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Grant N00014-89-J-1261

R&T Code 4131038

ONR Technical Report #24

Molecular Structure and Ordering of Phospholipids at a Liquid-Liquid Interface
by

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Langmuir, 13 (3070) 1997

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June 1998

19980623 047

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
1. AGENCY USE ONLY (Leave Blank)		2. REPORT DATE 30 May 1998		3. REPORT TYPE AND DATES COVERED Technical 6/1/97-5/31/98
4. TITLE AND SUBTITLE Molecular Structure and Ordering of Phospholipids at a Liquid-Liquid Interface			5. FUNDING NUMBERS N00014-89-J-1261	
6. AUTHOR(S) R. A. Walker, J. C. Conboy 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 #24	
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 Langmuir, 13 (3070) 1997				
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 Vibrational Sum Frequency Spectroscopy in Conjunction with Interfacial Pressure Measurements			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT	

Molecular Structure and Ordering of Phospholipids at a Liquid-Liquid Interface

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Summary:

Vibrational sum frequency spectroscopy in conjunction with interfacial pressure measurements provide direct information about the molecular structure of phosphocholine monolayers adsorbed to the interface between D_2O and carbon tetrachloride. Monolayers form from breakup at the interface of aqueous phase phosphocholine vesicles. For the saturated, symmetric, dialkyl phosphocholines used in this study, alkyl chain conformation as inferred from the relative intensity of CH stretch vibrational bands depends on both alkyl chain length and interfacial concentration. Temperature controlled experiments show the lipid bilayer gel to liquid crystalline phase transition temperature to play a pivotal role in determining interfacial coverage and alkyl chain structure. At equivalent interfacial coverages, longer chain phosphocholine species form more disordered monolayers than shorter chain phosphocholines.

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Unraveling the factors that affect phospholipid structure at interfaces has direct bearing on our understanding of cell membrane and vesicle properties.¹⁻³ Used as model systems for bilayers and biomembranes, phospholipid monolayers at the air-water interface have been the focus of extensive thermodynamic and spectroscopic studies.⁴⁻¹¹ This research has provided detailed information at both macroscopic and microscopic levels about compositions, phases and structures of various phospholipid monolayers. In contrast, experiments investigating phospholipid monolayers adsorbed to liquid-liquid interfaces are sparser and provide primarily macroscopic information. Examples include surface potential measurements¹ and Langmuir trough experiments¹²⁻¹⁴ done with phospholipid monolayers at the *n*-heptane-water interface, kinetic studies of enzymatic hydrolysis of phospholipid monolayers at the boundary of two immiscible electrolytic solutions¹⁵, and fluorescence microscopy experiments using labeled phospholipids adsorbed to a heptane-water interface to examine micron scale monolayer domain transitions.¹⁶

In this letter we provide direct *molecular level insight* into the structure of phospholipid monolayers at a liquid-liquid interface using vibrational sum frequency spectroscopy (VSFS). Spectra in the CH stretching region of several different phosphocholines adsorbed to the D₂O-carbon tetrachloride interface are recorded as a function of aqueous phosphocholine concentration. The monolayers form from a metastable equilibrium established between fully hydrated phosphocholines in their vesicle state and monomers at the interface. Relative intensities of CH stretching bands disclose information about monolayer structure and its dependence on such factors as interfacial concentration and hydrocarbon chain length. The data show two clear trends : a) order within a monolayer is correlated with interfacial concentration, and b) at a given interfacial concentration, shorter chain phospholipids form much more ordered monolayers than do longer chain phospholipids for chain lengths $12 \leq n \leq 18$. The lipid bilayer gel to liquid

crystalline phase transition plays a pivotal role in controlling interfacial monolayer concentration and structure.

Phospholipids used in this study belong to a family of saturated, symmetric, dialkyl phosphocholines (PC) having alkyl chain lengths of 12 carbon atoms (dilauroyl-PC or DLPC), 14 carbon atoms (dimyristoyl-PC or DMPC), 16 carbon atoms (dipalmitoyl-PC or DPPC) and 18 carbon atoms (distearoyl-PC or DSPC) (Fig. 1). The aqueous phase is prepared by dissolving a given phosphocholine (Avanti Polar Lipids) in a pH 7.0 D₂O-phosphate buffer solution (ionic strength of 10 mmolar in phosphate ion). The phospholipid suspension is sonicated above the lipid bilayer gel to liquid crystalline phase transition temperature (T_c), a procedure that results in the creation of small unilamellar vesicles.¹⁷ Using a platinum Wilhelmy plate/microbalance assembly, we measure pressure-concentration adsorption isotherms for the different phospholipids at the D₂O-CCl₄ interface. Interfacial tension measurements are recorded when the rate of change in the interfacial pressure (Π) falls to less than 2%/hr.

Adsorption isotherms under ambient conditions ($T = 23^\circ \pm 1^\circ \text{C}$) vary considerably for the phospholipids examined (Fig. 2) with different terminal surface pressures exhibited by the different molecules. DLPC shows the greatest interfacial activity, achieving a terminal interfacial pressure of $39 \pm 3 \text{ mN/m}$ at aqueous concentrations greater than 8 μmolar . DMPC has a terminal interfacial pressure of 30 mN/m at concentrations above 20 μmolar . DPPC and DSPC demonstrate the lowest interfacial activity with terminal pressures of less than 20 mN/m.

