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USE OF DEUTERATED PHOSPHOLIPIDS IN RAMAN SPECTROSCOPIC STUDIES OF MEMBRANE STRUCTURE

I. MULTILAYERS OF DIMYRISTOYL PHOSPHATIDYLCHOLINE (AND ITS -d_{54} DERIVATIVE) WITH DISTEAROYL PHOSPHATIDYLCHOLINE

R. MENDELSOHN and J. MAISANO

Department of Chemistry, Rutgers University, Newark College of Arts and Sciences, 73 Warren Street, Newark, N.J. 07102 (U.S.A.)

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Summary

The temperature dependence of the Raman spectrum has been studied for binary phospholipid mixtures of dimyristoyl phosphatidylcholine (and its chain deuterated -d_{54} derivative) with distearoyl phosphatidylcholine. Two distinct melting regions are observed for the 1 : 1 mole ratio mixture. The use of deuterated phospholipid permits the identification of the lower (≈22°C) transition with primarily the melting of the shorter chain component, and the higher (≈47°C) transition primarily with the melting of the longer chains. The C-H stretching vibrations of the distearoyl component respond to the melting of the dimyristoyl component, an apparent consequence of alterations in the lateral interactions of the distearoyl chains. These changes in the C-H spectral region suggest that phase separation does not occur in the gel state for this system. The results are in reasonable accord with recent calorimetric studies (Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3862—3866). The feasibility of using deuterated phospholipids to monitor the conformation of each component in a binary phospholipid mixture is demonstrated.

Introduction

The technique of Raman spectroscopy has become increasingly widespread for studies of phospholipid conformation in model membrane systems (refs. 1—9 and references contained therein). Vibrations of the hydrocarbon chains in several regions of the Raman spectrum undergo significant changes in position, linewidth, and intensity when the phospholipid is heated through its gel-liquid

crystal phase transition; hence the method is a useful probe of chain conformation.

A difficulty in applying the Raman technique to multicomponent systems (binary phospholipid mixtures, lipid · protein complexes, etc.) arises because the conformation-sensitive regions of the Raman spectrum near 1100 and 2900 cm^{-1} are overlapped with contributions from each component, thus rendering the determination of the contribution of a particular component to the spectrum difficult. In an attempt to overcome this problem, Mendelsohn et al. [7] have demonstrated that deuterated fatty acids inserted into phospholipid systems can monitor the hydrocarbon chain conformation of the latter. It was shown that the linewidth of the C-^{2}H stretching vibrations of the probe molecule increases significantly at the temperature of the phospholipid phase transition. As the C-^{2}H stretching modes occur in a spectral region (2100 cm^{-1}) which is not overlapped by contributions from other membrane components, the problem alluded to above was overcome.

The recent availability of deuterated phospholipids suggests a variety of interesting extensions of the above approach. In the current work, we report a Raman spectroscopic study of binary mixtures of the phospholipids dimyristoyl phosphatidylcholine (and its chain-deuterated d_{54} derivative) with distearoyl phosphatidylcholine. It was hoped to develop an approach by which the conformation and phase behaviour of each of the components in such a mixture could be directly monitored. Such a development would possess definite advantages over approaches commonly used in similar work, such as fluorescence spectroscopy, calorimetry, or electron spin resonance spectroscopy, and would be useful for the study of the interaction of protein with boundary lipid in reconstituted membranes, and lateral phase separation. The present system was chosen, aside from the availability of the materials, because a detailed phase diagram of the dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine system is available [10—12] and a direct comparison of the Raman data with the differential scanning colorimetry and spin-label experiments that have been used to construct the phase diagram would help to calibrate the Raman technique for future work. Furthermore, slightly differing molecular interpretations have been put forward to explain the observed phase behaviour [10—12] and it was felt that the Raman approach could yield insight into the molecular behaviour of the components in the binary mixture.

