Controlling the Morphology of Chiral Lipid Tubules

ARTICLE *in* LANGMUIR · MAY 1998

Impact Factor: 4.46 · DOI: 10.1021/la980086y

Controlling the Morphology of Chiral Lipid Tubules

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Received January 23, 1998. In Final Form: March 25, 1998

Several techniques for controlling the morphology of self-assembled lipid tubules are investigated by using circular dichroism spectroscopy and electron microscopy. These studies show that variations in the molecular structure of the diacetylenic phospholipid, lipid concentration, and solution conditions allow for control of the number of bilayers in the tubule walls, but not their diameter. Tubules formed in water and mixtures of alcohols adopt interesting morphologies and allow for further control of tubule structure. In addition, studies of lipids with different acyl chains show that tubule morphology is sensitive to the degree of order within the chains. Because of the chiral molecular architecture in lipid tubules, intense peaks in their circular dichroism spectra are observed. These peaks can be monitored to obtain information on the tubule morphology. This information is correlated to direct observations made using electron microscopy. Results of these studies have led to the optimization of large scale preparations of tubules for technological applications.

Introduction

Phospholipids self-organize in solution due to their amphiphilic structure. For diacyl phospholipids in excess water, the most common structure found is a spherical, bilayer aggregate known as a liposome.[1] In addition to being amphiphilic, glycerophospholipids are chiral, with the L-enantiomer predominant in nature. While the enantiomeric excess of L-α-amino acids and D-sugars in nature affects the structure and biological activity of proteins and nucleic acids, the chiral asymmetry of phospholipids does not typically manifest itself in the supramolecular structure of lipid biomembranes. One exception is the helical structures observed in saturated bile solutions, where lipids are apparently an essential ingredient.[2] Another exception is the unusual supramolecular aggregates formed from bilayers of synthetic phospholipids with diacetylenes in the acyl chains. These lipids are found to self-assemble into hollow, cylindrical structures, known as tubules, when the lipids are cooled into the gel phase.[3] Tubules differ from cochleate cylinders formed from anionic lipid bilayers because tubules are hollow while cochleate cylinders are filled with a rolled-up bilayer.[4] While formation of cochleate cylinders is induced by electrostatic interactions, formation of tubules is apparently induced by chiral interactions.[5] This chirality can be seen in the helical markings that are often observed when these tubules are decorated or stained.[6,7]

The most commonly studied diacetylenic phospholipid is 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine. This lipid, shown in Figure 1a, is designated $DC_{8,9}$-PC because it has eight methylenes between the ester and diacetylene group and nine methylenes between the diacetylene and the terminal methyl group. It forms tubules with a characteristic diameter of 0.5 μm and a characteristic length of 50−200 μm. The molecular architecture of tubules has been explained by theories based on molecular chirality.[5,8,9] In these theories, chiral interactions cause molecules to pack at a nonzero angle with respect to their nearest neighbors, as shown in Figure 1b. This chiral packing contributes to the twisting of the bilayer, which leads to the formation of a cylinder.

Phospholipid tubules have been extensively studied for use in technological applications, such as electroactive composites and controlled-release systems.[10] Those applications depend on coating tubules with a thin film of metal or ceramic to make them more rugged.[11] Figure 2 shows a scanning electron micrograph of a copper-plated lipid tubule. The tubules are observed to be hollow and contain spiral wrappings. To optimize tubules for applications, one may wish to manipulate the tubule morphology, e.g., the tubule length, diameter, and wall thickness. Control of length and diameter is important because those parameters determine the release rate in controlled-release systems and the electromagnetic properties of metallized cylinders in electroactive composites. Control of tubule wall thickness is important because the thickness determines whether the tubules can be coated. If the tubule walls are only one bilayer thick, then they are too fragile to be coated. On the other hand, tubule walls that are many bilayers thick make inefficient use

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S0743-7463(98)00086-9
Published on Web 05/29/1998

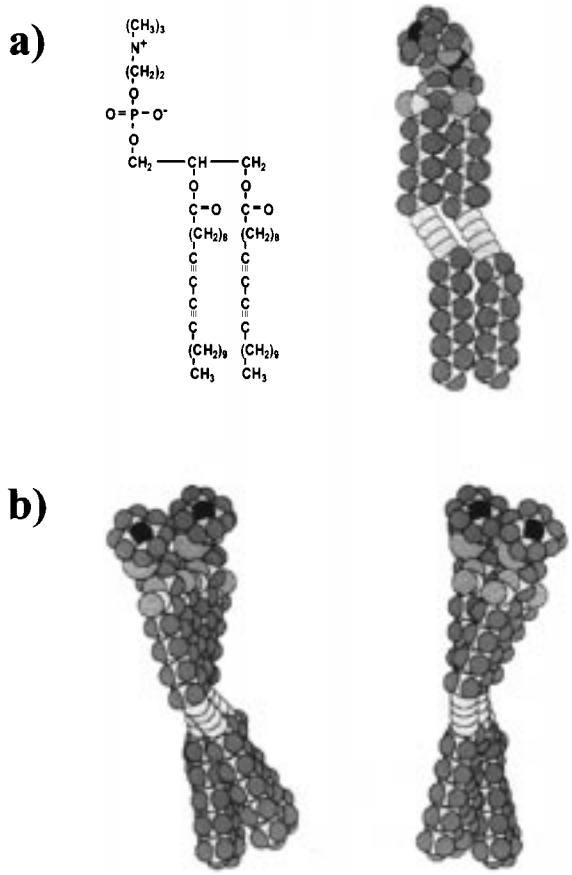

Figure 1. (a) The chemical structure of the diacetylenic lipid 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}$-PC), along with a model representing its three-dimensional structure. The diacetylene group introduces a kink into the acyl chain of the molecule, which imposes a steric hindrance to the molecules packing parallel to each other. (b) Nonparallel packing of the molecules can impart either a counterclockwise or clockwise twist to the lipid bilayer they form. Chirality of the molecules causes one orientation to be energetically preferable.

