratory have focused on the use of polarized IR external reflection–absorption spectroscopy to study biophysical monolayers [14]. In experiments with well-defined single component monolayers, we observed a band splitting in the methylene stretching vibrations in the C–H stretching region of the spectrum 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 [14] (Faucher and Dluhy, manuscript submitted).

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 infrared correlation spectroscopy (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 2D IR methods 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. The results of these experiments are the subject of this paper.

1.2. 2D IR correlation analysis: background

2D IR is a recently-developed analytical method that aids in the interpretation of complex spectra, especially spectra with the kind of broad, multiply overlapped peaks commonly encountered in condensed phase vibrational spectroscopy. As such, 2D IR may be considered as another tool for spectral resolution enhancement, much as curve fitting, spectral derivatives or deconvolution are mathematical techniques used to
identify the number and position of underlying bands in a complex bandshape [15,16]. In the case of 2D IR, the resolution of overlapping spectral features is enhanced by subjecting the sample to an environmental perturbation. The nature of this perturbation is arbitrary and can be any physical or chemical modification to the sample, the only requirement being that this modification must impart some kind of measurable change in the resulting spectrum. In 2D IR, the variations in the IR spectra obtained as a result of the sample perturbation are then mathematically cross-correlated to produce a 2D 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 external perturbation. Spectral resolution enhancement results when the sample perturbation affects the vibrational modes occurring at different wavelengths in a different manner.

The mathematical formalism for 2D IR was first introduced by Noda in 1986 [17]. In this original formalism, the correlation analysis was limited to macromolecular systems in which the external perturbation to the sample was restricted to having a simple time-dependent sinusoidal waveform [18]. In 1993, however, Noda introduced a generalized method for obtaining 2D correlation spectra using external perturbations having any arbitrary waveform that may be a function of time or other physical variable [19]. The mathematical formalism for this generalized method was somewhat more complicated than the original method, in that it required the complex Fourier transformation of dynamic spectra. Recently, a modification of the generalized method was introduced which uses the discrete Hilbert transform in place of the complex Fourier transform [20]. The new Hilbert transform approach substantially simplifies the computational difficulties associated with the generalized method.

The 2D IR correlation spectra are characterized by two independent wavenumber axes ($v_1$, $v_2$) and a correlation intensity axis. In general, two types of spectra are obtained, commonly referred to as the 2D synchronous spectrum and the 2D asynchronous spectrum. Vibrational modes that are significantly coupled, or whose transition dipole moments change in-phase at similar rates in response to the external sample perturbation (i.e. modes that are synchronized) appear in the 2D synchronous spectrum. Conversely, bands that are significantly decoupled, or whose transition dipole moments respond out-of-phase at different rates to the external sample perturbation (i.e. modes that are asynchronized) appear in the 2D asynchronous spectrum. The correlation intensity in the 2D synchronous and asynchronous maps reflects the relative degree of in-phase or out-of-phase response, respectively.

The 2D synchronous spectra are symmetric with respect to the diagonal line in the correlation map. Intensity maxima appearing along the diagonal are called autopeaks (corresponding to the autocorrelation of perturbation-induced molecular vibrations), and are always positive. Intensity maxima located at off-diagonal positions are called cross peaks (corresponding to the cross-correlation of perturbation-induced molecular vibrations at two different wavenumbers). A pair of cross peaks may be positive or negative.

The 2D asynchronous spectra are antisymmetric with respect to the diagonal line in the correlation map. Only cross peaks located at off-diagonal positions appear in asynchronous spectra; pair of cross peaks consist of two intensity maxima/minima, one of which is necessarily positive and the other necessarily negative. Asynchronous cross peaks represent a lack of strong chemical interactivity, since their presence reflects the mutually independent nature of the reorientation of the dipole moments of the molecule’s functional groups in response to an external perturbation.

