Published in final edited form as:

Vib Spectrosc. 2009 May 26; 50(1): 106–115. doi:10.1016/j.vibspec.2008.09.004.

Phase Behavior of Planar Supported Lipid Membranes Composed of Cholesterol and 1,2-Distearoyl-*sn*-Glycerol-3-Phosphocholine Examined by Sum-Frequency Vibrational Spectroscopy

Jin Liu and John C. Conboy^{*}

University of Utah, Department of Chemistry, 315 S. 1400 E. RM 2020, Salt Lake City, Utah, 84112

Abstract

The influence of cholesterol (CHO) on the phase behavior of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) planar supported lipid bilayers (PSLBs) was investigated by sum-frequency vibrational spectroscopy (SFVS). The intrinsic symmetry constraints of SFVS were exploited to measure the asymmetric distribution of phase segregated phospholipid domains in the proximal and distal layers of DSPC + CHO binary mixtures as a function of CHO content and temperature. The SFVS results suggest that cholesterol significantly affects the phase segregation and domain distribution in PSLBs of DSPC in a concentration dependent manner, similar to that found in bulk suspensions. The SFVS spectroscopic measurements of phase segregation and structure change in the binary mixture indicate that membrane asymmetry must be present in order for the changes in SFVS signal to be observed. These results therefore provide important evidence for the delocalization and segregation of different phase domain structures in PSLBs due to the interaction of cholesterol and phospholipids.

Keywords

vibrational spectroscopy; sum-frequency; phospholipids; cholesterol; planar supported lipid bilayers

Introduction

Cholesterol (CHO) is one of the major components of eukaryotic membranes and plays a key role in regulating the biological function and physical properties of these macromolecular assemblies.[1-3] For example, CHO is responsible for modulating the permeability, fluidity and mechanical strength of cell membranes.[4-9] CHO not only suppresses the phase segregation of phospholipids, but also eliminates phospholipid phase transitions at high CHO concentrations.[10-16] Studies have also suggested that CHO may combine with sphingolipids to form transient clusters, known as “lipid rafts”[17-19], which are believed to be involved in many important cellular processes, such as organizing and segregating membrane components for signaling and sorting.[19,20]

Various analytical techniques have been utilized to study the interaction of CHO with phospholipids. Fluorescence microscopy has been used to visualize phase segregation and

^{*}corresponding author: e-mail: conboy@chem.utah.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

domain formation in binary mixtures of phospholipids + CHO.[16,21,22] Differential scanning calorimetry (DSC) is a common method to study the effect of CHO on the phase transition of phospholipids by measuring the change in heat capacity as a function of temperature.[10-15] Nuclear magnetic resonance (NMR), Raman, and Fourier transform infrared spectroscopy (FTIR) have been used to investigate the effect of CHO on the conformation and order of the phospholipid acyl chains.[11,23-30]

In addition to these linear spectroscopic techniques and thermodynamic methods, a nonlinear spectroscopic technique, sum-frequency vibrational spectroscopy (SFVS), has been demonstrated to be ideally suited to study both the molecular orientation and phase behavior of phospholipids in a single planar supported phospholipid bilayer (PSLB).[31-33] For example, the effect of CHO on the conformational order of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in a monolayer at the water/air interface has previously been investigated by SFVS.[34] The theory of SFVS has been extensively reviewed in the literature.[35-37] Briefly, SFVS is a nonlinear optical spectroscopy which has the chemical selectivity of IR and Raman, and is inherently surface specific in nature. SFVS is forbidden in a medium with inversion symmetry but occurs at an interface between two isotropic media where the inversion symmetry is broken. SFVS is performed by overlapping both spatially and temporally a visible and tunable IR coherent light source at an interface where they combine to produce a photon at the sum of the incident frequencies. The sum-frequency intensity (I_{SF}) is proportional to the square of the susceptibility tensor $\chi^{(2)}$: [35-37]

$$I_{SF} \propto \left| \tilde{f}_{SF} f_{Vis} f_{IR} \chi^{(2)} \right|^2 \quad (1)$$

where $\tilde{f}_{SF}$ and f_{Vis}, f_{IR} are the nonlinear and linear Fresnel coefficients for the generated SF and incident electric fields respectively.[35-37] In the above equations $\chi^{(2)}$ is the sum of a resonant (R) and nonresonant (NR) contributions given by:

$$\chi^{(2)} = \chi_{NR} + \chi_R \quad (2)$$

with the resonant contribution defined as:

$$\chi_R = N \left(\sum_{x=1}^m \frac{\langle A_k M_{ij} \rangle_x}{\omega_{v_x} - \omega_{ir} - i\Gamma_{v_x}} \right) \quad (3)$$

where N is the population density of molecules at the interface, m is the number of vibrational transitions, A_k and M_{ij} are the IR and Raman transition probabilities respectively, ω_{ir} is the frequency of the input IR field, ω_{v_x} is the normal mode vibrational frequency of the transition being probed, and Γ_v is the line width of the transition. As SFVS is a coherent process, phase interference between the nonresonant background (χ_{NR}) and the resonant signal (χ_R) can exist. In order to take this interference into consideration, Equation 2 can be re-written as:

$$\chi^{(2)} = \chi_R e^{i\phi} + \chi_{NR} \quad (4)$$

where ϕ is the phase angle between the resonant vibrational transition (x) and nonresonant background (χ_{NR}), and m is the number of individual resonances. Substitution Equations 3 and 4 into Equation 1, the SFVS intensity can then be expressed as:

$$I_{SFVS} \propto \left| \tilde{f}_{SF} \tilde{f}_{vis} \tilde{f}_{IR} \left(N \left(\sum_{x=1}^m \frac{\langle A_k M_{ij} \rangle_x}{\omega_{v_x} - \omega_{ir} - i\Gamma_{v_x}} \right) e^{i\phi + \chi_{NR}} \right) \right|^2 \quad (5)$$

A SFVS spectrum is obtained by tuning the IR frequency through the vibrational resonances of the molecules at the interface and measuring the intensity of the generated sum-frequency light. The resulting spectrum can then be fit using Equation 5 to obtain information regarding the frequency, linewidth and phase angle of a particular vibrational resonance.

