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Chiral Self-Assembly of Nanotubules and Ribbons from Phospholipid Mixtures

Mark S. Spector,*,† Alok Singh,† Phillip B. Messersmith,‡ and Joel M. Schnur†

Center for Bio/Molecular Science and Engineering, Code 6900, Naval Research Laboratory, Washington, D.C. 20375-5348, and Biomedical Engineering Department, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

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ABSTRACT

Nanoscale structures have been found to self-assemble in a binary mixture of a long-chain, diacetylenic phospholipid and a short chain, saturated lipid. We observe unusual chiral-optical signatures of nanotubule, twisted ribbon, and microtubule morphologies that can be used to monitor their temporal and thermal stability. Circular dichroism results suggest that chiral packing of the lipids drives formation of these aggregates. The ease of formation, stability, and robust behavior of these lipid cylinders suggest that they have potential use in applications requiring high aspect ratio nanomaterials.

The self-assembly of biologically based amphiphiles into potentially useful nanostructures has been the subject of intense study in recent years. Many types of amphiphilic molecules self-assemble to form cylindrical tubules and helical ribbons.[1−4] In most cases, these systems consist of a single chiral amphiphile in an aqueous environment. One system, diacetylenic phospholipids, self-assemble into hollow, cylindrical aggregates with diameters of 0.5 μm and lengths of 50−200 μm.[5,6] These microtubules have attracted interest due to potential applications in controlled release, electroactive composites and multifunctional materials.[1] Theoretical work suggesting that the formation of helical morphologies is driven by twisting of the amphiphile bilayer due to symmetry breaking in the packing of chiral molecules[7,8] has been supported by circular dichroism (CD) studies.[9,10] When molecules form chiral aggregates, nonchiral molecular absorptions can become chirally active, with differential absorption of left- and right-handed circularly polarized light. CD peaks in such chiral structures can be quite large and provide useful structural information about their molecular architecture.[11] Lipid tubules are found to have intense peaks in their CD spectra, indicating large chiral correlations in molecular packing.[10] This chiral order can no longer be maintained when the chains become disordered and the tubules melt, leading to a decrease in CD peak intensity by 4 orders of magnitude.

Addition of short chain lipid spacers to diacetylenic lipid tubules has been found to significantly enhance polymerization efficiency when the length of the acyl chains in the spacer lipid is matched to the number of methylenes in the upper segment (closer to the headgroup) of the diacetylenic acyl chains.[12] This is probably due to geometric considerations in the mixed lipid bilayer, namely an increase in the average area-to-volume ratio of the lipids. Surprisingly, a gel-like network of twisted fibers was found to form in an equimolar mixture of 1,2-bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) and 1,2-bis(dinonanoyl)-*sn*-glycero-3-phosphocholine (DNPC).[13] Further studies found that a new, nanotubular morphology, consisting of cylinders with diameters around 50 nm, preceded the formation of twisted ribbons.[14] While these nanotubules were transformed into the ribbon-gel after a few hours at ambient temperature, they appeared to be stable at 4 °C. Such structures have prospective use as substrates for fabrication of electroactive composites based upon the incorporation of nanofibers.[15] There also appear to be potential applications of colloidal suspensions of nanotubules in biochemical sensing, energy transduction, and catalysis applications. However, it will be important to first learn how to prepare large-scale colloidal suspensions purely of nanotubules. In addition, surface modifications are often required in order to metallize nanotubules or to perform further functionalization for use in the above-mentioned applications.[16] In this paper, we report on the thermal behavior and stability of nanotubules as characterized by circular dichroism spectroscopy, electron microscopy, and NMR.

* Corresponding author: Phone (202) 404-6051, Fax (202) 767-9594, E-mail: spector@cbmse.nrl.navy.mil.
† Naval Research Laboratory.
‡ Northwestern University.

