Thermodynamics of Phospholipid Tubules in Alcohol/Water Solutions

Mark S. Spector,* Jonathan V. Selinger, and Joel M. Schnur

Contribution from the Center for Bio/Molecular Science and Engineering, Code 6900, Naval Research Laboratory, Washington, D.C. 20375-5348

Received May 21, 1997[⊗]

Abstract: Circular dichroism and calorimetry have been used to study the melting of self-assembled phospholipid tubules in alcohol/water solutions. The tubules are found to melt either continuously or discontinuously, depending on the type of alcohol, the alcohol/water ratio, and the lipid concentration. At high proportions of alcohol and low lipid concentrations, the ellipticity continuously decreases with temperature and a very broad peak in the specific heat is observed. In solutions with lower proportions of alcohol or large lipid concentrations, a large drop in ellipticity is accompanied by a sharp peak in the specific heat when the acyl chains disorder. The CD and specific heat measurements can be explained in terms of two distinct thermodynamic processes: the discontinuous melting is a first-order transition of the lipid bilayers from the ordered $L_{\beta'}$ phase to the disordered L_α phase, while the continuous melting reflects an increase in the lipid solubility with temperature. These results show that the solvent has an important effect on the thermodynamics of lipid tubules, and that circular dichroism is a sensitive probe of tubule thermodynamics.

Introduction

Lipid bilayers self-assemble into a variety of microstructures in solution.[1] In particular, certain synthetic phospholipids with diacetylenic moieties in the acyl chains form cylindrical microstructures, known as tubules.[2,3] Tubules are hollow cylinders with a characteristic diameter of 0.5 μm and a characteristic length of 50−200 μm. They have been studied extensively as paradigms for molecular self-assembly. They also have the potential for use in several technological applications, such as electroactive composites and controlled-release systems.[3] To explain the formation of tubules, several investigators have developed theories based on molecular chirality.[4] These theories show that chiral interactions cause the lipid molecules to pack at a nonzero angle with respect to their nearest neighbors. This chiral packing induces a twist in the bilayer, which leads to the formation of a cylinder. Recent experiments with circular dichroism (CD) have confirmed that tubules have a chiral structure.[5,6]

In spite of this theoretical work, some significant aspects of tubule formation and stability remain unexplained. One important issue concerns the thermodynamics of tubules. It is known from X-ray scattering experiments that tubules form at the transition from the higher temperature chain-melted L_a phase to the $L_{\beta'}$ phases of lipid bilayers, in which the lipid chains are ordered and tilted.[7] As the temperature is increased, tubules transform into other types of aggregates, such as micelles or spherical vesicles, or into monomeric lipid. This transformation is generally called melting, although we will argue below that it actually involves more than one thermodynamic process. (We will use the term melting to refer to any such process.) Two key open questions are the following: What determines the nature of the melting process? Is it related to the structure of the tubules?

A possible answer to these questions, proposed by Nounesis et al., is that the melting process is controlled by the thickness of tubule walls.[8] Detailed microscopy studies have found that tubules can have either single-bilayer or multiple-bilayer walls, depending on the solvent and on the lipid concentration. Tubules formed in ethanol/water solutions have five to ten bilayers in the walls.[9] By contrast, tubules formed in methanol/water solutions have predominantly single-bilayer walls at low lipid concentration[10] and two- to four-bilayer walls at high lipid concentration.[6,8] Using magnetic birefringence and calorimetry, Nounesis et al. found that single-bilayer tubules melt continuously while multiple-bilayer tubules melt discontinuously.[8] They attributed this change in the thermodynamics of tubule melting to a crossover from two-dimensional to three-dimensional melting as a function of the wall thickness. Although this dimensional crossover is an interesting possibility, until the present work it had not been tested by experiments at a wide range of solvents and lipid concentrations.

In this paper, we present a detailed study of the structure and thermodynamics of tubules. We investigate tubules in methanol/water and ethanol/water solutions over a wide range of alcohol volume fraction and lipid concentration, exploring a broad range of the phase diagram. In this study, we use two techniques, CD and calorimetry. These two techniques give complementary types of information about tubule melting. The

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1997.

(1) Cevc, G.; Marsh, D. *Phospholipid Bilayers: Physical Principles and Models*; John Wiley and Sons: New York, 1987.

(2) Yager, P.; Schoen P. E. *Mol. Cryst. Liq. Cryst.* **1984**, *106*, 371−381.

(3) Schnur, J. M. *Science* **1993**, *262*, 1669−1676**.**

(4) Helfrich, W.; Prost, J. *Phys. Rev. A* **1988**, *38*, 3065−3068. Zhongcan, O.-Y.; Ji-xing, L. *Phys. Rev. A* **1991**, *43*, 6826−6836. Nelson, P.; Powers, T. *J. Phys. II (France)* **1993**, *3*, 1535−1569. Selinger, J. V.; MacKintosh, F. C.; Schnur, J. M. *Phys. Rev. E* **1996**, *53*, 3804−3818.

(5) Schnur, J. M.; Ratna, B. R.; Selinger, J. V.; Singh, A.; Jyothi, G.; Easwaran, K. R. K. *Science* **1994**, *264*, 945−947.

(6) Spector, M. S.; Easwaran, K. R. K.; Jyothi, G.; Selinger, J. V.; Singh, A.; Schnur, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12943−12946.

(7) Thomas, B. N.; Safinya, C. R.; Plano, R. J.; Clark, N. A. *Science* **1995**, *267*, 1635−1638.

(8) Nounesis, G.; Ratna, B. R.; Shin, S.; Flugel, R. S.; Sprunt, S. N.; Singh, A.; Litster, J. D.; Shashidhar, R.; Kumar, S. *Phys. Rev. Lett.* **1996**, *76*, 3650−3653.

(9) Georger, J. H.; Singh, A.; Price, R. R.; Schnur, J. M.; Yager, P.; Schoen, P. E.; *J. Am. Chem. Soc.* **1987**, *109*, 6169−6175.