Materials and Methods

(1) Raman spectroscopy

The Raman spectra were obtained on a Jarrell-Ash model 25-400 Raman spectrometer equipped with a Spectra-Physics Model 164 argon-ion laser, photon counting detection and strip-chart recording. Samples for Raman scattering were injected into 1.0 mm internal diameter "Kimex" melting point capillaries, sealed, and placed into a thermostatted cell similar to that described by Thomas and Barylski [13]. The samples were examined in the transverse mode using a laser power of about 350 mW at 5145 Å. Samples with dimyristoyl phosphatidylcholine-d_{54} contained significant amounts of fluorescent impurity which gave rise to an intense background emission upon

which the Raman spectrum is superimposed. The background decreased more or less exponentially with time of irradiation until it stabilized at a constant level after about 90 min. All spectra reported for dimyristoyl phosphatidylcholine-d_{54}-containing preparations were obtained from samples "conditioned" in this way. The data so obtained were highly reproducible. Data points for melting curves were the average of 3—5 measurements. Typical uncertainties (standard deviations) were: intensity ratios ±5%, linewidths ±0.5 cm^{-1}, and frequency shifts ±2 cm^{-1} for narrow features.

Temperature calibration of the system was accomplished by insertion of a thermocouple into an unsealed capillary as close as feasible to the position of the laser focus. The local heating of the sample by the laser was estimated by monitoring T_m for dipalmitoyl phosphatidylcholine as measured from the temperature-dependent changes of its Raman spectrum [9]. Appropriate corrections were then made for other samples. It is pertinent to note that the local heating effect for samples containing fluorescent impurities (such as dimyristoyl phosphatidylcholine-d_{54}) is difficult to estimate, since the absorption of the laser light by the impurity leads to a temperature rise. The uncertainty in the sample temperature for such preparations may be as much as 2—3°C.

(2) Phospholipids

Samples of distearoyl phosphatidylcholine and dimyristoyl phosphatidylcholine were obtained from Sigma Chemical Co. Analysis with thin-layer chromatography in chloroform/methanol/water (65 : 25 : 4, v/v) showed only one spot with an R_F value of about 0.3 [14]. The samples were used without further purification. The sample of dimyristoyl phosphatidylcholine-d_{54}, obtained from Serdary Research Laboratory, London, Ontario, Canada, contained about 5% impurity as judged from thin-layer chromatography. Due to scarcity of the material, no attempt was made at further purification.

Samples of binary phospholipid mixtures were prepared by solvent evaporation of a chloroform solution containing both components. Final traces of solvent were removed by pumping on the sample for 2 h in a vacuum dessicator. Aqueous dispersions of lipid were prepared by addition of water to the dried, intimately mixed components, followed by extensive shaking on a vortex mixer at temperatures above T_m for distearoyl phosphatidylcholine. A cream-like suspension resulted. Samples were then injected into the melting point capillaries and packed slightly by centrifugation in a hematocrit centrifuge. Final concentrations of total lipid were about 30% by weight.

Results

(1) Spectra of dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine mixtures

Typical spectra at various temperatures for dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine (1 : 1, mole ratio) mixtures are shown in Fig. 1 for the region 900—1500 cm^{-1}. Of interest in the current work is the spectral region 1050—1150 cm^{-1} which contains the skeletal optical (primarily C-C stretching) vibrations of the hydrocarbon chains [3] along with a small under-

lying contribution from a stretching mode of the phosphate group [15]. Increasing the temperature with the concomitant formation of *gauche* configurations leads to drastic alteration of this spectral region (Fig. 1). The mode at 1130 cm^{-1}, assigned to C-C vibrations of hydrocarbon chains in the *all-trans* conformation, loses intensity in comparison with the broad feature in the 1080—1100 cm^{-1} region, which is assigned to a C-C stretching vibration in chain conformations containing *gauche* rotamers [3]. The intensity ratio I (1130)/(1080) is therefore a convenient measure of the *trans/gauche* population ratio [1,2,8,9]. The origin of the slight frequency shifts for these modes observed on chain melting has been discussed [3,6]. For the current work, the intensity referred to is the height at the position of maximum signal. The plot of the I (1130)/(1080) ratio of a dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine (1 : 1) binary mixture is shown in Fig. 2. Virtually identical curves result for heating or cooling cycles. The presence of two sigmoid features with melting temperatures of about 22 and 47°C is clearly shown. The pure phases of dimyristoyl phosphatidylcholine and distearoyl phosphatidylcholine in excess water have gel-liquid crystal transitions at 23.9 and 54.9°C, respectively, as measured by calorimetric techniques [12].

The spectral features observed in Fig. 1 and plotted in Fig. 2 derive intensity from both lipids in the binary mixture. Studies of dimyristoyl phosphatidylcholine-d_{54} and its 1 : 1 binary mixture with distearoyl phosphatidylcholine were undertaken in order to demonstrate the feasibility of monitoring the conformation of each component in the mixture.