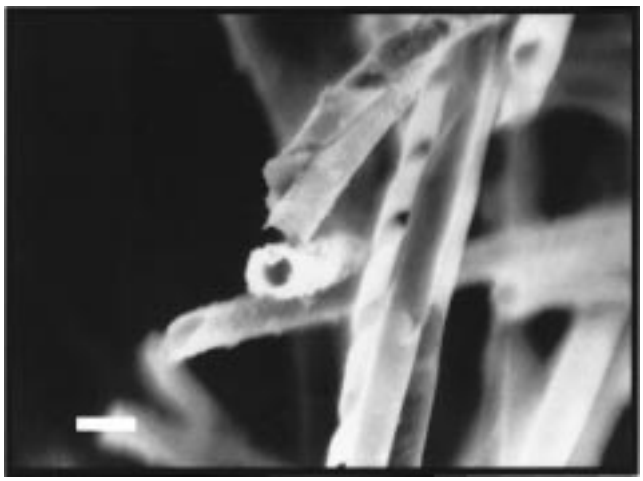

Figure 2. Scanning electron micrograph of copper-coated $DC_{8,9}$-PC tubules. The tubules are hollow with a diameter of approximately 0.5 μm. The helical wrappings evident in some tubules are all right-handed. Bar = 0.5 μm.

of the expensive lipid; the optimum thickness for applications requiring electroless metallization is just two bilayers.

Previous studies have used various approaches to modify the morphology of tubules. One approach for controlling tubule length and wall thickness has been to change the solvent. Tubules can be produced by first dissolving the lipids in alcohol and then mixing with water above the chain melting temperature T_m and slowly cooling the mixture.[12] Tubules formed this way are longer and less fragile than tubules formed in pure water. Detailed microscopy studies have found that the length distribution and thickness depend on the alcohol used, while the diameter does not. In particular, tubules formed in methanol/water solution consist predominantly of single-bilayer walls and are of maximal length at 85 vol % methanol (~100 μm), while tubules formed in ethanol/water solution have multiple bilayers and are longest at 70% ethanol (~70 μm).[13] Another approach for controlling tubule length and wall thickness is to change the rate of cooling through T_m. Decreasing the cooling rate has been found to increase greatly the average length of tubules and decrease the thickness of the walls in ethanol/water solutions.[14] However, the tubule diameter appears independent of the cooling rate and alcohol/water solvent. The only approach that has changed the diameter has been to alter the molecular structure of the lipid headgroup. The diameter of tubules formed from negatively charged diacetylenic lipids, synthesized by enzymatically replacing the choline portion with glycols, could be altered by varying the pH and ionic strength of the solution and the anion of added salt.[15] Depending on the exact conditions, single populations of either larger or smaller diameter tubules as well as tubules with bimodal distributions of diameters could be formed. However, due to the sensitivity of these lipids to their ionic environment and the difficulty of working with these lipids in mixed alcohol/water solvents, this approach has not yet proven practical for industrial application.

In our recent research, we have found that circular dichroism (CD), the difference in absorption of right and left circularly polarized light, is a very useful tool for studying tubule structure.[16−18] When the lipid $DC_{8,9}$PC is in nontubular form in solution, it has a small CD signal. By contrast, when the same lipid forms tubules, its CD signal increases by a factor of at least 10^4. This increase provides experimental evidence that molecular packing of lipid molecules in tubules is chiral, as implied by several theories.[5,8,9] Additional information comes from looking at the CD spectrum as a function of wavelength. Tubules have two distinct peaks in the CD spectrum: a peak at 195 nm that can be associated with chiral packing of the diacetylene groups within a single bilayer and a peak at 202−205 nm that can be associated with chiral ordering of headgroups between adjacent bilayers.[17] By measuring the relative amplitudes of those two peaks, we can see the crossover from single-bilayer tubules to multiple-bilayer tubules as a function of the lipid concentration and the nature of the solvent. We therefore used CD to investigate the thermodynamics of tubule melting in methanol/water and ethanol/water solutions.[18]

In this paper, we use CD as a tool to investigate four

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possible approaches for changing tubule morphology. First, we study the wall thickness of tubules in methanol/water solutions. By adjusting the lipid concentration and methanol/water ratio, we identify a protocol for making tubules with the optimum thickness of two bilayers. Second, we look at tubules in mixed methanol/ethanol/water solutions. We find that the mixed solvent leads to complex tubule morphologies, with bilayer ribbons helically wrapped around tubules. Third, we explore the effects of changing the lipid chain length. These results show that tubule ordering is sensitive to the degree of order within the lipid chains. Finally, we investigate mixtures of D- and L-lipid, following up on preliminary experiments reported in ref 16. These experiments show that tubules composed of opposite enantiomers produce CD spectra of opposite sign, indicating that molecular packing in tubules reflects the underlying molecular chirality.

