OFFICE OF NAVAL RESEARCH

Grant N00014-89-J-1261

R&T Code 4131038

ONR Technical Report
#32

Alkyl Chain Ordering of Asymmetric Phosphatidylcholines Adsorbed at a Liquid-Liquid Interface

by

B. L. Smiley and G. L. Richmond

J. Phys. Chem., submitted

Department of Chemistry
1253 University of Oregon
Eugene, OR 97403

June 1998

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	30 May 1998	Technical 3/1/97- 5/31/98

4. TITLE AND SUBTITLE
Alkyl Chain Ordering of Asymmetric Phosphatidylcholines Adsorbed at a Liquid-Liquid Interface

5. FUNDING NUMBERS
N00014-89-J-1261

6. AUTHOR(S)
B. L. Smiley and G. L. Richmond

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dept of Chemistry
University of Oregon
Eugene, OR 97403

8. PERFORMING ORGANIZATION REPORT NUMBER

ONR Technical Report
#32

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Dr. Peter Schmidt
Office of Naval
Research Physical
Science and Technology, ONR 331
800 North Quincy Street
Arlington, VA 22217-5000

10. SPONSORING/MONITORING AGENCY

11. SUPPLEMENTARY NOTES

J. Phys. Chem., submitted

12A. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release: distribution unlimited

12B. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Please see attached
abstract

14. SUBJECT TERMS
Interfacial Adsorption and Desorption of Phosphatidylcholine (PC) Monolayers

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	

Alkyl Chain Ordering of Asymmetric Phosphatidylcholines Adsorbed at a Liquid-Liquid Interface

B.L. Smiley and G.L. Richmond

Department of Chemistry, University of Oregon, Eugene, OR 97403

condensed title: *Alkyl Chain Ordering of PC Monolayers*

keywords: *vibrational sum frequency spectroscopy; oil-water interface; phospholipids; monolayer; hydrocarbon chain ordering*

ABSTRACT

Interfacial adsorption and desorption of phosphatidylcholine (PC) monolayers is an important mechanism by which complex biological systems dynamically control physical and biochemical surface properties. We present vibrational spectroscopic investigations of hydrocarbon chain ordering in PC monolayers adsorbed from aqueous solution to an oil (i.e. carbon tetrachloride)-water interface as a quantitative measure of molecular-level organization pertinent to the surface characteristics displayed by these films. In a series of saturated symmetric and asymmetric chain PCs, both symmetric PCs with 16 or fewer carbons per acyl chain and highly asymmetric PCs produced relatively disordered films at the liquid-liquid interface. The longest chain PCs studied, 1,2-distearoyl-sn-PC (C_{18}:C_{18}), 1-stearoyl-2-palmitoyl-sn-PC (C_{18}:C_{16}) and 1-palmitoyl-2-stearoyl-sn-PC (C_{16}:C_{18}), formed well-ordered crystalline phase monolayers at room temperature. The results can be explained in terms of enhanced chain-chain interactions among the longer, nearly symmetric hydrocarbon chains which occur only in the absence of intercalating solvent. Properties of the neat oil-water interface which may influence the formation of these well-ordered monolayer phases are discussed.

INTRODUCTION

Surfactant monolayers adsorbed at oil-water interfaces in biological organisms are involved primarily in the formation and stabilization of emulsions which allow water-insoluble fats to be consumed

and metabolized in an aqueous environment. The best known examples include bile salts secreted into the intestinal tract for the digestion of dietary fats (Prince, 1973), and plasma lipoproteins (PLs), which include chylomicrons from the small intestine and very low density lipoproteins (VLDLs) produced by the liver (Handa, et al., 1990). PLs are the means by which digested fats are transported through the blood stream. They consist mostly of a triglyceride core surrounded and stabilized by a phospholipid monolayer composed primarily of phosphatidylcholines (PCs) and a small percentage of surface-adsorbed protein (Miller and Small, 1987). Proteins in the blood bind to the PL surface and begin to hydrolyze the triglyceride core and only a small fraction of the phospholipids, such that the core volume shrinks and the surface monolayer begins to flatten and transform into bilayer structures (Schumaker and Lembertas, 1992). The efficiency with which surface-adsorbed proteins are able to hydrolyze the triglyceride core has been shown to depend on the acyl chain composition of monolayer PCs (Redgrave, et al., 1992). Other than bulk thermodynamic properties, however, little is known on the molecular level about the organization of PC monolayers at a liquid-liquid interface which would lead to different metabolic effects observed as a function of hydrocarbon chain composition.

Interest in the commercial use of natural phosphatidylcholines (or lecithins) as emulsifiers, including their use in delivery of water-insoluble drugs (Davis, et al., 1985) and in food preparations (Krog, et al., 1985), arises from the unusual natural surface properties of PCs (Ogino and Onishi, 1981). The ability of PCs to form condensed, relatively stable monolayers at an oil-water interface in comparison to monolayers formed by other surfactants (e.g. phosphatidylserine, phosphatidylethanolamine, cholesterol, oleic acid) is well recognized (Ogino and Onishi, 1981; Handa et al., 1990). A surfactant's ability to form a liquid-condensed film at the oil-water surface is in fact considered prerequisite for the formation of a thermodynamically stable microemulsion (Prince, 1973). The role of PCs as an essential component in lung surfactant monolayers has also been well recognized (Goerke, 1974), while PCs and other phospholipids in bilayer form (i.e. liposomes) are perpetually being studied as model cell membranes. Our aim in the described work has been to shed further light on the molecular-level structure of PCs adsorbed at an oil (CCl_4)-water interface looking specifically at ordering of the hydrocarbon chains.

The large majority of biological phospholipids contain two dissimilar hydrocarbon chains per molecule. The two chains may differ in length and degree of unsaturation such that a highly complex mixture of phospholipid structures is present in a particular bilayer membrane, allowing other structural elements such as integral membrane proteins which are distributed asymmetrically across a bilayer, to be accomodated without disruption of bilayer integrity (Huang and Mason, 1986). In spite of the asymmetry typical of phospholipids in vivo, bilayer and particularly monolayer investigations aimed at understanding the structural organization and properties of these molecules have focused mostly on saturated, symmetric chain phospholipids, with some exceptions (Evans, et al., 1987; Ali, et al., 1998). Little is therefore known about asymmetric chain phospholipids in bilayers, and even less is known about their monolayer properties.

