Anionic Glucophospholipids—A New Family of Tubule-Forming Amphiphiles

Françoise Giulieri, Frédéric Guillod, Jacques Greiner, Marie-Pierre Krafft,* and Jean G. Riess*

Abstract: A new family of amphiphiles that form tubules (i.e., hollow cylindrical bilayer-based microstructures) by self-aggregation has been identified, namely, anionic glucophospholipids of type **1**, in which a double-tailed hydrophobe is grafted through a phosphate linkage to the O-6 position of a polar glucose head group. Compounds **1a–c** self-assemble into stable, hollow tubular microstructures when dispersed in water and cooled below the temperature at which the transition between crystal and liquid-crystal phases takes place (estimated from change in turbidity). The diameter of the microstructures appears to depend on the nature of the hydrophobic tail, significantly smaller diameters being obtained for fluorinated tails. No tubules were obtained when galactose (**2a–c**, pH < 11) or mannose (**3a–c**) derivatives were used instead of glucose derivatives, or when glucose was derivatized at the O-3 (**4b**) rather than O-6 position; in these cases only vesicles were formed. Tubules made of **1** converted rapidly into giant vesicles when heated; they spontaneously formed again upon cooling. The presence of a fluorinated chain, as in **1b** and **1c**, increased the temperature at which the tubule–vesicle interconversion occurred to above room temperature. Because the amphiphiles are negatively charged, the formation of tubules is pH-dependent and is favored at higher pH. These findings support the view that hydration of and hydrogen bonds between polar heads play a major role in tubule formation. Hydration of the sugar-derived head groups decreases as the number of intermolecular hydrogen bonds increases; this favors membrane crystallization and tubule formation.

Keywords
amphiphiles · glucophospholipids · self-assembly · tubules · vesicles

Introduction

Glycolipids are important naturally occurring compounds.[1] Almost all living cells have, for example, a surface glycocalyx consisting of glycoproteins and glycolipids, often terminated by sialic acid residues. The carbohydrates present on the cell surface are involved in a variety of receptor and signaling phenomena. Phosphorylated lipopolysaccharides are major components of gram-negative bacteria such as *E. Coli* and *Salmonella typhimurium*.[2] They constitute the prime targets of the antibodies produced by the immune system in response to bacterial infection. In drug delivery and targeting, oligosaccharides constitute potential recognition sites for carbohydrate-mediated interactions between cells and drug carriers (such as liposomes) which bear such site-directing molecules.[3] Sugar derivatives can be present as co-amphiphiles in vesicles made of phospholipids (monosialoganglioside GM 1, for example),[4, 5] or can be covalently linked onto vesicles.[6] The formation of vesicles (liposomes) from various glycolipids,[7] including fluorinated glycolipids[8] and phosphoglycolipids,[9] has been reported.

Microtubules fulfill essential functions in eukaryotic cells.[10] Tubules, together with microfilaments and intermediate filaments, are the major constituents of the cytoskeleton. They are the main components of stable assemblies such as the axoneme, centrosome, and other complex structures found in protozoa. They participate actively in various processes at the cellular level, notably in cell division, cell motility and intracellular transport. They also interact with a large number of proteins, including kinesin and dynein, the motor proteins, and cross-linking proteins such as nexin.[11–13] Other naturally occurring lipid tubules are present on the surface of pine needle stomata responsible for CO_2/O_2 exchange.[14] These tubules are mainly composed of 10-nonacosanol[15] and their purpose is to filter off dust particles, which would otherwise clog the transpiration pores on the leaf's surface. It has also been shown that wax filaments consisting of tubular crystals are secreted by insects.[16]

Synthetic nanotubes have been obtained from novel forms of carbon, discovered since the mid-1980's. They include tubular fullerenes,[17, 18] and graphitic and single-layer carbon nanotubes.[19, 20] Other tubules result from the self-assembly of amphiphiles. With very few exceptions, the latter are chiral and/or can bind by means of hydrogen bonds between polar heads. These tubules usually consist of coiled single or multiple bilayer sheets; the volume of their aqueous core ranges from more to less significant. Tubules are generally considered to be quasi-crystalline assemblies, more ordered than vesicles.[21] The number of tubule-forming molecules identified so far is still relatively small and principally includes certain alkylaldonamides,[22] aminoacids,[23, 24] glutamates,[25] glycolipids,[8] phospholipid–nucleoside conjugates,[26, 27] diacetylenic phospholipids,[28] lipid–biotin conjugates,[29] and porphyrin derivatives.[30]