Materials and Methods

The L-enantiomer, 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine (L-$DC_{8,9}PC$), was purchased from JP Laboratory (Middlesex, NJ). The D-enantiomer (D-$DC_{8,9}PC$) was synthesized by hydrolyzing 2,3-bis(palmitoyl)-*sn*-glycero-1-phosphocholine (D-DPPC, Sigma Chemical Co.) with tetrabutylammonium hydroxide to form D-glycerophosphocholine, followed by reaction with tricosa-10,12-diynoic anhydride in the presence of 4-*N*,*N*-(dimethylamino)pyridine.[11,19] Other lipids were synthesized by reacting l-glycerophosphocholine with the appropriate diacetylenic anhydride. We drop the L designation when referring to these lipids for brevity. Diacetylenic fatty acids were synthesized by coupling the appropriate ω-alkynoic acid with iodoalkynes following the procedure of ref 19, with the exception of tricosa-10,12-diynoic acid, which was purchased from Farchan Laboratories (Gainesville, FL). All lipids were purified by column chromatography using silica gel and checked for purity by thin-layer chromatography using a chloroform/methanol/water (65:25:4, v/v/v) solvent system in both cases. The purified lipids were a white powder. Tubules were prepared by dissolving the lipid in HPLC-grade methanol or ethanol (Sigma) and mixing with Milli-Q water (Millipore Corp.) at 65 °C. On cooling the solution at 3 °C/h through the transition temperature, tubules are formed.[12] CD studies were performed on a JASCO J-720 spectropolarimeter operated between 175 and 600 nm. Solvent absorption limited the effective range of study to above 188 nm. Samples were placed in water-jacketed quartz cells with path lengths of 0.1−0.5 mm, with temperature control supplied by a water circulator (Neslab) providing thermal stability of about 0.2 °C. The spectrometer was calibrated with ammonium-*d*-camphorsulfonate ($[\theta]_{291}$ = 7910 deg cm²/dmol) and D-pantoyllactone ($[\theta]_{219} = -16140$ in water, $[\theta]_{223} = -12420$ in methanol).[20] Samples for electron microscopy were negative stained with 1% uranyl acetate and examined in a Zeiss EM-10C transmission electron microscope operating at 60 kV.

Results

Our first approach to controlling the morphology of diacetylenic lipid tubules was to vary the lipid concentration. It is already known that the thickness of the tubule wall depends on the lipid concentration as well as the alcohol length. Although tubules formed in ethanol/water have multiple-bilayer walls at all lipid concentrations, tubules formed in methanol/water have single-bilayer walls at low lipid concentration,[13] but multiple-bilayer walls at higher lipid concentrations.[21] This increase in thickness is accompanied by an increase in the CD signal

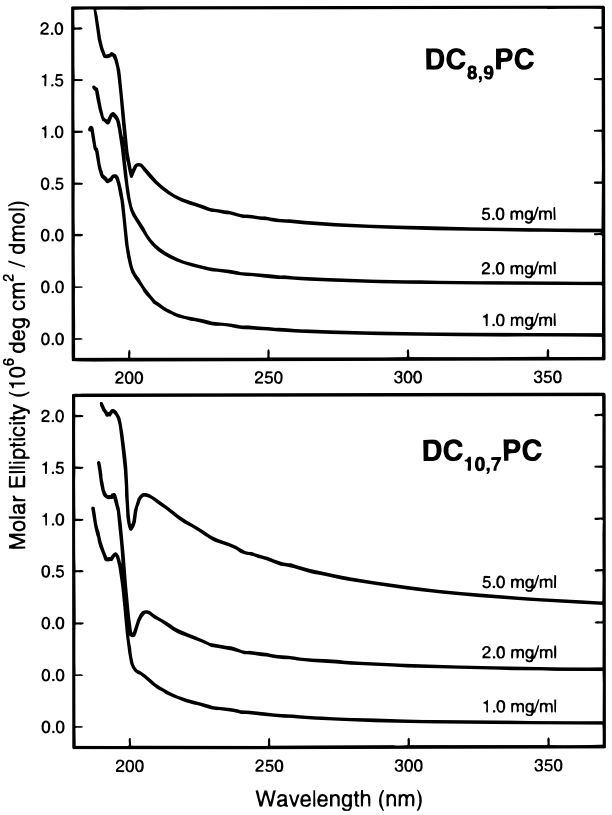

Figure 3. Circular dichroism spectra of $DC_{8,9}PC$ (top) and $DC_{10,7}PC$ (bottom) tubules prepared in 80:20 methanol/water at lipid concentrations of 1.0, 2.0, and 5.0 mg/mL taken at 25 °C. The curves have been offset vertically for display. The increase in ellipticity at 205 nm with increasing lipid concentration indicates formation of multiple-bilayer tubules at higher concentrations.

at 205 nm.[17] Figure 3 shows the concentration dependence of the CD spectra for two diacetylenic lipids. In the top panel we show the CD spectra from $DC_{8,9}PC$ tubules in 80:20 (v/v) methanol/water at three different lipid concentrations. The peak at 205 nm is prominent in the 5 mg/mL sample, whereas it only appears as a small elbow in the spectra at lower concentrations. The size of the 195 nm peak is similar at all concentrations. These results suggest a crossover from single-bilayer to multiple-bilayer tubules near a lipid concentration of 5 mg/mL in 80:20 methanol/water. As we previously reported, the crossover concentration depends on the methanol/water ratio, with the crossover occurring at higher concentrations as this ratio increases.[18]

Examining the tubule morphology at the crossover concentration, we found that most (>90%) of the tubules have double-bilayer thick walls in a 5 mg/mL sample prepared in methanol/water 85:15. A transmission-mode electron micrograph of such a tubule is shown in Figure 4. A complete cross section of this tubule is shown in the top panel, with an enlargement of the edge shown in the bottom panel. The wall thickness for the double-bilayer tubule in this micrograph is 16 ± 2 nm, implying a single-bilayer thickness of about 8 nm. This is slightly larger than the bilayer thickness of 6.6 nm determined by X-ray diffraction from multiple-bilayer tubules.[22] This may be an artifact of the staining process or may indicate that there is some swelling of the bilayers in these methanol/