In the bilayer state below the gel-to-liquid crystalline phase transition temperature, the packing arrangement adopted by a particular type of PC is largely determined by the amount of chain mismatch between two saturated hydrocarbon chains (Huang and Mason, 1986). An intermediate degree of chain length mismatch, between that of well-ordered PC bilayers with high and low chain mismatch, actually produces the most disordered bilayer state of the hydrocarbon chains due to the nature of chain-chain interactions between opposing monolayers (Huang and Mason, 1986). We expect PC monolayers in the absence of an opposing monolayer to exhibit distinctly different trends as a function of chain mismatch. Our measurements at the oil-water interface utilizing nonlinear optical vibrational spectroscopy to selectively probe the adsorbed monolayers indicate clear trends in chain ordering not anticipated based on available thermodynamic data.

THEORY

Vibrational Sum Frequency Spectroscopy

Vibrational sum frequency spectroscopy (VSFS) is a surface-specific nonlinear optical technique which has been widely applied in the study of molecular-level ordering at many types of gas, liquid, and solid interfaces, including buried interfaces (Shen, 1994; Bell, et al., 1996; Eisenthal, 1996; Conboy, et al., 1997). Sum frequency generation, as a second-order process, is symmetry forbidden in isotropic

media under the electric dipole approximation and is therefore allowed only at interfaces where the inversion symmetry is necessarily broken. Surface-specific information may therefore be gained using VSFS even at buried interfaces where adsorbed molecules are in equilibrium exchange with the surrounding bulk solutions.

Spatial and temporal overlap of two intense optical fields at frequencies ω_1 and ω_2 on a surface induces a polarization, P_{SF}, in the surface region at the sum frequency of the two fields, $\omega_{SF} = \omega_1 + \omega_2$. As given in Eq. 1, the induced polarization generates an electric field, E_{SF}, proportional to the product of the amplitudes of the incident electric fields, and to the second order nonlinear susceptibility, $\vec{\vec{\chi}}^{(2)}$, of the surface region on which the fields are incident.

$$E_{SF} \propto P_{SF} = \vec{\vec{\chi}}^{(2)} E_{VIS} E_{IR} \qquad [1]$$

In these experiments, the more intense field, $E(\omega_1) = E_{VIS}$, is in the visible region, and the second field, $E(\omega_2) = E_{IR}$, is tunable across the IR region of interest. The SF signal intensity is measured in reflection as shown schematically in Fig. 1. The signal generated at the sum frequency, ω_{SF}, is resonantly enhanced due to an increased molecular polarizability when ω_{IR} is tuned across allowed molecular vibrational transitions. Our measurements are sensitive only to this resonant component of the second order nonlinear susceptibility, $\vec{\vec{\chi}}_R^{(2)} = N\langle \alpha^{(2)} \rangle$, which is proportional to the molecular hyperpolarizability, $\alpha^{(2)}$, and the surface density, N, of interfacial molecules contributing to the sum frequency signal. As described in the Results and Discussion section, the latter is not necessarily equal to the number density of molecular species present at the interface since molecular ordering can render some vibrational modes sum frequency inactive. An average is taken over the distribution of molecular orientations in the surface region as indicated by the brackets. Eq. 2 describes the frequency dependence of $\alpha^{(2)}$,

$$\alpha^{(2)} = \sum_n \frac{A_n}{\omega_n - \omega_{IR} - i\Gamma_n} \qquad [2]$$

where, summing over all vibrational modes, ω_n is the frequency of the n^{th} vibrational mode, Γ_n is the width of the transition, and A_n is an amplitude coefficient proportional to the product of the Raman and IR transition moments (Du, et al., 1993). For a vibrational mode to be sum frequency active, A_n must be nonzero which requires that both the Raman and IR transition moments are nonzero for the particular vibration. As ω_{IR} approaches the frequency of an allowed vibrational transition, $\alpha^{(2)}$ in Eq. 2 becomes very large and a relatively large sum frequency signal is generated from the interface. Judicious choice of polarization conditions for the ω_{VIS} and ω_{IR} fields incident on the sample surface and selection of the polarization of the SF field measured using an analyzer allows SF active vibrational modes either normal to or in the plane of the surface to be separated. Data reported are for vibrational modes with a component normal to the interface plane.

MATERIALS AND METHODS

Materials

Phosphatidylcholines of all chain permutations were obtained from Avanti Polar Lipids (Alabaster, AL) in powder form, purity greater than 99%, and used as received. The racemic mixture of DSPC, 99% purity, was obtained from Sigma Chemical Company (St. Louis, MO). Carbon tetrachloride (CCl_4), 99.9+%, HPLC grade, was from Sigma-Aldrich. 10 mM phosphate buffer, pH 7.0 prepared in deuterium oxide (D_2O), 99.9%, HPLC grade, either from Aldrich (Milwaukee, WI) or Cambridge Isotope Laboratories (Andover, MA) was used as the aqueous phase and for preparation of phospholipid stock solutions.

Sample Preparation

The sample cell and all glassware were soaked in Nochromix glass cleaner, rinsed in water from a Barnstead Nanopure filtration system, and dried in an oven prior to use. Phospholipid stock solutions were prepared at concentrations less than 1 mM by prewarming the powder in D_2O buffer to above the gel to liquid crystalline phase transition temperature, T_m, for the particular lipid followed by bath sonication, also above T_m, for a time period at least sufficient to form a homogeneous solution. Following sonication, stock solutions were allowed to cool to room temperature before use. The sample cell was prepared with

CCl_4 as the bottom phase, and D_2O buffer as the top phase and allowed to sit for at least two hours prior to addition of the phospholipid in order to allow any possible surface-active contaminants to adsorb at the liquid-liquid interface. The sample cell was recleaned and prepared again with fresh solutions if surface contamination was evident at the neat CCl_4-D_2O buffer interface. Phosphatidylcholine monolayers were then prepared as follows: 1) an injection of the PC stock solution was made into the bulk aqueous phase using a Hamilton syringe, 2) PC adsorption to the CCl_4-D_2O interface was allowed to occur overnight, and 3) in general, a second injection from the same stock phospholipid solution was made following the overnight equilibration period. This second injection was made vigorously such that the partially formed PC monolayer was physically perturbed by the addition. The sample cell was allowed to sit for several hours following the second injection before beginning VSFS measurements. The second injection was necessary to promote room temperature formation of close packed monolayers given that PCs which are below their respective gel-to-liquid crystalline phase transition temperatures under ambient conditions do not form close packed monolayers from a single concentrated injection (Walker, et al., 1997). For some of the PCs, particularly those found to be less well ordered by VSFS, the second stock solution injection did not significantly affect monolayer ordering. Therefore, data for monolayers prepared by both single and double stock solution injections are included for these points.