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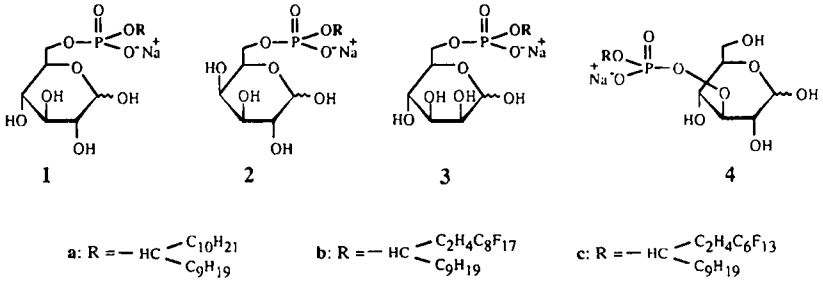
Some of these structures may provide models for enzyme clefts[22] or have potential as microcontainers for controlled release (antifouling, drug delivery).[28, 31] It has been suggested that they may constitute organic reaction media dispersible in an aqueous medium, with unique substrate adsorption/desorption characteristics.[22] Tubules have also been utilized as templates for metallization, leading to applications in material sciences.[28] Examples of the latter applications include the fabrication of cathodes for vacuum field emission and metallic microvials for controlled release.

A range of new amphiphiles, many of which were fitted with highly hydrophobic fluorinated tails,[32-34] have recently been synthesized in our laboratory. These amphiphiles are intended to serve as components of supramolecular systems destined for drug targeting and delivery, and other medical and nonmedical uses.[35-37] Tubules were observed to form from various fluorinated double-tailed glycolipids with polar heads derived from galactose.[38] It was also shown that fluorinated *single*-chain dimorpholinophosphates form multibilayer sheetlike membranes which roll-up into tubular structures.[39, 40] The latter amphiphiles, which possess no chiral center, no rigid rod, and no possibility of establishing hydrogen bonds between polar heads, illustrate the propensity of fluorinated chains to promote the self-assembly of amphiphiles into supramolecular systems more organized than vesicles.

We report here the formation of tubular microstructures from anionic glucophospholipids **1a–c** (Scheme 1) dispersed in water. These amphiphiles have either an entirely hydrogenated double tail (**1a**) or a mixed hydrophobic double chain consisting of one fluorinated chain and one hydrogenated chain (**1b,c**),



Scheme 1. Molecular structure of hydrogenated and mixed hydrogenated/fluorinated glycophospholipids.

grafted to D-glucose in the O-6 position. The influence of the nature of the sugar head will be established by comparison with glycophospholipids derived from D-galactose (**2**) and D-mannose (**3**). The impact of the fluorinated part of the tail will be discussed by comparing **1b** and **1c** with **1a**. Compound **4b**, in which the double tail is grafted in the O-3 position of glucose was also examined. The reversible temperature-dependent transformation that occurs between tubules and vesicles was investigated, as well as the pH-dependence of the formation of the tubules.

Results

Tubules versus vesicles—temperature of conversion: When hydrated in pure water at a 50 mM concentration at high temperature (>60 °C), the glycophospholipids **1–4** all formed a L_α lamellar phase, as indicated by polarization optical microscopy (presence of "malteze crosses"). No phase transition could, however, be detected by differential scanning calorimetry (DSC) measurements between −30 and 90 °C.[9] The phase-transition temperatures were therefore evaluated by monitoring the change in turbidity of the samples as a function of temperature.[9] A microcrystalline dispersion of a surfactant displays a higher turbidity than a liquid-crystalline dispersion.[41] Assuming that the temperature at which the change in turbidity occurs can be attributed to a gel-to-fluid transition, then the latter occurs in the 10–20 °C range for **1a**, 40–55 °C for **1b**, 35–40 °C for **1c**, and below 10 °C for **4b**.[9]