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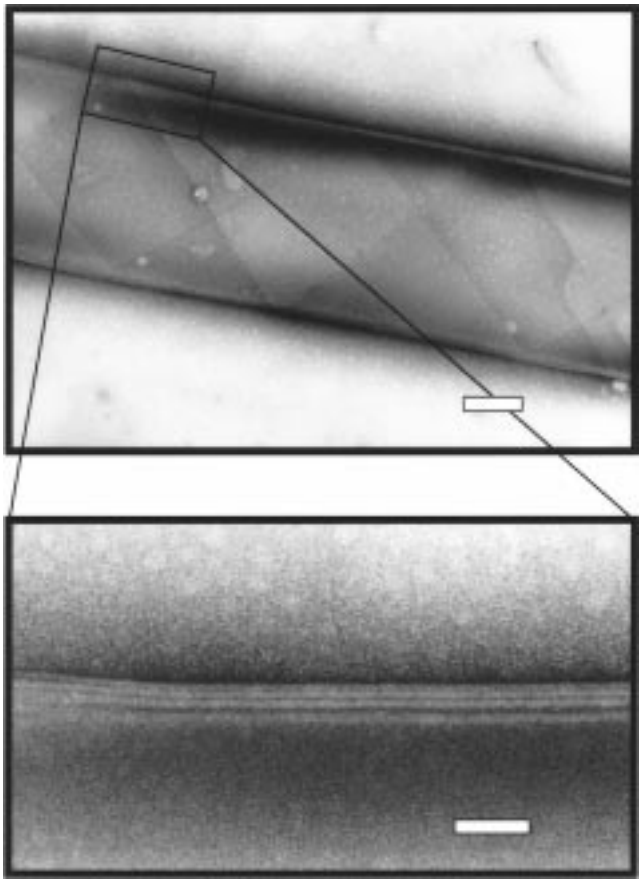

Figure 4. Negative stained electron micrograph of a $DC_{8,9}PC$ tubule from a 5 mg/mL sample in methanol/water (85:15). This sample predominantly contains double-bilayer tubules evidenced in the enlargement of the tubule edge shown in the bottom panel. Bar = 200 nm (top); 50 nm (bottom).

water tubules. These tubules also exhibit a very high aspect ratio. We have been able to use electroless plating to metallize these double-bilayer tubules, which we were unable to do with single-bilayer tubules.[23] This provides a significant cost savings over metallizing ethanol/water tubules which typically have 10 bilayers.[13] Unfortunately, this process results in highly thixotropic suspensions, which are difficult to process for large quantity applications. Diluting the sample leads to single-bilayer tubules that we are unable to coat with metal. As discussed below, this problem has been overcome through the use of mixed alcohol solvents.

We note that the double-bilayer tubule in Figure 4 shows clear helical markings. Such markings are characteristic of multiple-bilayer ethanol/water tubules but are not seen in single-bilayer methanol/water tubules. These markings may be associated with defects in the tilt direction of the lipid molecules on the tubules[5] or they may be the edges of helical ribbons wrapped around the inner tubule core. Although it is sometimes difficult to differentiate the helical markings from the top and bottom bilayers, observation of the tubule ends or taking stereopairs of micrographs occasionally allows unambiguously the determination of the handedness of the helical markings. Only right-handed helices are observed for the L-enantiomer.

As a second approach, we investigated the morphology of $DC_{8,9}PC$ tubules in solutions containing water and a

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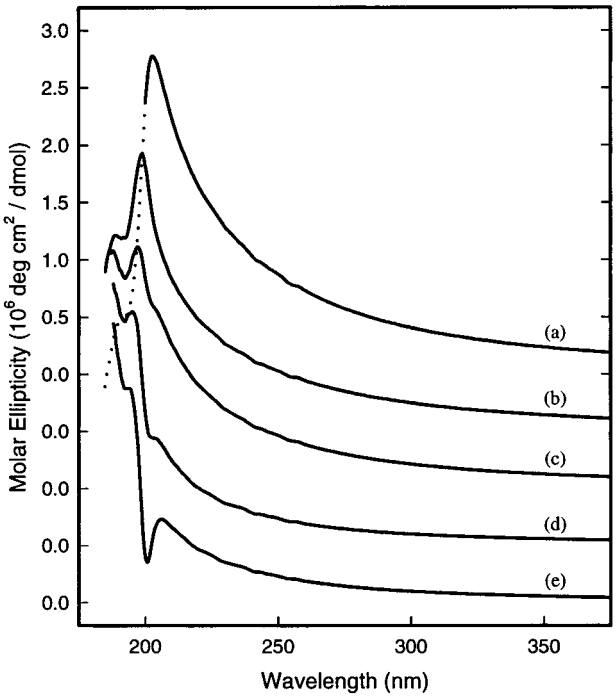

Figure 5. Circular dichroism spectra of mixed alcohol tubules in methanol/ethanol/water solutions at ratios of (a) 0:70:30, (b) 20:50:30, (c) 35:35:30, (d) 50:20:30, and (e) 70:0:30. All samples are prepared at a $DC_{8,9}PC$ concentration of 2.0 mg/mL, and the spectra are recorded at 25 °C. The curves have been offset vertically for display.

mixture of alcohols. Because variations of the alcohol chain length lead to structural changes in lipid tubules, forming tubules in multicomponent solutions may also lead to new morphologies. We studied this effect by making tubules in solutions of water, methanol, and ethanol, keeping the volume fraction of water fixed at 30% and varying the methanol/ethanol ratio. Figure 5 shows CD spectra of $DC_{8,9}PC$ tubules in these three-component solutions at 25 °C and a lipid concentration of 2 mg/mL. In the methanol/water (7:3) solution, the CD spectra are characterized by peaks at 205 and 195 nm, with another peak below the instrumental cutoff at 188 nm. As ethanol is added to the solution, the 195 nm peak appears to broaden and red-shift slightly. In a solution with equal amounts of methanol and ethanol, the lower peak shifts to 197 nm and the 205 nm peak still appears as a distinct elbow in the data. Figure 6 shows an electron micrograph of the tubules formed in this solution, 35:35:30 methanol/ethanol/water. These tubules appear to be quite different than those previously observed. They are composed of a single-bilayer inner wall, with no observable helical markings, and another partial bilayer helically wrapped around the outside. Almost all of the tubules observed in this solution have that appearance, although the width of the partial bilayer has a large variance. In the bottom-right corner of this picture, we see some tubules where the partial bilayer is coming unwrapped from the tubule. This may be an artifact introduced in the drying process.

