Functionalized Lipid Tubules as Tools for Helical Crystallization of Proteins

Philippe Ringler, Wolfgang Müller, Helmut Ringsdorf, and Alain Brisson*

Abstract: The development of functional supramolecular devices built by self-assembly of elementary molecules and with bioactive properties arouses considerable interest in the field of nanotechnology and new materials. We report here the formation of a new class of lipid tubules exhibiting both properties of molecular recognition and crystal formation for the protein streptavidin. These lipid tubules, made of biotin-containing dioctadecylamine molecules, are straight hollow cylinders with a constant diameter of 27 nm and variable length up to several micrometers. They are unilamellar with an inner diameter of about 16 nm, as shown by cryoelectron microscopy. Streptavidin binds to the biotinylated tubules and assembles spontaneously into ordered helical arrays at the tube surface. These crystals exhibit regular order up to about 1.5 nm resolution. In addition, the helical streptavidin arrays act as functionalized supramolecular devices that bind a wide variety of biotinylated objects, as demonstrated here with proteins and liposomes.

Keywords
helical structures · lipids · liposomes · self-assembly · tubules

Introduction

The properties of molecular recognition and self-organization possessed by biological molecules together with the wide variety of their functions explain the efforts devoted to the use of these materials for the development of supramolecular systems with bioactive properties, attempting to bridge the gap between life science and materials science.[1] The rational design of new molecular tools with selected properties depends on our understanding of the relationship between molecular structure and supramolecular assembly, which evolves from experiments conducted on model systems and theoretical considerations.[2]

The structures formed by lipids in aqueous solutions have been extensively studied to gain insight into more complex natural biological membranes. The self-organization properties of lipids have also been used to produce interfacial or Langmuir films, as well as micelles or liposomes for various applications such as the controlled delivery of therapeutic agents.[3] Although most amphiphiles form spheroidal supramolecular assemblies upon hydration, several amphiphiles have been shown to exhibit tubular morphology.[4–7] These lipid tubules have attracted special attention both from a theoretical point of view[8, 9] and for potential applications.[10] Theories of tube formation have been proposed, and it is considered that the chirality of the constitutive molecules is an essential parameter for the formation of tubes.[5, 11, 12] Although the relevance of lipid tubules in vivo remains to be established, the formation of membrane tubules has been reported under several conditions. For example, tubular structures are formed by some natural lipids, such as brain galactocerebrosides,[13] which also constitute the deposit found in several lipid storage diseases.[14] Intracellular tubular structures made of lipid–protein mixtures have also been found in cells overproducing membrane-bound proteins and interpreted as a specialization of biological membranes containing ordered arrays of these proteins.[15] The formation of membrane tubules of endoplasmic reticulum has also been shown to be inducible by kinesin-driven microtubule movement.[16]

In this report we extend the scope of lipid tubules by conferring upon the constituent molecules the property of specific binding towards proteins. This gives the functionalized tubes the ability to form protein crystals. This concept is presented here in the case of a biotinylated lipid, called DODA-EO_2-biotin, and of streptavidin. Streptavidin is a protein isolated from *Streptomyces avidinii* that consists of four identical subunits, each containing a single biotin (vitamin H) binding site.[17] The high affinity between streptavidin and biotin has made this system very popular in the field of labeling and affinity techniques. The new tool presented here uses the tubular morphology of self-assembled lipid molecules to induce the spontaneous formation of ordered protein arrays.

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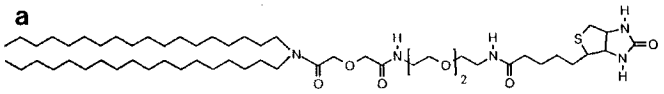
Results

Lipid tubules of DODA-EO_2-biotin: Liposome solutions of DODA-EO_2-biotin, a biotinylated dioctadecylamine lipid molecule containing ethylene oxide spacers (Figure 1 a), were prepared by solubilization in the presence of *n*-octyl-*β*-D-glucopyranoside (*β*-OG) followed by detergent dialysis. These solutions contained mainly tubular structures when observed by transmission electron microscopy (TEM) (Figure 1 b). The lipid tubules presented a highly homogeneous morphology, appearing as straight hollow cylinders with a constant diameter of about 27 nm. Their length was variable and could reach several tens of micrometers. The hollow nature of the tubules was suggested by the presence of stain at their extremities (Figure 1 b arrowheads) and along the length of some tubes (Figure 1 b arrow). The