Vibrational Spectroscopy

The optical system used for VSFS measurements has been described previously (Conboy, et al., 1996). Only a brief description is therefore given here. The visible field at 532 nm is generated by frequency doubling a portion of the 1064 nm output of a Nd:YAG laser producing 12 nanosecond pulses at 10 Hz. The other portion of the 1064 nm Nd:YAG output generates 1-2 mJ IR pulses tunable in the range 3.2-3.7 μm via a $LiNbO_3$ optical parametric oscillator. The laser system has a 6 cm^{-1} bandwidth. The 532 nm and IR beam are directed at the sample surface at their critical angles, as shown in Fig. 1, to enhance the SF signal generated. The SF signal from the interface is measured in reflection. D_2O, rather than H_2O, is used as the aqueous phase to reduce absorption of the IR pulse energy by water at the sample interface which can cause boiling.

RESULTS AND DISCUSSION

Spectra from 1-myristoyl-2-stearoyl-sn-PC (MSPC), 1-palmitoyl-2-stearoyl-sn-PC (PSPC) and 1,2-distearoyl-sn-PC (DSPC) monolayers adsorbed at the CCl_4-D_2O interface for S-polarized sum frequency signal, S-polarized visible and P-polarized IR fields (i.e. SSP) are shown in Fig. 2 as a function of the IR energy. Close packed monolayers were formed at the CCl_4-D_2O interface for these samples as evidenced by a complete relaxation of the meniscus between the two liquids with the exception of the DSPC monolayer which displayed a small amount of residual surface curvature. Representative surface pressure measurements of similarly prepared samples indicated terminal surface pressures of approximately 42 mN/m in agreement with other studies (Walker, et al., submitted). The MSPC (C_{14}:C_{18}) spectrum in Fig. 2(a) shows a prominent methylene symmetric stretch (CH_2-SS) peak centered at 2850 cm^{-1} next to the more narrow methyl symmetric stretch (CH_3-SS) peak at 2875 cm^{-1} (Snyder, et al., 1982; Conboy et al., 1997). These two peak assignments have been confirmed by recent studies on selectively deuterated surfactants (Conboy et al., 1997). A vibrational resonance peaked at approximately 2932 cm^{-1} is thought to include contributions from methylene asymmetric stretches (CH_2-AS), with the shoulder on the high energy side at approximately 2944 cm^{-1} assigned to a methyl Fermi resonance (CH_3-FR) (Snyder et al., 1982; Conboy et al., 1997). The broad structure centered at approximately 2905 cm^{-1} has been assigned to a methylene Fermi resonance (Snyder et al., 1982; Conboy et al., 1997)

The SSP polarization condition selects only sum frequency active vibrational modes with a component of the transition moment normal to the interface plane. In the SSP spectra, we use the area ratio of the CH_3-SS to the CH_2-SS to provide a relative measure of monolayer hydrocarbon chain ordering (Bell et al., 1996). For very well ordered monolayers with all *trans* chain conformations the CH_2-SS is symmetry forbidden in VSFS and is therefore absent (Guyot-Sionnest, et al., 1987). As monolayer disorder increases, the CH_2-SS becomes sum frequency active to a degree related to the degree of hydrocarbon chain disorder. The CH_3-SS mode shows increased intensity with increased monolayer ordering as the fields couple to a less random and more upright orientation of the CH_3 symmetry axis. Consequently the area ratio CH_3-SS/CH_2-SS becomes very large for well ordered monolayers and is small

for disordered monolayers from a combined effect. For MSPC (C_{14}:C_{18}), the calculated ratio CH_3-SS/CH_2-SS is 0.74 ± 0.15 as determined from experiments on multiple samples. The prominent signal from the CH_2-SS mode indicates that this is a relatively disordered monolayer. In Fig. 2(b), a representative SSP spectrum for PSPC (C_{16}:C_{18}) which has two more methylene units in the sn-1 chain than MSPC is shown with a much less prominent CH_2-SS mode and a strong CH_3-SS mode compared to MSPC. The spectrum for symmetric chain DSPC (C_{18}:C_{18}) shows little or no CH_2-SS. As given in Table 1, the calculated ratios for PSPC and DSPC are 3.08 ± 0.93 and 4.59 ± 1.38, respectively. The larger error inherent in the measurements for the more ordered monolayers stems from difficulty in determining the area of a very small CH_2-SS peak overlapping the tail of the CH_3-SS mode. Nevertheless, it is clear that both PSPC and DSPC produce extremely well ordered monolayers with few gauche chain conformations which, on the basis of the strong CH_3-SS mode, likely have chains oriented near normal to the surface plane. This observation is consistent with increased chain-chain interactions expected for the longer alkyl chains as has been observed in monolayers at the air-water interface (Evans et al., 1987; Ali et al., 1998; Walker, et al., submitted). At room temperature, all three PCs are in their respective gel states (Marsh, 1990).

The ordering of a series of symmetric chain PC monolayers adsorbed at similar interfacial concentrations at the CCl_4-D_2O interface was determined by VSFS as a function of diacyl chain length. As shown in Fig. 3, the calculated CH_3-SS/CH_2-SS peak area ratio is nearly constant or slightly decreasing for DLPC (C_{12}:C_{12}) through DPPC (C_{16}:C_{16}), followed by a sharp increase in chain ordering as previously noted for DSPC (C_{18}:C_{18}) in Fig. 2(c). An abrupt increase in chain ordering from relatively disordered DPPC monolayers, T_m = 41°C (Marsh, 1990) , to very well ordered DSPC monolayers, T_m = 55°C (Marsh, 1990), by the addition of only two methylene units per chain is evidence for a distinct difference in the monolayer phases below the respective main transition temperatures which will be discussed further. Walker et al. (submitted) have made similar measurements on symmetric PCs adsorbed above the respective T_ms at the CCl_4-D_2O interface using a single injection technique rather than the double injection, room temperature adsorption used here. Those results are similar for DLPC (C_{12}:C_{12}) through DPPC (C_{16}:C_{16}) where the CH_3-SS/CH_2-SS ratio shows a slight decrease with chain length at the CCl_4-

D_2O interface. However, in contrast to our results, the authors did not observe a significant change in chain ordering for DSPC as compared to DPPC. The slight decrease in chain ordering with increasing chain length was attributed to enhanced solvation of the longer acyl chains by CCl_4 (Walker et al., submitted). Excluding DLPC which is above T_m at room temperature, monolayer adsorption below T_m using the technique described has been shown to result in more ordered monolayers than those produced on adsorption above T_m (Smiley, et al., 1998). It is possible that CCl_4 is not able to penetrate between the hydrocarbon chains of the gel-state phospholipid during room temperature adsorption. The 55 $Å^2$ per PC molecule estimated from surface pressure measurements by Walker et al. (submitted) for adsorption at the CCl_4-water interface above T_m also indicates that, in spite of the high final surface pressures obtained in these measurements which would otherwise indicate formation of a close-packed monolayer, the PCs may be in either a liquid expanded or phase coexistence region of the isotherm (Mingins, et al., 1982).