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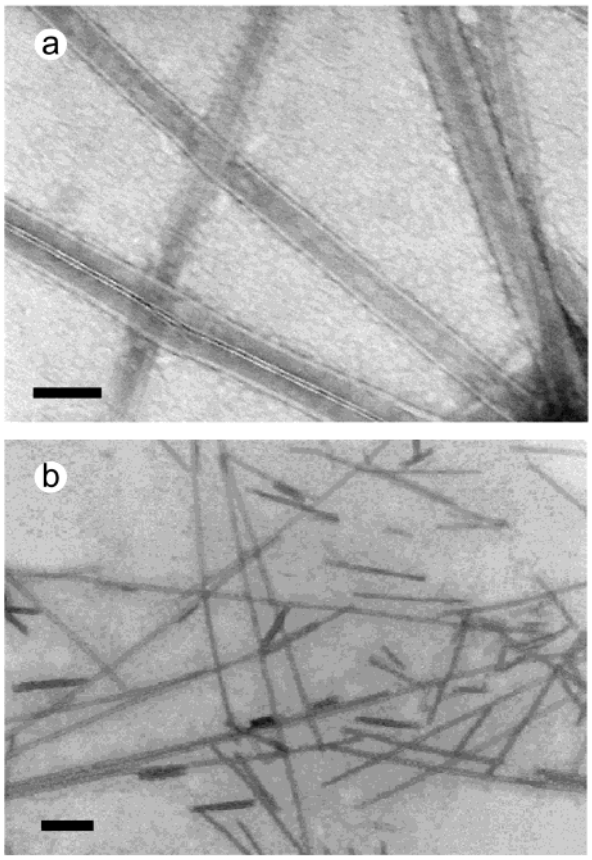

Figure 1. Negative stained transmission electron micrograph of nanotubules formed from an equimolar mixture of $DC_{8,9}PC$ and DNPC (2 mM total lipid concentration) (a) stored at 4 °C for 10 h and (b) heated to 20 °C for 2 h. Bar = (a) 100 nm, (b) 500 nm.

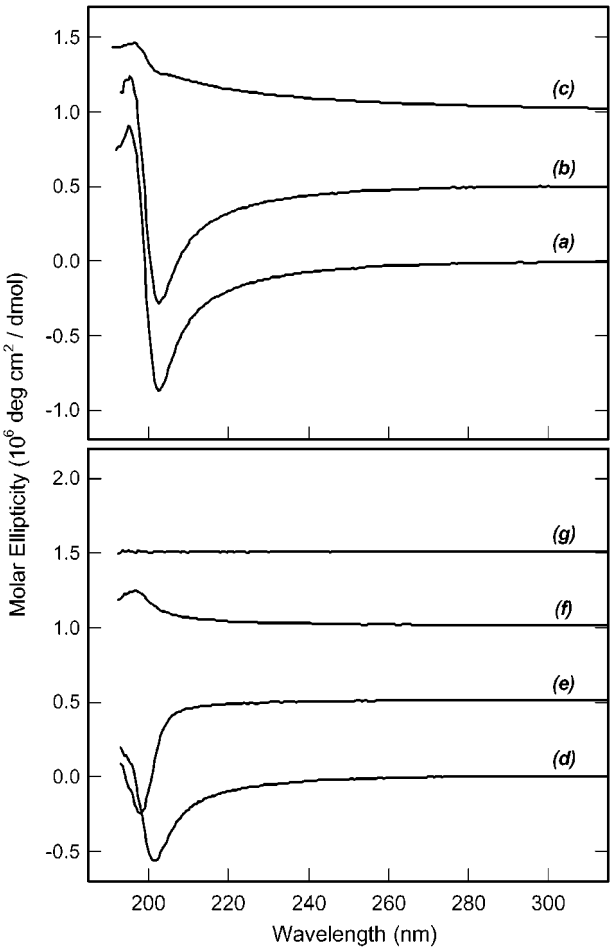

Figure 2. CD spectra of an equimolar mixture of $DC_{8,9}PC$ and DNPC (2 mM total lipid concentration) in the nanotubule phase. Top panel: In the nanotubule phase at (a) 4 °C and (b) 20 °C. (c) Also shown is the spectra from pure $DC_{8,9}PC$ microtubules in water at 20 °C. Bottom panel: (d) in the nanotubule phase at 25 °C, (e) in the twisted ribbon phase at 26 °C, (f) in the microtubule phase at 38 °C, and (g) completely melted at 40 °C. Sample absorption prevents data acquisition below 192 nm. The curves have been offset vertically for display.