Molecular areas of DLPC, DMPC, DPPC, and DSPC at saturation monolayer coverages are calculated using adsorption isotherm data (Fig. 2) and the Gibbs equation.¹⁸ At its terminal interfacial pressure, the DLPC monolayer exhibits the closest packing with a molecular area of $50 \pm 8 \text{ \AA}^2/\text{molec}$. This value corresponds to a tightly packed monolayer and agrees quite well with limiting areas obtained from Langmuir trough ($44 \text{ \AA}^2/\text{molec}$)¹¹ and neutron scattering ($51 \text{ \AA}^2/\text{molec}$)⁸ experiments on phosphocholines at the air-water

interface. DMPC monolayers at terminal surface pressure are slightly expanded with molecular areas of $70 \pm 15 \text{ \AA}^2/\text{molec}$. DPPC and DSPC form expanded monolayers at the $\text{D}_2\text{O}-\text{CCl}_4$ interface with molecular areas greater than $100 \text{ \AA}^2/\text{molec}$ under ambient conditions.

Vibrational spectra of the phospholipids adsorbed to the $\text{D}_2\text{O}-\text{CCl}_4$ interface are acquired using vibrational sum frequency spectroscopy in a total internal reflection geometry. Given its molecular and interfacial specificity, VSFS has developed into a powerful technique for probing solid-liquid, solid-air, liquid-air and, recently, liquid-liquid interfaces.¹⁹⁻²² The method involves two coherent optical fields - typically one fixed frequency visible and one tunable infrared - converging spatially and temporally on the interface. When the infrared radiation is resonant with a vibration of a molecule at the interface, the two waves interact through the resonant component of the second order nonlinear susceptibility (χ^2) to create a third optical field equal in energy to the sum of the visible and infrared energies.²³ In a total internal reflection geometry the visible and infrared beams pass through the high index medium (CCl_4) and the sum frequency (SF) signal is collected in reflection. Spectra appearing in this report show s-polarized SF signal arising from s-polarized visible (532 nm, 5-10 mJ, 12 ns) and p-polarized infrared (3.3-3.7 μm , 1-3 mJ) This choice of polarizations probes vibrational modes which have their transition moments perpendicular to the $\text{D}_2\text{O}-\text{CCl}_4$ interface.

Spectra of the different phospholipids at their terminal surface pressures (Fig. 3) reflect the disparities observed in the Π -concentration isotherms (Fig. 2). From the spectral data comes direct, unambiguous information about the molecular structure of the monolayers which could only be inferred from the interfacial pressure measurements. Our method for evaluating order of the alkyl chains within a phospholipid monolayer compares relative intensities of CH_3 and CH_2 symmetric stretch bands.¹⁹ In highly ordered monolayers where the hydrocarbon chains pack together tightly in an all *trans* configuration, selection rules and the electric dipole approximation render methylene

vibrational transitions SF inactive. Spectra of highly ordered monolayers, therefore, contain only the symmetry allowed CH stretching modes from the terminal methyl group. In a disordered monolayer, one in which the hydrocarbon chains possess defects, methylene vibrations become SF active and, along with the terminal methyl modes, contribute to the spectrum. Information about monolayer structure can thus be obtained from the relative intensities of the CH_3 symmetric stretch (at 2872 cm^{-1}) and the CH_2 symmetric stretch (at 2850 cm^{-1}).²⁴ A large CH_3/CH_2 ratio implies a high degree of order and a primarily all-*trans* conformation of the alkyl chains, whereas a small ratio suggests greater disorder in the form of *gauche* conformations.

DLPC at its terminal interfacial pressure forms the most ordered monolayer with a very large CH_3/CH_2 ratio of 2.8 ± 0.1 (Fig. 3). This ratio of 2.8 is larger than ratios obtained for tightly packed single chain C_{12} surfactants (i.e. a ratio of 1.4 for sodium *n*-dodecyl sulfate and 2.4 for *n*-dodecyl ammonium chloride) at the $\text{D}_2\text{O}-\text{CCl}_4$ interface.²⁵ This large ratio may result from either tighter packing of the phospholipids due to diminished headgroup repulsion between the zwitterionic choline headgroups or simply having two short alkyl chains in close proximity to each other by virtue of being attached to the same headgroup. DMPC with its less tightly packed monolayer ($70\text{ \AA}^2/\text{molec}$) shows a smaller ratio of 1.3 ± 0.2 , indicating greater conformational disorder within the hydrocarbon chains. The two longer chain phosphocholines, DPPC and DSPC, form the least ordered - and most expanded - monolayers with ratios of 0.8 ± 0.1 .