(10) Ratna, B. R.; Baral-Tosch, S.; Kahn, B.; Rudolph, A. S.; Schnur, J. M. *Chem. Phys. Lipids* **1992**, *63*, 47−53.

S0002-7863(97)01666-1

CD signal gives information about *intermolecular* chiral order. As we discussed in earlier papers,[5,6] the large CD signal in tubules is an indication of chiral molecular packing. At high temperature, when the tubules melt into other aggregates or into monomers, the chiral packing disappears and the CD signal goes to approximately zero. Thus, the CD signal can be regarded as an order parameter for tubule melting. By contrast, the large specific heat peaks associated with tubule melting reflect mostly the *intramolecular* order of the chains in the lipid molecules. The magnitude of the melting enthalpy, 25−30 kcal/mol,[8,11] is much greater than the enthalpy associated with typical phase transitions in thermotropic liquid crystals, in which only the intermolecular order changes, so it must be associated with the disordering of the chains.

The main result of these experiments is a pair of phase diagrams for tubules in methanol/water and ethanol/water solutions, expressed in terms of alcohol fraction and lipid concentration. These phase diagrams show whether the tubules have single or multiple bilayers and whether the tubules melt continuously or discontinuously. In particular, the phase diagrams show that the crossover from continuous to discontinuous melting does not occur at the same alcohol fraction and lipid concentration as the crossover from single-bilayer to multiple-bilayer tubules. Rather, both single- and multiple-bilayer tubules exhibit both continuous and discontinuous melting, depending on the alcohol fraction and lipid concentration. These results cannot be explained by the dimensional-crossover argument.

As an alternative explanation, we suggest that these experiments show two distinct thermodynamic processes, melting of the lipid bilayers into the disordered lamellar L_α phase and melting of the lipid molecules into solution. In this scenario, the observed discontinuous melting is a first-order $L_{\beta'}$ to L_α transition. The observed continuous melting is *not* a second-order phase transition, but rather a gradual process of transferring lipid molecules from tubules into solution. At low temperature, most of the lipid is in tubules, but a small amount of lipid is dissolved in small aggregates or monomers. That amount is limited by the solubility of the lipid in the alcohol/water solvent, or equivalently, by the critical aggregation concentration. As the temperature increases, that solubility increases, so more lipid is transferred from the tubules into solution. If the solubility reaches the total lipid concentration before the temperature reaches the $L_{\beta'}$ to L_α transition temperature, then *all* the lipid goes into solution through this gradual process, and hence the tubules melt continuously. By contrast, if the temperature reaches the $L_{\beta'}$ to L_α transition temperature before the solubility reaches the total lipid concentration, then the lipid remaining in the tubules undergoes a first-order transition into the L_α phase, and hence the tubules melt discontinuously.

The plan of this paper is as follows. In the Results section, we present CD and specific-heat data for the melting of tubules in methanol/water and ethanol/water solutions, for a wide range of alcohol fraction and lipid concentration. These experimental results lead to phase diagrams for tubules in methanol/water and ethanol/water solutions. In the Discussion section, we interpret these results in terms of the two thermodynamic processes of melting into an L_α phase and melting into a lipid solution. We show that this interpretation explains both qualitative and quantitative features of our data.

Materials and Methods

The lipid 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) was purchased from JP Laboratory (Middlesex, NJ) and purified by column chromatography. The tubules were prepared by dissolving $DC_{8,9}PC$ in HPLC-grade methanol or ethanol and mixing with water at 60 °C. On cooling the solution at 3 °C/h through the transition temperature, tubules are formed.[9] CD studies were performed on a Jasco J-720 spectropolarimeter operating between 175 and 700 nm. The samples were placed in water-jacketed quartz cells with path lengths of 0.1 to 0.5 mm. Temperature control was provided by a water circulator, which provided thermal stability of about 0.2 °C. The spectrometer was calibrated with ammonium-*d*-camphorsulfonate ($[\theta]_{291}$ = 7910 deg cm^2/dmol) and D-pantoyllactone ($[\theta]_{219} = -16\,140$ in water, $[\theta]_{223} = -12\,420$ in methanol).[12] Specific heat measurements were performed on a Microcal MC-2 scanning calorimeter at a heating rate of 10 °C/h. The specific heat contribution from the solvent was subtracted.

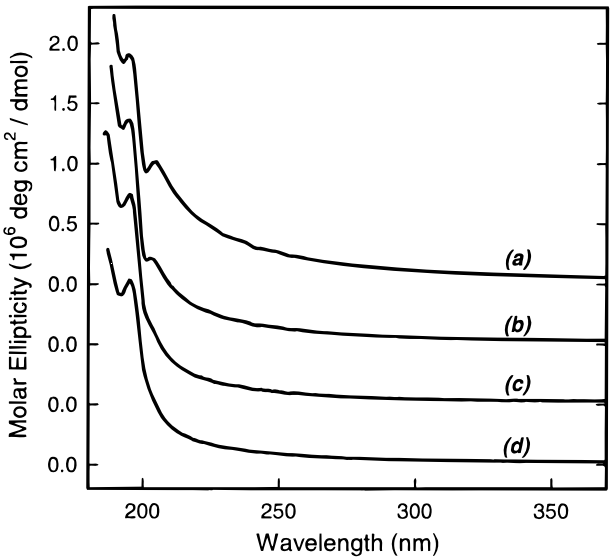

Figure 1. Circular dichroism spectra of $DC_{8,9}PC$ tubules prepared in methanol/water at ratios of (a) 60:40, (b) 70:30, (c) 80:20, and (d) 90:10 at a lipid concentration of 1 mg/mL. The spectra were taken roughly 15 °C below the melting temperature. The curves have been offset vertically for display. The increase in ellipticity at 205 nm with decreasing methanol fraction indicates formation of multi-bilayer tubules in 60:40 methanol/water.