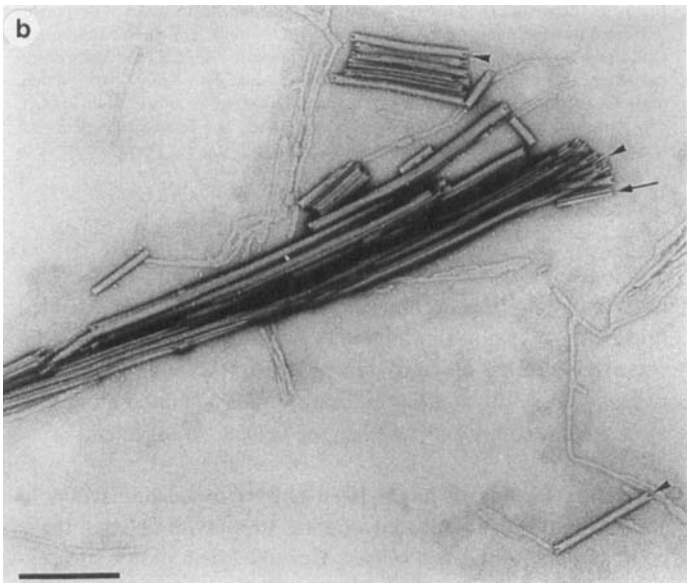


Figure 1. Lipid tubules of DODA-EO_2-biotin. a) Chemical structure of DODA-EO_2-biotin. The three asymmetric carbon atoms in the natural d-biotin are in a *cis–cis* configuration.[42] b) Electron micrograph of lipid tubules made of pure DODA-EO_2-biotin (negative staining in NaPTA). The hollow nature of the tubes is suggested by the characteristic presence of a stain deposit observed either at their extremities (arrowheads) or all along their length (arrow). The packing of the lipid tubules in bundles is likely to have been induced by drying. Scale bar: 0.4 μm.

formation of lipid tubules was unexpected, as most lipids form uni- or multibilayer structures with a vesicular shape when subjected to a similar treatment. When mixtures of DODA-EO_2-biotin and dioleoylphosphatidylcholine (DOPC) (molar ratios 1:4 and 1:10) were analyzed, a mixed population of round vesicles and tubules was observed (data not shown). We concluded therefore that the formation of the tubular structures was due to genuine self-assembly of the chiral DODA-EO_2-biotin molecules.

The uni- or multilamellar nature of DODA-EO_2-biotin tubules was investigated by cryoelectron microscopy. An example of a lipid tubule in the frozen hydrated state is shown in