When plotted versus concentration, the SF intensity ratio curves (Fig. 4) resemble the isotherms of the corresponding phosphocholines (Fig. 2). DLPC shows the greatest dynamic range with a ratio of ~ 1.0 at sub- μmolar phospholipid concentrations and a terminal value of 2.8 at phospholipid concentrations of $8\text{ }\mu\text{molar}$ and above. The DMPC order ratio rises more gradually with concentration, the ratio rising from 0.8 at $1\text{ }\mu\text{molar}$ up to 1.3 at $20\text{ }\mu\text{molar}$. Alkyl chain order for both DSPC and DPPC show little dependence on aqueous concentration.

The SF spectra together with interfacial pressure measurements provide a clear picture of how phospholipid monolayer structure at a liquid-liquid interface depends on interfacial concentration. Still unresolved, however, is the question of why phospholipids differing only in the length of their hydrocarbon chains exhibit such markedly different behavior. The answer hinges on the physical properties of the different phosphocholine vesicles in aqueous solution. Differential scanning calorimetry²⁶, Raman²⁷, and infrared²⁸ experiments have all identified two phases of vesicle lipid bilayers, gel and liquid crystalline. This transition from the well ordered gel phase to the more fluid liquid crystalline phase with increasing temperature emerges quite naturally in molecular dynamics simulations.³ DLPC has a phase transition temperature (T_c) of -1°C . For the room temperature experiments described above, the vesicle bilayers of DLPC existed in a disordered, liquid crystalline state with relatively weak intermolecular forces between the hydrocarbon chains. DPPC and DSPC have T_c equal to 41°C and 55°C , respectively. These vesicles were in a gel state held together tightly by strong intermolecular forces. Spectral and isotherm data suggest that monolayer structure is correlated with T_c ; a low T_c (e.g. -1°C for DLPC) leads to high interfacial concentration and a high degree of order and vice versa. We now explicitly demonstrate the relationship between vesicle bilayer phase and alkyl chain conformation within the monolayer at the $\text{D}_2\text{O-CCl}_4$ interface using DSPC as an example.

Interfacial pressure measurements of DSPC show a strong dependence on temperature (Fig 5). Below the gel to liquid crystalline transition temperature, the interfacial pressure of a $20\ \mu\text{molar}$ solution of DSPC slowly and monotonically rises with increasing temperature. At the 55°C transition temperature the surface pressure abruptly and irreversibly climbs to $40 \pm 3\ \text{mN/m}$, the same terminal surface pressure of DLPC ($T_c = -1^\circ\text{C}$). Further studies investigating the temperature window of this transition are currently underway. Interfacial pressure remains constant at temperatures above T_c indicating formation of a tightly packed monolayer. From isotherm data taken above T_c ,

we calculate a molecular area for the tightly packed DSPC monolayer equal to that of DLPC at room temperature ($50 \text{ \AA}^2/\text{molec}$). We also note that monolayer formation appears to be irreversible as evidenced by a constant interfacial pressure even when the temperature is reduced below T_c . A large reduction in the phospholipid free energy from solvation of the hydrocarbon chains in CCl_4 ($\Delta H_{\text{solv}} \sim 4.5 \text{ kJ/mol per CH}_2 \text{ unit}$ ²⁹) apparently introduces an insurmountable kinetic barrier to phosphocholine desorption from the interface.

Spectra of tightly packed DSPC monolayers show a corresponding change in alkyl chain structure with temperature. Because the 55° C transition temperature of DSPC brings the interface too close to the boiling point of CCl_4 for spectra to be recorded, we make use of irreversible monolayer formation to create a tightly packed DSPC monolayer above T_c and then cool the interface to room temperature. We refer to this process as annealing the interface. Annealing the DSPC interface leads to a slightly more ordered monolayer as determined spectroscopically than the corresponding DSPC system at similar aqueous concentration and room temperature. (Figure 6) At aqueous concentrations above $10 \text{ }\mu\text{molar}$, the CH_3/CH_2 ratio reproducibly rises from 0.8 up to 1.1 after annealing, reflecting a modest increase in alkyl chain order with an increase in surface coverage. The three phospholipids with experimentally accessible transition temperatures (DMPC, DPPC, and DSPC) all show a similar effect of temperature on interfacial coverage (Table 1). Above the bilayer transition temperature, DMPC, DPPC and DSPC form tightly packed monolayers ($\sim 50 \text{ \AA}^2/\text{molec}$). The annealed monolayers show modest increases in alkyl chain order, but in no instance do the CH_3/CH_2 ratios of the tightly packed monolayers of DMPC (1.7), DPPC (1.2), or DSPC (1.1) show the same degree of order as that of DLPC (2.8). The degree of order observed in the alkyl chain structure is independent of temperature over the experimentally accessible temperature range ($2^\circ \text{ C} \leq T \leq 50^\circ \text{ C}$). Thus for equivalent interfacial concentrations, alkyl chain conformation in the phospholipid monolayers is highly dependent upon chain length for $12 \leq n \leq 18$. The longer chain

phospholipids, even when tightly packed, will sustain greater conformational disorder within the monolayer.