Results

We first present our results for $DC_{8,9}PC$ tubules prepared in methanol/water solution. Previously, tubules formed at methanol fractions larger than 70% and lipid concentrations below 1 mg/mL were found to have single-bilayer walls.[10] However, it was recently found that tubules with multiple-bilayer walls can form at higher lipid concentrations at the same methanol/water ratios.[6,8] We began by studying the effect of changing the relative proportions of methanol and water at a fixed lipid concentration. Figure 1 shows CD spectra of $DC_{8,9}PC$ tubules as the methanol/water ratio is increased from 60:40 to 90:10. These spectra are from samples at a lipid concentration of 1 mg/mL and taken roughly 15 °C below the melting temperature (see below). The spectra all contain peaks at 195 nm and show an increasing ellipticity below 190 nm. A sharp increase in solvent absorption below 186 nm limits our ability to resolve the lower peak. In addition, a peak at 205 nm seen in the 60:40 methanol/water sample becomes smaller as the methanol/water ratio is increased. Previous measurements of spectra as a function of lipid concentration found that the crossover from single- to multiple-bilayer tubules is marked by an increase in the 205 nm CD peak.[6] The spectra in Figure 1 indicate the presence of some multiple-bilayer tubules in the 60:40 methanol/

(11) Rudolph, A. S.; Testoff, M. A.; Shashidar, R. *Biochim. Biophys. Acta* **1992**, *1127*, 186−190.

(12) Schippers, P. H.; Dekkers, H. P. J. M. *Anal. Chem.* **1981**, *53*, 778−782.

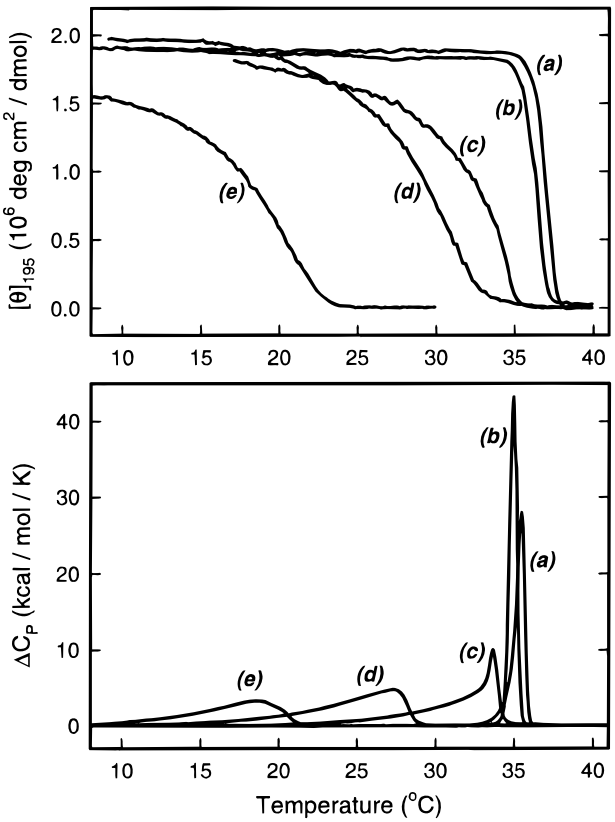

Figure 2. Temperature dependence of the CD spectra at 195 nm (top) and specific heat (bottom) of $DC_{8,9}PC$ tubules prepared in methanol/water at ratios of (a) 60:40, (b) 70:30, (c) 80:20, (d) 85:15, and (e) 90:10 at a lipid concentration of 1 mg/mL. These results show a crossover from discontinuous to continuous melting as the methanol fraction is increased.

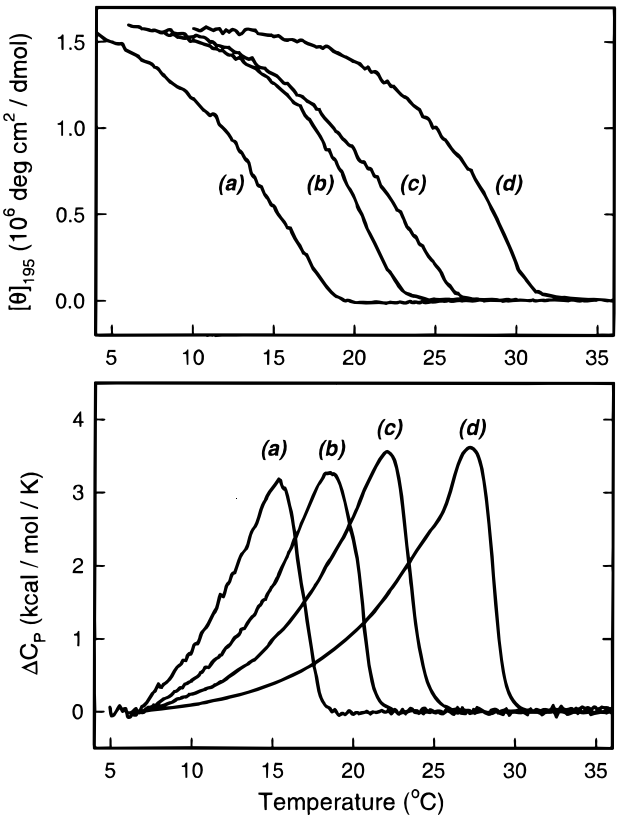

Figure 3. Concentration dependence of the CD spectra at 195 nm (top) and specific heat (bottom) of $DC_{8,9}PC$ tubules prepared in methanol/water (90:10) at lipid concentrations of (a) 0.5, (b) 1.0, (c) 2.0, and (d) 5.0 mg/mL. The melting temperature is observed to change from 18 °C to 30 °C as the lipid concentration is increased.

water sample, while the 80:20 sample consists entirely of single-bilayer tubules. Prior observations of the 70:30 sample with electron microscopy indicate it consists almost entirely of single-bilayer tubules.[6] Thus, the average number of bilayers decreases with increasing methanol fraction, in addition to increasing with lipid concentration.