The susceptibility element $\chi^{(2)}$ is also directly related to the sum of the individual molecular hyperpolarizabilities, β_{ijk} , through a statistical average over all molecular orientations:

$$\chi^{(2)} = \frac{N}{\epsilon_0} \langle \beta_{ijk} \rangle. \quad (6)$$

In addition to the interfacial symmetry requirements of SFVS, the symmetry of the molecular species comprising the interface also dictates the observed SFVS response. For example, we have previously shown that the terminal CH_3 groups of the phospholipid acyl chains can be used as an intrinsic probe of the symmetry of the bilayer.[31-33,38,39] For a symmetric phospholipid bilayer, cancellation of the methyl symmetric stretch ($\text{CH}_3 \nu_s$) transition dipoles from the termini of the phospholipid acyl chains in the upper and lower layers will occur resulting in a reduction in the measured SFVS $\text{CH}_3 \nu_s$ oscillator strength. An increase in membrane asymmetry will also result in an increase in the intensity of the $\text{CH}_3 \nu_s$ as the local symmetry is relaxed.⁴⁷

We have used the $\text{CH}_3 \nu_s$ to directly monitor the intrinsic rate of phospholipid transbilayer migration (or flip-flop) by preparing asymmetric phospholipid bilayers in which one leaflet is comprised of perdeuterated phospholipids and the other, perhydrogenated phospholipids.[38, 39] The normal components of the vibrations are probed selectively by examining the s -polarized sum-frequency output with input s -polarized visible, and p -polarized IR. Destructive interference occurs for the normal component of opposing $\text{CH}_3 \nu_s$ transition dipole moments due to the coherent nature of SFVS. However, for an asymmetric arrangement in which a deuterated phospholipid monolayer opposes a perhydrogenated phospholipid layer, no such interface is observed due to the difference in resonant frequencies of the CD_3 and $\text{CH}_3 \nu_s$. By utilizing this symmetry constrain imposed on the measured $\text{CH}_3 \nu_s$ SFVS intensity, the asymmetry of a PSLB can be measured as functions of time and temperature to deduce the kinetics of phospholipid flip-flop without the need for a fluorescent or spin labeled phospholipid probe.

More subtly, this same interference phenomenon can be exploited to measure small perturbations in the distribution of phospholipids and phospholipid domains in the proximal and distal leaflets of a PSLB.[33] For a phospholipid bilayer composed of a single phospholipid species at the phase transition, *gel* and *liquid-crystalline* (*l.c.*) domains are in phase coexistence due to disparities in the phospholipid structures and differences in phospholipid lateral diffusion in these two phases.[40-42] Near the T_m , the presence of structural discontinuities between the *gel* and *l.c.* domains present in the distal and proximal layers of a PSLB results in

a local break in the symmetry of the bilayer. This break in the bilayer symmetry is “sensed” by the $\text{CH}_3 \nu_s$ due to local discontinuities in the membrane structure, dielectric and mobility, [43] resulting in an increase in the $\text{CH}_3 \nu_s$ intensity.[32,33] Utilizing the sensitivity of the $\text{CH}_3 \nu_s$ to membrane asymmetry, the intensity of this resonance as a function of temperature has been used to measure the phase behavior of PSLBs of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DPPC, DHPC (1,2-diheptadecanoic-*sn*-glycero-3-phosphocholine), and DSPC by SFVS.[32,33] Similarly, the local break in membrane asymmetry due to the presence of phase segregated phospholipid domains in the phospholipid mixture of DMPC+DSPC and DOPC+DSPC have also been investigated as a function of the phase state of the membrane.[33]

The current study addresses the effect of CHO on the structure and phase behavior of a PSLB composed of binary mixtures of CHO and 1,2-distearoyl-*sn*-glycerol-3-phosphocholine (DSPC). The SFVS results show that CHO significantly affects the phase segregation and domain distribution in PSLBs of DSPC in a concentration dependent manner, similar to the behavior observed in solution phase liposomes of CHO and DSPC measured by DSC. The SFVS results are consistent with the thermodynamic phase diagram of CHO + DSPC. What is surprising is that at high CHO concentration in the membrane (>40 mol%), a spontaneous asymmetric distribution of phospholipids and CHO is generated. This asymmetry was investigated by exploiting the coherent nature of the SFVS as a means to ascertain the origins of the molecule distribution in each leaflet of the PSLB. The SFVS results presented here provide important evidence for the delocalization of phase segregated domain structures and the induction of phospholipid and CHO asymmetry in DSPC + CHO PSLBs.

Experimental

Materials

Cholesterol (CHO, 99+ %) was purchased from Aldrich. Deuterated CHO (25, 26, 26, 26, 27, 27, 27-D7, CHO-*d*₇, 98 %) and D₂O (99.9 %) were obtained from Cambridge Isotope Laboratories. Gas chromatography grade CHCl_3 was purchased from Mallinckrodt. 1,2-distearoyl-*sn*-glycerol-3-phosphocholine (DSPC), 1,2-distearoyl-D70-*sn*-glycerol-3-phosphocholine (DSPC-*d*₇₀) and 1,2-distearoyl-D70-*sn*-glycerol-3-phosphocholine-1,1,2,2-D4-N,N,N-trimethyl-D9 (DSPC-*d*₈₃) were purchased from Avanti Polar Lipids. All materials were used as received. The molecular structures of CHO, CHO-*d*₇, DSPC, DSPC-*d*₇₀ and DSPC-*d*₈₃ are shown in Figure 1. An IR/UV grade fused silica hemicylindrical prism (Almaz Optics, Marlton, NJ) was used as the substrate for all the SFVS experiments unless otherwise stated, in which case a sapphire hemicylindrical prism (Harrick Scientific) was used.

Preparation of Phospholipid + CHO Bilayers and CHO Monolayers

The Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) method was used to assemble the DSPC and DPSC+CHO bilayers using a KSV Minitrough (KSV Instruments). CHO and DSPC were dissolved individually in chloroform at a concentration of 1 mg/mL. For the DSPC+CHO or DSPC+CHO-*d*₇ bilayers, the CHO was premixed with DSPC at 5, 10, 15, 25, 40, and 60 mol %. The solutions of DSPC, DSPC+CHO and DSPC+CHO-*d*₇ were spread at the air/water interface of the LB trough and compressed to a surface pressure of 30 mN/m at 23 °C.

For the bilayer samples, the first layer of the bilayer was deposited on the prism via a vertical pull of the substrate from the aqueous subphase into air using the LB method. The second layer of the bilayer was deposited by a horizontal dip into the subphase by the LS method. The lipid bilayer membranes were maintained in an aqueous environment after deposition and transferred to a fluid flow cell.

Monolayers of CHO without phospholipids were also prepared in order to compare the spectral signatures of the phospholipids with those arising from CHO. CHO and CHO- d_7 were dissolved individually in chloroform at 1 mg/mL concentration. The LB method was used to prepare the monolayers of both CHO and CHO- d_7 at the air/water interface at a surface pressure of 30 mN/m and a temperature of 23 °C.

SFVS Experiments

Detailed information on the SFVS instrument and experimental setup can be found elsewhere. [33] Temperature control of the cell was accomplished by heating the Teflon cell block with an external circulating water bath (HAAKE PHOENIX II, Thermo Electron Corporation). A type K thermocouple with a resolution of 0.05 °C and an accuracy of 0.2 °C was used to measure the temperature of the solution above the bilayer. SFVS spectra were collected with *s*-polarized sum-frequency, *s*-polarized visible, and *p*-polarized IR (*ssp*). Data points were collected every 2 cm⁻¹ by averaging 100 laser pulses. For the temperature scans, the temperature was increased at a rate of 0.24 °C per minute unless otherwise stated. The data were obtained by continuously monitoring the CH₃ ν_s intensity at 2876 cm⁻¹ with *s*-polarized sum-frequency, *s*-polarized visible, and *p*-polarized IR. The CH₃ ν_s intensity was averaged for 5 seconds (50 laser pulses).