Important factors in controlling alkyl chain order within a phospholipid monolayer at the D_2O-CCl_4 interface include both interfacial phospholipid concentration and phospholipid alkyl chain length. The interfacial pressure and VSFS experiments discussed above demonstrate that the forces within the bilayers of the aqueous vesicles control the interfacial concentration and hence the structure of phospholipids adsorbed to the interface. Weaker intermolecular forces found in the liquid crystalline state of vesicle bilayers reduce the barrier to vesicle breakup at the interface and the corresponding tightly packed monolayers evince a higher degree of order amongst the alkyl chains. Conversely, stronger cohesive forces within bilayers inhibit phospholipid deposition at the interface, and the alkyl chains in these expanded monolayers show spectroscopic evidence of greater disorder. At equivalent interfacial concentrations alkyl chain structure of phospholipid monolayers shows a strong dependence on alkyl chain length. DLPC, DMPC, DPPC and DSPC above their respective bilayer phase transition temperatures will form monolayers at the D_2O-CCl_4 interface having similar interfacial concentrations. Monolayers composed of longer chain phospholipids, however, show considerably greater alkyl chain disorder suggesting that the organic subphase effectively screens chains from each other and reduces barriers to intramolecular motion.

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Acknowledgements: We gratefully acknowledge financial support from the National Science Foundation (CHE 9416856) and the Office of Naval Research. We thank Julie Gruetzmacher and Elizabeth Raymond for their assistance with interfacial pressure measurements and data analysis; we thank Rebekka Wachter for insightful discussions.

Figure captions:

Figure 1. Molecular structure of dialkyl phosphocholines used in this study.

$R = C_{n-1}H_2CH_3$ where $n = 12$ is dilauroylphosphocholine (DLPC), $n = 14$ is dimyristoylphosphocholine (DMPC), $n = 16$ is dipalmitoylphosphocholine (DPPC), and $n = 18$ is distearoylphosphocholine (DSPC).

Figure 2. Pressure (Π)-aqueous concentration adsorption isotherms for DLPC, DMPC, DPPC, and DSPC taken at the D_2O-CCl_4 . Interfacial pressures were calculated by subtracting the value of the measured interfacial tension from the interfacial tension of a neat D_2O-CCl_4 interface (45 mN/m). Lines are shown as guides for the eye.

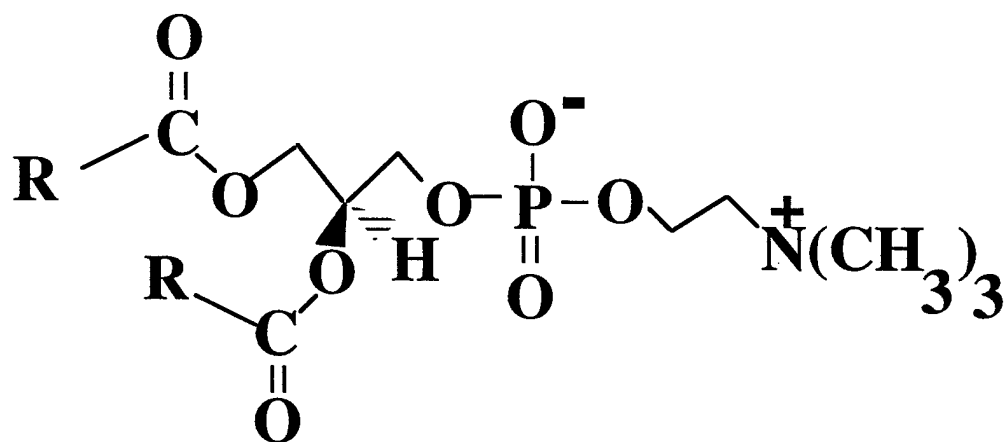
Figure 3. VSFS spectra of DLPC, DMPC, DPPC, and DSPC at saturated monolayer coverage under ambient conditions ($T = 22^\circ C$). Spectra contain both the methylene and methyl symmetric stretches (CH_2 ss at 2850 cm^{-1} and CH_3 ss at 2872 cm^{-1}), a broad Fermi resonance assigned to overtones of CH_2 bending motion (CH_2 FR, $2885 - 2915\text{ cm}^{-1}$) and the CH_2 asymmetric stretch (CH_2 as at 2930 cm^{-1}). Solid lines represent fits to the spectra using Voigt profiles for the different bands. Relative band positions are accurate to $\pm 3\text{ cm}^{-1}$.

Figure 4. Intensity ratios of the CH_3 symmetric stretch to the CH_2 symmetric stretch for DLPC, DMPC, DPPC, and DSPC versus bulk aqueous phospholipid concentration. Lines are shown as guides for the eye.

Figure 5. Interfacial pressure of a $20\text{ }\mu\text{M}$ aqueous solution of DSPC versus temperature. Shown are the gel to liquid crystalline phase transition temperature (T_c) for DSPC and the terminal interfacial pressure (40 mN/m) of DLPC at room temperature.

Figure 6. Spectra of DSPC monolayers in the CH symmetric stretch region. The bottom spectrum shows the CH_2 and CH_3 symmetric stretches from a monolayer in metastable equilibrium with a solution of DSPC vesicles at $23^\circ C$ (molec area of $120\text{ }\text{\AA}^2/\text{molec}$). The upper spectrum, also taken at $23^\circ C$, shows the change in spectral intensities that accompanies formation of a tightly packed monolayer (molec area of $50\text{ }\text{\AA}^2/\text{molec}$).