The thermal characteristics of the tubules were studied by following the peak CD signal as the tubules were heated through the melting temperature. The top panel of Figure 2 shows the temperature dependence of the ellipticity at 195 nm for $DC_{8,9}$-PC tubules prepared at a lipid concentration of 1 mg/mL. At 70:30 methanol/water, the ellipticity is constant between 10 °C and the melting temperature, approximately 36 °C, where it drops sharply to near zero. Above that temperature, no clear CD signal is observed; we are able to put an upper limit of 200 deg cm^2 dmol^{-1} on the ellipticity, which is 10^4 smaller than that observed in tubules. The thermal stability of our sample cell does not allow a more detailed analysis of the region around the melting temperature, but the data are consistent with discontinuous melting. The ellipticity at 205 nm shows the same temperature dependence as at 195 nm in the 60:40 sample. This indicates that the number of bilayers in the tubules does not change as the melting temperature is approached. Similar results are obtained at higher concentrations in the 70:30 methanol/water sample where multiple-bilayer tubules are also observed. By contrast, tubules prepared in solutions with a higher methanol/water ratio show a continuous decrease in the ellipticity as the melting temperature is approached. In addition, the melting temperature is observed to decrease with increasing proportion of methanol. The crossover from discontinuous to continuous melting is also seen in differential scanning calorimetry studies of the same samples. The bottom panel of Figure 2 shows specific heat measurements on the same 1 mg/mL tubules. In samples with a higher proportion of water, the steep drop in ellipticity at the melting temperature is accompanied by a sharp endothermic peak in the excess specific heat, ΔC_p. Samples with a continuously decreasing ellipticity have a very broad, asymmetric peak in ΔC_p.

The concentration dependence of the melting curves for tubules prepared in 90:10 methanol/water is shown in Figure 3. For all concentrations studied, the tubules have single-bilayer walls and have no apparent peak in their CD spectra at 205 nm. However, we are unable to study samples with concentrations greater than 5 mg/mL because the CD signal exceeds the limits of the spectrometer at the shortest available path length. The top panel of Figure 3 shows the temperature dependence of the 195 nm CD peak. We observe continuous melting at all concentrations. The corresponding specific heat measurements, shown in the bottom panel of Figure 3, give broad peaks. Integrating these peaks yields an enthalpy of about 25 kcal/mol at all concentrations. The melting temperature of these tubules is found to depend strongly on the lipid concentration, decreasing from 30 °C at 5.0 mg/mL to 18 °C at 0.5 mg/mL. Similar measurements of the tubules prepared in 70:30 methanol/water show discontinuous melting at all concentrations and no concentration dependence of the melting temperature.

Figure 4 shows melting curves at an intermediate ratio of 85:15 methanol/water. At lipid concentrations of 2 mg/mL and smaller, the ellipticity at 195 nm shown in the top panel continuously decreases as the tubules are heated, in a similar manner as seen at 90:10 methanol/water in Figure 3. Again, the melting temperature is found to depend on lipid concentration. However, at higher concentrations of lipid the CD has a

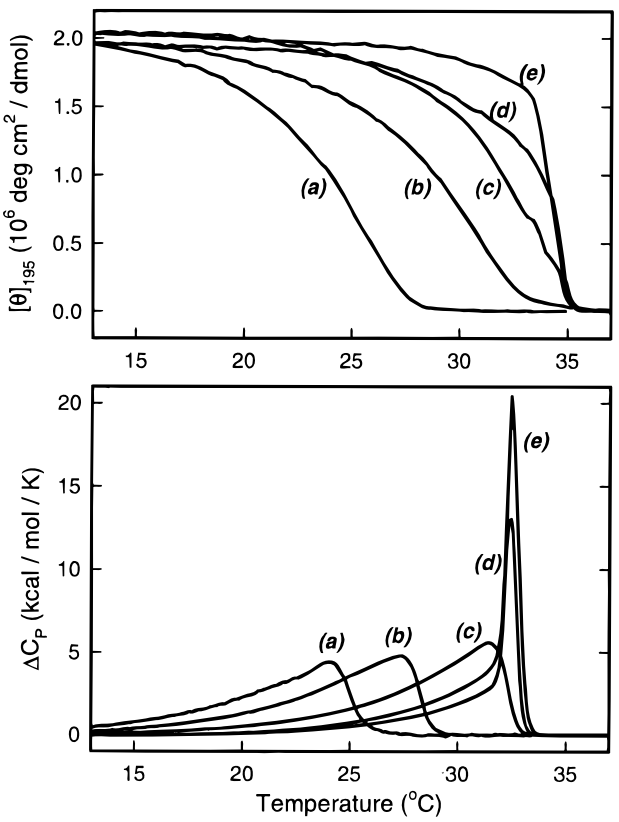

Figure 4. Concentration dependence of the CD spectra at 195 nm (top) and specific heat (bottom) of $DC_{8,9}PC$ tubules prepared in methanol/water (85:15) at lipid concentrations of (a) 0.5, (b) 1.0, (c) 2.0, (d) 3.0, and (e) 5.0 mg/mL. These results show a crossover from continuous to discontinuous melting as the lipid concentration is increased.

much sharper drop at the melting temperature. At a concentration of 5 mg/mL, this drop is nearly discontinuous. This crossover in melting behavior is reflected in the specific heat peaks shown in the bottom panel of Figure 4, which are broad at lower concentrations and become sharp as the concentration is increased.

Similar experiments were performed on tubules prepared in ethanol/water solutions. Figure 5 shows CD spectra of $DC_{8,9}$-PC tubules as the ethanol/water ratio is varied from 50:50 to 80:20. These spectra are from samples at a lipid concentration of 1 mg/mL and taken approximately 15 °C below the melting temperature. These spectra are all characterized by a broad peak centered about 205 nm, in contrast with the methanol/water tubules which had a number of sharper peaks. Another peak near 195 nm is suggested by an elbow seen in some of the spectra in Figure 5. A number of small ripples are observed in all these spectra between 230 and 250 nm where small absorptions from the diacetylene are known to be.[6] The main peak is much sharper at an ethanol/water ratio of 80:20, becoming smaller and broader as the proportion of water is increased.