Although there is always one CH_3 group per chain which may contribute to the VSF signal, the possible number of gauche conformations per chain, which produce a local break in inversion symmetry among adjacent CH_2 units making them SF active, increases with increasing hydrocarbon chain lengths. An increased CH_2-SS mode intensity observed at the CCl_4-water interface in expanded surfactant monolayers with increasing hydrocarbon chain lengths was attributed to a greater number of gauche conformations per chain (Conboy, et al., submitted). Normalization of our SF data to account for differences in the number of CH_2 contributors to the SF spectrum was therefore considered. However, where significant changes in ordering are apparent our data show a clear decrease in the CH_2-SS mode intensity (i.e. larger ratio) with increasing hydrocarbon chain length which is inconsistent with a greater number of SF active CH_2 units in the longer hydrocarbon chain PCs. Normalization of our data to the number of methylene units present was therefore not appropriate.

Peak area ratios calculated from VSFS measurements on monolayers of 1-stearoyl-2-capryl-sn-PC (SCPC; C_{18}:C_{10}), 1-stearoyl-2-lauroyl-sn-PC (SLPC; C_{18}:C_{12}), 1-stearoyl-2-myristoyl-sn-PC (SMPC; C_{18}:C_{14}), 1-stearoyl-2-palmitoyl-sn-PC (SPPC; C_{18}:C_{16}) and DSPC (C_{18}:C_{18}) adsorbed at room temperature to the liquid-liquid interface are given in Table 1 and plotted as a function of 2-acyl chain

length in Fig. 4. CH_3-SS/CH_2-SS peak ratios less than 1 were obtained for SCPC, SLPC and SMPC, SPPC being significantly more ordered and DSPC most ordered of the series. The difference in chain length, or chain mismatch, between the sn-1 and sn-2 acyl chains of the PC molecules clearly influences the monolayer packing arrangement. The mismatched portion of the longer hydrocarbon chains might be expected to have greater disorder proportional to the degree of mismatch resulting from reduced chain-chain interactions, consistent with our observations. In bilayers, differences in length of the two PC hydrocarbon chains, and particularly the mismatch between them, are thought to produce different degrees of interdigitation with resulting differences in ordering of the hydrocarbon chains (Huang and Mason, 1986). In the same series SCPC (C_{18}:C_{10}), SLPC (C_{18}:C_{12}), SMPC (C_{18}:C_{14}), SPPC (C_{18}:C_{16}) and DSPC (C_{18}:C_{18}) below their respective T_ms, the least ordered bilayer was SMPC as deduced from Raman spectroscopy measurements. SCPC and SLPC are thought to form mixed interdigitated bilayers, DSPC a noninterdigitated bilayer, and SPPC likely forms a partially interdigitated bilayer, all of which are reasonably well ordered (Huang and Mason, 1986). SMPC, because of its intermediate degree of chain mismatch, can form neither a partially interdigitated nor a mixed interdigitated layer structure and is therefore the most disordered (Huang and Mason, 1986). Consistent with our VSFS data, we might expect a single monolayer in the absence of an interdigitated opposing monolayer to exhibit average ordering of the hydrocarbon chains which is more directly a function of differences in chain length.

In Fig. 4, an increase in chain ordering is also observed for the more symmetric sn-1-palmitoyl (C_{16}) PCs, PSPC (C_{16}:C_{18}) being significantly more ordered than 1-palmitoyl-2-lauroyl-sn-PC (PLPC; C_{16}:C_{12}), 1-palmitoyl-2-myristoyl-sn-PC (PMPC; C_{16}:C_{14}) and symmetric chain DPPC (C_{16}:C_{16}). The effective chain mismatch of DPPC is actually greater than that for asymmetric PSPC due to the conformation of the PC backbone, as will be discussed below. The sn-1-myristoyl (C_{14}) PCs show slightly decreased ordering with increasing sn-2 chain length for monolayers which are all fairly disordered, possibly as a combined effect of a shorter effective chain length and differences in chain mismatch.

In bilayers, chain mismatch has been quantified in a parameter $\Delta C/CL$, in which $\Delta C = | n_1 - n_2 + 1.5 |$ is the mismatch, in number of carbon-carbon bond lengths, between the sn-1 chain with n_1 carbon atoms and the sn-2 chain with n_2 carbon atoms. The number 1.5 comes from the chain mismatch present in a

symmetric chain PC (i.e. $n_1 = n_2$) in which the sn-2 chain extends an estimated 1.5 carbon-carbon bond lengths less than the sn-1 chain into the hydrophobic region (Huang and Mason, 1986). CL is the length, in number of carbon-carbon bonds, of the longer of the two hydrocarbon chains, (n_1 - 1) or (n_2 - 2.5) (Huang and Mason, 1986). Applying a similar analysis to our studies, the CH_3-SS/CH_2-SS ratio is shown vs. calculated chain mismatch, $\Delta C/CL$, in Fig. 5 for the PC monolayers. The most disordered monolayers in the sn-1-stearoyl (C_{18}) series, as measured by VSFS, have a $\Delta C/CL$ greater than 0.3. A clear increase in hydrocarbon chain ordering is observed for SPPC (C_{18}:C_{16}) and DSPC (C_{18}:C_{18}) which have progressively lower values of the chain mismatch parameter. This is consistent with the surface pressure measurements of Ali, et al. (1998) for room temperature sn-1-stearoyl PC monolayers at the air-water interface in which smaller molecular areas were observed for the more symmetric (i.e. longer sn-2 acyl chain) molecules with lower chain mismatch at "membrane-like" surface pressures, which are thought to correspond to approximately 30 mN/m (Blume, 1979). The PSPC (C_{16}:C_{18}) monolayer, which has a lower chain mismatch than DPPC (C_{16}:C_{16}) as previously mentioned, is far better ordered than any of the other sn-1-palmitoyl (C_{16}) PCs at the CCl_4-D_2O interface, including DPPC. This is again consistent with results from the air-water interface in which saturated sn-1-palmitoyl PCs with the sn-2 acyl chain 16 or more carbons in length have smaller molecular areas indicative of more tightly packed layer (Evans et al., 1987). It is interesting that, in spite of the different calculated chain mismatch for PC isomers having the same two chains (e.g. C_{16}:C_{18} vs. C_{18}:C_{16}), the monolayer chain ordering as deduced from VSFS does not seem to depend on the position, sn-1 or -2, to which the longer acyl chain is bound. Although a larger number of samples would be necessary to better define this trend, it is possible that the 1.5 carbon-carbon bond length chain mismatch defined for bilayer systems of symmetric PCs (Huang and Mason, 1986) is not appropriate for PC monolayers adsorbed at the CCl_4-D_2O interface.