When aqueous dispersions (pH 7) of glycophospholipids **1–4** were sonicated above their assumed T_c, small unilamellar vesicles (SUVs) were obtained.[9] While the SUVs made of the galactose (**2a–c**) and mannose (**3a–c**) derivatives were stable at room temperature, or tended to evolve to form large multilamellar fluid vesicles (malteze crosses visualized by polarization microscopy) at room temperature, those made of the glucose derivatives (**1a–c**) were not. Within hours or days, depending on the amphiphile and experimental conditions, elongated microstructures were observed to develop progressively. At the same time, the viscosity of the samples increased and the dispersions turned into a gel. The structure of these fibrous assemblies was investigated and a rationale for the observed differences in aggregation behavior was sought.

When vesicles made of the hydrogenated glucophospholipid **1a** were allowed to stand at 4 °C, that is, below the amphiphile's T_c (assumed to be between 10 and 20 °C), well-developed fibers were obtained. They were observed at 4–6 °C by phase-contrast and dark-field optical microscopy (Fig. 1a,b). Their typical dimensions were about 1 μm in diameter and 100 μm in length.

Freeze-fracture electron micrographs of these self-assembled structures (Fig. 2) showed that they are hollow tubules formed by multiple bilayers wrapped around an aqueous internal core. We have no explanation yet for the unique polyhedral shape of the two tubules of **1a** shown in Figure 2; it is unlikely to be an artefact that occured during freezing. One can, however, imagine that the bilayers may have reached a degree of crystallinity that does not allow them to be bent into a circular section.

These tubules transformed into large multilamellar vesicles when the temperature was increased. The temperature of conversion from tubules to vesicles (T_{TV}) was found to depend on the temperature at which the tubules had been stored and on storage time. For example, when the tubules were maintained at 4 °C for only one day, T_{TV} was reproducibly measured to be around 15 °C. After one week at the same tem-

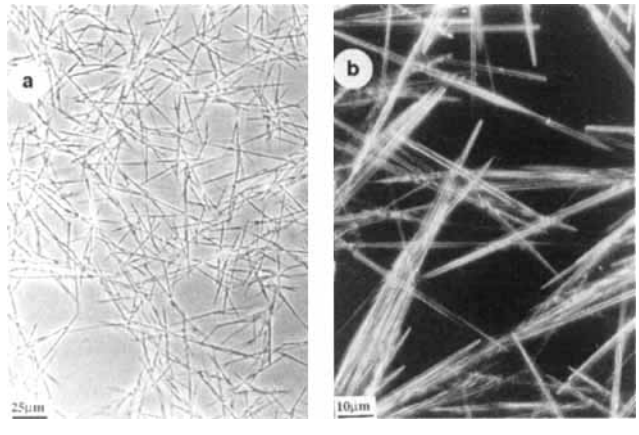


Fig. 1. Tubules formed by the hydrogenated glucophospholipid **1a**, observed by optical microscopy using phase contrast (a) and dark-field illumination (b).

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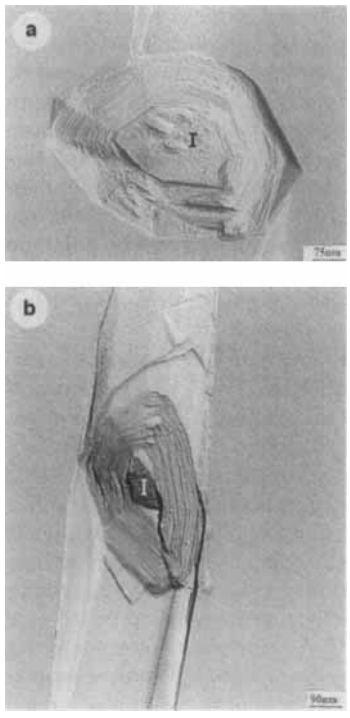

Fig. 2. Freeze-fracture electron micrographs of a tubule made from the hydrogenated glucophospholipid **1a**: a) frontal cross-section showing the internal aqueous core (I); b) oblique section showing the multiple bilayers and the elongated shape of the aggregate.