(II) Dimyristoyl phosphatidylcholine-d_{54}

It has been shown previously that the linewidth of the C-^{2}H stretching vibra-

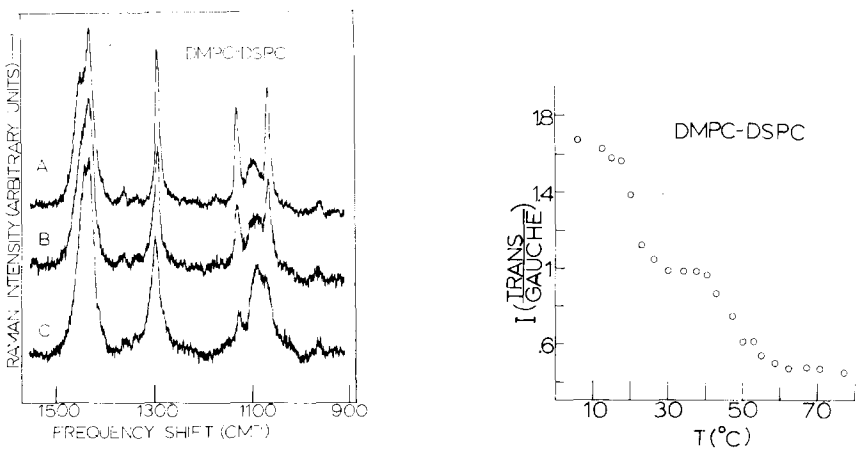

Fig. 1. Raman spectra in the 900—1500 cm^{-1} region for a dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine (DMPC-DSPC) (1 : 1) binary mixture at A, 7°C; B, 27°C; C, 58°C. Total lipid concentration, 30% by weight. Resolution, about 5 cm^{-1}.

Fig. 2. Temperature dependence of the relative *trans-gauche* population ratio of a dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine (DMPC-DSPC) 1 : 1 binary mixture. The nature of the ordinate measurement is discussed in the text.

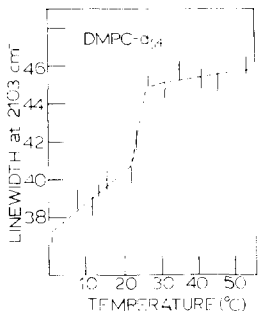

Fig. 3. Temperature variation of the linewidth of the C-^{2}H stretching vibrations at 2103 cm^{-1} for dimyristoyl phosphatidylcholine (DMPC)-d_{54}. The ordinate measurement refers to the full width at half-maximum. The uncertainty in the halfwidth is ±0.5 cm^{-1}, and is indicated by the vertical line at each data point.

tions in deuterated fatty acids responds to conformational changes of the hydrocarbon chain [7]. For the current study, it is necessary to demonstrate that the same parameter for dimyristoyl phosphatidylcholine-d_{54} undergoes a reversible variation with temperature when the deuterated phospholipid melts. The temperature dependence of the linewidth of the C-^{2}H stretching vibrations at 2103 cm^{-1} for dimyristoyl phosphatidylcholine-d_{54} is shown in Fig. 3. The linewidth increases sharply from 40.5 to 45 cm^{-1} with a transition temperature of about 24°C. The presence of fluorescent impurities introduces some uncertainty into the temperature determination. The relatively rapid formation of *gauche* rotamers below T_m is also evident from Fig. 3 as measured by the variation in the linewidth from 36.8 cm^{-1} at 1.5°C to 39.8 cm^{-1} at 16°C. Similar effects were noted for dimyristoyl phosphatidylcholine by Yellin and Levin [8]. It is clear from Fig. 3 that the variation in the linewidth at 2103 cm^{-1} is a suitable probe of phospholipid conformation in deuterated systems.

(III) Dimyristoyl phosphatidylcholine-d_{54}/distearoyl phosphatidylcholine binary mixtures

Spectra typical of those from 1 : 1 binary mixtures of dimyristoyl phosphatidylcholine-d_{54} and distearoyl phosphatidylcholine are shown in Fig. 4. The spectral region shown (1900—3800 cm^{-1}) includes the C-^{2}H stretching modes of the deuterated component near 2100 cm^{-1}, the C-H stretching vibrations near 2900 cm^{-1} arising primarily from distearoyl phosphatidylcholine (with a slight contribution of about 5% from the non-deuterated head group of the dimyristoyl phosphatidylcholine-d_{54}) and the O-H stretching vibration of the solvent appearing as a broad feature centered at 3300 cm^{-1}. The sloping background (decreasing with increasing frequency shift) is due to fluorescence.