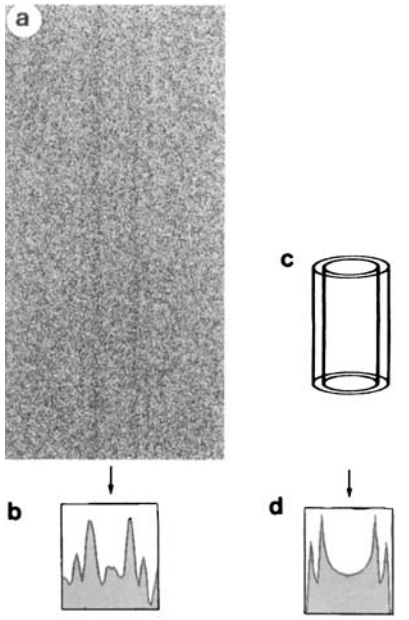


Figure 2. Unilamellar structure of a frozen hydrated lipid tubule of DODA-EO_2-biotin. a) Cryoelectron micrograph of a lipid tubule observed in the frozen hydrated state. The tube diameter is about 27 nm. The tube presents a characteristic contrast pattern made of four high-density stripes. Scale bar: 27 nm. b) Graph of the projected density emphasizing the four-striped pattern. The image (a) was digitized with a step size of 25 μm, corresponding to a pixel size of 0.56 nm. A straight portion (175 nm) of the lipid tubule was boxed and the projected density was calculated by summing the pixel values along the tube direction with programs from the IMAGIC package.[41] c) Model of a unilamellar cylindrical liposome in which the two concentric black annuli symbolize the lipid polar headgroups, characterized by higher electron scattering properties. d) Projected density of the model (c), calculated as in (b), exhibiting four peaks with shape and intensities similar to the projected density calculated for the lipid tubule in (b).

Figure 2. The tube presents four stripes along its length, two grey outer stripes and two darker inner stripes. This contrast pattern is characteristic of unilamellar lipid vesicles[18] and is a result of the greater electron-scattering ability of the lipid polar headgroups as compared with the hydrocarbon chains. The inner diameter of lipid tubules, measured as the distance between the two inner darker stripes, is about 16 nm.

Binding of streptavidin to DODA-EO_2-biotin liposomes: Lipid tubules made of DODA-EO_2-biotin are functional structures, bearing biotin sites that confer on them the property of molecular recognition for streptavidin. When streptavidin was added to pure DODA-EO_2-biotin suspensions or to lipid mixtures made of DODA-EO_2-biotin and DOPC, a noticeable aggregation developed within a few minutes. This effect can be explained by the symmetric distribution of biotin sites on opposite sides of tetravalent streptavidin molecules, which can thus interact with two neighboring liposomes and induce the formation of an intricate liposome network.[19] This provided macroscopic evidence that biotin sites were accessible on lipid structures made of DODA-EO_2-biotin.

In order to characterize quantitatively the interaction between streptavidin and DODA-EO_2-biotin aggregates, a polyacrylamide gel electrophoresis assay (PAGE) based on a modification of the electrophoretic migration properties of proteins induced by their specific binding to liposomes or micelles was performed.[20] When streptavidin was mixed with an excess of

DODA-EO_2-biotin and the mixtures were characterized by PAGE in the presence of sodium dodecyl sulfate (SDS), the streptavidin band was totally shifted towards a fast-migrating position (Figure 3a). In the example presented here, 80 pmol streptavidin was titrated against 210 pmol DODA-EO_2-biotin.

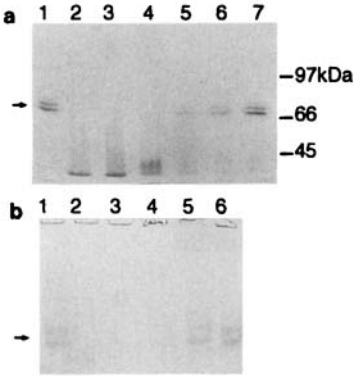


Figure 3. Titration of streptavidin binding by PAGE in denaturing (a) and nondenaturing (b) conditions. a) Streptavidin (80 pmol) was mixed with DODA-EO_2-biotin/DOPC liposomes (1:10 mol/mol) containing the following amounts of DODA-EO_2-biotin (from lanes 1 to 7): 0, 2100, 700, 210, 70, 20, and 0 pmol. The samples were mixed with SDS before deposition on the gel. Titration is complete with about 210 pmol of DODA-EO_2-biotin. The arrow indicates the bands corresponding to the streptavidin tetramer. The position of molecular weight markers is indicated on the right. b) Streptavidin (200 pmol) was mixed with DODA-EO_2-biotin/DOPC liposomes (1:10 mol/mol) containing the following amounts of DODA-EO_2-biotin (from lanes 1 to 6): 0, 2100, 700, 210, 70, and 0 pmol. The samples were deposited on the gel without further treatment. Titration is achieved with about 2100 pmol of DODA-EO_2-biotin.