A major advantage of 2D IR correlation spectroscopy is the possibility for enhanced resolution observed in the asynchronous spectra. If two IR dipole transition-moments change orientations independently of each other at different rates, overlapped bands appear as two cross peaks in the asynchronous spectrum. This is true even for heavily overlapped bands for which the corresponding cross peaks may appear close to the diagonal [21].
The temporal, or phase, relationship, between two cross-correlated IR bands changing intensity under a time-dependent perturbation determines the sign of the crosspeaks. Two positive crosspeaks (intensity maxima) are observed in the 2D synchronous spectrum when two transition-moments change orientations identically, and in-phase. Two negative crosspeaks (intensity minima) are observed in the 2D synchronous spectrum when two transition-moments change orientations identically, but anti-phase. A positive and a negative cross peak are observed in the 2D asynchronous spectrum when two transition dipole moments change orientations out-of-phase; specifically, a maximum and a minimum occur when the orientations are 90° out-of-phase.

The interpretation of 2D IR correlation maps can be difficult. The very reason that IR spectroscopy is valuable in molecular analysis, i.e. the highly sensitive nature of vibrational spectra to local environment, means that the 2D synchronous and asynchronous spectra can be complex, even for simple systems. Several articles have appeared that explore the current state-of-the-art for 2D IR spectral interpretation. The effect of commonly encountered changes in IR band parameters, such as frequency, bandwidth, intensity changes and errors in band position have been described by Gericke et al. [22]. Other common complications encountered in spectral analysis, including the effect of noise and baseline fluctuations on 2D spectra, were investigated by Czarnecki [23]. However, neither of these studies specifically addresses the assumptions and complications encountered in the use of 2D IR correlation analysis for external reflection–absorption spectroscopy of Langmuir monolayers. Using computer simulations, we address these questions in this article, and show how this analysis can be used to confirm our polarized monolayer data.

2. Materials and methods

2.1. Surface chemistry

The phospholipid used in these experiments was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC); this material was obtained from Avanti Polar Lipids (Alabaster, AL) at 99+% stated purity and used without further purification. 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⁻¹. The lipid sample was spread onto a Nima 601M Series Langmuir film balance (Nima, Coventry, UK) and allowed to settle for at least 10–15 min before compression began. Subphase water was obtained from a Barnstead (Dubuque, IA) RO pure/Nanopure reverse osmosis/deionization system having a nominal resistivity of 18.3 MΩ cm and a pH of approximately 5.6.

2.2. Fourier transform infrared external reflectance measurements

Infrared external reflection–absorbance spectra of monolayers at the A/W interface were collected using a Perkin-Elmer Spectrum 2000 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), and through an infrared bandpass filter (OCLI, Santa Rosa, CA) onto the surface of a Nima 601M film balance (Coventry, UK) at an incidence angle of 30° to the surface normal. The IR beam reflected off of the subphase, sampling the film, and was recollimated by a second parabolic mirror that directed it onto the focusing mirror of a liquid N₂-cooled narrow band HgCdTe detector. The IR bandpass filter (3300–2000 cm⁻¹) was placed just above the water to reduce any localized heating effect [24]. For polarized spectra, an Al wire-grid polarizer on KRS-5 (Model IGP225, Molelectron Detector Inc., Portland, OR) was used. 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 with the correct polarization characteristics was used as the background spectrum. The sub-
phase 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 25°C with a relative humidity of 70%. The lipid sample in CHCl₃ 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 0.7 to a maximum of 50.0 mN m⁻¹.

External reflection–absorption spectra were collected with 1024 scans at 4 cm⁻¹ resolution, apodized with a Norton-Beer (medium) function, and was Fourier transformed with one level of zero filling. All monolayer IR spectra are presented as reflection–absorption spectra, i.e. $A = \log(\frac{R}{R_0})$ 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.

Fig. 1. Infrared external reflection–absorption spectra of DPPC monolayer films at the A/W interface. Reflection spectra were collected as a function of monolayer surface pressure and were acquired with (A) unpolarized; and (B) parallel ($R_p$) polarized radiation.
using the GRAMS/32 (Galactic Industries, Salem, NH) program, but have not otherwise been smoothed or further processed.