Mixed alcohol solutions also allowed greater control over tubule thickness to overcome the processing problems described above. By producing tubules in a solution of 64:16:20 methanol/ethanol/water at a lipid concentration of 5 mg/mL, we were able to obtain very high aspect ratio tubules. TEM analysis showed these tubules contained between two and four lipid bilayers. This system proved to be less thixotropic and we were able to process these

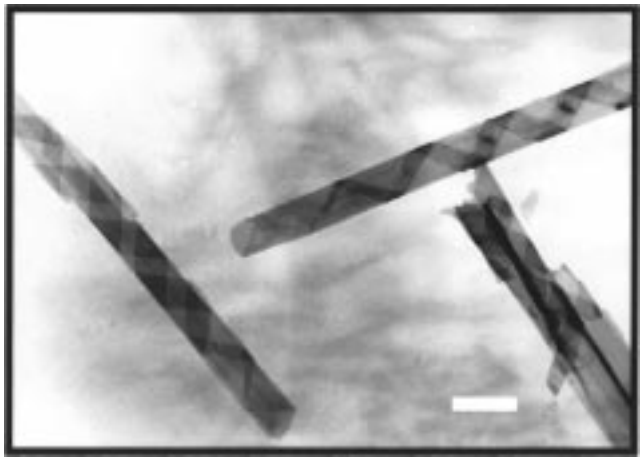

Figure 6. Negative stained electron micrograph of $DC_{8,9}PC$ tubules prepared in methanol/ethanol/water (35:35:30) at a lipid concentration of 2 mg/mL. These tubules are composed of a single-bilayer tubule, with another partial bilayer helically wrapped around it. Bar = 1.0 μm.

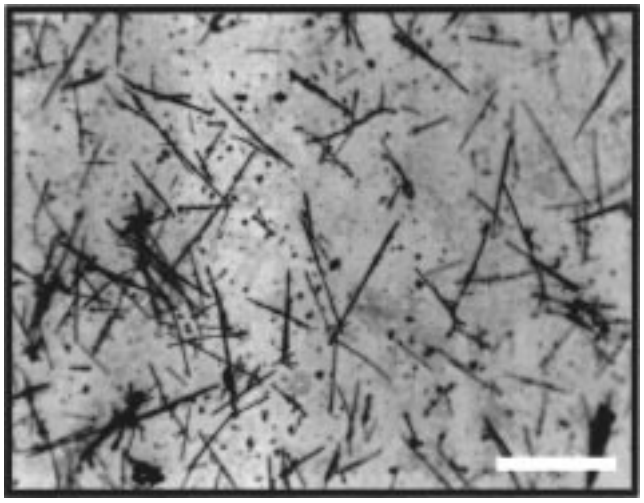

Figure 7. Optical micrograph of copper-coated tubules obtained by metalizing $DC_{8,9}PC$ tubules formed in a solution of 64:16:20 methanol/ethanol/water at a lipid concentration of 5 mg/mL. The tubules are in an acrylic polymer film and have an average length of about 60 μm with a standard deviation of 35 μm. Bar = 100 μm.

tubules by using electroless plating techniques to obtain long metallized tubules. Figure 7 shows an optical micrograph of copper-coated tubules prepared in this manner and embedded in an acrylic polymer film. The average length of these tubules is 60 μm with a standard deviation of 35 μm. Tubules formed in ethanol/water solutions tend to be thicker and require significant agitation to disperse them during processing. This shear leads to breakage and a decrease in the average length of the metal-coated tubules to 15 μm with a standard deviation of 9 μm. Tubules formed in the 64:16:20 solution are not severely thixotropic and require much less agitation. This enables us to produce high aspect ratio (>100:1) metallized tubules.

Our third approach to controlling tubule morphology is to evaluate effects of variations in the acyl chain on tubule morphology, beginning with the lipid 1,2-bis(tricosa-12,14-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{10,7}PC$). The only difference between this lipid and $DC_{8,9}PC$ is that the diacetylene group is two carbons closer to the end of the acyl chain in $DC_{10,7}PC$. CD spectra from $DC_{10,7}PC$ tubules in 80:20 methanol/water are shown as a function