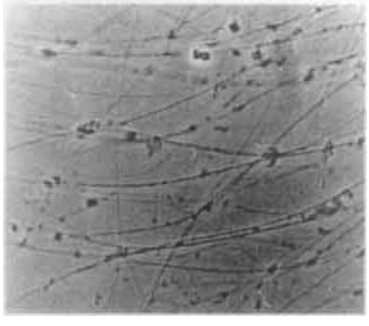

Fig. 3. Phase-contrast optical micrograph, taken at 11–13 °C, of very long tubules formed from the hydrogenated phosphoglucolipid **1a** (1 cm = 50 μm).

perature, T_{TV} was 22 °C; the tubules were then stable at 20 °C for several months, probably as a result of a reduction in the number of defects or because of a crystalline reorganization. The tubule-to-vesicle conversion, which occurred almost instantaneously, was found to be reversible. However, the reverse transformation, back to tubules upon cooling, was slow and could take several hours.

Interestingly, the storage temperature was also found to have a strong impact on the length of the tubules. For example, if freshly produced vesicles of **1a** were stored around 11–13 °C, that is, at a temperature closer to T_c, rather than at 4 °C, very long tubules, of up to a few millimeters in length, were observed by optical microscopy (Fig. 3). Their average diameter remained, however, comparable to that of the tubules formed at 4 °C. Some vesicles were present together with the long tubules. A very slight increase in temperature was sufficient to convert the latter into vesicles (T_{TV} = 15 °C). The length of the tubules was found to be controlled by the rate at which the sample was cooled: slower cooling gave longer tubules, as has also been reported for other tubules.[28]

Tubules were also observed to form from the fluorinated glucophospholipids **1b** and **1c**, but the conditions of formation differed significantly. The introduction of a fluorinated tail allowed the tubules to form and to be stable above room temperature. Vesicles formed from **1b** and **1c** were observed to convert slowly into tubules when stored at room temperature, that is, below the amphiphiles' assumed T_c. The fluorinated tubules were characterized by negative staining and freeze–fracture electron microscopy (Fig. 4a,b). Under the same experimental conditions, the length of these tubules was comparable to that of the hydrogenated ones (ca. 100 μm). Their average diameter was, however, significantly smaller (0.1–0.2 μm). Because of their small diameter, their observation by optical microscopy was difficult; optical micrographs could, however, be obtained by using dark-field microscopy. Fluorinated tubules made of **1b** and **1c** also converted reversibly and rapidly into vesicles at temperatures above 60 °C for **1b** and 45 °C for **1c**.

Very similar tubular self-aggregates could also be obtained directly (i.e., without first preparing well-defined SUVs) from both hydrogenated and fluorinated glucophospholipids, by dispersing them in water with gentle hand-shaking above their assumed T_c, then allowing this crude dispersion to stand at 4 °C (**1a**) or at room temperature (**1b,c**). The morphology of these tubules was very similar to that of the tubules formed from SUVs, as assessed by freeze–fracture electron microscopy. The diameter of the fluorinated tubules was, again, much smaller than that of the hydrogenated ones (ca. 0.1–0.2 vs. 1 μm).

Contrary to the vesicles made from the glucophospholipids **1**, those from the fluorinated galactophospholipids **2b,c** and mannophospholipids **3b,c** were all very stable. They could even be heat-sterilized and stored for several months at room temperature without significant change in their average particle size. The vesicles made from hydrogenated galacto- and mannophospholipids **2a** and **3a** were less stable and tended to evolve into large lamellae and multiwalled vesicles.[9] No tubules, however, were observed to form even when dispersions of **2** or **3** were maintained at 4 °C for one month. Stable vesicles (for at least 20 days) were also obtained from the fluorinated glucose derivative **4b**, the isomer of **1** in which the hydrophobic tail is grafted in the O-3 position.