The temperature dependence of the ellipticity and the specific heat of $DC_{8,9}PC$ tubules prepared at a lipid concentration of 1 mg/mL in varying ratios of ethanol/water are shown respectively in the top and bottom panels of Figure 6. These data qualitatively show the same trends as the methanol/water tubules. At ratios near 1:1 of ethanol and water, tubule melting is discontinuous, with a steep drop in the CD accompanied by a sharp endothermic peak in the excess specific heat. At larger proportions of ethanol, the CD goes continuously to zero, while the specific heat has a broad, asymmetric peak. Comparing

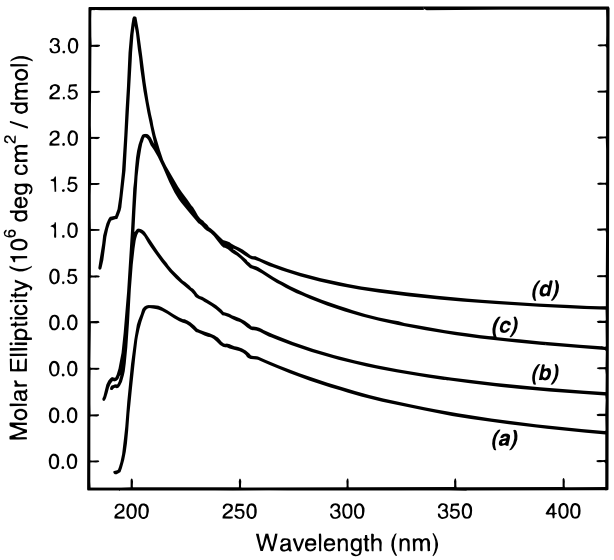

Figure 5. Circular dichroism spectra of $DC_{8,9}PC$ tubules prepared in ethanol/water at ratios of (a) 50:50, (b) 60:40, (c) 70:30, and (d) 80:20 at a lipid concentration of 1 mg/mL. The spectra were taken roughly 15 °C below the melting temperature. The curves have been offset vertically for display. The peak becomes larger and sharper as the ethanol fraction is increased.

the ethanol/water and methanol/water data, we observe that the ellipticity well below the melting temperature depends on the ethanol/water ratio in Figure 6, while it is relatively independent of the methanol/water ratio in Figure 2. This corresponds to the sharpening of the CD peak observed at higher proportions of ethanol in Figure 5. This sharpening is accompanied by a shift of the peak in the CD from 210 nm at 50:50 ethanol/water to 202 nm at 80:20 ethanol/water. In the methanol/water tubules, the 195-nm peak does not shift with alcohol/water ratio.

The concentration dependence of the melting curves for tubules prepared in 70:30 ethanol/water is shown in Figure 7. This behavior is qualitatively similar to that shown in Figure 4 from tubules prepared in 85:15 methanol/water. A crossover from discontinuous to continuous melting is seen in both the ellipticity and specific heat as the lipid concentration is lowered from 5 mg/mL. The melting temperature is again seen to decrease with lipid concentration.

In summary, for both methanol/water systems and ethanol/water systems, an increase in the lipid concentration leads to an increase in the melting temperature, and to a change from continuous to discontinuous melting. For methanol/water systems, an increase in the lipid concentration also leads to a change from single-bilayer tubules to multiple-bilayer tubules, as has previously been reported.[6,8] For ethanol/water systems, the tubules always have multiple bilayers, regardless of the lipid concentration. Similarly, for both methanol/water systems and ethanol/water systems, an increase in the alcohol fraction leads to a decrease in the melting temperature, and to a change from discontinuous to continuous melting. In addition, for methanol/water systems, an increase in the alcohol fraction leads to a change from multiple-bilayer tubules to single-bilayer tubules.

Figure 8 shows these experimental results as phase diagrams in terms of lipid concentration and alcohol fraction for both methanol/water (top panel) and ethanol/water (bottom panel) systems. In these phase diagrams single-bilayer tubules are indicated by circles, while multi-bilayer tubules are shown as squares. Filled symbols show points where discontinuous melting is observed, while unfilled symbols indicate continuous melting. Points close to the crossover, for example curve d in Figure 4, are denoted by a gray symbol. On the methanol/water

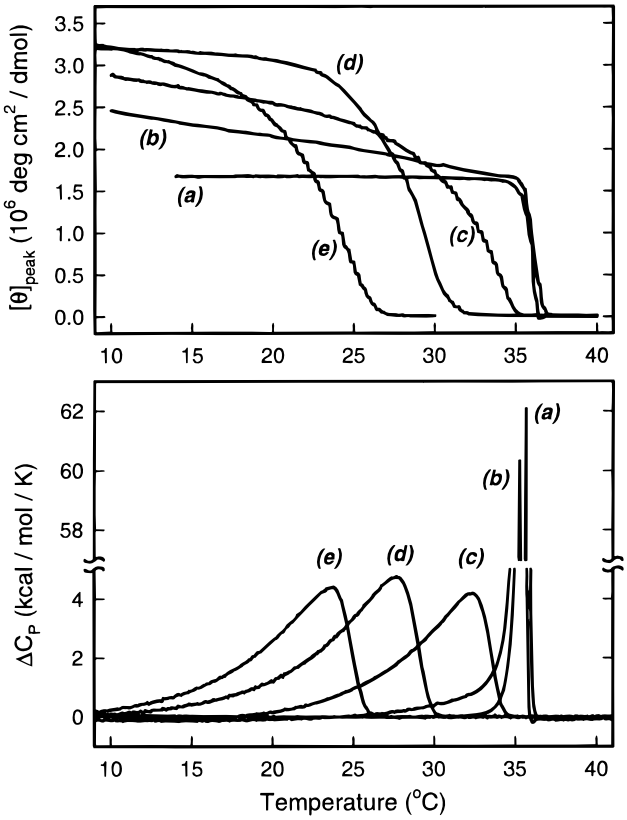

Figure 6. Temperature dependence of the peak molar ellipticity (top) and specific heat (bottom) of $DC_{8,9}PC$ tubules prepared in ethanol/water at ratios of (a) 50:50, (b) 60:40, (c) 70:30, (d) 75:25, and (e) 80:20 at a lipid concentration of 1 mg/mL. These results show a crossover from discontinuous to continuous melting as the ethanol fraction is increased, in a similar manner as seen for methanol/water tubules in Figure 2. However, here the amplitude of the CD signal also increases with alcohol fraction.