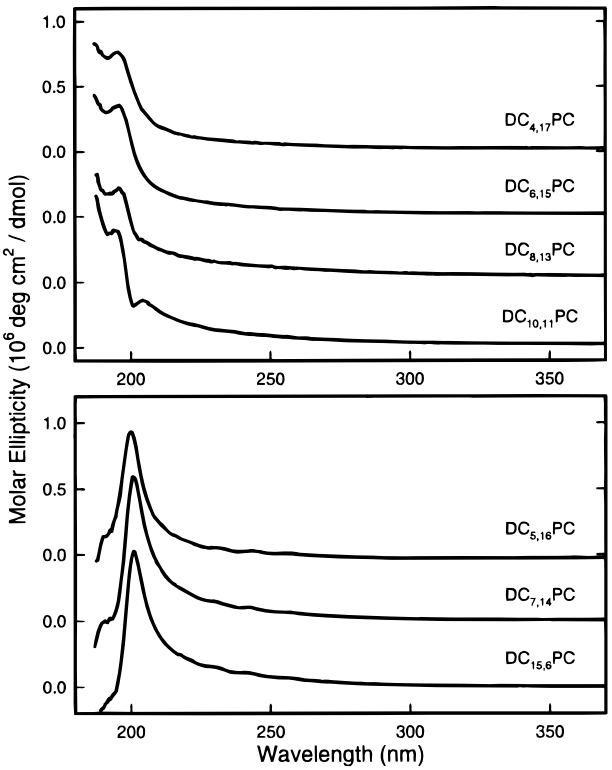

Figure 8. Circular dichroism spectra of tubules containing positional isomers of the lipid 1,2-bis(heptacosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{m,n}PC$). The top panel shows spectra for lipids with *m* even, $DC_{4,17}PC$, $DC_{6,15}PC$, $DC_{8,13}PC$, and $DC_{10,11}PC$. The increase in ellipticity at 205 nm as the diacetylene is moved down the acyl chain indicates formation of multiple-bilayer tubules. The bottom panel shows spectra for lipids with *m* odd, $DC_{5,16}PC$, $DC_{7,14}PC$, and $DC_{15,6}PC$. The spectra appear independent of diacetylene position. All samples are prepared in 80:20 methanol/water at lipid concentrations of 1.0 mg/mL, and the spectra are taken at 25 °C. The curves have been offset vertically for display.

of lipid concentration in the bottom panel of Figure 3. Again, we find the CD peak at 205 nm is larger at higher concentrations, but here the crossover occurs at 2 mg/mL. At higher concentrations, the peak continues to grow but also becomes much broader. Electron microscopy observations confirm that samples prepared at a concentration of 1 mg/mL $DC_{10,7}PC$ in 80:20 methanol/water consist of almost entirely single-bilayer tubules, while those with a lipid concentration of 5 mg/mL contain tubules with 6−10 bilayers. Previous Fourier transform infrared (FTIR) studies have found a sharpening of the CH_2 wagging peaks as the diacetylene is moved down the chain toward the terminal methyl group.[24] This was attributed to an increase in the order of the acyl chain (fewer gauche conformers) and of the headgroup packing. Here we find that the 205 nm peak also increases as the diacetylene is moved down the chain with the lipid concentration and solvent remaining the same.

We further probed this effect by studying a series of positional isomers of the diacetylenic group in the lipid 1,2-bis(heptacosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{m,n}PC$). These lipids have four more carbons than $DC_{8,9}PC$ in their acyl chain, with *m* methylenes between the ester and diacetylene group and *n* between the diacetylene and terminal methyl group. Figure 8 shows CD spectra from tubules composed of seven such lipids in

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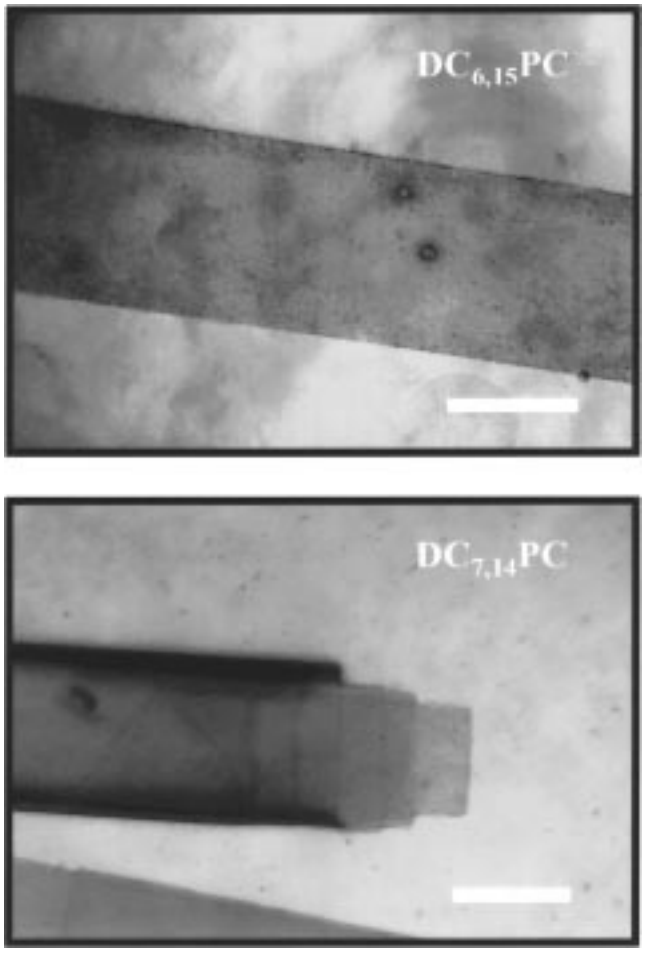

Figure 9. Negative stained electron micrograph of a $DC_{6,15}PC$ tubule (top) and a $DC_{7,14}PC$ tubule from a 1 mg/mL sample in methanol/water (8:2). Changing the position of the diacetylene by one carbon in the acyl chain causes a transformation from single- to multiple-bilayer tubules. Bars = 0.5 μm.

methanol/water (80:20) at a lipid concentration of 1 mg/mL. The top panel shows the spectra for tubules with *m* even. All of these spectra show a similar peak at 195 nm, while the 205 nm peak is observed only in the $DC_{10,11}PC$ tubules where the diacetylene is closest to the terminal methyl group. This trend is the same as that seen by comparing the 2 mg/mL tubules from $DC_{8,9}PC$ and $DC_{10,7}$-PC in Figure 3.