The temperature variation of the linewidth at 2103 cm^{-1} for the dimyristoyl phosphatidylcholine-d_{54} component is shown in Fig. 5. Also plotted is the ratio of the intensities of the antisymmetric to the symmetric C-H stretching vibrations at 2880 and 2850 cm^{-1}, respectively, for the distearoyl phosphatidylcholine. This ratio has previously been shown to be sensitive to hydrocarbon conformation [9,16,17]. The linewidth at 2103 cm^{-1} undergoes a sigmoid shaped variation, indicating the presence of a gel-liquid crystal phase transition

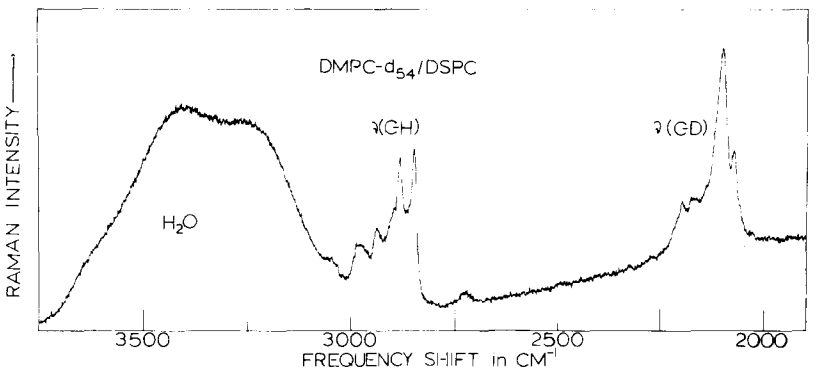

Fig. 4. Typical spectrum of a dimyristoyl phosphatidylcholine (DMPC)-d_{54}/distearoyl phosphatidylcholine (DSPC) binary mixture (1 : 1, mole ratio). Resolution approx. 4 cm^{-1}. Intensity of the $C^{-2}H$ (C-D) stretching mode at 2100 cm^{-1} corresponds to approx. 8000 counts per s. Data shown are for 2°C.

with $T_m \approx 21°C$ in the dimyristoyl phosphatidylcholine-d_{54} component of the binary mixture. As in multilayers of dimyristoyl phosphatidylcholine-d_{54}, the presence of significant *gauche* rotamer formation below T_m is evident from Fig. 5 (compare Figs. 3 and 5). No further transition in the linewidth at 2103 cm^{-1} is noted within the error of measurement (±0.5 cm^{-1}). If it is assumed that chain deuteration does not drastically effect the phase behaviour of dimyristoyl phosphatidylcholine (see ref. 18 for some discussion of this point) then it is clear from a comparison of Figs. 1 and 5 that the dimyristoyl phosphatidylcholine component takes part primarily in the lower transition, and the upper transition at 47°C seen in Fig. 1 is therefore due primarily to the distearoyl phosphatidylcholine.

The I (2880)/(2850) ratio of the distearoyl phosphatidylcholine component, surprisingly, seems to respond to the melting of the dimyristoyl phosphatidylcholine-d_{54}. The ratio increases initially and has a maximum value at about 18°C, close to the transition temperature of the other component. This is

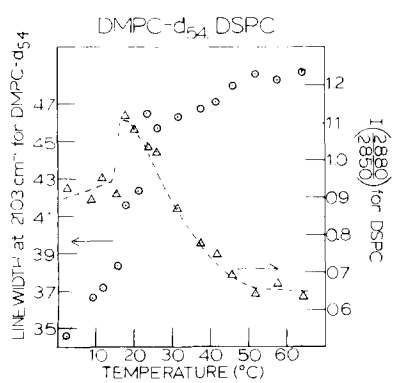

Fig. 5. Temperature variation of (i) the linewidth at 2103 cm^{-1} (○.....○) for the dimyristoyl phosphatidylcholine-d_{54}/distearoyl phosphatidylcholine (DSPC) binary mixture. Left-hand ordinate scale used. (ii) The I (2880)/I (2850) intensity ratio of the antisymmetric to the symmetric C-H stretching vibrations in the distearoyl phosphatidylcholine component of the mixture (△-----△). Right-hand ordinate scale used.