When freshly prepared dispersions containing tubules of **1a** and **1b,c** were observed by cross-polarization microscopy at 6 and 20 °C, respectively, no malteze crosses were seen; this indicates that the $L\alpha$ phase disappears as the tubules form. On the other hand, malteze crosses, characteristic of the fluid lamellar phase, were consistently seen at 6 and 20 °C for **2** and **3**.

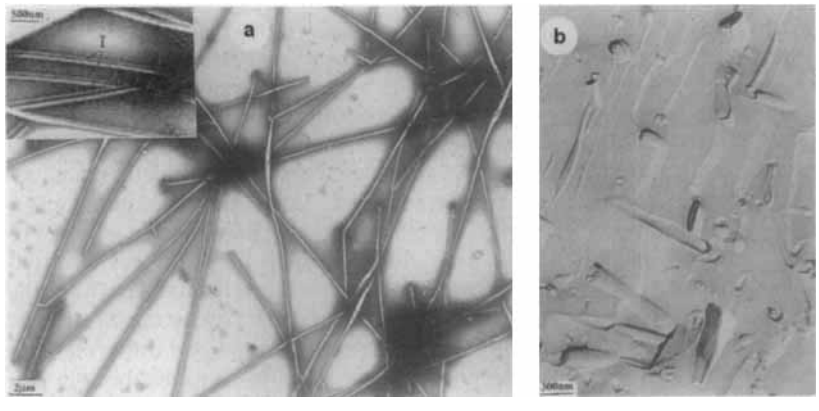

Fig. 4. Electron micrographs of tubules formed from the mixed fluorinated/hydrogenated glucophospholipid **1b**: a) general view and detail showing the internal aqueous core (I) (negative staining); b) wrapped bilayers and internal core as seen after freeze fracture.

Influence of pH: In order to assess the influence of pH, dispersions of the fluorinated glycophospholipids **1b**, **2b**, and **3b** were prepared above their T_c and their pH was adjusted to pH 2, 4 (HCl), and 11 (NaOH). At pH 2 and 4, vesicles were obtained from all three compounds. All three samples remained fluid for at least two months at 4 and 20 °C, and were shown to contain only vesicles. No tubules were seen to form.

The pH-dependence of tubule formation was established in the case of **1b** by the following experiment. The pH of a tubule-containing dispersion of **1b**, initially adjusted to 7, was decreased to 2 at room temperature. Tubules were

then found to transform into vesicles within approximately one hour. When the pH of this vesicle-containing dispersion was increased to 11, tubules formed again within around three hours. When vesicles were produced from **1b** at pH 11 and were stored at room temperature, the first tubules began to appear one hour later, significantly faster than at pH 7 (12 h).

At pH 11, some tubules were also observed to form from fluorinated mannophospholipid **3b**, but only after two weeks and at 4 °C; none were obtained at room temperature. When heated at room temperature, they immediately converted into vesicles. Their temperature of conversion (T_{TV}) did not appear to depend on storage time.

Discussion

In bilayers an equilibrium sets in between attractive hydrophobic forces and repulsive hydrophilic forces. The difference in phase-transition temperatures and phase behavior observed here between the various hexose derivatives bearing the same hydrophobic moiety implies that there are differences in their hydrophilic interactions. The sugar was seen to exert a decisive influence. The glucose derivatives having their hydrophobic tails grafted in the O-6 position all readily gave tubules below the assumed T_c, while none were obtained when the head group was derived from galactose, for which the membranes remained fluid throughout the range of temperature investigated (>6 °C). For the mannose derivative **3a**, tubules were only seen when the pH was high and the temperature low.