Results and Discussion

Spectral Assignments

SFVS spectra (Figure 2a) were taken of symmetric PSLBs of DSPC with and without CHO- d_7 at 23 °C, well below the *gel* to *liquid-crystalline* (*l.c.*) phase transition temperature (T_m) of DSPC (54.5 °C).[10] The spectra were recorded in the frequency range from 2800 cm⁻¹ to 3050 cm⁻¹ which encompasses the C-H vibrational modes of the alkyl chains of DSPC and the C-H resonances from CHO- d_7 . The SFVS spectra of a pure DSPC bilayer and those containing 5, 10, 15, 25, 40 and 60 mol% CHO- d_7 all have similar resonances. The peaks at 2848 cm⁻¹ and 2876 cm⁻¹ are assigned to the CH₂ ν_s and CH₃ ν_s from the fatty acid chains of the phospholipid, while the small and broad peak centered at 2964 cm⁻¹ is a combination band of the CH₃ antisymmetric stretch (ν_{as}) from the terminal methyl groups of phospholipid alkyl chains and from undefined resonances arising from CHO,[31,32,44-48] which was verified by the following experiments.

In order to distinguish the SFVS resonances of the phospholipid matrix from those of CHO, several control experiments were performed. Of particular importance is the origin of the peaks at 2876 cm⁻¹ and 2964 cm⁻¹, as the amplitudes of these resonances increase with increasing CHO content in the membrane as seen in the spectrum of 60 mol% CHO- d_7 in a DSPC/DSPC bilayer compared to that of a pure DSPC bilayer. As a control, the spectrum of 60 mol% CHO- d_7 in a deuterated DSPC- d_{83} /DSPC- d_{83} bilayer was also obtained, Figure 2b. The difference spectra of 60 mol% CHO- d_7 in a DSPC/DSPC bilayer and 60 mol% CHO- d_7 in a DSPC- d_{83} /DSPC- d_{83} bilayer are shown in Figure 2c. Figure 2c reveals that the peak at 2876 cm⁻¹ is predominantly due to DSPC, while the peak at 2964 cm⁻¹ is from CHO- d_7 . [47] Also apparent is a peak at 2940 cm⁻¹ arising from the Fermi resonance of the terminal CH₃ groups on the phospholipid alkyl chains. This peak is negative in the difference spectrum due to interference with the adjoining peak at 2964 cm⁻¹.

In order to prevent any spectral congestion in the C-H stretching region of the phospholipids, in particular to avoid overlap of the symmetric stretch of the terminal methyl groups of the alkyl chains with resonances from CHO, selectively deuterated CHO (CHO- d_7), was used in all the studies presented here. To illustrate the importance of using CHO- d_7 , the spectra of a monolayer of CHO and CHO- d_7 at the SiO₂/air interface are presented in Figure 3. For CHO, the CH₂ ν_s at 2856 cm⁻¹ and the CH₃ ν_s at 2880 cm⁻¹ are visible. In addition, the CH₂ ν_{as} at

2912 cm^{-1} is also observed.[45-47,49] A large broad peak centered at 2964 cm^{-1} is also seen, and most likely arises from the unresolved C-H vibrations of the rings and side chains.[47, 48] The spectrum of CHO- d_7 is similar to that of CHO except for the absence of the $\text{CH}_3 \nu_s$ at 2880 cm^{-1} . The use of CHO- d_7 effectively reduces the SFVS response due to CHO in the region of the phospholipid $\text{CH}_3 \nu_s$ at 2876 cm^{-1} . Contributions from CHO- d_7 in this spectral region are not completely eliminated as examination of Figure 3 reveals a small resonance at 2876 cm^{-1} ; however, this represents less than 5 % of the total signal when compared to the $\text{CH}_3 \nu_s$ of DSPC alone. A more detailed spectroscopic analysis of the vibrational resonances arising for CHO is also presented below.

CHO+DSPC SFVS Results

The use of a vibrational spectroscopic method for the characterization of the phase behavior of phospholipid+CHO bilayers usually requires a unique spectral signature to be identified which is correlated to structural changes in the membrane. Previous IR and Raman studies have used the C-H and C-C vibrational resonances of the acyl chains to obtain information on the structure of the phospholipid matrix.[24-27,50-52] Since SFVS is a vibrational spectroscopic technique it would seem appropriate to use these same resonances for characterization of the phase behavior of a DSPC+CHO PSLB. However, the changes in these resonances are complicated by the symmetry constraints imposed on SFVS and care must be taken in the interpretation of the spectral changes observed.[33] The symmetry restrictions of SFVS can, however, be used to provide direct information on the structure of the bilayer assembly; or more precisely, the asymmetry existing between the upper (distal) and lower (proximal) layers of the membrane. [31-33,38,39]

In this study, the effect of CHO concentration on the phase behavior of binary DSPC+CHO mixtures in PSLBs was examined by continuously monitoring the SFVS intensity of the $\text{CH}_3 \nu_s$ (2875 cm^{-1}), originating from the terminal methyl group of the DSPC alkyl chains, as functions of temperature and CHO content in the membrane. Figure 4 shows the $\text{CH}_3 \nu_s$ intensity recorded as a function of temperature for 5, 10, 15, 25, 40 and 60 mol% of CHO- d_7 in a DSPC bilayer using a polarization combination of *ssp*. For comparison, the SFVS intensity of the $\text{CH}_3 \nu_s$ as a function of temperature for a pure DSPC bilayer is also shown in Figure 4. [32] Data were collected from low to high temperature at a temperature scan rate of 0.24 $^{\circ}\text{C}/\text{min}$. This scan rate was determined to provide a reproducible temperature dependent response which also allowed for an optimal data acquisition time.

For 0, 5, 10, and 15 mol% CHO- d_7 , a broad peak is observed in the $\text{CH}_3 \nu_s$ intensity as a function of temperature with a maximum observed near the thermodynamic phase transition of DSPC ($T_m = 54.5^{\circ}\text{C}$). [10] Further analysis of the temperature dependent SFVS data for 5 mol% CHO- d_7 reveals two components, one with a maximum at $55.1 \pm 0.5^{\circ}\text{C}$ and the other at $57.3 \pm 0.3^{\circ}\text{C}$. The maxima were determined by using a combination of a nonlinear least-squares fit of the temperature dependent data with two Gaussian line shapes and by obtaining the second derivative of the experimental data. Similar analysis for 10 and 15 mol% CHO- d_7 +DSPC are presented in Table 1. Comparison with DSC measurements reveals that the maximum in the $\text{CH}_3 \nu_s$ intensity at lower temperature observed by SFVS correlates extremely well with the maximum in the heat capacity measured for solution phase vesicles of the same composition,[10] see Table 1. In addition, the shift to lower temperature with increasing CHO content is consistent with previous DSC, NMR, and vibrational spectroscopy measurements. [11,14,24,53-56] The second, higher temperature peak at approximately 57°C is observed for 0, 5, 10, and 15 mol% CHO- d_7 in the DSPC bilayers. This maximum in the $\text{CH}_3 \nu_s$ occurs 3°C above the thermodynamic phase transition of the phospholipid alkyl chains as determined by DSC.[10] This behavior can be correlated to the CHO+DSPC phase diagram, and is discussed in more detail below. At 25 mol% CHO- d_7 , the $\text{CH}_3 \nu_s$ SFVS intensity increases

over a much broader temperature range, 45 °C to 70 °C, compared to pure DPSC, 50 °C to 62 °C. Above 25 mol% CHO, the measured $\text{CH}_3 \nu_s$ intensity does not exhibit a discernable increase in intensity near the T_m of DPSC. In fact, the $\text{CH}_3 \nu_s$ intensity is at a maximum at low temperature and decreases upon heating.