This result is in agreement with the fact that each streptavidin can bind up to four biotinylated lipid molecules. It also indicates that biotin groups are freely accessible in the micellar form of the aggregates. In nondenaturing conditions, streptavidin gave two bands that were absent when an excess of DODA-EO_2-biotin-containing liposomes was added. The total disappearance of the bands corresponding to the streptavidin tetramer (arrow in Figure 3b) can be explained by the fact that the liposomes are too large to enter the gel.[20] Titration of 200 pmol streptavidin was complete with 2100 pmol DODA-EO_2-biotin. Taking into account the facts that a) only half of the lipids are accessible in intact unilamellar vesicles, b) DODA-EO_2-biotin/DOPC liposomes with a 1:10 molar ratio were used in the present experiment, c) one streptavidin molecule covers a surface equivalent to forty lipid molecules,[21] and d) streptavidin molecules can bind liposomes by their two opposite sides, titration of 200 pmol streptavidin is expected to require between 800 and 1600 pmol of DODA-EO_2-biotin. This value is in satisfactory agreement with the results obtained. These results indicate that streptavidin binds in a titrable manner to both DODA-EO_2-biotin liposomes and micelles.

Helical crystallization of streptavidin on DODA-EO_2-biotin tube surface: We found by TEM that streptavidin assembles spontaneously into ordered helical arrays at the tube surface (Figure 4). Following streptavidin binding, the diameters of lipid tubules increased from 27 nm to 38 nm, an increase corresponding to about twice the thickness of the streptavidin molecule, as known from X-ray analysis.[21] This observation indicates that one single layer of protein covers the tube surface. Tubes presented striations extending perpendicularly to the tube axis and about 5.2 nm distant from each other. The helical symmetry of the protein arrangement was clear from the aspect of the corresponding diffraction patterns, which exhibited a characteristic

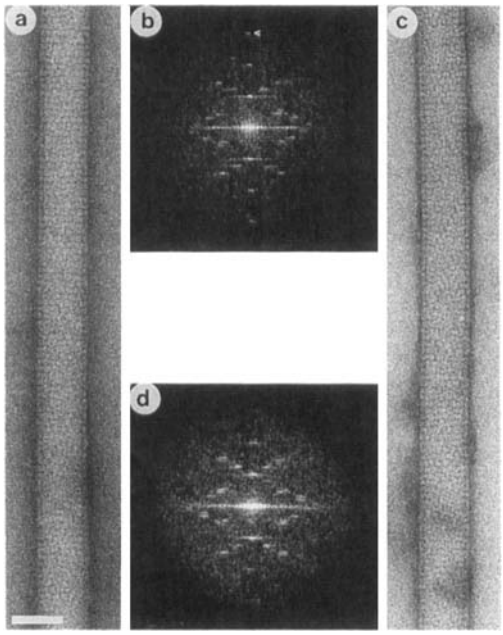


Figure 4. Images (a,c) and Fourier transforms (b,d) of helical crystals of streptavidin formed on lipid tubules containing DODA-EO_2-biotin. a,c) Stain striations extend along the tubules. Protein densities are particularly visible at tube edges, corresponding to streptavidin molecules viewed edge-on. Scale bar: 40 nm. (b,d) Distribution of Fourier transform amplitudes from the tubes shown in (a,c), corresponding to about 1700 streptavidin molecules. The finest spacing between layer lines indicates a helical repeat of 47 nm. Visible diffraction peaks extend up to 1.7 nm (arrowhead in (b)).

distribution of intensities confined to layer lines (Figure 4b,d).[22, 23] The resolution of the crystalline order extended up to about 1.5 nm. Binding and crystallization of streptavidin occurred within a few minutes, reflecting the high affinity between streptavidin and biotin groups and the limited molecular movements required to stabilize the helical arrangement.