The formation of tubules, that is, aggregates that are better organized than vesicles, suggests that bilayers made of glucophospholipids **1** are less hydrated than those formed by the galacto- and mannophospholipids **2** and **3**, or by the glucose-derived isomer **4**. This may mean that the negative charge is less exposed in **1**. Epimeric changes in the hexose head group have a substantial effect on the orientation and dynamics of this head group in a membrane[7] and have been reported to induce significant changes in phase behavior. Thus, for example, a lamellar phase was obtained, above T_c, for a nonionic glyceroglycolipid derived from D-glucose, while an inverse hexagonal phase was found for its mannose-derived analogue.[42] The importance of the position of the OH groups in the epimer was also indicated by the formation of micellar rods from *N*-octyl-D-gluconamide, of scrolled bilayers from *N*-octyl-D-mannonamide, and of twisted ribbons from *N*-octyl-D-galactonamide.[43] These differences were assigned to changes in the extent of hydrogen bonding. When hydrogen bonding between heads prevails, it is at the expense of hydration. Heat capacity measurements further indicated that water is more extensively bound to galactose than to glucose.[7]

Moreover, it is known that interactions between hydrogen-bond acceptor and donor can reduce ionic strength and increase the phase-transition temperature.[44, 45] The extent of hydrogen bonding depends on the pK of the amphiphile, and hence on pH. For phosphatidic acid derivatives the extent of dissociation is very important, and the transition temperature reaches a maximum when half the acid is dissociated.[44] The anionic phosphate linker of glycophospholipids **1**–**4** is a proton acceptor, and the hexose a proton donor. The formation of tubules from these amphiphiles was found to be pH-dependent. Vesicles were only obtained at low pH, and the rate of tubule formation increased with pH. This implies that there is less head-to-head hydrogen bonding involving a hydroxyl group of the sugar at low pH, when the phosphate group is protonated. It appears that, in the case of **1**, the hydration of the phosphate group is reduced, because the orientation of the OH groups favors the formation of head-to-head hydrogen bonds. In the case of galactose and mannose, the separation and/or relative orientation between the hydroxyl groups and the phosphate may be unfavorable to head-to-head bonding. Hydrogen bonds with water molecules from the aqueous phase would then form more readily; the polar head would then be more hydrated, and this would explain why tubules obtained. At high pH the presence of Na^+ as a counterion probably plays a significant role in the formation of tubular aggregates from mannophospholipid derivative **3b**. Indeed, it was found that, in the case of di-*n*-alkyl phosphates, increased counterion binding leads to less hydration of the head groups.[46]

Fluorinated chains strongly increase the ability of amphiphiles to form ordered membranes.[35–37] The aggregates formed by fluorinated surfactants are found to be more organized and more stable than those formed by their direct hydrocarbon analogues.[33–37, 47] A striking illustration of this is that even the short, single-chain perfluorooctylethylphosphocholine can form stable vesicles, which can be sterilized by heat, while only micelles are obtained from decylphosphocholine, its hydrogenated analogue.[48] It has also been shown that the presence of a fluorinated tail of sufficient length can significantly increase the transition temperature.[37, 49] As a direct consequence of this increase in T_c, fluorinated glucophospholipid **1b** form tubules that are stable at room temperature, and these tubules remain stable up to 60 °C; in contrast, the tubules formed from the hydrogenated analogue **1a** are only stable up to 22 °C (after an "annealing" period at 4 °C).

A significant and consistent difference in diameter was observed between hydrogenated and fluorinated tubules of otherwise identical amphiphiles; the diameter of the latter were smaller. A survey of the literature shows that, if the length of the tubules can be modified over a wide range by changing the conditions of preparation, the diameter remains nearly constant at ca. 1 μm, which is in line with the diameter of glucophospholipid **1a**. At first glance, one can assume that the aggregate's spontaneous curvature depends primarily on the geometry of the amphiphile. Indeed, fluorinated chains have much larger sections than hydrogenated ones (ca. 30 vs. 20 Å^2). Consequently, the presence of fluorocarbon rather than hydrocarbon chains results in a significant increase in the volume (V) of the amphiphile's hydrophobic moiety (liquid state: 560 vs. 680 Å^3 for **1a** and **1b**, respectively). The fact that tubules with a higher curvature and therefore a lower packing parameter P ($= V/a_0 l_c$) are obtained with fluorinated amphiphiles may indicate that the surface area of their polar head (a_0) is larger, since the extended chain length (l_c) is not significantly modified. One should note, however, that Israelachvili's packing parameter P is only relevant to fluid aggregates[50] and should not be used to predict amphiphile aggregation in quasi-crystalline aggregates such as tubules. Other factors are known to influence the curvature of tubular membranes, including chirality and molecular tilt.[51] It has been proposed that an intrinsic bending force operates in anisotropic tilted membranes made of chiral molecules.[52] The smaller diameter of fluorinated tubules may mean that the fluorinated chains induce a larger torsional angle within the membrane. The tendency of fluorinated chains to line up adjacent to one another[49] also induces a specific arrangement of mixed fluorinated/hydrogenated lipids such as **1b** and **1c** within the membrane; this may also have an impact on the curvature. The presence of both the chiral polyhydroxylated head group and the mixed hydrophobic chain increases considerably the system's complexity. Tubule-forming amphiphiles with much simpler molecular design will be needed in order to attempt an assignement of the observed effects to specific structural features.