In general, the SFVS results suggest that membrane asymmetry is present over a much broader temperature range compared to the sharp change in heat capacity measured by DSC.[10] This is apparent by the much wider temperature range over which a measurable change in the $\text{CH}_3 \nu_s$ intensity is seen compared with the narrow thermodynamic heat capacity change observed by DSC.[10] Unlike DSC, which measures the enthalpy of melting the phospholipid fatty acid chains, SFVS measures the asymmetry between the distal and proximal layers of the PSLBs. The SFVS data therefore suggest that there is considerable membrane asymmetry present before and after the onset of the chain melting transition as measured by DSC. This observation is supported by fluorescence microscopy measurements of phase segregation in PSLBs and large unilamellar vesicles,[16,21,22,57-59] where discrete domains are observed over a much larger temperature range than defined by the thermodynamics of chain melting measured by DSC. The melting of the phospholipid acyl chains is best described by a pseudo first-order process[60], with the formation of domains within the two dimensional membrane driven by line tension forces and electrostatic interactions between phase segregated phospholipid domains.[61] This results in a broader temperature region of phase coexistence for the *l.c.* and *gel* states than would be expected based on DSC results alone.

In addition to a correlation between the temperature at which a maximum in the $\text{CH}_3 \nu_s$ intensity is observed and the phase transition measured by DSC, there is also a correlation between the increase in the full-width at half maximum (FWHM) measured by DSC and SFVS. The DSC results show an increase in the FWHM of the heat capacity of DPSC+CHO liposomes with increasing CHO content up to a concentration of 25 mol%.[10] Above 30 mol% CHO, a complete disappearance of a definable change in heat capacity is observed as a broad featureless temperature dependent change in the heat capacity.[10] The same trend is observed in the FWHM of the SFVS data, in which an increase in intensity of the $\text{CH}_3 \nu_s$ with temperature occurs over a much broader temperature range with increasing CHO content and a definable maximum associated with the T_m of the phospholipids is completely abolished above 25 mol % CHO-*d*₇.

It should be noted that the pre-transition, at 50.5 °C for DPSC, as observed by DSC in the absence of CHO,[10] cannot be seen in the SFVS data of Figure 4. IR and Raman spectroscopy have been used to measure this transition by monitoring the changes in the CH_2 twist at $\sim 1300 \text{ cm}^{-1}$, CH_2 band at $\sim 1440 \text{ cm}^{-1}$, the C-H stretching region between 2800 and 3000 cm^{-1} and the C-C stretching region between 1000-1200 cm^{-1} . [62-66] A possible reason for the absence of this transition in the SFVS data is that the pre-transition does not produce a significant change in the membrane asymmetry to be visible. It could also be that the pre-transition may be present but merged into the broad temperature dependent peak seen in Figure 4. The possibility also exists that the pre-transition does not occur for membranes confined to a surface such as the PSLBs used here. Additional experiments are being conducted to explore these possibilities.

CHO+DPSC Phase Diagram

A number of NMR and DSC studies have been conducted to determine the phase behavior of the binary CHO+phospholipid system.[5,11] The combined results of these experiments have been used to produce the composite phase diagram for CHO+DPSC illustrated in Figure 5. [5,11] Superimposed on the phase diagram are the vertical paths traversed at the fixed CHO-*d*₇ concentrations of 5, 10, 15, 25, 40 and 60 mol% for the SFVS experiments described in Figure 4.

At 5 and 10 mol% CHO- d_7 in the temperature range of 30 to 52 °C, the composition of the PSLB is characterized by a single solid-ordered phase (*so*) in which the acyl chains of DSPC are highly ordered in an *all-trans* configuration with a lateral phospholipid mobility of 2×10^{-10} cm²/s [5,11,67-70] In this temperature range, the intensity of the CH₃ ν_s is at a minimum. The weak CH₃ ν_s measured is consistent with the PSLB being in the homogenous *so* region of the phase diagram in which the highly symmetric nature of the bilayer results in the cancellation of dipole transition moments of the terminal methyl groups of the alkyl chains between the top and bottom layers, Figure 6. As the membrane is heated between 52 °C and 62 °C, there is a narrow region of *so* and liquid-disordered (*ld*) phase coexistence centered near the T_m of DSPC,[5,11] (see Figure 5). In this region of the phase diagram an increase in the CH₃ ν_s intensity is observed. As reported earlier, two maxima are observed in the CH₃ ν_s intensity, see Table 1. These maxima correlate with the phase boundaries illustrated in Figure 5, and represent the limits of the *so+ld* phase coexistence region. Similar behavior has been previously measured by SFVS for binary phospholipid mixtures of DMPC+DSPC in which two maxima were observed defined by the T_m of the two phospholipid components.[33] The increase in CH₃ ν_s intensity in this region of the phase diagram is attributed to disparate phase segregated domains in the proximal and distal layers of the bilayer, see Figure 6. Above 62 °C the intensity of CH₃ ν_s returns to the same intensity measured below 52 °C as the bilayer is returned to a symmetric structure characterized by a single *ld* phase, (Figure 6).

For 15 and 25 mol% CHO- d_7 in DSPC, examination of the phase diagram in Figure 5 reveals that in the temperature range of 30 °C to 52 °C the membrane is in a state of *so+lo* (*liquid-ordered*) phase coexistence. The measured CH₃ ν_s intensity in this temperature region for both 15 and 25 mol% CHO- d_7 is comparable to that observed for pure DSPC and DSPC with 5 and 10 mol% CHO- d_7 . Based on our previous measurements of phase segregated phospholipid membranes, it would seem likely that an increase in the CH₃ ν_s intensity would be visible in this region of the phase diagram due to the presence of the *so* and *lo* phase segregated domains, which have been observed by fluorescence microscopy.[57-59] However, the sensitivity of SFVS lies in its ability to discriminate between differences in the structure of the phospholipid acyl chains as probed by the CH₃ ν_s . Although DSPC is present in the CHO rich *lo* phase and CHO poor *so* phase, these phases possess identical structure with regards to the composition of the fatty chains, namely a nearly *all-trans* configuration since DSPC is below its main phase transition, Figure 6.[55,71] It might be possible that variations in the CHO content could give rise to an asymmetry in the membrane, but studies of the rate of CHO exchange between the proximal and distal layers suggest that this rate is very rapid and would be time averaged over the course of the measurements made here.[72-77] Although phase segregation is occurring as observed by fluorescence microscopy, the similarity in the structure of the aliphatic phospholipid tails precludes the use of SFVS from detecting any membrane asymmetry in this region of the phase diagram.

For 15 mol% CHO- d_7 , as the bilayer passes through the *so+lo* to the *ld* phase boundary there is an increase in the intensity of the CH₃ ν_s . It should be noted that this increase occurs over a narrower temperature range compared to 5 and 10 mol% CHO- d_7 , which is consistent with the phase diagram in Figure 5, as the *so+ld* phase coexistence region is not present in this concentration range of CHO. As the membrane is heated above 55 °C and enters the homogenous *ld* state, the CH₃ ν_s intensity returns to a minimum as seen with 5 and 10 mol% CHO- d_7 .