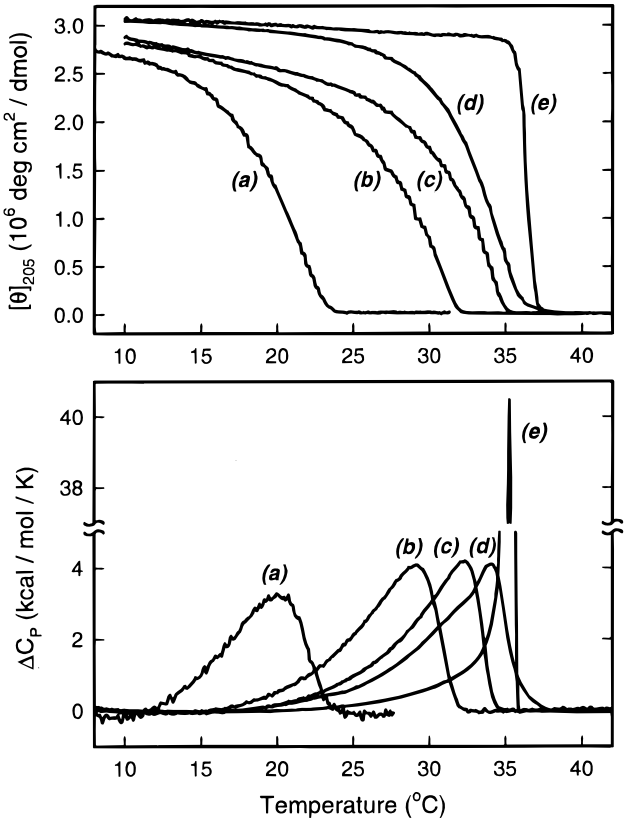

Figure 7. Concentration dependence of the CD spectra at 205 nm (top) and specific heat (bottom) of $DC_{8,9}PC$ tubules prepared in ethanol/water (70:30) at lipid concentrations of (a) 0.2, (b) 0.5, (c) 1.0, (d) 2.0, and (e) 5.0 mg/mL. These results show a crossover from continuous to discontinuous melting as the lipid concentration is increased.

phase diagram, two curves are shown: the solid curve is the boundary between discontinuous and continuous melting and the dashed curve is the boundary between single-bilayer and multiple-bilayer tubules. On the ethanol/water phase diagram, only the boundary between discontinuous and continuous melting is shown. The second boundary is omitted because no single-bilayer tubules are observed.

Discussion

Our experiments show that tubules can melt either discontinuously or continuously, depending on the type of alcohol in the solvent, the volume fraction of alcohol, and the concentration of lipid. In previous work, Nounesis *et al.* found that single-bilayer methanol/water tubules melt continuously, while multiple-bilayer methanol/water tubules melt discontinuously.[8] They attributed this difference to a dimensional crossover, arguing that single-bilayer tubules are effectively two-dimensional systems that melt through a second-order defect-unbinding transition,[13] while multiple-bilayer tubules are three-dimensional systems that melt through a conventional first-order transition. Our experimental results agree with their results at the alcohol fraction and lipid concentrations that they studied. However, our results for a broader range of alcohol fraction and lipid concentration disagree with the dimensional-crossover argument in two ways. First, the two boundaries in the methanol/water phase diagram do not lie on top of each other, as one would expect from the dimensional-crossover argument. Rather, for a certain range of methanol fraction, there are single-bilayer tubules that melt discontinuously. Second, in the ethanol/water phase diagram there is a boundary between discontinuous and continuous melting, but there is no boundary between single-bilayer and multiple-bilayer tubules. Instead, for a wide range of ethanol fraction, there are multiple-bilayer tubules that melt continuously.

Here, we propose an alternative explanation for our experimental results. This explanation is based on the concept that the lipid is in two coexisting phases, the tubule phase and a disordered phase composed of small aggregates or monomers dissolved in the alcohol/water solvent. Only the lipid in the tubule phase is packed in a chiral structure, and hence only that lipid contributes to the CD signal. Because the molar ellipticity is normalized by the total amount of lipid, not just the amount in tubules, it is a measure of the fraction of lipid in the tubule phase. The maximum amount of lipid in solution is given by the solubility of the lipid in the alcohol/water solvent; the rest of the lipid goes into tubules. Thus, by analogy with theories of micelle formation,[14] this maximum solubility can be called the critical aggregation concentration (CAC) for tubule formation. The solubility or CAC(T) is expected to increase as the temperature T increases. At low T, where CAC(T) is small, most of the lipid is in tubules. As T increases, CAC(T) also increases, so more of the lipid is transferred out of tubules into solution. For that reason, the molar ellipticity gradually decreases. That decrease continues until either (a) the temper-

(13) Nelson, D. R. In *Phase Transitions and Critical Phenomena*; Domb, C., Lebowitz, J. L., Eds.; Academic Press: New York, 1983; Vol. 7. Strandburg, K. J. *Rev. Mod. Phys.* **1988**, *60*, 161−207.

(14) Hiemenz, P. C. *Principles of Colloid and Surface Chemistry*; Marcel Dekker: New York, 1986; Chapter 8. Ben-Shaul, A.; Gelbart, W. M. In *Micelles, Membranes, Microemulsions, and Monolayers*; Gelbart, W. M., Ben-Shaul, A., Roux, D., Eds.; Springer-Verlag: New York, 1994.