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Experimental Procedure

Materials and methods: All the amphiphiles investigated (**1a–c**, **2a–c**, **3a–c**, **4b**) were synthesized and purified according to previously published methods [53, 54]. The final steps of their preparation were the dissolution in a water/methanol (1:1) mixture and adjustment of the pH to 7 with 0.2 M NaOH. The compounds were then concentrated and precipitated in acetone, and the resulting solids were purified by trituration with ether and the color removed by treatment with activated charcoal. Water was deionized with an Elgastat purification system. Differential scanning calorimetry (DSC) was performed on a Setaram DSC92 apparatus, and turbidity measurements on a Philips PU 8650 spectrometer equipped with a thermoregulated cell. Sonication was achieved with a Branson B30 sonifier fitted with a 3 mm titanium probe (50% pulse time, dial 3). Samples were observed by phase-contrast, dark-field polarization and interference contrast optical microscopy. The optical microscope (Olympus BH2) was equipped with a thermoregulated stage (Physitemp Instruments TS4ER, Clipton, NJ). Samples were also observed by transmission electron microscopy after negative staining or freeze fracture (Philips CM12). In the negative staining procedure a drop of the dispersion, diluted ten times, was applied on a formvar-coated copper grid for 1 min, dried with a filter paper and stained with phosphotungstic acid (2%, pH 7) [55]. In the freeze-fracture procedure, glycerol (20%) was added to the sample just prior to freezing. Samples were frozen at −110 °C in liquid propane and fractured under vacuum in a Balzers cryofract (BAF 301, 2×10^{-6} Torr). After Pt/C and rotary carbon depositions, replicas were picked up on a 400 mesh grid, after being washed successively with 20% aqueous sodium dodecyl sulfate, ethanol/water (1:1), and distilled water.

Preparation of hydrogenated tubules: The hydrogenated glucophospholipid **1a** (50 mM) was hydrated for 30 min at 40 °C and dispersed by sonication for 2 min at room temperature. The pH of the dispersion was measured to be 7. Small unilamellar vesicles (SUV, 30–70 nm) were then obtained. The sample was refrigerated and maintained at 4 °C. The vesicles were then observed to transform slowly into tubules; conversion was complete within one week. The tubules were stable at 4 °C for at least 6 months. It was also observed that samples that had been stored at 4 °C for one week could be allowed to return to room temperature (20 °C) without losing their tubular structure; these tubules were stable at 20 °C without noticeable change in morphology for at least 1 month.

Tubules were also obtained by dispersing **1a** at room temperature by using an Ultra-Turrax mixer model T25 (Ika-Labotechnik) for 1 min; SUVs did not form under these conditions. The resulting coarse dispersion was stored at 4 °C. Tubules were then formed within 1 day, i.e., more rapidly than with the first procedure, in which well-formed SUVs were prepared first. These tubules were stable at 4 °C for at least 1 month. Again, if they were annealed at 4 °C for one week, they then became stable at room temperature for at least 1 month.

Preparation of fluorinated tubules: Fluorinated tubules were obtained from **1b** and **1c** (50 mM) by using either of the two procedures described above. However, it was necessary to sonicate or disperse **1b** and **1c** at 60 °C, i.e., above their phase-transition temperature. The fluorinated tubules were observed to be stable for at least 6 months at room temperature.

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