followed by a gradual decrease until about 50°C when a plateau is reached (Fig. 5). The observation of a rapid increase in I (2880)/(2850) with temperature between 15 and 18°C as shown in Fig. 5 is not the normal direction of the temperature variation expected for this parameter [9,16,17] which usually decreases with increasing temperature. For example, I (2880)/(2850) for distearoyl phosphatidylcholine multilayers in H_2O decreases monotonically from 1.16 at 3°C to 1.03 at 28°C (Mendelsohn, R. and Maisano, J., unpublished). The apparently anomalous behaviour of I (2880)/(2850) in the mixture is considered below.

Discussion

The calorimetric studies of Phillips et al. [10] and the spin-label studies of Shimshick and McConnell [11] produced rather similar phase diagrams for the dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine system. It was suggested that this system behaves in monotectic fashion. In such a case, the components of binary mixtures crystallise separately as the system is cooled and therefore lateral phase separation occurs in the gel state. In addition, the gel to liquid crystal transition (melting) temperature for the lower melting component is independent of its mole fraction in the binary mixture. More recently, however, the high sensitivity differential scanning colorimetry studies of Mabrey and Sturtevant [12] indicated that there was no region of truly isothermal melting such as that expected for a monotectic system. Instead, a gradual increase in the melting temperature for the lower melting component from 25 to 28°C was observed as its mole fraction in the binary mixture was decreased from 90 to 50%. However, the dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine system phase diagram deviated markedly from ideal behavior. At 0.36 mole fraction of distearoyl phosphatidylcholine, two main peaks are noted in the plot of excess heat capacity versus temperature (Fig. 3A in ref. 12). The onset temperature of the lower transition (at about 1 : 1 mol ratio) is about 28°C while the completion temperature of the higher melting transition is about 47°C. It is evident from Fig. 3A of ref. 12 that a region of reduced excess heat capacity occurs between the two main transitions and appears at about 35°C. The calorimetric results and the Raman data presented here are therefore in good agreement as to the shape of the melting curve (Fig. 1).

The current observation of two distinct transitions, in itself is consistent with either of the afore-mentioned phase diagrams for the dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine system. The Raman data cannot discriminate between the required 100% participation of the dimyristoyl phosphatidylcholine component in the lower transition for a monotectic system and the necessarily extensive (say 95%) participation of the dimyristoyl phosphatidylcholine component required to describe the non-ideal phase behaviour proposed by Mabrey and Sturtevant [12]. Furthermore, the temperatures for the observed calorimetric transitions are obtained by an extrapolation procedure and may not be directly comparable with the melting temperatures derived from alterations in the *trans/gauche* population ratio as measured from the Raman experiment.

Three lines of evidence further demonstrate the lack of any extensive participation of distearoyl phosphatidylcholine in the lower transition as required from the calorimetric data. (1) The absence of any significant frequency shift in the vibration at 2880 cm^{-1} when dimyristoyl phosphatidylcholine-d_{54} melts suggests that no *gauche* rotamers form in the distearoyl phosphatidylcholine component. Formation of *gauche* rotamers causes about a 10 cm^{-1} increase in this frequency [9]. (2) The similarity of the melting profiles for dimyristoyl phosphatidylcholine-d_{54} alone and in the binary mixture (compare Figs. 3 and 5) strongly suggests that this component in the binary mixture does not contain a significant amount of distearoyl phosphatidylcholine. (3) When the formation of *gauche* rotamers occurs in a given phospholipid, a decrease in I (2880)/(2850) is noted. Exactly the opposite behaviour is observed in Fig. 5 for the distearoyl phosphatidylcholine.