Monolayer ordering of PCs, and other surfactants, adsorbed at an oil-water interface is not generally expected to exhibit the same trends as at an air-water interface. Surface pressure vs. area isotherms, especially at the lower molecular surface densities, are expanded at the oil-water as compared to the air-water interface due to hydrocarbon chain solvation by the oil phase (Mingins et al., 1982). Experimental investigations involving VSFS measurements of symmetric chain PC monolayers have

indicated a decrease in hydrocarbon chain ordering with increasing chain length at the liquid-liquid interface, while complimentary measurements at the air-water interface show an opposite trend (Walker, et al., submitted). However, isotherms at the two interfaces overlap at the higher surface pressures and similar molecular areas (Alexander and Teorell, 1939; Mingins et al., 1982; Evans et al., 1987) where oil intercalating between PC hydrocarbon chains in more expanded states at the oil-water interface is presumably forced out.

From the preceding results, it is evident that the monolayer ordering of some PCs adsorbed at the CCl_4-D_2O interface is related to the degree of chain mismatch, but the formation of very well ordered monolayers was observed only for PCs with at least one stearoyl (C_{18}) chain, the second chain being either stearoyl or palmitoyl (C_{16}). Comparison of the sn-1-stearoyl and sn-1-palmitoyl data of Fig. 5 suggests that, as the minimum thickness of the hydrocarbon region of the monolayer (i.e. the effective length of the shorter hydrocarbon chain) is increased, the monolayer can maintain a well-ordered state with a greater degree of chain mismatch up to a point. It is also not likely to be coincidental that the three PCs which form well ordered monolayers also have the highest T_ms, each at least 20°C above room temperature, noting that, although transition temperatures are generally reported for bilayers or aqueous dispersions, monolayer transition temperatures at the oil-water interface have been reported to be comparable (Small, et al., 1988). It has been reported that DPPC and DSPC dispersions in water undergo subtransitions from a crystalline to a gel state at 22°C and 29°C, respectively, following incubation at 0°C for several days (Finegold and Singer, 1984; Huang and Mason, 1986). Many saturated, mixed chain PCs have also been reported to undergo subtransitions. In a series of PCs having a C_{18} chain in the sn-1 or sn-2 position and the chain in the other position varying between 10 and 18 (even numbers) carbons in length, PSPC, DSPC and SPPC had the highest subtransition temperatures (Mattai, et al., 1987). It is possible that the longer chain PC monolayers adsorbed at the CCl_4-D_2O interface undergo a similar transition into a crystalline state below their respective subtransition temperatures, which would acount for the large increase in hydrocarbon chain ordering observed at room temperature for DSPC as compared to DPPC. It has also been reported in some sources that racemic PC mixtures do not exhibit this crystalline-to-gel state subtransition (Boyanov, et al., 1983; Koynova, et al., 1995), while another study on hydrated

DPPC revealed the presence of a DL-DPPC subtransition, only with a much lower excess enthalpy than that measured for L-DPPC (Kodama, et al., 1985). If the longer chain L-PC monolayers undergo a gel-to-crystalline subtransition to form the well-ordered monolayers observed, we would therefore expect a monolayer composed of a racemic mixture of the same long chain PC to be less well ordered, consistent with the lack of a crystalline phase or the presence of a less stable low temperature phase with a lesser degree of monolayer ordering.

Fig. 6(a) shows the spectrum obtained by VSFS for a monolayer of DL-DSPC adsorbed at the CCl_4-D_2O interface by the same method of preparation used for other samples. It is clear from the very large CH_2-SS peak near 2850 cm^{-1} that this monolayer formed by the racemic DSPC mixture is extremely disordered with respect to the hydrocarbon chains. The calculated CH_3-SS/CH_2-SS area ratio for this sample is 0.51 $\pm$ 0.22. It should also be noted that only a scant monolayer was formed at the liquid-liquid interface for this sample (i.e. the interface meniscus was not substantially reduced by addition of the phospholipid). However, using higher bulk aqueous phospholipid concentrations and/or longer adsorption periods up to five days, DL-DSPC did eventually form very well-ordered monolayers at the CCl_4-D_2O interface as evidenced in the spectrum shown in Fig. 6(b). The calculated CH_3-SS/CH_2-SS peak area ratio of 4.55 $\pm$ 1.36 indicates that the DL-DSPC monolayer becomes at least as well-ordered as L-DSPC monolayers allowing sufficient time. These observations suggest that, although both L-DSPC and DL-DSPC adsorbed at the CCl_4-D_2O interface do form crystalline monolayer phases as evidenced by VSFS of the hydrocarbon chains, differences in the rate of monolayer formation are apparent. Especially for condensed assemblies, the same packing arrangements found in monolayers and bilayers of PC L-enantomers are not likely to be geometrically favorable in the mixture. Consistent with our results, it has also been suggested that differences in molecule chirality are more likely to be reflected in packing of the polar headgroup region rather than of the hydrophobic chains (Boyanov et al., 1983).

The observation that DL-DSPC forms well-ordered monolayers less readily than does L-DSPC argues in favor of a gel-to-crystalline subtransition occurring in the long chain PCs studied to produce well-ordered DSPC, SPPC and PSPC monolayers at room temperature. As previously mentioned, subtransitions have been observed typically in enantomerically pure saturated PCs after low temperature

incubation near 0°C for several days to months (Finegold and Singer, 1984; Mattai et al., 1987). It is therefore quite intriguing to have obtained crystalline monolayer formation in less than 24 hours at 22°C. A possible explanation for this phenomenon is provided by the "ice-like" structure of water (D_2O) molecules which has been experimentally observed at an oil-water interface (Gragson and Richmond, 1997). Although molecular-level processes required for the formation of the crystalline (L_c) phase in PCs have not been elucidated, the near freezing water in which PCs are incubated leading to formation of the L_c phase is most certainly "ice-like." The interfacial water structure both in the PC dispersions and at the liquid-liquid interface may serve to nucleate the L_c phase. It is noteworthy that emulsion particles, modeling plasma lipoproteins, which were stabilized by long chain symmetric PCs are metabolized in vivo at a much reduced rate compared to emulsions stabilized by a more fluid unsaturated PC closer to the natural composition (Redgrave et al., 1992). The well-ordered layers of the symmetric, long-chain PCs may therefore overstabilize the emulsion, forming a barrier to normal metabolic processes.