For 25 mol% CHO- d_7 in DSPC, as the bilayer is heated above 50 °C the CH₃ ν_s intensity increases as the phase boundary between the *so+lo* and *ld+lo* phases is reached. The CH₃ ν_s intensity reaches a maximum at ~55 °C. As the temperature is increased above 55 °C, the bilayer enters the *ld+lo* phase coexistence region characterized by CHO poor *ld* domains containing DSPC with an increasing amount of gauche defects in the acyl chains, while the

CHO rich *lo* phase, being more restricted by the presence of CHO, contains fewer gauche conformations, Figure 6.[5,11] The disparity between the structures of the *lo* and *ld* phases and their apparent discontinuity in the proximal and distal leaflets of the bilayer is what most likely gives rise to the increase in SFVS intensity in this temperature range. Above 60 °C, the bilayer enters the homogenous *ld* phase which is characterized by a decrease in the measured SFVS intensity.

When the CHO-*d*₇ concentration is increased to 40 and 60 mol% the phase transition of DSPC as measured by SFVS is completely absent. These results suggest that only one phase exists above 40 mol% CHO in DSPC in the temperature range examined, which is consistent with the homogenous *lo* phase illustrated in Figure 6. These results are also in good agreement with previous DSC measurements which showed that the addition of CHO eliminates the normally sharp thermal transition of phospholipids between the *gel* and *lc* phases.[10] However, it is noted that a higher CH₃ *v*_s intensity is observed at temperatures < 60 °C for 40 and 60 mol% CHO-*d*₇.

DSPC and CHO Asymmetry

In the *lo* phase at temperatures below 60 °C for 40 and 60 mol% CHO in DSPC, the phospholipids should be in a highly symmetric arrangement and therefore produce little CH₃ *v*_s intensity. One possible interpretation for the increase in the CH₃ *v*_s at low temperatures (30 °C to 50 °C) is that CHO and DSPC are asymmetrically distributed within the top and bottom layers of the bilayer. This hypothesis can be tested by examining the spectra of 40 and 60 mol % CHO-*d*₇ in a DSPC bilayer at 23 °C, Figure 2a. The intensities at 2876 cm⁻¹ from the terminal methyl group of DSPC and the resonances at 2964 cm⁻¹ from CHO are much higher than for CHO concentrations below 25 mol% in the bilayer. If the number density of phospholipids and CHO were identical in each layer, the SFVS intensity should remain invariant due to cancellation of opposing dipoles in each layer. However, a clear increase in SFVS intensity is observed for both the phospholipid and CHO vibrational resonances suggesting the introduction of membrane asymmetry.

The percent asymmetry (%AS) of DSPC in the bilayer produced by the inclusion of 60 mol% CHO-*d*₇ at 23 °C can be determined directly from the baseline corrected CH₃ *v*_s intensity. We have shown previously that the %AS can be calculated using Equation 7:[33]

$$\%AS = \frac{\sqrt{I_{AS}^{CH_3 v_s}}}{\sqrt{I_{max}^{CH_3 v_s}}} \times 100\% \quad (7)$$

where $I_{max}^{CH_3 v_s}$ is the maximum CH₃ *v*_s intensity of an asymmetric DSPC bilayer[33] and $I_{AS}^{CH_3 v_s}$ is the CH₃ *v*_s intensity of 60 mol% CHO-*d*₇ in DSPC bilayer obtained from the data in Figure 2a. As we are dealing with a homogenous *lo* phase at 60 mol% CHO, which has been verified by fluorescence microscopy (data not shown), the asymmetry measured in the PSLB is presumably due solely to a net asymmetry in the phospholipid and CHO content in the distal and proximal leaflets of the bilayer and not do to asymmetry arising from lateral domain mismatch as discussed above and observed previously for PSLBs composed of a single phospholipid component.[33] The calculated %AS for DSPC from the data in Figure 2a is 51% in the presence of 60 mol% CHO. That is, there is a population inversion of DSPC in the bilayer in which one leaflet contains a greater number of phospholipids (75.5% of total DSPC phospholipid density) versus the other leaflet (24.5% of total DSPC). The phospholipid asymmetry is presumably associated with a corresponding asymmetric distribution of CHO in

the membrane, as suggested by the spectra in Figure 2. In order to investigate the asymmetric distribution of both CHO and phospholipids in the bilayer, several SFVS interference measurements were performed.

By exploiting the coherent nature of the SFVS response, nonresonant interference was used to determine the origins of the phospholipid and CHO asymmetry in the membrane.[33,78] PSLBs of DSPC containing 60 mol% *CHO-d₇* were prepared on a sapphire (Al₂O₃) substrate. Al₂O₃ has a large nonresonant SFVS background due to its anisotropic hexagonal structure. [79,80] Interference between the nonresonant background and the resonant contribution from the vibrational modes of the phospholipids and CHO can be used to deduce the concentration asymmetry in each leaflet of the bilayer independently. The SFVS intensity is proportional to the square of the second order susceptibility, $\chi^{(2)}$ which is the sum of the resonant (*R*) and nonresonant (*NR*) contributions and is given by Equation 2:

$$\chi^{(2)} = \chi_R + \chi_{NR} = \frac{N_{distal}}{\epsilon_0} \langle \beta^{CH_3 \nu_s} \rangle - \frac{N_{proximal}}{\epsilon_0} \langle \beta^{CH_3 \nu_s} \rangle + \chi_{NR} \quad (6)$$

where *N* is the population density of molecules at the interface, ϵ_0 is the permittivity of a vacuum, and β is the second-order hyperpolarizability expressed in terms of the molecular hyperpolarizability and the orientation averages for the CH₃ ν_s from DSPC in the distal and proximal leaflets of the bilayer.[33] The sign of the molecular hyperpolarizabilities for the CH₃ ν_s in the distal and proximal leaflets are opposite due to the opposing nature of the transition dipole moments. It is noted, however, that the signs of the hyperpolarizabilities are arbitrary and may not reflect their true polarity.