Synthetic spectra were calculated using an Array Basic program written in our laboratory for the GRAMS/32 environment (Dluhy, unpublished). All synthetic spectra were calculated as 50% Lorentzian and 50% Gaussian peak shapes with full widths at half maximum equal to 20 cm$^{-1}$. No noise was added to the synthetic spectra.

2.3. Calculation of 2D IR correlation spectra

The 2D IR synchronous and asynchronous spectra were calculated using 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 that replaces Fourier transforms with Hilbert transforms [20]. The 2D IR correlation maps were calculated from the unpolarized surface pressure-resolved IR monolayer spectra shown in Fig. 1A. The average spectrum was subtracted from each spectrum in the set prior to the cross correlation analysis. The synchronous spectrum, $\Phi(v_1, v_2)$, and the asynchronous spectrum, $\Psi(v_1, v_2)$, were then calculated using Eqs. (1) and (2).

$$
\Phi(v_1, v_2) = \frac{1}{n-1} \sum_{j=1}^{n} y(v_1, P_j)y(v_2, P_j)
$$

$$
\Psi(v_1, v_2) = \frac{1}{n-1} \sum_{j=1}^{n} y(v_1, P_j) \sum_{k=1}^{n} M_{jk}y(v_2, P_k)
$$

In Eqs. (1) and (2), $v_1$ and $v_2$ represent two independent frequencies, $n$ represents the number of spectra used in the calculation, and $M_{jk}$ is the Hilbert transform matrix, which is defined in Eq. (3).

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

To reduce the effect of noise in the experimental 2D IR spectra, correlation intensities less than 1% of the full scale were cut, as previously described [25].

3. Results and discussion

3.1. 2D IR spectroscopy at the air–water interface: experiments

Fig. 1A presents the unpolarized external reflection IR spectra of a monomolecular film of
DPPC in the C–H stretching region between 3000–2800 cm\(^{-1}\). To acquire these spectra, the monolayer film was applied to the surface of the film balance, allowed to equilibrate, and then compressed step-wise over a range of surface pressures from 0.7 to 50.0 mN m\(^{-1}\) with spectra collected during the interval between each successive compression. Fig. 1A presents monolayer IR spectra obtained with unpolarized incident radiation. Clearly evident in the spectra are the antisymmetric (\(v_a\)) C-H\(_2\) stretching band (\(\sim 2920\) cm\(^{-1}\)), the symmetric (\(v_s\)) C-H\(_2\) stretching band (\(\sim 2850\) cm\(^{-1}\)) and the antisymmetric C-H\(_3\) stretching band (\(\sim 2960\) cm\(^{-1}\)). The symmetric methyl vibration (\(\sim 2860\) cm\(^{-1}\)) is barely apparent as a slight shoulder on the symmetric C-H\(_2\) 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 \(v_a\) CH\(_2\) band maximum shifts from \(\sim 2925\) to \(2919\) cm\(^{-1}\) while the \(v_s\) CH\(_2\) band maximum shifts from \(\sim 2855\) to \(2851\) cm\(^{-1}\). The shift of the CH\(_2\) peak frequency to lower wavenumbers has long been used to distinguish hydrocarbon order in a variety of alkane systems \[26\]. The magnitude of the wavenumber shifts for the 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 \[2,3\].

Fig. 1B also presents the C–H region of external reflection IR spectra of DPPC monolayers at the A/W interface, but unlike the spectra shown...
in Fig. 1A, these spectra were acquired with parallel polarized \( (R_p) \) incoming radiation. Even taking into account the lower signal-to-noise ratio of the polarized spectra, a comparison of Fig. 1A and B clearly show a subtle but reproducible splitting of the \( v_a \) \( \text{CH}_2 \) vibration at 2920 cm\(^{-1}\). Two sub-bands are observed at low surface pressures that are directly related to the populations of ordered and disordered conformational states [14] (Faucher and Dluhy, manuscript submitted). As seen in Fig. 1B, these sub-bands appear to merge at intermediate pressures. At higher surface pressures, the overall band shape appears to shift to lower wavenumber, indicating a mostly ordered conformational state in the monolayer. Using curve-fitting procedures based on these polarized spectra, the fractional conformational state of the monolayer as a function of surface pressure can be determined [14].