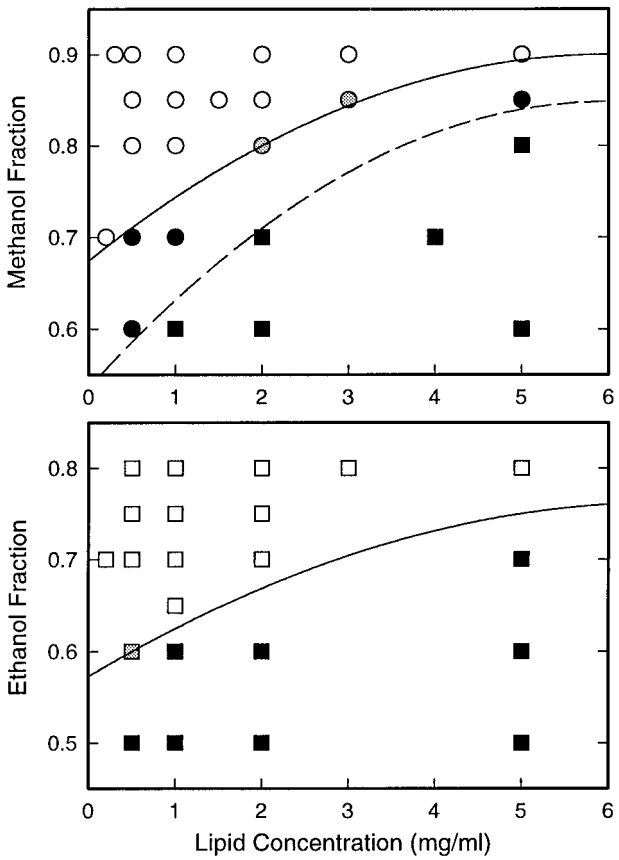

Figure 8. Phase diagrams for methanol/water (top) and ethanol/water (bottom) systems. Each of the points where we have characterized tubules indicates whether the tubules have single (circles) or multiple (squares) bilayers, and whether the melting is discontinuous (filled) or continuous (unfilled). Points close to the crossover are denoted by a gray symbol. The solid lines denote the approximate boundaries between discontinuous and continuous melting, while the dashed line denotes the boundary between single- and multiple-bilayer tubules in methanol/water.

ature T reaches the $L_{\beta'}$ to L_α transition temperature T_m or (b) CAC(T) reaches the total lipid concentration c. If T reaches T_m before CAC(T) reaches c, then the lipid chains within the tubules disorder, and hence the lipid cannot remain in the highly ordered tubule structure. In this case, the tubules melt discontinuously into another morphology, such as micelles or spherical vesicles, through a first-order phase transition. By contrast, if CAC(T) reaches c before T reaches T_m, then all of the lipid is transferred out of the tubule phase into the solution. In that case, the tubules appear to melt continuously, but they do not undergo a second-order phase transition. Rather, the continuous melting is just a gradual process of transferring the lipid into solution.

We have only limited information about the solution. Because the CD signal above the melting temperature is very low, we can infer that the lipid in this phase is not packed in a chiral structure. Furthermore, because the solution above the melting temperature is not turbid, we can infer that this phase does not have any large aggregates of lipid. Thus, this phase is probably composed of monomers or small lipid aggregates, such as micelles or small vesicles.

Our proposed scenario, with the two thermodynamic processes of melting into a disordered lamellar phase and melting into a lipid solution, explains several features of our experimental results. First, it explains the dependence of the melting behavior on the lipid concentration c. At low c, CAC(T) reaches c before T reaches T_m, so the tubules melt continuously. As c increases, the system must go to a higher temperature before CAC(T) reaches c, so the melting temperature increases. When c becomes large enough, T reaches T_m before CAC(T) reaches c, so the system crosses over from continuous to discontinuous melting. In the regime of discontinuous melting, the tubules always melt at T_m, and hence the melting temperature no longer depends on c. In addition, our scenario also explains the dependence of the melting behavior on the alcohol fraction. As the alcohol fraction increases, the solubility of the lipid in the alcohol/water solvent increases, and hence CAC(T) reaches the lipid concentration c at a lower temperature. Thus, as the alcohol fraction increases, the system crosses over from discontinuous to continuous melting. In the regime of continuous melting, the melting temperature decreases with increasing alcohol fraction. Finally, our scenario explains the difference in melting behavior between methanol/water and ethanol/water systems. If the lipid is more soluble in ethanol than in methanol, then the crossover between discontinuous and continuous melting is shifted to lower alcohol fraction in ethanol/water than in methanol/water systems, for the same lipid concentration. This shift is seen in the phase diagrams of Figure 8.

The dependence of melting temperature on lipid concentration can be expressed quantitatively. Standard theories of ideal solutions,[15] or equivalently theories of micelles,[14] show that CAC(T) varies as

$$\frac{\partial(\ln \mathrm{CAC}(T))}{\partial(1/T)} = -\frac{\Delta H}{R} \qquad (1)$$

where ΔH is the molar enthalpy of melting. In our proposed scenario, tubules melt continuously into a lipid solution when CAC(T) reaches the lipid concentration c. Thus, eq 1 predicts a relationship between the continuous melting temperature and the lipid concentration, $\partial(\ln c)/\partial(1/T) = -\Delta H/R$. To test this prediction, we compare the melting temperature with the lipid concentration in Figure 9. In this figure, lipid concentration is plotted on a log scale versus inverse temperature for tubules in methanol/water (top panel) and ethanol/water (bottom panel). The vertical line denotes the boundary between continuous melting (unfilled symbols) and discontinuous melting (filled symbols) at the $L_{\beta'}$ to L_α transition temperature: 35.6 °C for methanol/water and 37.5 °C for ethanol/water. The points representing continuous melting lie on straight lines. The slopes of these lines yield enthalpies of 32 ± 1 kcal/mol for methanol/water and 38 ± 2 kcal/mol for ethanol/water systems. For comparison, by integrating the corresponding specific heat measurements, we obtain enthalpies of 25 ± 2 kcal/mol for methanol/water and 32 ± 2 kcal/mol for ethanol/water systems. Although the enthalpies determined from Figure 9 are not exactly the same as the enthalpies determined from the specific heat measurements, they are reasonably close, and they show the same increase when methanol is replaced by ethanol. The discrepancy is probably due to the fact that the lipid/alcohol/water solution is not ideal. Thus, this quantitative test supports our proposed explanation of the experimental results.