The temperature dependence of I (2880)/I (2850) for the distearoyl phosphatidylcholine component of the binary mixture is the opposite of that normally observed, as noted above. The origin of this observed increase from 0.91 at 15°C to 1.12 at 18°C is not entirely clear but some evidence pertinent to this point has recently appeared [9]. It has been shown that the I (2880)/I (2850) ratio is sensitive to lateral interaction changes in the hydrocarbon chains [4,9,15] as well as to the intramolecular formation of *gauche* rotamers [15,16]. The sensitivity of this ratio to lateral packing was clearly demonstrated by Gaber and Peticolas [9]. They monitored I(2880)/I(2850) for solid hexadecane in matrices with varying mol fractions of perdeuterated hexadecane. The experiments were therefore done at a more or less constant *trans/gauche* population ratio. At 100 mol % hexadecane, I (2880)/I (2850) was 2.2 while at 20 mol % it dropped to about 1.5, a decrease of about one-third. This effect was attributed to destruction of intermolecular vibrational coupling between adjacent chains as a result of the vibrational frequencies in the deuterated derivatives being greatly altered. Part of the Raman intensity at 2880 cm^{-1} derived from Fermi resonance was thereby lost, leading to the observed reduction in the intensity ratio. In the current study, the interaction of light hydrocarbon chains in the distearoyl phosphatidylcholine component of the binary mixture is reduced by the presence of deuterated chains of the dimyristoyl phosphatidylcholine-d_{54}. At temperatures below the melting of the dimyristoyl phosphatidylcholine-d_{54}, this effect results in a reduction of the I (2880)/I (2850) ratio in the binary mixture compared with the pure component (0.93 in the mixture of 2°C, vs. 1.16 in distearoyl phosphatidylcholine multilayers at 3°C). At temperatures close to T_m for dimyristoyl phosphatidylcholine-d_{54}, it appears that the segregation of each of the individual phases leads to increased lateral interactions of the distearoyl phosphatidylcholine chains, and concomitant restoration of that part of the Raman intensity derived from interchain vibrational coupling effects. At 20°C, therefore, I (2880)/I (2850) for distearoyl phosphatidylcholine in the mixture is the same within experimental error as for the pure component (1.13 vs. 1.10). The C-H intensity at 2880 cm^{-1} is therefore fully restored.

If the current interpretation for the observed variation in I(2880)/I(2850) is correct, it provides further evidence supporting the view of Mabrey and Sturtevant [12] that lipid phase separation does not occur in the gel phase for

this system. The initially low value for I (2880)/I (2850) in the gel phase suggests that significant intermixing occurs between the non-deuterated chains of the distearoyl phosphatidylcholine with the deuterated ones of dimyristoyl phosphatidylcholine. Extensive phase separation (and concomitant restoration of the I (2880)/I (2850) ratio to values approaching pure distearoyl phosphatidylcholine multilayers) only occurs when most of the dimyristoyl phosphatidylcholine has melted.

The temperature variation of I (2880)/I (2850) for distearoyl phosphatidylcholine above 20°C in the binary mixture (as shown in Fig. 5) does not indicate the presence of a reasonably cooperative phase transition for this component such as that monitored via the skeletal optical modes in Fig. 1. Since the different spectral parameters measured in the two cases respond, in part, to different aspects of hydrocarbon chain conformation, the two spectral regions need not have the same temperature dependence. As Gaber and Peticolas [9] have shown, temperature variations in inter- and intramolecular effects are not necessarily coupled. Further evidence of the independence of changes in inter- and intramolecular structure in the current study comes from examination of the variation in the frequency of the 2880 cm^{-1} vibration. The frequency has been shown to increase from 2880 to 2890 cm^{-1} as a result of *gauche* rotamer formation [9]. The temperature variation of this frequency in the current work has a significantly different shape than the variation in I (2880)/I (2850) shown in Fig. 5 and is consistent with the presence of a phase transition in the distearoyl phosphatidylcholine component at about the same temperature as observed via the 1100 cm^{-1} region. A detailed study of the relationship between changes in inter- and intrachain effects and the appearance of the C-H stretching region will be presented in the future.

The advantage of the Raman spectroscopic approach outlined here is inherent in the ability of the technique to directly monitor conformation in both ordered and disordered phases without adding a reporter molecule. Other methods are limited in this respect. For example, it has been shown that stearic acid spin labels migrate to the more fluid phases in multiphase systems [19] and it is known that certain types of fluorescent probes may partition into ordered or disordered regions, depending on the probe structure [20]. In such cases, measurements of phospholipid fluidity may not be representative of the entire sample. With the approach outlined here, it will be possible to prepare samples in which (for example) the fluid regions contain deuterated chains and the ordered regions contain light chains. The conformation of each may then be individually monitored. Such a technique will be used to study the conformation of both boundary as well as bulk lipid in reconstituted systems containing intrinsic membrane proteins. Studies along these lines are under way.

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