In order to ascertain which leaflet of bilayer contains an excess of CHO and DSPC a SFVS spectrum of 60 mol% *CHO-d₇* in a DSPC-*d₇₀* bilayer on a sapphire substrate (Figure 7b) was obtained and compared to the spectrum of a monolayer of *CHO-d₇* on sapphire (Figure 7a). Upon initial inspection there appears to be no correlation between the two spectra. However, spectral analysis of the individual vibrational resonance reveals a striking similarity between the two spectra. The spectra in Figure 7 were fit using Equation 5. When fitting the spectra in Figure 7, the same nonresonant background (χ_{NR}), resonant peak amplitudes and widths were used for both spectra. The only thing which was varied between the spectra was the phase angle between the resonant signal and nonresonant background (ϕ) which was taken as either 0 (in phase with χ_{NR}) or π (out of phase with χ_{NR}). The resulting fits and individual peaks are shown in Figure 7, with the parameters listed in table 2. An excellent correlation between the vibrational frequencies observed by SFVS and those measured by Raman spectroscopy, for various forms of CHO, are observed.[47,48] Using the LB deposited CHO film as a point of reference, the hydroxyl group of CHO should be pointing towards the sapphire surface (see insert of Figure 7a), based on the orientation of CHO at the air/water interface prior to transfer. Using this convention, the resonance at 2828, 2866, 2910 and 2990 cm⁻¹ are assigned as in phase with the underlying substrate while the remaining resonance at 2843, 2978, 2939 and 2955 cm⁻¹ are out of phase. The most sticking result of the spectral analysis is that the spectrum of *CHO-d₇* in a DPSC-*d₇₀* bilayer appears to be the exact inverse of the spectrum of a *CHO-d₇* monolayer prepared by the LB method, as noted by the change in sign of the various resonances. The exception being the resonance at 2843 cm⁻¹, which is presumably due to the CH₂ ν_s of CHO.[44,46,81] This would indicate that CHO is asymmetrically distributed in the upper, distal leaflet of the PSLB with the -OH group oriented towards the bulk aqueous phase, as illustrated in Figure 7b. Based on mass balance, the displaced DPSC phospholipids would then be preferentially located in the proximal leaflet. It should be noted that an exact determination of the origins of the vibrational resonances for CHO is problematic due to the

fact that a definitive classification of the vibrational transitions of CHO is not present in the literature.

The asymmetric distribution of CHO and phospholipid molecules in the bilayer could possibly be driven by the chemically and physically different nature of the substrate and water interfaces which the proximal and distal layers are in contact. At high CHO loads in the membrane the chemical potential difference between the two layers may be sufficient to result in preferential partitioning of CHO in one layer over the other. It is also important to note, however, that this partitioning appears to be abolished with heating and is restored upon cooling as seen in Figure 4.

Reversibility of Phase Behavior

The reversibility of the SFVS response measured for the CHO+DSPC PSLBs upon heating and cooling was examined in detail. Initially, data were collected from low to high temperature at a temperature scan rate of 0.24 °C/min, as shown in Figure 4. After the bilayer had reached a temperature of 70 °C, the system was then returned to 30 °C using a rapid cooling rate of ~1 °C/min, and allowed to reach equilibrium for 30 minutes. The bilayer was then heated again at a rate of 0.24 °C/min, Figure 4. As an additional control, data were also collected from high to low temperature using a slow cooling rate of 0.24 °C/min (shown only for 10 and 40 mol% CHO, Figure 4). It is important to note that the heating/cooling curves are nearly identical within experimental error. These results are in agreement with the DSC endotherms and exotherms obtained upon heating and cooling respectively which show no observable hysteresis.[10] This suggests that there are no significant irreversible structural changes occurring in the PSLB as a result of heating or laser excitation. This result might not be expected for PSLBs as there are significant changes in the area per molecule upon heating and cooling.[82] The reproducible SFVS response provides strong evidence that the PSLBs are stable over the temperature range examined and no major irreversible structural defects with regards to membrane asymmetry are induced during the course of heating or cooling.

Conclusions

SFVS was used for the first time to directly determine the asymmetric distribution of CHO and phospholipid domains in phase segregated PSLBs. The interference of the terminal methyl groups ($\text{CH}_3 \nu_s$) at 2875 cm^{-1} originating from the phospholipid alkyl chains was used to probe the symmetry change of PSLBs composed of DSPC and CHO as functions of CHO concentration and temperature. SFVS data reveals that CHO significantly affects the phase segregation and domain distribution in PSLBs of DSPC in a concentration dependent manner. For 5 and 10 mol% CHO, an increase in the $\text{CH}_3 \nu_s$ intensity was observed near the *so+ld* phases coexistence region and was attributed to disparate phase segregated domains in the proximal and distal layers of the bilayer. The maxima in the $\text{CH}_3 \nu_s$ intensity recorded as a function of temperature for these systems correlates extremely well with bulk DSC results for solution phase liposomes of the same composition. For 15 and 25 mol% CHO, the $\text{CH}_3 \nu_s$ intensity increases as the phase boundary between *so+lo* and *ld+lo* phases is reached. It is the disparity between the structures of *so+lo* and *ld+lo* phases and their apparent discontinuity in the proximal and distal leaflets of the bilayer which gives rise to an increase in $\text{CH}_3 \nu_s$ SFVS intensity. For 40 and 60 mol% CHO, a weak $\text{CH}_3 \nu_s$ intensity was observed at the T_m of DSPC, indicating there is a symmetric distribution of phospholipids and CHO domains in PSLBs. The abolition of a discernable peak in the temperature dependent $\text{CH}_3 \nu_s$ data indicates that the phase transition of DSPC is eliminated, which is in a good agreement with previous DSC results. Interestingly, there is an increase in the $\text{CH}_3 \nu_s$ intensity below the T_m of DSPC for 40 and 60 mol% CHO, suggesting that CHO and DSPC are asymmetrically distributed in the PSLB at these concentrations and temperatures. SFVS interference measurements using

Al₂O₃ substrates of the PSLBs showed that there is more CHO and fewer phospholipids in the distal layer of the PSLB (in contact with the bulk aqueous phase), whereas less CHO and more phospholipids distribute in the proximal layer of the bilayer (in contact with the silica surface). This bilayer becomes symmetric with regards to CHO and DSPC distributions upon heating. The SFVS results presented here provide important evidence for the delocalization of phase segregated domain structures in DSPC + CHO PSLBs and illustrates the strength of a coherent vibrational spectroscopy, such as SFVS, in deciphering structural as well as molecular asymmetries in biological membranes.

Acknowledgments

Funding for this research was provided by the NSF (CHE 0515940) and NIH (GM068120). Thanks go to Prof. David J. Neivandt for the suggestion of performing a SFVS interference measurement for the determination of the absolute asymmetry in the phospholipid bilayers containing CHO (232nd ACS National Meeting, San Francisco, CA). Special thanks also go to Mr. Timothy Anglin and Mr. Michael Cooper for assistance in collecting the CHO spectra on sapphire.