Table 1. Phase transition temperatures (T_c) and terminal CH_3/CH_2 intensity ratios below T_c and of the annealed (tightly packed) monolayers at $23^\circ C$.



1,2-Dialkyl-*sn*-Glycero-3-Phosphocholine

Figure 1
Walker, et al.

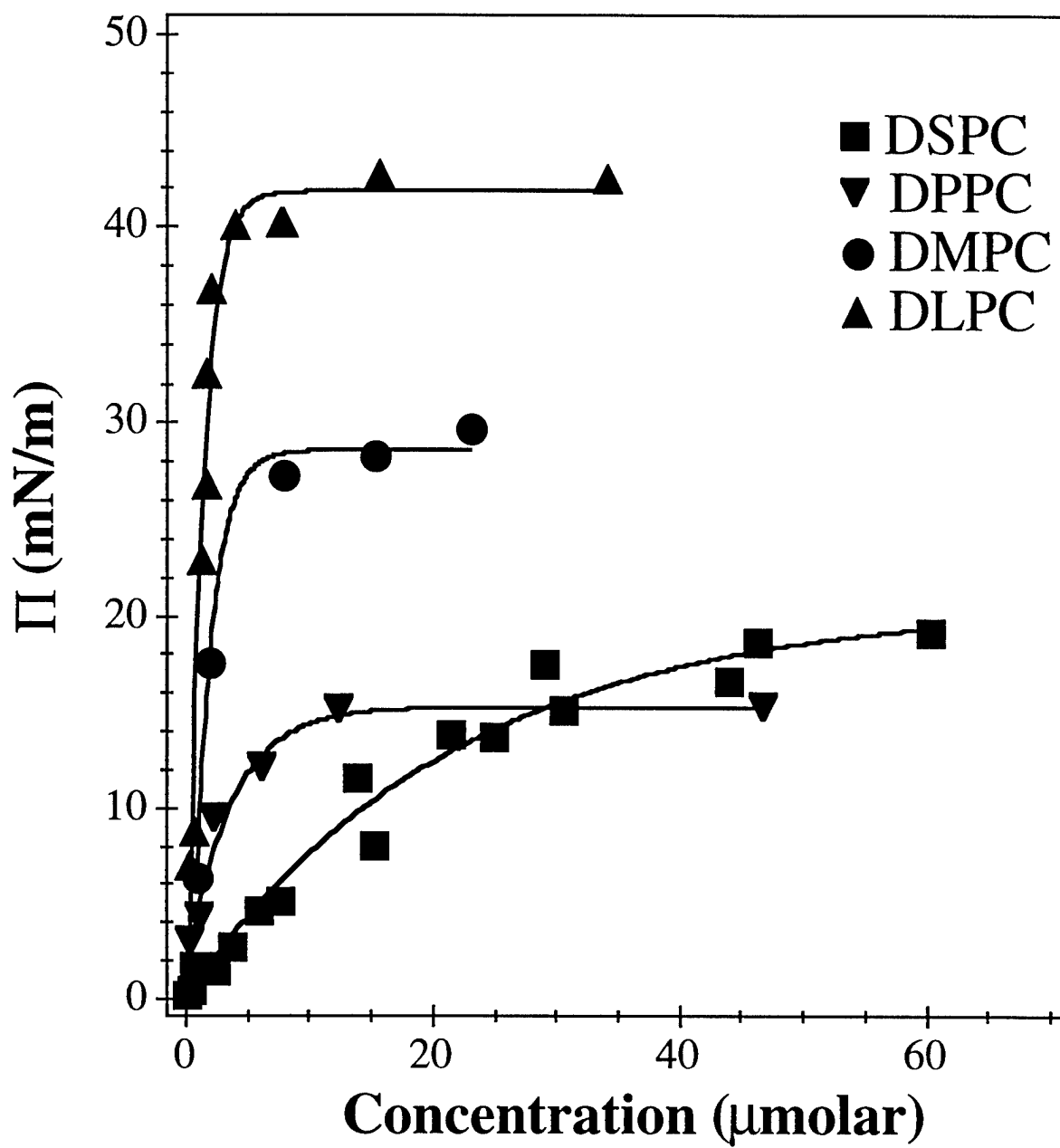


Figure 2
Walker, et al.