As a final point, we can compare our results with studies of bilayers of saturated phospholipids in alcohol/water solutions. In those systems, small concentrations of alcohol induce interdigitation of the chains.[16] The alcohol preferentially partitions to the lipid bilayer interface, displacing water and changing the nature of head group interactions. At low alcohol concentrations, this leads to an increased distance between head

(15) Murrell, J. N.; Jenkins, A. D. *Properties of Liquids and Solutions*, 2nd ed.; Wiley: New York, 1994; Chapter 6.

(16) Rowe, E. S. *Biochemistry* **1983**, *22*, 3329−3305. Simon, S. A.; McIntosh, T. J. *Biochim. Biophys. Acta* **1984**, *773*, 169−172.

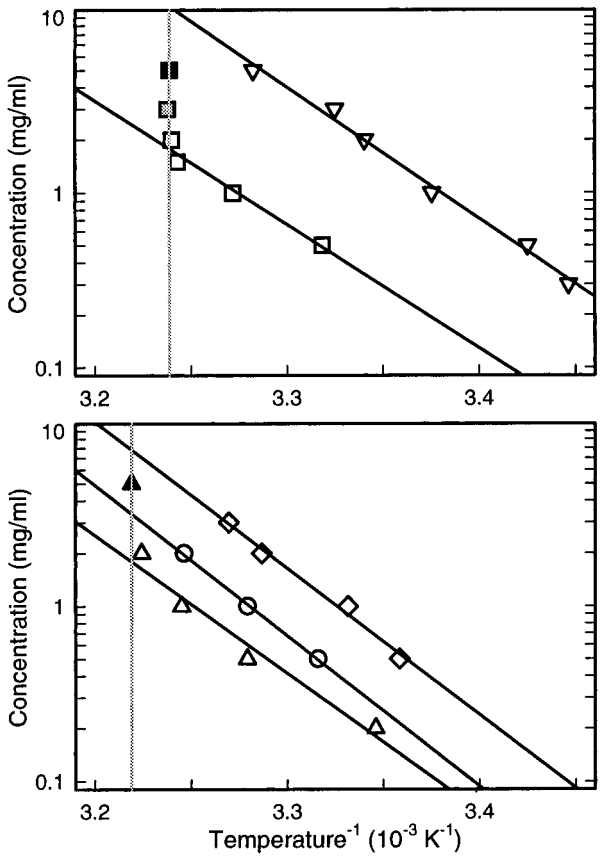

Figure 9. Lipid concentration as a function of inverse melting temperature determined from CD measurements. The top panel shows results from tubules prepared in 85:15 (□) and 90:10 (▽) methanol/water, while the bottom panel shows data from tubules prepared in 70:30 (△), 75:25 (○), and 80:20 (◇) ethanol/water. The vertical line denotes the boundary between continuous melting (unfilled symbols) and discontinuous melting (filled symbols).

groups and a corresponding increase of molecular tilt in the $L_{\beta'}$ phase.[17] As the alcohol concentration increases, the molecules reach a maximum tilt angle and instead began to interdigitate. At intermediate alcohol concentrations (2.5 to 5.5 percent by volume for ethanol and saturated phosphocholines),[17] the interdigitated phase $L_{\beta i}$ coexists with the normal $L_{\beta'}$ phase. At higher alcohol fractions, the bilayers are completely in the $L_{\beta i}$ phase. In our case, packing constraints do not allow tubules to form in this interdigitated phase, so the lipids dissolve into a disordered phase. Both Raman and IR studies indicate the $L_{\beta'}$ phase of $DC_{8,9}PC$ is very highly ordered relative to that of saturated phosphocholines.[18,19] In addition, the large melting enthalpies observed in our samples imply the tubule phase is highly ordered. This may account for the observation that much higher concentrations of alcohol are necessary to disorder $DC_{8,9}$-PC tubules.

In conclusion, we have used circular dichroism and specific heat measurements to study the melting of phospholipid tubules formed in solutions of alcohol and water. We find that tubules can melt either continuously or discontinuously, depending on the type of alcohol, the volume fraction of alcohol, and the concentration of lipid. This suggests that the nature of the melting is governed by changes in the lipid solubility in relation to the $L_{\beta'}$ to L_{α} transition temperature. The solubility is strongly influenced by the type of alcohol and the volume fraction of alcohol. Finally, we have shown that circular dichroism can be a useful tool in studies of the thermodynamics of lipid tubules, and it should be useful for studies of other chirally ordered supramolecular structures.

Acknowledgment. We acknowledge valuable discussions with K. Easwaran, S. Leslie, R. Price, J. Prost, B. Ratna, A. Rudolph, A. Singh, R. Shashidhar, and H. Wickman. This research was supported by the Office of Naval Research. M. Spector is supported by a National Research Council−Naval Research Laboratory Research Associateship.

JA9716665

(17) Vierl, U.; Löbbecke, L.; Nagel, N.; Cevc, G. *Biophys. J.* **1994**, 67, 1067−1079. Löbbecke, L.; Cevc, G. *Biochim. Biophys. Acta* **1995**, *1237*, 59−69.

(18) Schoen, P. E.; Yager, P. *J. Polym. Sci., Polym. Phys. Ed.* **1985**, *23*, 2203−2216. Rudolph, A. S.; Singh, B. P.; Singh, A.; Burke, T. G. *Biochim. Biophys. Acta* **1988**, *943*, 454−462.

(19) Schoen, P. E.; Nagumo, M.; Singh, A. *Chem. Phys. Lipids* **1994**, *69*, 251−257.