The bottom panel of Figure 8 shows similar spectra for tubules composed of lipids with *m* odd. These spectra differ from those with *m* even in two ways. First, the spectra have a large peak at 201 nm and a small elbow near 195 nm. This is qualitatively more similar to spectra from multiple-bilayer $DC_{8,9}PC$ tubules in ethanol/water[17] than those in methanol/water (Figure 3). Second, the spectra look similar regardless of the position of the diacetylene group. This holds even for $DC_{15,6}PC$ where the diacetylene is close to the end of the acyl chain. Figure 9 shows electron micrographs comparing the structure of tubules with even and odd *m* prepared under the same conditions as those from which the CD spectra in Figure 8 were obtained. The top panel of Figure 9 shows the cross section of a typical $DC_{6,15}PC$ tubule, which shows that these tubules have walls composed of a single bilayer, while the bottom panel shows the end of a typical $DC_{7,14}$-PC tubule, revealing multiple-bilayer walls. This odd−even difference in the tubule structure is reflected in the CD spectra shown in Figure 8 and indicates a significant difference in the lipid packing arrangement when the

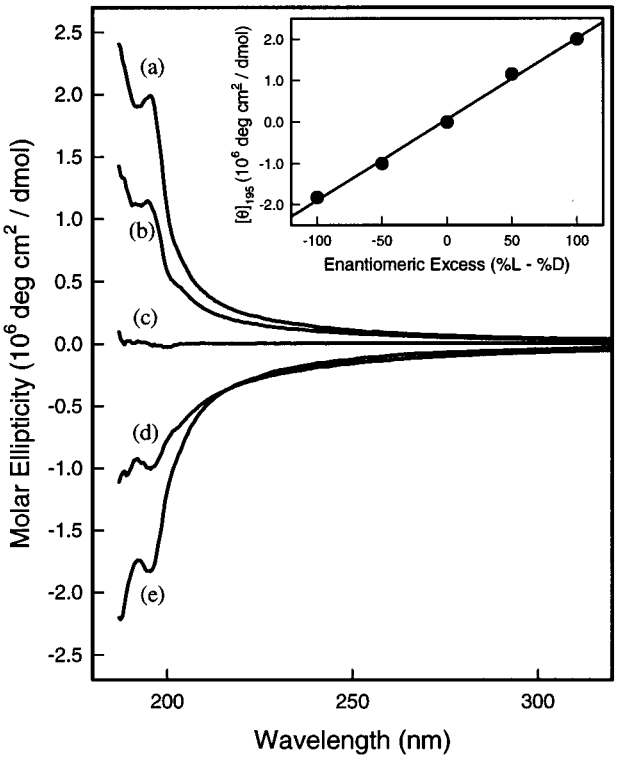

Figure 10. Circular dichroism spectra of tubules made from mixtures of opposite enantiomers of $DC_{8,9}PC$. The spectra shown correspond to mixtures of (a) 100:0, (b) 75:25, (c) 50:50, (d) 25:75, and (e) 0:100 L-/D-$DC_{8,9}PC$. All samples are prepared in 80:20 methanol/water at a total lipid concentration of 1.0 mg/mL, and the spectra are recorded at 25 °C. The inset shows the dependence of the peak molar ellipticity on the enantiomeric excess along with a linear fit of the data.

diacetylene is moved by only one position in the acyl chain. We also find that the tubules containing lipids with odd *m* melt at a lower temperature than those with even *m*, in agreement with previous work for tubules in pure water.[24] We find the tubules undergo a discontinuous melting transition at 48 °C for *m* odd or 52 °C for *m* even (except for $DC_{4,17}PC$ which melts at 59 °C).

Finally, we studied the effects of mixing opposite enantiomers of $DC_{8,9}PC$ on tubule morphology. Figure 10 shows the CD spectra from tubules composed of mixtures of L- and D-enantiomers prepared in a solution of 80:20 methanol/water and a lipid concentration of 1 mg/mL. Spectra from tubules made purely from the L- and D-enantiomers are nearly identical, but of opposite sign. This reflects the opposite handedness of packing in tubules composed of lipids with opposite chirality. Our previous work showed similar results over a more limited wavelength range for ethanol/water tubules.[16] In addition, we find that tubules made from mixtures of opposite enantiomers show comparable spectra with the magnitude and sign scaling with the enantiomeric excess. Figure 10 shows spectra from mixtures of 75:25, 50:50, and 25:75 D-/L-$DC_{8,9}PC$, in addition to the pure enantiomers. The similar shape of the spectra indicates no structural changes other than packing direction as the overall chirality is changed. The inset to Figure 10 shows the dependence of the molar ellipticity at 195 nm on the enantiomeric excess of the lipid mixture, along with a linear fit of the data. Optical microscopy shows the mixed enantiomer tubules to be approximately the same diameter, length, and density. Even the racemic mixture, which shows no CD signal, has the same turbidity, indicating the same tubule density. Indeed, electron microscopy

observations of the racemic mixture in ethanol/water solutions show formation of left- and right-handed tubules.[6] Thus, the change in magnitude of the CD can be interpreted as a change in the enantiomeric excess of tubules.

Discussion

The results of our first approach to changing tubule morphology, by varying the lipid concentration, are straightforward to interpret and apply. These results show that the thickness of the tubule walls changes gradually as a function of lipid concentration in methanol/water solution. At low concentration the tubules have single-bilayer walls, while at high concentration they have multiple-bilayer walls. Thus, by working near the crossover concentration, we can prepare tubules with double-bilayer walls, which are thick enough to be coated with metal but are not so thick that they waste lipid. Although we were able to metal plate these tubules, the solution proved to be too thick to process on a large scale.

Our experiments on tubules in mixed methanol/ethanol/water solutions show that the solvent also has a substantial effect on tubule structure. This effect is too great to be explained by a simple change in the lipid solubility or other physical properties of the solvent. Rather, we must consider effects of alcohol preferentially partitioning to the lipid bilayer interface. This would change the nature of the hydration layer surrounding the bilayer and, thus, alter the spacing and packing of the lipid headgroups.[26] It is also possible that the alcohol enters the bilayer causing a larger change in the molecular packing. Expansion of the intermolecular spacing may allow neighboring molecules to rotate more freely with respect to each other. By the theoretical argument of Harris et al.,[27] this rotation should average out part of the chiral interaction between neighboring molecules, thus reducing the intermolecular order in the membrane. These effects would become stronger when methanol is replaced by ethanol, which has a longer acyl chain. Combining our results from variations in lipid concentration with those from mixed alcohol solutions, we were able to optimize the solution conditions for large quantity processing. Using a solution of 64:16:20 methanol/ethanol/water and a lipid concentration of 5 mg/mL, we were able to produce high aspect ratio metallized tubules that were not thixotropic.