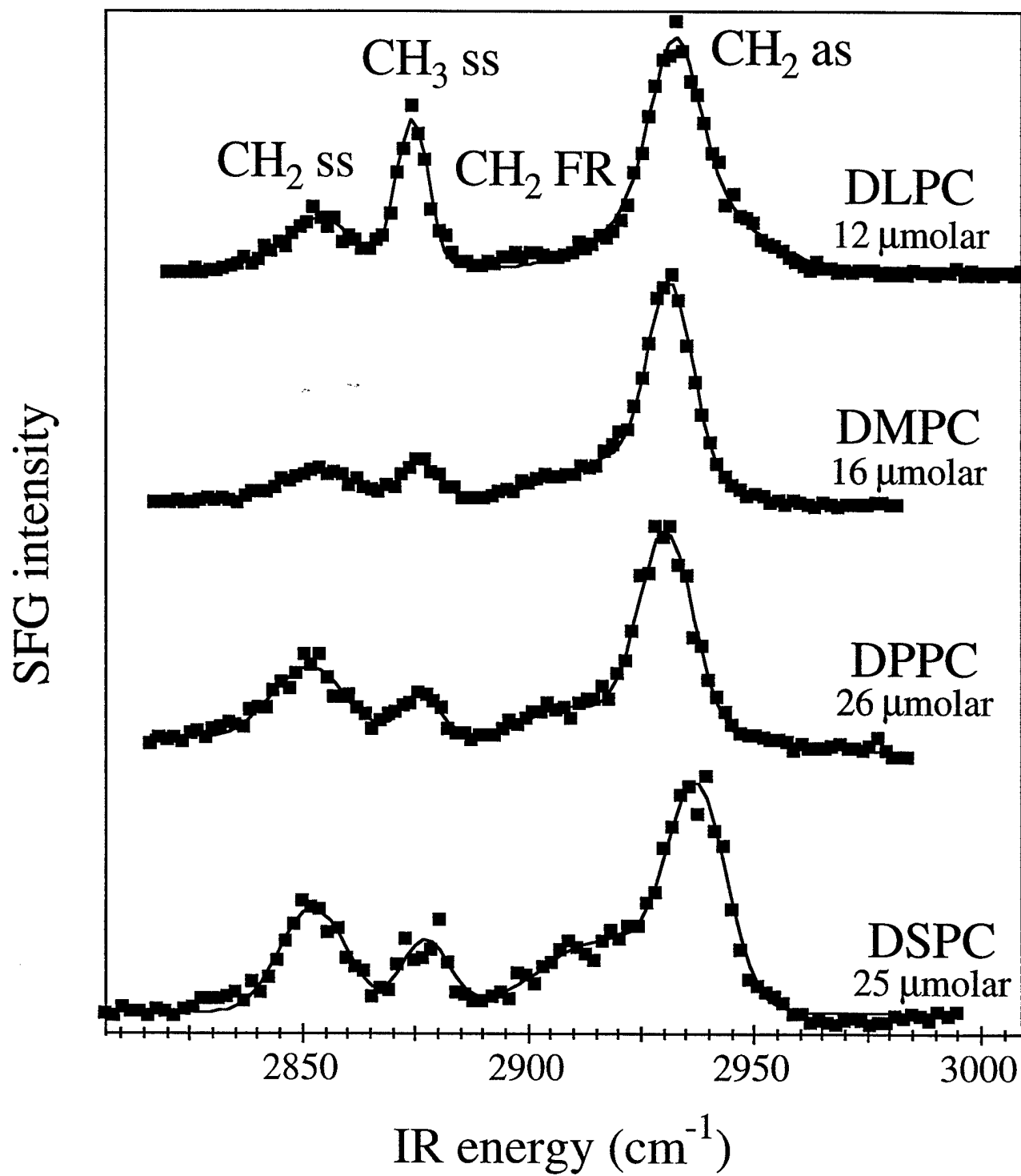


Figure 3
Walker, et al.