An equimolar dispersion of $DC_{8,9}PC$ and DNPC (Avanti Polar Lipids) was prepared by forming a dried lipid film from stock chloroform solutions of each lipid, followed by hydration at 60 °C with ultrapure water. Lipids were dispersed by intermittent vortex mixing while maintaining the temperature at 60 °C for 3 h until a homogeneous dispersion was formed. The samples were slowly cooled to 4 °C over a period of approximately 4 h and stored at least 10 h at 4 °C before characterization. Quickly transferring a small amount of sample from a refrigerator to a carbon-coated electron microscopy grid confirmed that this procedure results in an abundance of nanotubules as seen in Figure 1.[17] Only cylindrical aggregates with diameters of approximately 50 nm are observed in these micrographs. While all diameters were observed to fall in a narrow range (45−55 nm), the lengths of the tubules were sensitive to sample preparation conditions and were observed up to tens of microns. No other aggregate morphologies were observed in the suspension at 4 °C. Helical markings, which are often observed in pure $DC_{8,9}PC$ microtubules,[6] are absent in the nanotubules shown in Figure 1. Such markings in microtubules suggest that chirally induced twisting of the lipid bilayer is driving their formation and is consistent with the CD spectroscopy results discussed above.

To ascertain the chiral order in mixed lipid nanotubules, we performed CD measurements. A large positive peak at 195 nm and a negative peak at 202 nm, as shown in the top panel of Figure 2, characterize the CD spectra of $DC_{8,9}PC$/DNPC nanotubules. This spectrum is indicative of a splitting in the $\pi-\pi^*$ excitation of the diacetylene moiety which usually shows a UV absorption at 198 nm.[10] The bisignate nature of this peak reveals strong coupling of the electric transition moments of neighboring diacetylenic groups.[18] Such exciton coupling is not observed in $DC_{8,9}PC$ microtubules, as shown in curve *c* of Figure 2, which suggests tighter packing in the mixed lipid nanotubules. The composition of the nanotubule phase was estimated by NMR spectrometry after separation from the supernatant by centrifugation followed by freeze-drying. The measured ratio of 3:4 between methyl and methylene protons indicates the presence of an equimolar ratio of the two lipids in nanotubules. The observation of large CD peaks in the nanotubular phase suggests that chiral packing is an important factor in their formation.[8]

The thermal behavior of the mixed lipid aggregates was studied by monitoring the temperature dependence of the

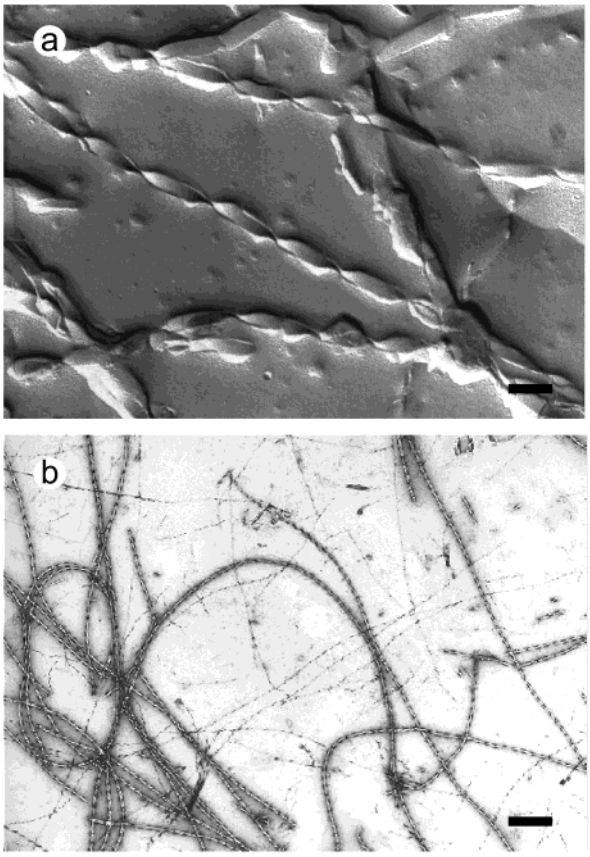

Figure 3. Electron micrographs of twisted ribbons formed from a mixture of $DC_{8,9}PC$ and DNPC at 27 °C prepared using (a) freeze-fracture and (b) negative staining techniques. Bar = (a) 100 nm, (b) 250 nm.