Our results on variations in the lipid chain length are not as directly applicable to industrial processing, but they give important information on the connection between intramolecular order in the lipids and intermolecular chiral order in the tubules. To see this connection, consider again the molecular structure of $DC_{8,9}PC$. The most striking feature of this structure is that the diacetylene group introduces a kink into the acyl chain of the molecule, which is evident in the space-filling model shown in Figure 1a. This kink has two effects. First, it orders the acyl chains by reducing the number of gauche conformers. Both FTIR[24] and Raman[25] studies indicate that $DC_{8,9}PC$ is in a highly ordered, all-trans configuration when tubules form. Second, the kink imposes a steric hindrance to the molecules packing parallel to each other. Hence, we argue the optimum packing of two neighboring molecules can have either of the two arrangements shown in Figure 1b. If the molecule had no chiral center, those two arrangements would be degenerate. Because the molecule has a chiral center, the degeneracy between the two arrangements is lifted, and one of them is favored. That favored packing of the molecules causes the favored twist of the lipid bilayer, which leads to tubule formation, as predicted theoretically. This argument implies that a kink in the molecular structure is very important for tubule formation because it determines the possible arrangements of neighboring molecules, and the molecular chirality only selects between those possible arrangements.

The effects of moving the position of the diacetylene in the acyl chain on tubule morphology are two-fold. First, as noted earlier, FTIR studies show that the CH_2 wagging peaks become sharper as the diacetylene is moved away from the headgroup, toward the terminal methyl group. This sharpening shows an increase in the intramolecular order of the acyl chain (a reduction in the number of gauche conformers) and of the headgroup. Our results show that the CD peak at 205 nm *also* increases as the diacetylene is moved down the chain. Interestingly, this is only true when the number of methylenes between the ester and diacetylene group is even. We previously argued that this CD peak is associated with chiral interactions between headgroups in adjacent bilayers.[17] Thus, we see that there is a correlation between increasing order within lipid molecules and increasing order in the molecular packing. Second, as the number of carbons between the headgroup and the diacetylene alternates between even and odd, the orientation of the kink should alternate back and forth. By the argument in the preceding paragraph, the favored packing between neighboring molecules should also alternate between two arrangements—not the two arrangements related by a mirror symmetry in Figure 1b, but two quite different arrangements. Thus, the alternation between even and odd segment lengths can have a substantial effect on the molecular packing that leads to tubule formation. This effect is seen in the CD spectra of Figure 8.

Finally, our experiment on making tubules of mixed L- and D-enantiomers was an attempt to change the tubule diameter. Theories of tubule formation predict that mixing opposite enantiomers, or mixing chiral lipids with similar but achiral analogues, should dilute the chiral interaction between lipid molecules.[9] For that reason, the diameter of the tubules should increase as the enantiomeric excess decreases. This predicted dependence on enantiomeric excess should be especially pronounced near the racemic point, where the tubule diameter should diverge. However, we did not observe any dependence of tubule diameter on enantiomeric excess. Instead, we found that the mixture of L- and D-enantiomers forms tubules with approximately the same diameter, length, and density as pure chiral tubules and that the net CD signal of the racemic mixture approaches zero.

There are two ways to reconcile the observation that a racemic mixture forms tubules with our earlier observation that the molecular packing of lipid molecules in tubules is chiral. One possible interpretation of this experiment is that opposite enantiomers phase separate almost completely, so that each tubule is nearly pure L- or D-lipid. An alternative explanation is that the lipid undergoes a spontaneous symmetry, breaking between the two possible molecular arrangements shown in Figure 1b, which may be accompanied by a partial phase separation of opposite enantiomers.[28] In either case, the mixed lipid would give tubules with approximately the same diameter as a pure enantiomer. The main distinction between these two

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scenarios is the prediction for how the magnitude of the CD peak should depend on enantiomeric excess. If the lipid phase-separates nearly completely, then the difference in the fraction of right- and left-handed tubules should be proportional to the enantiomeric excess, and hence the CD peak should be proportional to the enantiomeric excess. By contrast, if the lipid undergoes a spontaneous symmetry breaking, then even a slight enantiomeric excess should bias the system in favor of right- or left-handed tubules, and hence the CD peak should increase sharply as one moves away from the racemic point. The inset to Figure 10 shows that the CD peak scales linearly with enantiomeric excess over the full range that was studied. Thus, the most likely explanation of our results is a nearly complete phase separation of the L- or D-lipid.

From the perspective of applications, the main result of this experiment is that mixing opposite enantiomers is not a useful tool to change tubule morphology. It is still possible, however, that mixing chiral lipids with achiral analogues could increase the tubule diameter, if one could find achiral molecules that mix well with the chiral lipids. This possibility remains a subject for further research.

In conclusion, we have used circular dichroism and electron microscopy to study the morphology of diacetylenic lipid tubules formed in alcohol/water solutions. We are able to control the number of bilayers in the tubule wall, but not the diameter, through changes in the lipid concentration, alcohol/water ratio, and position of the diacetylene group in the acyl chain. We are working to obtain greater control over the length of the tubules. These studies allow for production of tubules for technological applications with improved efficiency.

Acknowledgment. We acknowledge valuable discussions with K. Easwaran, B. Ratna, A. Rudolph, and R. Shashidhar. This research was supported by the Office of Naval Research. J.M.R. was supported by the NRL/NSF Summer Research Internship Program.

LA980086Y

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