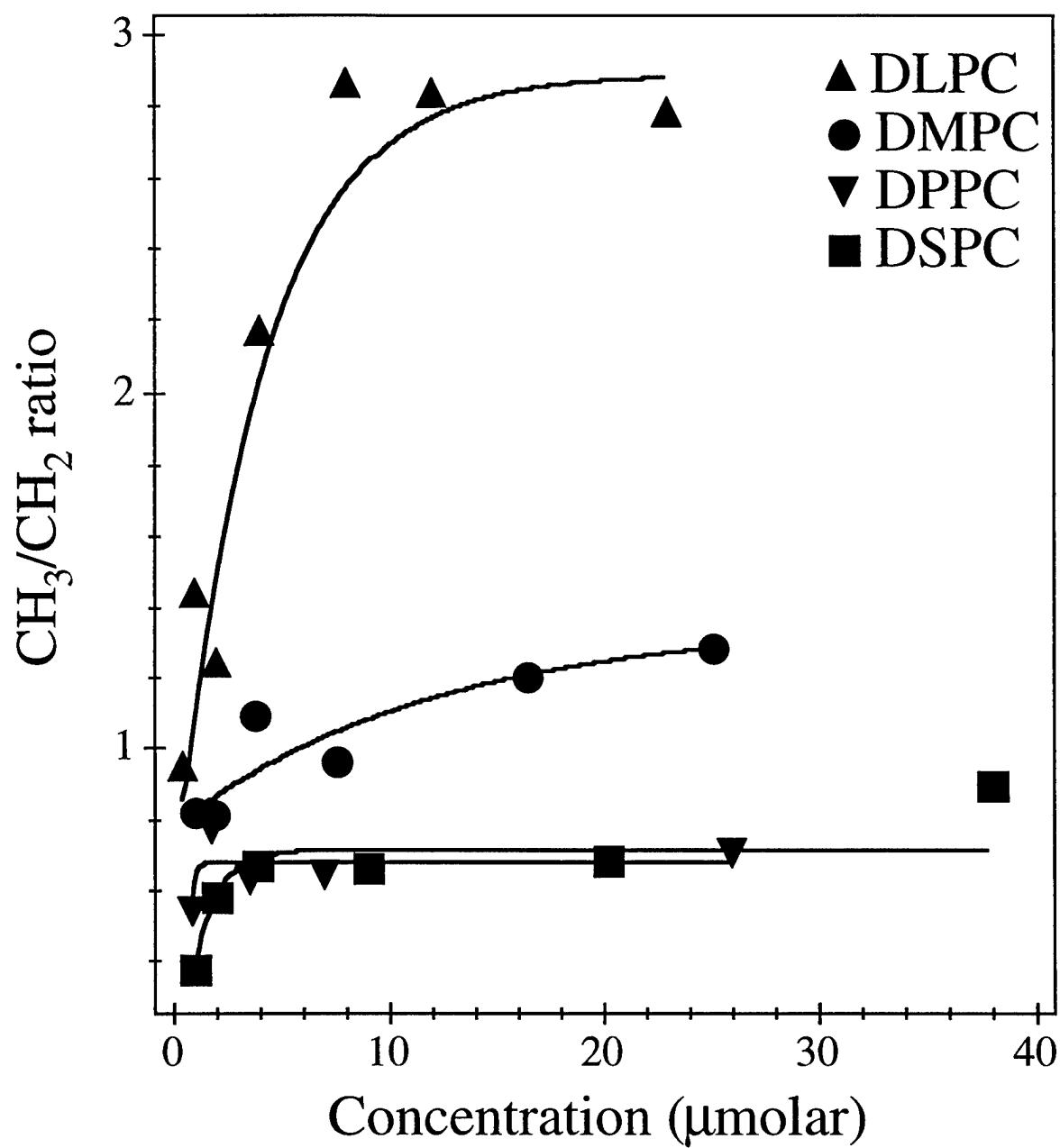


Figure 4
Walker, et al.

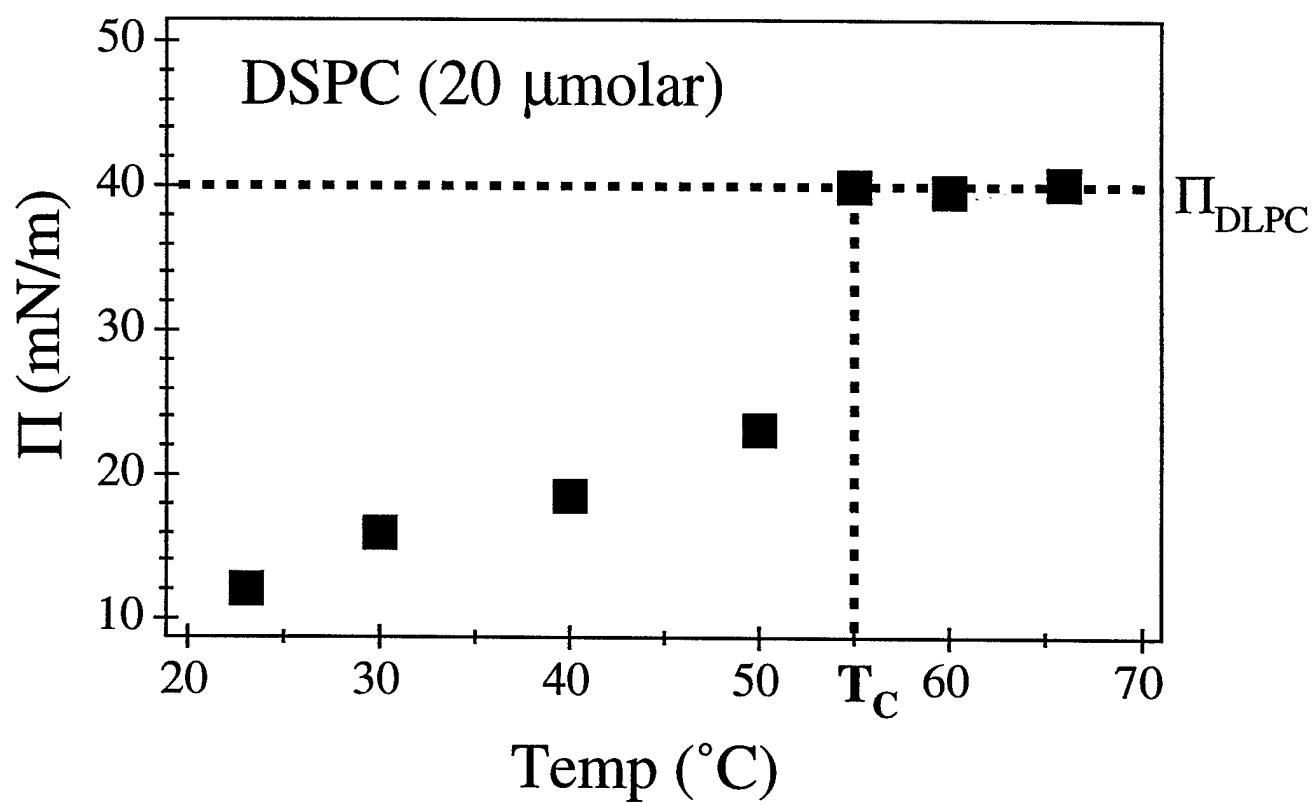


Figure 5
Walker, et al.