CD spectra. The bottom panel of Figure 2 shows representative spectra at higher temperatures. As the nanotubules are heated, the peak positions remain constant up to 25.5 °C, where a discontinuous change in the spectrum is observed. Above this transition temperature, one negative peak is observed at 198 nm (curve *e*). The loss of exciton coupling at the transition temperature indicates a substantial change in the molecular packing of the twisted ribbon phase. This is consistent with earlier calorimetric studies where a change in morphology from nanotubules to twisted ribbons was identified at 25.4 °C.[14] Figure 3 shows electron micrographs of the sample above the transition temperature. Only right-handed ribbons with 25 nm diameters and pitches about 120 nm are observed. The freeze-fracture micrograph (Figure 3a) shows that these ribbons consist of long, twisted strips of membrane with uniform diameters. The length of membrane edge exposed to water indicates this morphology differs significantly from the closed structure of tubules where the edge is only in contact with water at the ends of the cylinder.

It is interesting to compare these results to the work by Oda and co-workers on charged gemini surfactants with chiral counterions.[19] They found that a helical ribbon morphology is favored for long-chain surfactants, while twisted ribbons are favored for shorter-chain surfactants. The helical aggregates are topologically equivalent to a tubule, where the membrane has mean or cylindrical curvature. On the other hand, a twisted ribbon has Gaussian curvature, but no mean curvature. Oda et al. developed a theoretical model which predicts that a twisted ribbon always has a lower free energy than a helical ribbon for a membrane in a fluid phase, while helical ribbons or tubules require crystalline membranes.[19] Based upon these predictions, we hypothesize that the transition from nanotubules to twisted ribbons is driven by a change in the molecular packing of the two lipids, rather than by a change in the relative amounts of the two lipids in the membrane. Indeed, the NMR results described above indicate an equimolar ratio of $DC_{8,9}PC$ and DNPC in the ribbons, as well as nanotubules. We speculate that in the twisted ribbon phase, the DNPC chains are disordered and act to fluidize the entire membrane.

Upon further heating, the magnitude of the 198 nm CD peak decreases continuously to zero at 37 °C. The peak position remains constant up to 33 °C, above which it shifts gradually to higher wavelengths. Above 37 °C, the CD spectra (Figure 2, curve *f*) are consistent with a multilamellar microtubule phase characterized by positive peaks at 196 and 205 nm.[10] A mixture of twisted ribbons and microtubules is observed in electron micrographs taken from a 35 °C sample. We believe that as the temperature is increased from 25 °C, the DNPC is solubilized as micelles. At the same time, micron-sized tubules predominantly containing $DC_{8,9}PC$ are formed. Above 37 °C, only the microtubules contribute to the CD signal. At this point, the DNPC has completely transformed into micelles that do not have an easily measurable CD signal. The microtubular phase completely melts at 39.5 °C, and no CD signal is observed at higher temperatures (Figure 2, curve *g*).

The spectroscopy results described above clearly show that CD is a powerful tool for monitoring morphological changes in chiral, self-assembled systems. While it is difficult to obtain quantitative information from CD spectroscopy, the following observations can made about our system. The bisignate CD signal observed in the nanotubular phase has a negative sign- that is, the higher energy (lower wavelength) state has a positive peak and the lower energy state has a negative peak.[18] This is consistent with the negative Cotton effect observed in the twisted ribbon phase where the chromophores are not coupled. The micron-sized tubule phase previously studied has a positive Cotton effect.[9,10] Interestingly, electron microscopy shows that both the microtubules and the twisted ribbons are right-handed, while the handedness of the nanotubules could not be determined. The changes in molecular morphology that cause the inversion of the CD signal in the mixed lipid aggregates are yet to be explained.