CONCLUSIONS

We have reported original observations of differences in hydrocarbon chain ordering among a series of saturated symmetric and asymmetric chain PC monolayers adsorbed at a CCl_4-D_2O interface which depend on the width of the hydrophobic region of the monolayer and degree of hydrocarbon chain length mismatch within the PC molecule. Three of the PCs studied, specifically SPPC (C_{18}:C_{16}), DSPC (C_{18}:C_{18}) and PSPC (C_{16}:C_{18}), formed extremely well-ordered monolayers at the liquid-liquid interface by room temperature adsorption from the aqueous phase. Highly asymmetric PCs formed relatively disordered monolayers, as might be expected from the reduced chain-chain interactions among the mismatched portions of the longer chains. The shorter chain PC monolayers (<16 carbons/chain) were also relatively disordered. The greater disorder seen in the shorter chain PCs, irrespective of chain mismatch, is again a likely consequence of reduced chain-chain interactions over a smaller hydrophobic layer thickness.

It is noteworthy that the main differences measured in monolayer organization could not be predicted based on the respective gel-to-liquid crystalline (bilayer) phase transition temperatures.

Disordered monolayers were observed both for PCs above and below their respective T_ms at room temperature. The most pronounced differences in monolayer organization (i.e. the well-ordered long chain PCs) may result from monolayer subtransitions. The apparent ability of the oil-water interface to nucleate the formation of adsorbed crystalline PC monolayers in the absence of extended low temperature equilibration points to differences between bilayer and oil-water monolayer PC assemblies distinct from chain solvation effects. Further interface-specific characterizations using VSFS will help to shed light on additional potentially unique features of PCs and other natural surface-active molecules adsorbed at liquid-liquid interfaces.

ACKNOWLEDGEMENTS

The authors thank Dr. R.A. Walker for helpful discussions and T. E. Hannon for preliminary studies with the asymmetric PCs. Financial support from the National Science Foundation (CHE 972575) and the Office of Naval Research is gratefully acknowledged.

REFERENCES

Alexander, A. E. and T. Teorell. 1939. A study of films at the liquid-liquid interface. *Trans. Faraday Soc.* 35:727-737.

Ali, S., J. M. Smaby, M. M. Momsen, H. L. Brockman and R. E. Brown. 1998. Acyl chain length asymmetry alters the interfacial elastic interactions of phosphatidylcholines. *Biophys. J.* 74:338-348.

Bell, G. R., C. D. Bain and R. N. Ward. 1996. Sum-frequency vibrational spectroscopy of soluble surfactants at the air/water interface. *J. Chem. Soc., Faraday Trans.* 92(4):515-523.

Blume, A. 1979. A comparative study of the phase transitions of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta* 557:32-44.

Boyanov, A. I., B. G. Tenchov, R. D. Koynova and K. S. Koumanov. 1983. Absence of subtransition in racemic dipalmitoylphosphatidylcholine vesicles. *Biochim. Biophys. Acta* 732:711-713.

Conboy, J. C., M. C. Messmer and G. L. Richmond. 1996. Investigation of surfactant conformation and order at the liquid-liquid interface by total internal reflection sum-frequency vibrational spectroscopy. *J. Phys. Chem.* 100(18):7617-7622.

Conboy, J. C., M. C. Messmer and G. L. Richmond. 1997. Dependence of alkyl chain conformation of simple ionic surfactants on head group functionality as studied by vibrational sum-frequency spectroscopy. *J. Phys. Chem. B* 101(34):6724-6733.

Davis, S. S., J. Hadgraft and K. J. Palin 1985. Medical and Pharmaceutical Applications of Emulsions. *In* Encyclopedia of Emulsion Technology. P. Becher, editor. Marcel Dekker, Inc., New York. 159-238.

Du, Q., R. Superfine, E. Freysz and Y. R. Shen. 1993. Vibrational spectroscopy of water at the vapor/water interface. *Phys. Rev. Lett.* 70(15):2313-2316.

Eisenthal, K. B. 1996. Liquid interfaces probed by second-harmonic and sum-frequency spectroscopy. *Chem. Rev.* 96:1343-1360.

Evans, R. W., M. A. Williams and J. Tinoco. 1987. Surface areas of 1-palmitoyl phosphatidylcholines and their interactions with cholesterol. *Biochem. J.* 245:455-462.

Finegold, L. and M. A. Singer. 1984. Phosphayidylcholine bilayers: Subtransitions in pure and mixed lipids. *Chem. Phys. Lipids* 35:291-297.

Goerke, J. 1974. Lung Surfactant. *Biochim. Biophys. Acta* 344:241-261.

Gragson, D. E. and G. L. Richmond. 1997. Comparisons of the structure of water at neat oil/water and air/water interfaces as determined by vibrational sum frequency generation. *Langmuir* 13(18):4804-4806.

Guyot-Sionnest, P., J. H. Hunt and Y. R. Shen. 1987. Sum-frequency vibrational spectroscopy of a Langmuir film: Study of molecular orientation of a two-dimensional system. *Phys. Rev. Lett.* 59(14):1597-1600.

Handa, T., H. Saito and K. Miyajima. 1990. Phospholipid monolayers at the triolein-saline interface: Production of microemulsion particles and conversion of monolayers to bilayers. *Biochemistry* 29:2884-2890.

Huang, C. and J. T. Mason. 1986. Structure and properties of mixed chain phospholipid assemblies. *Biochim. Biophys. Acta* 864:423-470.

Kodama, M., H. Hashigami and S. Seki. 1985. Static and dynamic calorimetric studies on the three kinds of phase transition in the systems of L- and DL-dipalmitoylphosphatidylcholine/water. *Biochim. Biophys. Acta* 814:300-306.

Koynova, R., B. G. Tenchov, S. Todinova and P. J. Quinn. 1995. Rapid reversible formation of a metastable subgel phase in saturated diacylphosphatidylcholines. *Biophys. J.* 68:2370-2375.

Krog, N. J., T. H. Riisom and K. Larsson 1985. Applications in the Food Industry: I. *In* Encyclopedia of Emulsion Technology. P. Becher, editor. Marcel Dekker, Inc., New York. 321-365.

Marsh, D. 1990. CRC Handbook of Lipid Bilayers. CRC Press, Inc., Boston.

Mattai, J., P. K. Sripada and G. G. Shipley. 1987. Mixed-chain phosphatidylcholine bilayers: Structure and properties. *Biochemistry* 26:3287-3297.

Miller, K. W. and D. M. Small 1987. Structure of triglyceride-rich lipoproteins: an analyis of core and surface phases. *In* Plasma Lipoproteins. A. M. J. Gotto, editor. Elsevier, New York. 1-75.

Mingins, J., J. A. G. Taylor, B. A. Pethica, C. M. Jackson and B. Y. T. Yue. 1982. Phospholipid monolayers at non-polar oil/water interfaces. Part 3- Effect of chain length on phase transitions in saturated di-acyl lecithins at the n-heptane/aqueous sodium chloride interface. *J. Am. Chem. Soc., Faraday Trans. 1* 78:323-339.