DSPC (20 μmolar)

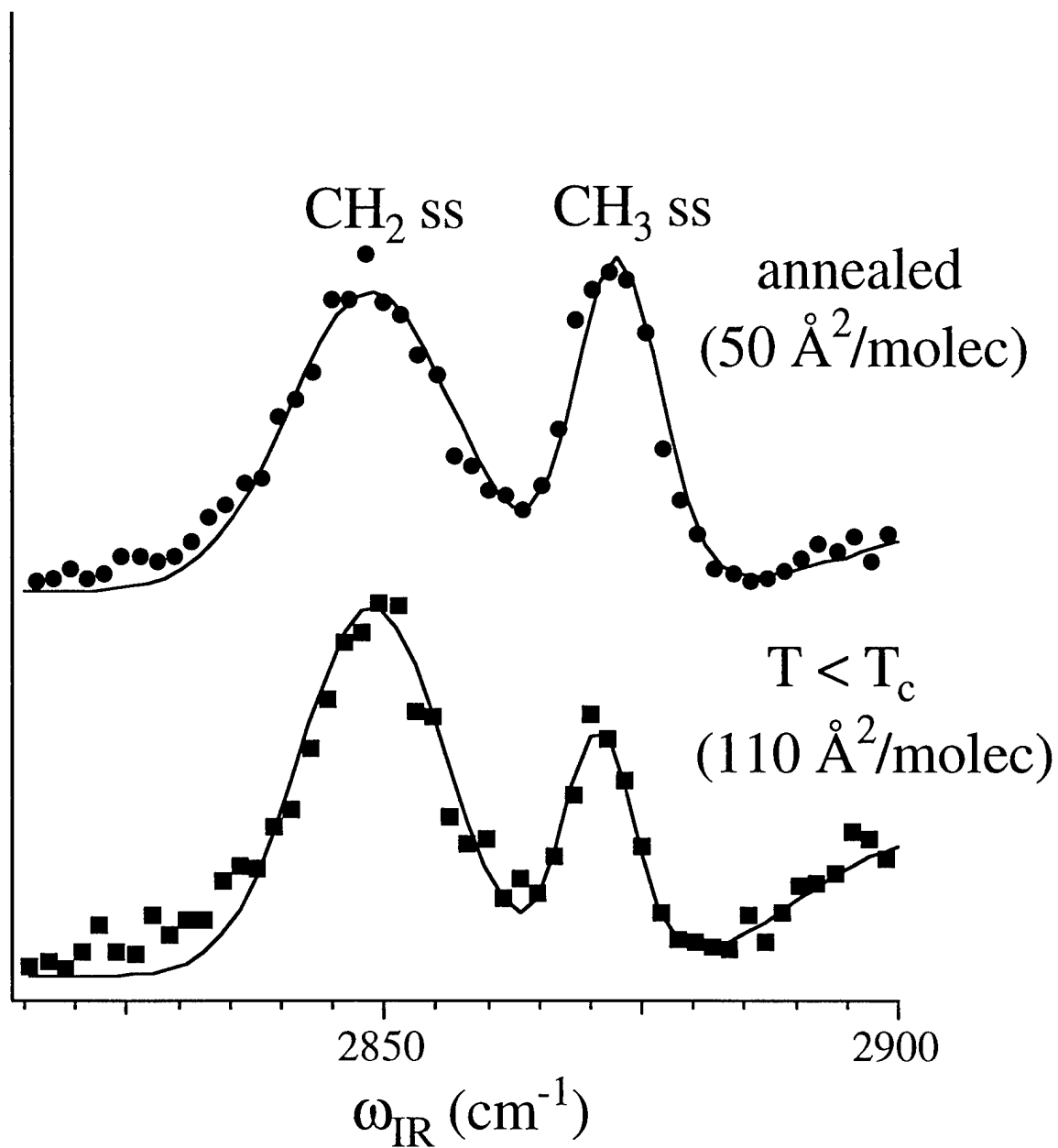


Figure 6
Walker, et al.

Table 1

Molecule	T_c (°C)	CH ₃ /CH ₂ ratio	
		T < T _c	annealed
Dilauroylphosphocholine (DLPC)	-1	—	2.8 ± 0.1
Dimyristoylphosphocholine (DMPC)	23	1.3 ± 0.2	1.7 ± 0.2
Dipalmitoylphosphocholine (DPPC)	41	0.8 ± 0.2	1.2 ± 0.3
Distearoylphosphocholine (DSPC)	55	0.8 ± 0.1	1.1 ± 0.1