Secondary binding of biotinylated objects to tubular arrays of streptavidin: We investigated whether streptavidin-coated tubes could bind biotinylated objects through their biotin sites exposed to the aqueous environment. They did indeed bind biotinylated proteins, as shown here in the case of ferritin, a 500 kDa iron-bearing protein that is easily recognizable by electron microscopy (Figure 5a). A similar result was obtained with other biotinylated proteins, such as linear polymers of streptavidin or alkaline phosphatase (data not shown). The streptavidin tubular crystals also bound liposomes containing lipid species with different specificities, which could be used for a second molecular recognition process. In the experiment presented in Figure 5b, liposomes containing DOPC, monosialoganglioside G_{M1} and *N*-(6-((biotinoyl)amino)hexanoyl)dipalmitoylphosphatidylethanolamine (biotin-LC-DPPE), a biotinylated lipid different from DODA-EO_2-biotin, are shown to interact with a tubular array of streptavidin. These liposomes wrap around the streptavidin tube and present a smooth surface. After addition of the B_5 moiety of cholera toxin, a protein exhibiting a high affinity for G_{M1}-gangliosides,[24] the surface of the liposomes becomes rough and covered with particles presenting the characteristic rosette shape of the B_5 particle (Figure 5c).[25] The possibility of targeting liposomes of different

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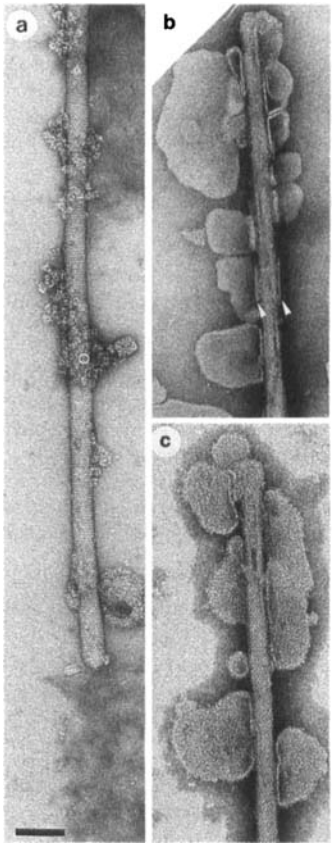

Figure 5. Binding of biotinylated objects to helical arrays of streptavidin. a) Polybiotinylated horse spleen ferritin molecules bound to a streptavidin tubular crystal. Many globular ferritin molecules (white circle) are seen associated with the helical array of streptavidin molecules. The presence of small ferritin aggregates is certainly due to the polybiotinylation of the protein and the presence of free streptavidin molecules. Binding was specific as no ferritin molecules were bound after preincubation of the streptavidin tubes with an excess of biotin. b) Lipid vesicles made of DPPE-LC-biotin/G_{M1}/DOPC (1:1:10 molar ratio) bound to a tubular array of streptavidin. Some lipid vesicles wrap around the tube surface like sleeves maintained by an extended zipper structure. The smooth surface of the vesicles is characteristic of the absence of bound proteins. c) As for b) after further incubation with the B_5 moiety of cholera toxin. The lipid vesicles are now covered with particles exhibiting the characteristic annular structure of B_5 pentamers. Scale bar: 100 nm.

specificities at the same place is of potential interest for various applications such as initiation of membrane fusion processes or diffusion-limited reactions.

Discussion

The formation of lipid tubules with DODA-EO_2-biotin constitutes a new example of amphiphile-forming supramolecular structures with helical or tubular morphologies.[7, 26] These tubes are, to our knowledge, the first which are functionalized, being able to bind streptavidin by their biotin headgroups. In addition, the unilamellar nature of DODA-EO_2-biotin tubes constitutes a marked difference from the multilamellar nature of many other lipid tubules previously reported. For example, tubular structures obtained with either polymerizable diacetylenic lecithins[27] or aldonamides[5, 6] have diameters measured in hundreds of nanometers.