Ogino, K. and M. Onishi. 1981. Interfacial action of natural surfactants in oil/water systems. *J. Colloid Interface Sci.* 83(1):18-25.

Prince, L. M. 1973. Emulsions. *In* Biological Horizons in Surface Science. L. M. Prince and D. F. Sears, editors. Academic Press, New York. 353-366.

Redgrave, T. G., V. Rakic, B.-C. Mortimer and J. C. L. Mamo. 1992. Effects of sphingomyelin and phosphatidylcholine acyl chains on the clearance of triacylglycerol-rich lipoproteins from plasma. Studies with lipid emulsions in rats. *Biochim. Biophys. Acta* 1126:65-72.

Schumaker, V. and A. Lembertas 1992. Lipoprotein metabolism: Chylomicrons, very-low-density lipoproteins and low density lipoproteins. *In* Molecular Genetics of Coronary Artery Disease. Candidate Genes and Processes in Atherosclerosis. A. J. Lusis, J. I. Rotter and R. S. Sparkes, editors. Karger, New York. 98-139.

Shen, Y. R. 1994. Surfaces probed by nonlinear optics. *Surf. Sci.* 299/300:551-562.

Small, D. M., J. W. Steiner, A. Derksen and S. B. Clark. 1988. Thermal transitions of phosphatidylcholines on the surface of lipoprotein-like emulsion particles. *Biophys. J.* 53:211a.

Smiley, B. L., R. A. Walker, D. E. Gragson, T. E. Hannon and G. L. Richmond 1998. Vibrational sum frequency spectroscopy of surfactants and phospholipid monolayers at liquid-liquid interfaces. Laser Techniques for Condensed Phase and Biological Systems, SPIE Vol. 3273, San Jose, CA, in press.

Snyder, R. G., H. L. Strauss and C. A. Elliger. 1982. C-H stretching modes and the structure of n-alkyl chains. 1. Long, disordered chains. *J. Phys. Chem.* 86:5145-5150.

Walker, R. A., J. C. Conboy and G. L. Richmond. 1997. Molecular structure and ordering of phospholipids at a liquid-liquid interface. *Langmuir* 13(12):3070-3073.

TABLE 1 Phosphatidylcholine chain ordering at the CCl_4-D_2O interface.

PC molecule (C_{sn-1}:C_{sn-2})	peak ratio	$\Delta C/C_L$	T_m(°C)*
SCPC (18:10)	0.56 ± 0.11	0.56	20
SLPC (18:12)	0.60 ± 0.14	0.44	18
SMPC (18:14)	0.80 ± 0.16	0.32	30
SPPC (18:16)	2.60 ± 0.78	0.21	44
DSPC (18:18)	4.59 ± 1.38	0.09	55
PLPC (16:12)	0.78 ± 0.16	0.37	10
PMPC (16:14)	0.93 ± 0.19	0.23	27
DPPC (16:16)	1.17 ± 0.24	0.10	41
PSPC (16:18)	3.08 ± 0.93	0.03	49
DMPC (14:14)	1.10 ± 0.22	0.12	23
MPPC (14:16)	0.83 ± 0.17	0.04	36
MSPC (14:18)	0.74 ± 0.15	0.16	40
DLPC (12:12)	1.30 ± 0.26	0.14	-1

* from Marsh, 1990

FIGURE 1 Schematic representation of VSFS from the CCl_4-water interface utilizing a total internal reflection geometry. ω_{VIS} and ω_{IR} are incident on the liquid-liquid interface at their respective critical angles. Monolayer formation occurred by adsorption of PCs from the top aqueous phase according to the method described in the text.

FIGURE 2 VSFS spectra for (a) MSPC (C_{14}:C_{18}), (b) PSPC (C_{16}:C_{18}) and (c) DSPC (C_{18}:C_{18}) adsorbed at the CCl_4-water interface shown as a function of IR wavenumber for S-polarized sum frequency, S-polarized visible and P-polarized IR fields. The aqueous phase was 10 mM phosphate buffer, pH 7.0 in D_2O. Peak identifications are as given in the text.

FIGURE 3 CH_3-SS/CH_2-SS peak area ratios, which provide a measure of the degree of hydrocarbon chain ordering, for VSFS peaks obtained from the symmetric chain PC monolayers as a function of the number of carbon atoms per alkyl chain.

FIGURE 4 Peak area ratios for sn-1-stearoyl (C_{18}; --O--), sn-1-palmitoyl (C_{16}; --▲--) and sn-1-myristoyl (C_{14}; --□--) PCs are shown as a function of 2-acyl chain length in number of carbon atoms.

FIGURE 5 Hydrocarbon chain ordering, as indicated by the CH_3-SS/CH_2-SS peak area ratio calculated from VSFS measurements, as a function of the chain mismatch parameter, $\Delta C/CL$ for sn-1-stearoyl (C_{18}; --O--) and sn-1-palmitoyl (C_{16}; --▲--) PCs.

FIGURE 6 Measured SSP sum frequency intensity vs. IR energy for adsorbed monolayers of the racemic DSPC mixture. Representative spectra are shown for (a) an incomplete monolayer prepared by the standard method, and (b) a tightly packed monolayer observed using either higher bulk PC concentrations and/or longer equilibration times. Peak identifications are as given in the text.