To be of practical use, the mixed lipid nanotubules must not only be stable for extended periods at refrigerator temperatures, but must also be stable long enough to allow for further processing at ambient temperatures. We have found that samples left at 4 °C still show a predominance of nanotubules after 2 months. Bringing the sample above 25 °C results in a slow conversion of nanotubules to twisted ribbons. Svenson and Messersmith performed a detailed study on the concentration and temperature dependence of

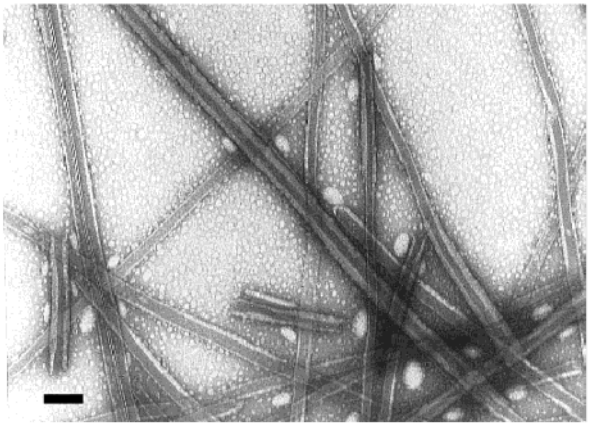

Figure 4. Electron micrograph from a mixture of $DC_{8,9}PC$ and DNPC incubated at 34 °C for 2 h showing a coexistence of nanotubules and small vesicles. Bar = 100 nm.

this transformation and found it could be quite slow at low concentrations (below 3 mM).[14] We probed the stability of nanotubules by transferring them from the refrigerator to a water bath set at various temperatures. In our 2 mM solutions, we also find that conversion from nanotubules to higher temperature morphologies occurs very slowly, even at bath temperatures of 34 °C. Figure 4 shows an electron micrograph of a sample incubated at 34 °C for 2 h, which shows coexistence of nanotubules and small vesicles. This experiment was repeated many times, and no helical ribbons were observed at the 2 h point. After approximately 24 h, a mixed system of twisted ribbons and microtubules is observed. The apparent contradiction between this result and the CD scans, where a very sharp thermal transition from nanotubules to ribbons was observed, may be due to the CD scans being performed in thin (0.5 mm) cells that equilibrate quicker.

These studies have provided new structural information about the molecular architecture of mixed lipid nanotubules. The results give experimental evidence suggesting that chiral packing of the lipids drives both nanotubule and twisted ribbon formation. NMR results show that the molecular composition remains unchanged during the transition between these morphologies, while CD spectroscopy suggests a significant change in molecular packing. It will be interesting to vary the acyl chain length in both the diacetylenic and spacer lipids to see if this affects molecular packing and leads to changes in nanotubule morphology. Our results indicate that bulk solutions of lipid nanotubules are sufficiently robust to survive the required handling for functionalization, purification, and scale-up procedures necessary for practical applications.

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(17) Stained samples for transmission electron microscopy were prepared by drying the suspension on a carbon-coated Formvar grid and then negatively staining with 1% uranyl acetate. Freeze-fracture samples were rapidly frozen by slamming them against a copper plate at −150 °C (Gentleman Jim). Fracture and deep etching were performed on a Cressington CFE 40. Electron microscopy was performed on a Zeiss EM-10C or a JEOL 120 CX. Circular dichroism studies were carried out on a Jasco J-720 spectropolarimeter. Samples were removed from the refrigerator and immediately placed in a water-jacketed quartz cell (0.5 mm path length) that had been precooled to 4 °C by a water circulator (Neslab). Spectra were recorded every 0.5 °C while heating at a rate of 4 °C/hour. A 400 MHz, Bruker DRX-400 spectrometer was used to record the NMR spectra. The ratio of lipids was determined by comparing the number of terminal methyl protons (δ0.88 ppm, triplet) from both lipids to the total number of methylene protons in the α-position to the diacetylenic group (δ2.27 ppm, multiplet). In an equimolar mixture, this ratio should be 12:16.
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