While these experiments show that polarized IR spectra are capable of distinguishing monolayer conformational states, the relatively poor signal-to-noise ratio in these polarized spectra limits our ability to use IR spectroscopy to more fully quantify monolayer properties. In order to increase our ability to interpret IR monolayer spectra, we have applied 2D IR correlation methods to study the surface-pressure induced dynamical changes apparent in the IR spectra of DPPC monolayers at the A/W interface. In particular, we studied whether 2D IR methods are able to confirm the band splitting observed in the C–H region of polarized IR monolayer spectra.

For all the 2D IR analyses described here, we used the unpolarized IR monolayer spectra from Fig. 1A. The results of these 2D calculations for the DPPC monolayer IR spectra are shown in Figs. 2 and 3. Fig. 2A represents the synchronous correlation map obtained from the cross-correlation of the C–H stretching region (3000–2800 cm\(^{-1}\)) of the DPPC monolayer at differing surface pressures. Two auto peaks, six positive cross
peaks and two negative cross peaks are observed in the synchronous map shown in Fig. 2A. Of all the peaks observed in this 2D spectrum, the three main peaks at 2959, 2919 and 2851 cm\(^{-1}\) are readily assigned to the \(v_a\) CH\(_3\), \(v_a\) CH\(_2\), and the \(v_s\) CH\(_2\) stretching modes, respectively. The CH\(_2\) and CH\(_3\) cross peaks all have positive correlation intensities, indicating an in-phase reorientation of transition moments as the surface pressure increases for these modes. This agrees with the one-dimensional (1D) spectra in Fig. 1 where it is seen that these peaks increase in intensity as the surface pressure increases. The largest autopeaks and cross peaks appear at 2919 and 2851 cm\(^{-1}\) since the \(v_a\) and \(v_s\) CH\(_2\) stretching bands experience the largest relative intensity increase in the monolayer spectra [27].

The 2D IR asynchronous correlation spectrum for the C–H stretching region of the DPPC monolayer is shown in Fig. 2B. The most dominant cross peaks appear at 2923, 2916, 2854, and 2849 cm\(^{-1}\). As in the synchronous spectrum, these peaks can be assigned to the \(v_a\) and \(v_s\) CH\(_2\) stretching modes. Similarly, two cross peaks at 2960 and 2956 cm\(^{-1}\) are assigned to the \(v_a\) CH\(_3\) stretch. The peak at 2865 cm\(^{-1}\) may be attributed to the \(v_s\) CH\(_3\) mode but with a large degree of uncertainty because of the low correlation intensity. As well, the peaks at 2899 and 2890 cm\(^{-1}\) have very low correlation intensities and are of uncertain origin. Since the cross peaks at 2923, 2916, 2854, and 2849 cm\(^{-1}\), attributable to the \(v_a\) and \(v_s\) CH\(_2\) stretching modes, contain the highest correlation intensities, we will restrict our analysis to these peaks.

It is clear from the 2D IR asynchronous spectrum presented in Fig. 2B that both the \(v_a\) and \(v_s\) CH\(_2\) bands split into two components, in agreement with the polarized IR monolayer spectra of DPPC (Fig. 1B). These components are located at 2923 and 2916 cm\(^{-1}\) for the \(v_a\) CH\(_2\) band and at 2854 and 2849 cm\(^{-1}\) for the \(v_s\) CH\(_2\) band. The asynchronous map further shows that the band splitting results in one positive and one negative band, as predicted by 2D IR theory [21]. This band splitting, as well as the positive and negative bands, is further illustrated in Fig. 3A, which is a 3D wire frame representation of the asynchronous spectrum of the entire CH\(_2\) region shown in Fig. 2B. A similar 3D wire frame plot of only the \(v_a\) CH\(_2\) peak from the 2D IR asynchronous map is presented in Fig. 3B, providing a close up view of

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1 In all the 2D correlation maps presented in this article, a solid line indicates that the peak has a positive correlation intensity, while a dashed line indicates that the peak has a negative correlation intensity.
the band splitting and the positive–negative doublet for this peak.