FIGURE 1

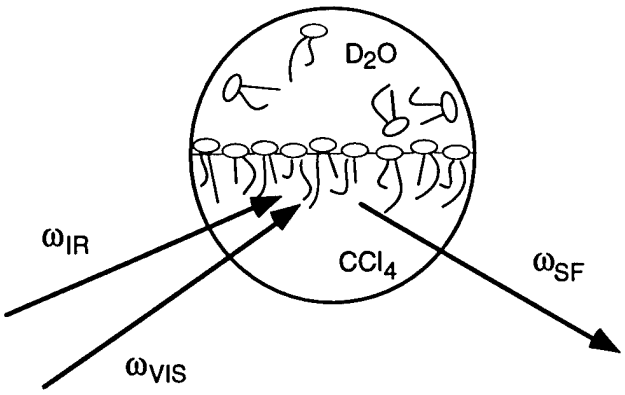


FIGURE 2

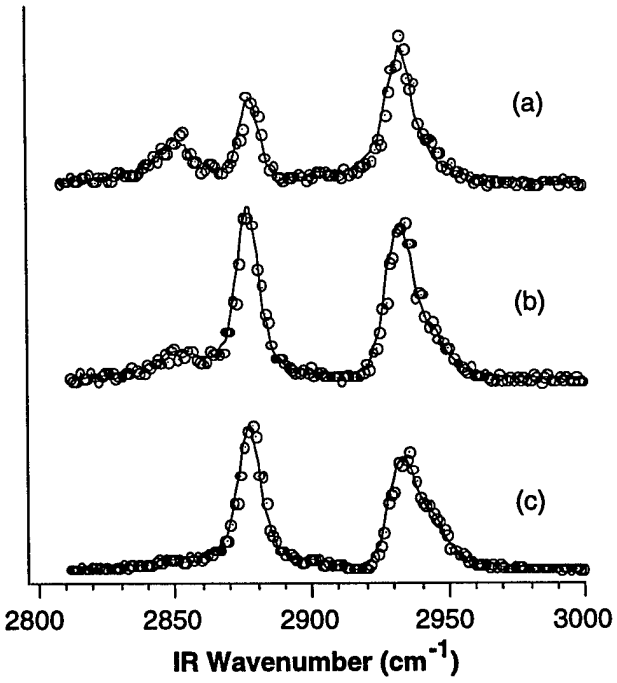


FIGURE 3

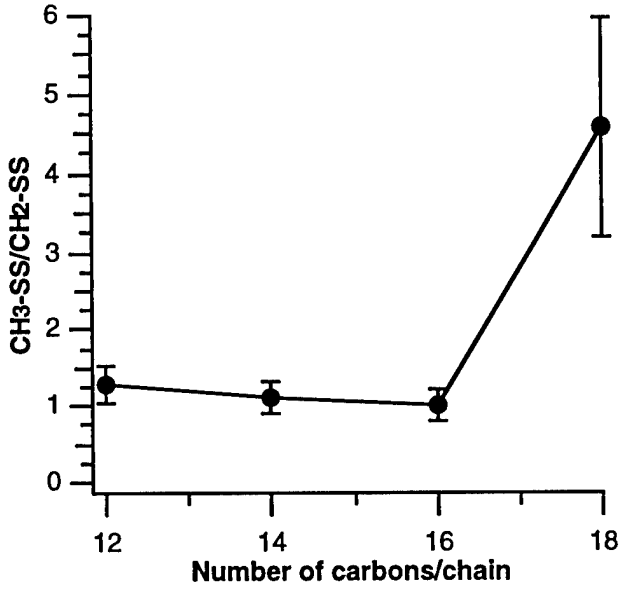


FIGURE 4

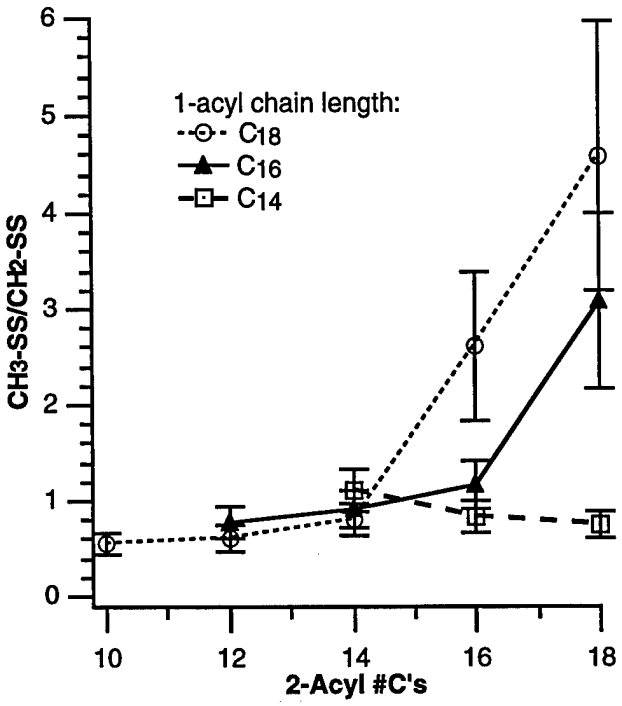

1-acyl chain length:
C18
C16
C14
CH3-SS/CH2-SS
0
1
2
3
4
5
6
10
12
14
16
18
2-Acyl #C's

FIGURE 5

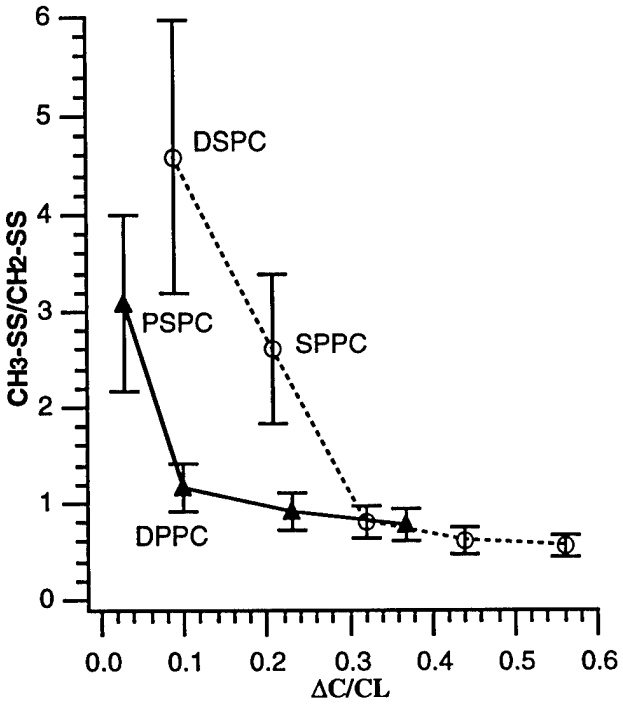
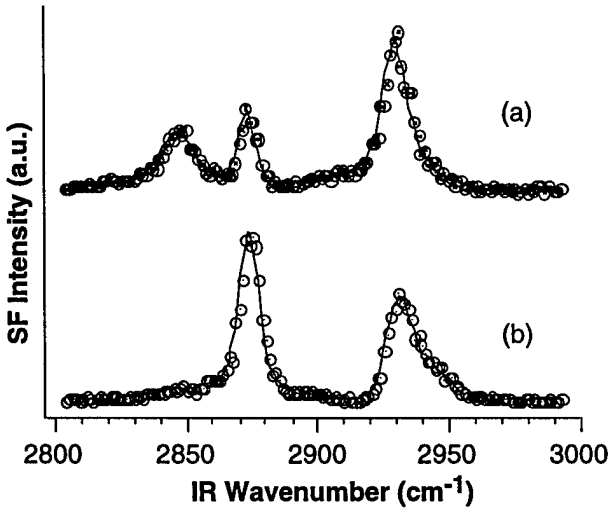

6
5
4
3
2
1
0
CH3-SS/CH2-SS
DSPC
PSPC
SPPC
DPPC
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ΔC/CL

FIGURE 6


SF Intensity (a.u.)
(a)
(b)
2800
2850
2900
2950
3000
IR Wavenumber (cm-1)