References

- Demel RA, De Kruffy B. *Biochimica et Biophysica Acta, Reviews on Biomembranes* 1976;457:109–32.
- Gennis, RB. *Biomembranes: Molecular Structure and Function*. Springer-Verlag; New York: 1989.
- Alberts B, Alexander J, Lewis J, Raff M, Roberts K, Walter P. *Molecular Biology of the Cell*, 4th Edition. 2004
- Presti FT. *Membr Fluid Biol* 1985;4:97–146.
- Ipsen JH, Karlstroem G, Mouritsen OG, Wennerstroem H, Zuckermann MJ. *Biochimica et Biophysica Acta* 1987;905:162–72. [PubMed: 3676307]
- Yeagle PL. *Biochimica et Biophysica Acta* 1985;822:267–87. [PubMed: 3904832]
- Simons K, Ikonen E. *Science* 2000;290:1721–6. [PubMed: 11099405]
- Radhakrishnan A, McConnell HM. *Biophysical Journal* 1999;77:1507–1517. [PubMed: 10465761]
- Sankaram MB, Thompson TE. *Proceedings of the National Academy of Sciences of the United States of America* 1991;88:8686–90. [PubMed: 1656453]
- McMullen TPW, McElhaney RN. *Biochemistry* 1997;36:4979–4986. [PubMed: 9125520]
- Vist MR, Davis JH. *Biochemistry* 1990;29:451–64. [PubMed: 2302384]
- Estep TN, Mountcastle DB, Biltonen RL, Thompson TE. *Biochemistry* 1978;17:1984–9. [PubMed: 580764]
- Mabrey S, Mateo PL, Sturtevant JM. *Biochemistry* 1978;17:2464–8. [PubMed: 581060]
- McMullen TPW, Lewis RNAH, McElhaney RN. *Biochemistry* 1993;32:516–22. [PubMed: 8422361]
- McMullen TPW, Lewis RNAH, McElhaney RN. *Biophysical Journal* 2000;79:2056–2065. [PubMed: 11023909]
- Loura LM, Fedorov A, Prieto M. *Biophysical journal* 2001;80:776–88. [PubMed: 11159445]
- Estep TN, Mountastle DB, Bareholz Y, Biltonen RL, Thompson TE. *Biochemistry* 1979;18:2112–2117. [PubMed: 435470]
- London E, Brown DA. *Biochimica et Biophysica Acta, Biomembranes* 2000;1508:182–195.
- Cottingham K. *Analytical Chemistry* 2004;76:403A–406A.
- Simons K, Ikonen E. *Nature (London)* 1997;387:569–572. [PubMed: 9177342]
- Loura LMS, Fedorov A, Prieto M. *Biochimica et Biophysica Acta, Biomembranes* 2001;1511:236–243.
- Worthman LAD, Nag K, Davis PJ, Keough KMW. *Biophysical Journal* 1997;72:2569–2580. [PubMed: 9168032]
- Veatch SL, Keller SL. *Physical Review Letters* 2002;89:268101/1–268101/4. [PubMed: 12484857]
- Sankaram MB, Thompson TE. *Biochemistry* 1990;29:10676–84. [PubMed: 2271675]

25. Davies MA, Schuster HF, Brauner JW, Mendelsohn R. *Biochemistry* 1990;29:4368–73. [PubMed: 2350543]
26. Pink DA, Green TJ, Chapman D. *Biochemistry* 1981;20:6692–8. [PubMed: 6895474]
27. Mendelsohn R, Davies MA, Brauner JW, Schuster HF, Dluhy RA. *Biochemistry* 1989;28:8934–9. [PubMed: 2605234]
28. Robinson AJ, Richards WG, Thomas PJ, Hann MM. *Biophysical journal* 1995;68:164–70. [PubMed: 7711238]
29. Veatch SL, Polozov IV, Gawrisch K, Keller SL. *Biophysical Journal* 2004;86:2910–2922. [PubMed: 15111407]
30. Anderson TG, McConnell HM. *Biophysical journal* 2001;81:2774–85. [PubMed: 11606290]
31. Liu J, Conboy JC. *Langmuir* 2005;21:9091–9097. [PubMed: 16171337]
32. Liu J, Conboy John C. *Journal of the American Chemical Society* 2004;126:8894–5. [PubMed: 15264810]
33. Liu J, Conboy JC. *J Phys Chem C* 2007;111:8988–8999.
34. Bonn M, Roke S, Berg O, Juurlink LBF, Stamouli A, Mueller M. *Journal of Physical Chemistry B* 2004;108:19083–19085.
35. Miranda PB, Shen YR. *Journal of Physical Chemistry B* 1999;103:3292–3307.
36. Shen, YR. *The Principles of Nonlinear Optics*. Wiley; New York: 1984.
37. Shen YR. *Nature (London, United Kingdom)* 1989;337:519–25.
38. Liu J, Conboy John C. *Journal of the American Chemical Society* 2004;126:8376–7. [PubMed: 15237984]
39. Liu J, Conboy JC. *Biophysical Journal* 2005;89:2522–2532. [PubMed: 16085770]
40. Wu SHW, McConnell HM. *Biochemistry* 1975;14:847–54. [PubMed: 163646]
41. Komura S, Shirotori H, Olmsted PD, Andelman D. *Europhysics Letters* 2004;67:321–327.
42. Borden MA, Martinez GV, Ricker J, Tsvetkova N, Longo M, Gillies RJ, Dayton PA, Ferrara KW. *Langmuir* 2006;22:4291–4297. [PubMed: 16618177]
43. Raudino A, Castelli F, Briganti G, Cametti C. *Journal of Chemical Physics* 2001;115:8238–8250.
44. MacPhail RA, Strauss HL, Snyder RG, Elliger CA. *Journal of Physical Chemistry* 1984;88:334–41.
45. Tamm LK, Tatulian SA. *Quarterly Reviews of Biophysics* 1997;30:365–429. [PubMed: 9634652]
46. Snyder RG, Strauss HL, Elliger CA. *Journal of Physical Chemistry* 1982;86:5145–50.
47. Bulkin BJ, Krishnan K. *Journal of the American Chemical Society* 1971;93:5998–6004.
48. Faiman R. *Chemistry and Physics of Lipids* 1977;18:84–104. [PubMed: 832340]
49. O'Leary TJ, Levin IW. *Journal of Physical Chemistry* 1984;88:1790–6.
50. Recktenwald DJ, McConnell HM. *Biochemistry* 1981;20:4505–10. [PubMed: 6269591]
51. Seelig A, Seelig J. *Biochemistry* 1974;13:4839–45. [PubMed: 4371820]
52. Lehnert R, Eibl HJ, Mueller K. *Journal of Physical Chemistry B* 2004;108:12141–12150.
53. Huang TH, Lee CWB, Das Gupta SK, Blume A, Griffin RG. *Biochemistry* 1993;32:13277–87. [PubMed: 8241184]
54. Filippov A, Oradd G, Lindblom G. *Biophysical Journal* 2003;84:3079–3086. [PubMed: 12719238]
55. Reinl H, Brumm T, Bayerl TM. *Biophysical Journal* 1992;61:1025–35. [PubMed: 19431823]
56. McMullen TP, Lewis RNAH, McElhaney RN. *Biophysical Journal* 1994;66:741–52. [PubMed: 8011906]
57. Pokorny A, Almeida PFF, Vaz WLC. *Biophysical Journal* 2001;80:1384–1394. [PubMed: 11222299]
58. Binder WH, Barragan V, Menger FM. *Angewandte Chemie, International Edition* 2003;42:5802–5827.
59. Kato T. *Science (Washington, DC, United States)* 2002;295:2414–2418.
60. Chen L, Johnson ML, Biltonen RL. *Biophys J* 2001;80:254–270. [PubMed: 11159399]
61. Hu Y, Meleson K, Israelachvili J. *Biophysical Journal* 2006;91:444–453. [PubMed: 16632506]
62. Fox CB, Harris JM. Unpublished Raman results.