3.2. 2D IR spectroscopy at the air–water interface: computer simulations

It is known that the coexistence of two overlapped sub-bands can result in the formation of cross peaks near the diagonal in the asynchronous spectrum [21]. However, just a simple frequency shift of one IR band can also result in the formation of cross peaks near the diagonal. For example, Gericke et al. have published synthetic 2D IR spectra for a peak undergoing a simple frequency shift [22]. Czarnecki has also published synthetic 2D spectra for a peak undergoing a frequency shift as well as for a peak containing two sub-bands that change intensity in the same direction with slightly different rates [23]. Both these studies show that a band splitting observed in the asynchronous 2D spectrum can be due solely to a frequency shift without the presence of underlying sub-bands.

While these previous studies provide valuable hints in how to interpret 2D IR spectra, they do not directly address the situation that pertains in the monolayer IR experiment. In particular, the effect of the simultaneous changes in the characteristic frequencies, intensities, bandwidths and number of sub-bands found in monolayer IR spectra has not been adequately addressed. To help determine the physical cause of the splitting in the 2D IR asynchronous spectrum of DPPC monolayers, we have simulated 2D IR synchronous and asynchronous spectra for two possible physical causes under the specific conditions pertaining to the monolayer experiment and compared the simulated 2D spectra with the experimental 2D spectra.
Our computer simulated monolayer spectra fall into two limiting cases, one in which a single band shifts frequency only, and one in which an IR band shape is composed of two sub-bands that do not change frequency, but only relative intensity. We refer to the simulated spectra for the case in which a single IR peak undergoes a simple frequency shift as the ‘frequency shifting’ model. We refer to the simulated spectra for the case in which one overall IR bandshape is composed two overlapping sub-bands as the ‘overlapped peaks’ model. In the ‘overlapped peaks’ model one sub-band decreases in intensity while the other increases in intensity as the simulated monolayer surface pressure increases. In our calculations we have simulated the C–H stretching region from 3000–2800 cm\(^{-1}\) as being composed of two major bands, corresponding to the \(v_a\) CH\(_2\) band at \(\sim 2920\) cm\(^{-1}\) and the \(v_s\) CH\(_2\) band at \(\sim 2850\) cm\(^{-1}\). To accurately simulate the experimental data, the following conditions were applied to the synthetic monolayer spectra. (1) IR band intensities increased as the surface area available to the monolayer decreases; (2) the intensity ratio of the \(v_a\) CH\(_2\) band to the \(v_s\) CH\(_2\) band was assumed to be 100:75; (3) spectral lineshapes used were 50% Gaussian and 50% Lorentzian in character; (4) 20 cm\(^{-1}\) linewidths (full width at half height) were used to simulate broadened condensed phase peaks; (5) the initial band position and the final band position in the ‘frequency shifting’ model corresponds to the positions of the two overlapped sub-bands in the ‘overlapped peaks’ model; and (6) spectral intensities increased proportionately to the decrease in surface area available to the monolayer as the simulated experiment proceeded (i.e. a 5% decrease in surface area resulted in a 5% increase in spectral intensity).

The simulated monolayer spectra used in the calculations for the ‘frequency shifting’ model are shown in Fig. 4. In these spectra, the frequency shift of the \(v_a\) CH\(_2\) band was calculated from 2926...
to 2918 cm\(^{-1}\) in 1.0 cm\(^{-1}\) increments. Conversely, the frequency shift of the \(v_a\) \(\text{CH}_2\) band was calculated from 2856 to 2850 cm\(^{-1}\) in 0.75 cm\(^{-1}\) increments.