The spontaneous crystallization of streptavidin on the tube surface constitutes a remarkable property of DODA-EO_2-biotin tubes, which is of potential interest in the field of structural biology. The crystallogenesis of helical arrays of soluble proteins by affinity binding to tubular lipid vesicles is similar in its principle to the growth of two-dimensional protein crystals induced by specific binding of proteins to lipids incorporated into planar lipid films.[28] However, this new method presents several notable advantages with respect to the formation of two-dimensional crystals at air–water interfaces. In protein crystals with helical symmetry, the repeat motif is viewed in many different orientations along the helix; this enables its three-dimensional structure to be calculated from one single image by Fourier–Bessel reconstruction methods.[22] In the case of two-dimensional crystals, the determination of a three-dimensional structure necessitates the recording and combination of many images of tilted specimens. This operation has intrinsic limitations resulting in a loss of resolution along the direction perpendicular to the crystal plane.[29] In addition, helical crystals form in solution and can be easily transferred onto electron microscopy grids. In contrast, the transfer of two-dimensional crystals from air–water interfaces, or of Langmuir films in general, is extremely tricky, inefficient, and prone to result in structural damage. The actual resolution of the streptavidin crystals, close to 1.5 nm, is likely to be limited by the use of negative stains. The ultimate resolution will depend on the intrinsic crystalline order and the number of averaged molecules. It has been shown that a resolution of 0.9 nm, sufficient to reveal the major elements of protein secondary structure, could be achieved by averaging 50000 molecules arranged in a helical crystal.[23] As this number of molecules corresponds to a length of 10 μm of a DODA-EO_2-biotin tube, it is possible that high resolution data could be obtained by image analysis of single tubes.

Streptavidin molecules are known to form various types of planar two-dimensional crystals by specific binding to biotinylated lipid layers.[30] The packing of streptavidin molecules at the tube surface, presently under study, is likely to derive from one of these crystalline forms by a slight rearrangement of molecular interactions, without implying any bending of the streptavidin molecule itself.

The tubular crystals presented here are distinct in nature from other protein tubular crystals already reported, such as those made of viral proteins,[31] membrane proteins,[32, 33] or membrane-bound proteins.[34] In these latter cases, tubular structures have resulted from the particular shape and spatial arrangement of the proteins and not from a preexisting tubular matrix. This is well substantiated in the case of the acetylcholine receptor tubes that form by spontaneous rearrangement of round native membrane vesicles.[32] In the present study, the helical array of streptavidin is induced by the preexisting morphology of DODA-EO_2-biotin lipid tubules. It is important to note that we have studied many biotinylated lipids which formed spheroidal liposomes and we have never observed, despite extensive investigations, the transformation of spheroidal liposomes covered with streptavidin into tubular crystals.

We anticipate that the new type of lipid tubule presented here could lead to interesting applications in the field of structural biology. The possibility of cocrystallizing complexes made of streptavidin and of a biotinylated protein of interest would constitute a powerful new approach in electron crystallography. The rational design of lipid tubules with selected protein specificities constitutes another direction to investigate, which first requires an understanding of which part of the molecule is responsible for the property of tube formation.[35] In addition, the tubular crystals of streptavidin act as *functional* systems that present interesting surface properties for secondary binding of a variety of biotinylated molecules. A wide range of biotinylated compounds, such as proteins, dyes, and polymers, are easily available, and biotin-ligand linkers have already been used for building mixed protein multilayers in an

organized manner.[36] Thus, potential applications in surface chemistry, catalysis, drug delivery, and nanotechnology in general are foreseen.

Experimental Section

Materials: DODA-EO_2-biotin was synthesized as previously described from d-biotin.[37] Streptavidin was purchased from either Jackson Immunoresearch or Boehringer Mannheim. β-OG, monosialoganglioside G_{M1}, biotin immobilized on agarose resin, biotinylated horse spleen ferritin and B_5 cholera toxin were purchased from Sigma. DOPC and biotin-LC-DPPE were obtained from Avanti Polar Lipids and Pierce, respectively. All other chemicals were the best available commercial grade.

Preparation of liposomes of DODA-EO_2-biotin: Liposomes were prepared by detergent dialysis.[38] DODA-EO_2-biotin and β-OG were first solubilized in $CHCl_3$ at 4 and 100 mg mL^{-1}, respectively, and mixed in 1:10 weight ratio. After evaporation of the solvent under reduced pressure, a volume of buffer made of NaCl (100 mM), EDTA (1 mM), NaN_3 (3 mM), sodium phosphate (20 mM), pH 6.5, was added to give a final lipid concentration of 4 mg mL^{-1}. The mixture was stirred at room temperature until it became clear and was then poured into Spectra/Por no. 1 dialysis tubing (Medicell, London (UK)) with a molecular weight cut-off of 6000–8000 Da. Dialysis was performed at 4 °C for 2 days until a noticeable turbidity was observed. The same procedure was used for preparing mixtures of DODA-EO_2-biotin and DOPC (1:4 and 1:10 (mol/mol)).