63. Csiszar A, Koglin E, Meier RJ, Klumpp E. *Chemistry and Physics of Lipids* 2006;139:115–124. [PubMed: 16413520]
64. Le Bihan T, Pezolet M. *Chemistry and Physics of Lipids* 1998;94:13–33.
65. Gaber BP, Peticolas WL. *Biochimica et Biophysica Acta* 1977;465:260–74. [PubMed: 16250339]
66. Okamura E, Umemura J, Takenaka T. *Vibrational Spectroscopy* 1991;2:95–100.
67. Seul M, Subramaniam S, McConnell HM. *Journal of Physical Chemistry* 1985;89:3592–5.
68. Hac AE, Seeger HM, Fidorra M, Heimburg T. *Biophysical Journal* 2005;88:317–333. [PubMed: 15501937]
69. Von Tscharner V, McConnell HM. *Biophysical Journal* 1981;36:421–7. [PubMed: 7306664]
70. Korlach J, Schwille P, Webb WW, Feigenson GW. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:8461–8466. [PubMed: 10411897]
71. Clarke JA, Heron AJ, Seddon JM, Law RV. *Biophysical Journal* 2006;90:2383–2393. [PubMed: 16537550]
72. Lange Y, Dolde J, Steck TL. *Journal of Biological Chemistry* 1981;256:5321–3. [PubMed: 7240138]
73. Backer JM, Dawidowicz EA. *The Journal of biological chemistry* 1981;256:586–8. [PubMed: 6935193]
74. Backer JM, Dawidowicz EA. *Biochimica et biophysica acta* 1979;551:260–70. [PubMed: 420833]
75. Rodriguez WV, Wheeler JJ, Klimuk SK, Kitson CN, Hope MJ. *Biochemistry* 1995;34:6208–17. [PubMed: 7742326]
76. Steck Theodore L, Ye J, Lange Y. *Biophysical journal* 2002;83:2118–25. [PubMed: 12324429]
77. Leventis R, Silvius JR. *Biophysical Journal* 2001;81:2257–2267. [PubMed: 11566796]
78. Holman J, Davies PB, Nishida T, Ye S, Neivandt DJ. *Journal of Physical Chemistry B* 2005;109:18723–18732.
79. Wu, WY.; Zee, RH. *Proceedings of the Intersociety Energy Conversion Engineering Conference*; 1991. p. 110-15.
80. Tani T, Miyamoto Y, Koizumi M, Shimada M. *Ceramics International* 1986;12:33–7.
81. Cates DA, Strauss HL, Snyder RG. *Journal of Physical Chemistry* 1994;98:4482–8.
82. Leonenko ZV, Finot E, Ma H, Dahms TES, Cramb DT. *Biophysical Journal* 2004;86:3783–3793. [PubMed: 15189874]

Figure 1.
Molecular structures of CHO, CHO-*d*₇, DSPC, DSPC-*d*₇₀ and DSPC-*d*₈₃.

Figure 2.

(a) SFVS spectra of DSPC PSLBs containing various concentrations of CHO- d_7 recorded at 23 °C. (b) SFVS spectra of 60 mol% CHO- d_7 in a DSPC bilayer (black) and 60 mol% CHO- d_7 in a DSPC- d_{83} bilayer (red) recorded at 23 °C. Spectrum (c) is the difference of the DSPC + 60 mol% CHO- d_7 spectrum minus the spectrum of DSPC- d_{83} + 60 mol% CHO- d_7 shown in (b). Also shown in blue are the individual peaks used in the fitting of the difference spectrum (spectra are offset for clarity). All SFVS spectra were collected with *s*-polarized sum-frequency, *s*-polarized visible, and *p*-polarized IR (*ssp*).

Figure 3. SFVS spectra of pure CHO (black) and CHO-*d*₇ (red) monolayers at the silica/air interface. The SFVS spectra were collected with *s*-polarized sum-frequency, *s*-polarized visible, and *p*-polarized IR (*ssp*).

Figure 4.

SFVS $\text{CH}_3 \nu_s$ intensity as a function of temperature for increasing concentrations of CHO-d_7 in a DSPC bilayer recorded with the *ssp* polarization combination. Black curves represent samples heated at a rate of $0.24^\circ\text{C}/\text{min}$. Red curves represent samples which were heated at a rate of $0.24^\circ\text{C}/\text{min}$ after the bilayer was heated to 70°C at a rate of $0.24^\circ\text{C}/\text{min}$ and rapidly cooled to 30°C at $1^\circ\text{C}/\text{min}$. Blue curves represent samples cooled with at a rate of $0.24^\circ\text{C}/\text{min}$ after the bilayer was heated to 70°C at a rate of $0.24^\circ\text{C}/\text{min}$.

Figure 5.

Composite phase diagram of a DSPC+CHO binary mixture extrapolated from the data of Vist et al., and Ipsen et al.[5,11] Definitions: *solid-ordered* (*so*), *liquid-disordered* (*ld*), and *liquid-ordered* (*lo*) phases. The blue arrow indicates the direction of increasing CHO concentration at 23 °C examined by SFVS. The red arrows indicate the temperature paths traversed at the fixed CHO concentration of 5, 10, 15, 25, 40 and 60 mol% for the SFVS experiments.

Figure 6.

Illustrations of CHO+DSPC structures: (a) symmetric distribution of CHO in a *so* (blue) and *ld* phases (red), (b) symmetric distribution of CHO in the *so+lo* phase coexistence region (blue and green respectively) and the *lo+ld* phase coexistence region (green and red respectively), and (c) an asymmetric distribution of CHO in the *lo* phase at low temperature and a symmetric distribution at high temperature.

Figure 7.

(a) SFVS spectrum of a CHO-*d*₇ monolayer on a sapphire substrate and (b) a spectrum of 60 mol% CHO-*d*₇ in a DSPC-*d*₇₀ bilayer on sapphire, both shown in gray. Also shown are the spectral fits to the data (solid black lines) and the individual peaks used to obtain the fits which have been offset for clarity. An illustration of the CHO arrangement in a monolayer and bilayer are shown as inserts to (a) and (b) respectively.

Table 1

Measurement of the phase transition of DSPC with and without CHO by SFVS and DSC.[10]

mol% CHO	SFVS Maxima Maximum #1 (°C)	Maximum #2 (°C)	DSC(T_m) (°C)[10,14]
0	none	57.5 ± 0.5	54.5
5	55.1 ± 0.5	57.3 ± 0.3	54.5
10	54.3 ± 0.2	57.0 ± 0.6	54.0
15	53.1 ± 0.3	57.6 ± 0.4	53.7

Parameters used to obtain the spectral fits shown in Figure 7 for a CHO- d_7 monolayer (M) and 60 mol% CHO in a DSPC- d_{70} bilayer (B) on a sapphire substrate.

Table 2

Raman Frequency $\omega(\text{cm}^{-1})$ [47,48]	SFVS Frequency $\omega(\text{cm}^{-1})$	Amplitude A	Width $\Gamma(\text{cm}^{-1})$	Phase ^M ϕ	Phase ^B ϕ
2827 $\pm$ 3	2828	4	8	0	π
2848 $\pm$ 4	2843	5	8	π	π
2866 $\pm$ 5	2866	7	10	0	π
2883 $\pm$ 3	2878	4	8	π	0
2906 $\pm$ 4	2910	3	7	0	π
2936 $\pm$ 6	2939	11	12	π	0
2950 $\pm$ 5	2955	4	10	π	0
2991 $\pm$ 19	2990	4	15	0	π