The 2D IR synchronous spectrum calculated for the computer simulated spectra corresponding to the ‘frequency shifting’ model is shown in Fig. 5A. All together, there are four autopeaks, four positive cross peaks, and eight negative cross peaks\(^2\). The negative cross peaks are present because certain frequencies change intensity in opposite directions. Specifically, the correlation intensities decrease for the highest wavenumbers and increase for the lowest wavenumbers of both peaks as the bands shift to lower wavenumbers. The appearance of negative cross peaks is in contrast to the measured synchronous spectrum for DPPC. This is due to the fact that the experimental IR monolayer intensities do not decrease in any portion of the methylene band region in the measured IR spectra.

The 2D IR asynchronous map calculated for the computer simulated spectra corresponding to the ‘frequency shifting’ model is shown in Fig. 5B. The most obvious feature of this correlation map is the presence of a quartet of cross peaks, two with positive intensities and two with negative intensities, in each quadrant of the spectrum. In addition, a curved elongation along the diagonal is associated with these asynchronous cross peaks. Similar features in simulated 2D asynchronous spectra have been previously described [22,23]. These features are diagnostic of a single band undergoing a frequency shift.

The 3D wire frame surface plots of the correlation intensities calculated from the 2D asynchronous spectrum of the ‘frequency shifting’ simulated spectra are shown in Fig. 6. Fig. 6A illustrates the entire simulated C–H region, while Fig. 6B presents a closer view of only the calculated, frequency-shifted, \(v_a\) \(\text{CH}_2\) band. The characteristic elongated quartet pattern is evident from this view, although the fourth small negative correlation peak is hidden behind the large positive peak.

The simulated monolayer spectra used in the calculations for the ‘overlapped peaks’ model are shown in Fig. 7. In these spectra the overall antisymmetric \(v_a\) \(\text{CH}_2\) bandshape was calculated as the sum of two individual sub-bands located at 2926 and 2918 cm\(^{-1}\). The peak at 2926 cm\(^{-1}\) was

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\(^2\) Two of the peaks described do not appear in the synchronous spectrum presented in Fig. 5A because of the number of contour levels used. If a larger number of contour levels are used, the peaks are observed; however, more contour levels further emphasise noise and baseline fluctuations, and the spectrum becomes more difficult to interpret.
Fig. 9. A 3D wire mesh surface plot representation of the 2D asynchronous correlation map for the ‘overlapped peaks’ computer simulated monolayer film spectra shown in Fig. 7. Data used for this plot was the same as used to calculate the 2D asynchronous correlation map in Fig. 8B. (A) Surface plot representation of entire C–H region between 3000 and 2800 cm$^{-1}$; (B) surface plot representation of the spectral region between 2980 and 2880 cm$^{-1}$ surrounding the antisymmetric $\nu_a$ CH$_2$ band at ~2920 cm$^{-1}$.

(corresponding to the disordered sub-band) began the calculations at maximum intensity and decreased in 10% increments to a minimum as the simulated surface pressure increased. In contrast, the peak at 2918 cm$^{-1}$ (corresponding to the ordered sub-band) began the calculations at minimum intensity and increased in 10% increments to a maximum as the simulated surface pressure increased. A similar situation existed in the calculations for the symmetric $\nu_s$ CH$_2$ bandshape. This band was also calculated as the sum of two individual sub-bands, now located at 2856 and 2850 cm$^{-1}$. As the simulated surface pressure increased, the peak at 2856 cm$^{-1}$ (corresponding to the disordered sub-band) began the calculations at maximum intensity and decreased in 10% increments to a minimum, while the peak at 2850 cm$^{-1}$ (corresponding to the ordered sub-band) began the calculations at minimum intensity and increased in 10% increments to a maximum. In these calculations only the individual sub-band intensities were varied; the frequencies of the sub-bands stayed constant.