Titration of streptavidin binding to DODA-EO_2-biotin liposomes or micelles by PAGE: Binding of streptavidin to biotinylated lipids either in liposomes or in micelles was measured by means of an electrophoretic migration shift assay performed in either nondenaturing or denaturing conditions.[20] A fixed quantity of streptavidin was incubated for 15 min at room temperature with increasing amounts of DODA-EO_2-biotin liposomes. For PAGE in denaturing conditions, the samples (25 µL maximal volume) were mixed with 5 µL of a solution containing bromophenol blue in 30% glycerol supplemented with 2% SDS, and deposited on SDS-containing gels. For PAGE in nondenaturing conditions, the samples were mixed with the bromophenol blue/glycerol solution without SDS and directly deposited on the gel. PAGE was performed in 0.8 mm thick slab gels. No stacking gels were used for PAGE in nondenaturing conditions. Separating gels contained 10% acrylamide and 0.4% bisacrylamide in 400 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.8, supplemented with 0.1% SDS in denaturing conditions. For PAGE in denaturing conditions, stacking gels contained 5% acrylamide and 0.13% bisacrylamide. The migration buffer was 25 mM Tris, 192 mM glycine, pH 8.3, supplemented with 0.1% SDS in denaturing conditions. Gels were stained with Coomassie brilliant blue according to standard procedures.

Binding of streptavidin to DODA-EO_2-biotin lipid tubules for TEM observations: Binding of streptavidin to lipid tubules obtained with pure DODA-EO_2-biotin or with a DODA-EO_2-biotin/DOPC lipid mixture (molar ratio 1:4) was induced by mixing a given volume of biotinylated lipid solution, at a final DODA-EO_2-biotin concentration of 90 µM, with a twofold molar excess of streptavidin from a 5 mg mL^{-1} solution. After incubation for 10 min at room temperature, the excess of free protein was eliminated by a 10-min incubation in the presence of agarose resin beads containing immobilized biotin groups. Samples were processed for TEM as described below.

Binding of biotinylated objects to helical arrays of streptavidin: Streptavidin-bound tubules were prepared as described before and adsorbed onto hydrophilic carbon-coated grids for 5 min. A grid was then placed for few minutes on top of a droplet containing the solution of interest: 1) a 10 µM solution of polybiotinylated horse spleen ferritin; 2) a 1 mg lipid per mL liposome solution made of biotinylated lipids (biotin-LC-DPPE), ganglioside G_{M1}, and DOPC (molar ratio: 1:1:10). In this latter case, some grids were washed and deposited on top of a 0.5 mg mL^{-1} B_5 cholera toxin solution for subsequent binding of B_5 to its receptor, the monosialoganglioside G_{M1}.[24]

TEM and image analysis: For electron microscopy of pure lipids, a 0.2–0.4 mg mL^{-1} lipid solution was deposited onto a carbon-coated grid, previously rendered hydrophilic by glow discharge in air.[39] Sodium phosphotungstate solution (NaPTA, 2%, pH 7.5) was used as a negative stain. Streptavidin-bound lipid tubules were negatively stained with 1% uranyl acetate, pH 3.5 (UA). Cryoelectron microscopy of frozen hydrated liposomes was carried out by adsorption of a droplet of a 0.1 mg mL^{-1} lipid suspension onto a hydrophilic carbon-coated grid and plunging of the grid into cooled liquid propane.[40] Observation was at about −170 °C with a Gatan-626 cryoholder.

Electron microscopy was performed on a Philips CM12 operating at 100 kV. Images of tubes exhibiting helical symmetry were selected by optical diffraction and digitized with a Kodak Eikonix Model 1412 CCD camera with a step size of 14 µm, corresponding to a pixel size of 0.32 nm. Fourier transforms of straight portions of tubes extending over a length of 450 nm were calculated by the IMAGIC image analysis package.[41]

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