The 2D synchronous and asynchronous correlation maps calculated for the computer-simulated spectra corresponding to the ‘overlapped peaks’ model is shown in Fig. 8. Fig. 8A presents the 2D synchronous spectrum for the ‘overlapped peaks’ model. It is clear from a comparison of Fig. 8A with Fig. 5A that the synchronous correlation maps for the ‘frequency shifted’ and the ‘overlapped peaks’ models are very similar. The resemblance of the 2D synchronous spectra for the two models is related to the inherent assumptions of the synchronous correlation analysis. That is, the intensity of the 2D synchronous spectrum becomes significant if the perturbation-induced spectral changes of the two IR wavenumber axes are
similar to each other. For the case of the two models under consideration here, the overall spectral bandshapes are very similar, regardless of the number of underlying sub-bands. Therefore, since the input spectra for the two models closely resemble each other, the synchronous correlation intensities will also be comparable, as seen in Figs. 5A and 8A.

The 2D asynchronous spectrum for the ‘overlapped peaks’ simulated model is shown in Fig. 8B. In contrast to the similarity observed in the case of the 2D synchronous spectra, it is clear that the ‘frequency shifted’ and ‘overlapped peaks’ models give very different 2D asynchronous spectra (i.e. compare Fig. 8B and Fig. 5B). For the ‘overlapped peaks’ model, a correlation intensity peak doublet, consisting of one positive intensity peak and one negative intensity peak, is observed in each quadrant of the 2D asynchronous spectrum (Fig. 8B). This contrasts with the case of the ‘frequency shifting’ simulation model, in which a correlation intensity peak quartet is observed (Fig. 5B). In further contrast with the ‘frequency shifting’ model, no curved elongation of the cross peaks along the diagonal is observed.

The 3D wire frame surface plots of the correlation intensities calculated from the 2D asynchronous spectrum of the ‘overlapped peaks’ simulated spectra are shown in Fig. 9. Fig. 9A illustrates the entire simulated C–H region, while Fig. 9B presents a closer view of only the calculated, overlapped, νa CH2 bands. The characteristic asynchronous doublet pattern associated with two overlapping sub-bands is evident from this view.

The computer simulations of 2D spectra for the two competing models (‘frequency shifting’, Figs. 5 and 6; and ‘overlapped peaks’, Figs. 8 and 9) can now be used to interpret the measured, experimental 2D spectra for the DPPC monolayer (Figs. 2 and 3). In Fig. 2B, the 2D asynchronous spectrum for DPPC, a clear doublet is observed in each quadrant of the correlation map, and no curved elongation of the cross peaks along the diagonal is seen. This asynchronous correlation doublet for DPPC is further viewed in the surface plots of Fig. 3. In comparison with the two model systems, it is apparent that the experimentally measured asynchronous 2D IR spectrum for DPPC closely resembles the computer-simulated spectra for the ‘overlapped peaks’ model. Therefore, the origin of the splitting in the νa CH2 and νs CH2 bands in the 2D asynchronous spectrum (Fig. 2B) is due to overlapping sub-bands that represent the ordered and disordered conformational states of the monolayer.

Although 2D IR correlation spectroscopy is a recent development, other groups have previously used this technique 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 [28]. 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(β-hydroxybutyrate) using a sinusoidal strain perturbation and a dynamic IR linear dichroism method [27]. In this case, the splitting of both the νa and νs CH2 vibrational modes was again seen in the 2D asynchronous correlation spectrum. The pattern of the CH2 splitting observed in the 2D asynchronous map in this study matches exactly 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 non-crystalline disordered regions in poly(β-hydroxybutyrate).

The presence of cross peaks in a 2D asynchronous spectrum requires that the correlated wavenumbers are responding out-of-phase with each other. The existence of a splitting in the DPPC monolayer 2D spectrum strongly suggests that it is the result of two populations of lipid in the monolayer that exist in slightly different environments, and which are affected differently by the external sample perturbation, i.e. an increase.
in surface pressure. This observation and interpretation is in agreement with the results and conclusions of previous polarized IR experiments reported by our laboratory [14] (Faucher and Dluhy, manuscript submitted).

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