

Relationship between molecular structure and supramolecular morphology of DODA-EO₂-biotin and related lipids

Philippe Huetz^a, Stephanie van Neuren^a, Philippe Ringler^c, Felix Kremer^a,
Jan F.L. van Breemen^a, Anno Wagenaar^b, Jan B.F.N. Engberts^b,
Johannes G.E.M. Fraaije^a, Alain Brisson^{a,*}

^a Department of Biophysical Chemistry, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, BIOSON Institute, Nijenborgh 4, 9747 Groningen, The Netherlands

^b Department of Chemistry, Organic and Molecular Inorganic Chemistry, University of Groningen, Nijenborgh 4, NL-9747 AG Groningen, The Netherlands

^c Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 Rue Laurent Fries, F-67404 Illkirch, France

Received 19 March 1997; received in revised form 19 June 1997; accepted 10 July 1997

Abstract

We have recently reported that a biotinylated lipid molecule, called DODA-EO₂-biotin, forms tubular lipid structures upon hydration, which act as a matrix for the formation of ordered helical arrays of streptavidin as well as for secondary macromolecular recognition reactions involving biotinylated structures (Ringler et al., 1997). In the present study, the supramolecular structures formed by the compounds obtained during the synthesis of DODA-EO₂-biotin and of compounds structurally related to DODA-EO₂-biotin were investigated by transmission electron microscopy, with the objective being to understand the relationship between molecular structure and supramolecular morphology. From the eight lipid molecules investigated, only DODA-EO₂-biotin formed tubular structures. Several structural parameters were identified as playing a role in the formation of DODA-EO₂-biotin tubes, such as the chirality of the biotin moiety, the saturated nature of the lipid chains, the presence of amide bonds and the correct length and structure of the hydrophilic spacer. In addition, helical crystals of streptavidin were only obtained upon binding of streptavidin to the supramolecular assemblies formed by DODA-EO₂-biotin. © 1997 Elsevier Science Ireland Ltd.

Keywords: Lipid tubules; Biotinylated lipids; Supramolecular aggregates; Liposomes; Helical crystallization of proteins

* Corresponding author. Tel.: +31 50 3634216; fax: +31 50 3634800; e-mail: Brisson@chem.rug.nl

1. Introduction

The formation of lipid tubules has raised considerable interest in the recent years due to their potential use as micro- or nano-structures (Schnur, 1993; Archibald and Mann, 1993a; Fuhrhop and Köning, 1994). A variety of compounds have been shown to act as building blocks for mesoscopic aggregates with helical or tubular morphology: synthetic compounds such as double- or single-chain amphiphiles with an amino-acid backbone (Nakashima et al., 1984; Yamada et al., 1984; Imae et al., 1992), polymerizable lecithins with diacetylenic fatty acyl chains (Yager and Schoen, 1984; Georger et al., 1987), octyl-al-donamides (Pfnemüller and Welte, 1985; Fuhrhop et al., 1987; Köning et al., 1993), single chain diacetylenic aldonamides (Frankel and O'Brien, 1994), diphenylglycoluril-based amphiphiles (van Nunen et al., 1994), non-ionic or fluorinated surfactants (Uchegbu and Florence, 1995; Giulieri et al., 1994), di-polyprenyl phosphates (Birault et al., 1996), anionic glucophospholipids (Giulieri et al., 1996) and natural lipids such as phospholipids (Papahadjopoulos et al., 1975), bile lipids (Kaplun et al., 1994) or galactocerebrosides (Archibald and Yager, 1992). Several structural parameters have been identified as playing an essential role in the formation of tubular assemblies (Schnur, 1993; Nakashima et al., 1984; Frankel and O'Brien, 1994; Singh et al., 1988; Fuhrhop et al., 1988; Kulkarni et al., 1995), such as a highly ordered conformation of the acyl tails (Nakashima et al., 1985; Burke et al., 1988; Chappell and Yager, 1991), the presence of a chiral head group (Nakashima et al., 1984; Singh et al., 1988; Fuhrhop et al., 1988), or the formation of intermolecular amide hydrogen bonds stabilizing the association of polar head groups (Fuhrhop et al., 1987). Theories have been elaborated to explain the formation of lipid tubules (Schnur, 1993) based on notions of spontaneous torsion of bilayer edges (Helfrich, 1986; Helfrich and Prost, 1988; Selinger et al., 1996), curvature energy (Lubensky and Prost, 1992), surface energy reduction (Köning et al., 1993), or chiral bilayer effect (Fuhrhop et al., 1987). The picture which has emerged from these papers is that the process of

tube formation is driven by the chirality of the constituting molecules and involves a sequential transformation of ribbons into wound sheets, open helices and finally closed lipid cylinders.

We have recently described a new type of lipid tubule formed by the DODA-EO₂-biotin molecule (Ringler et al., 1997). These molecules extend the field of supramolecular structures with tubular morphology by adding the property of molecular recognition for proteins. The functionalized tubes not only bind the streptavidin protein, but also induce its spontaneous organization into ordered helical crystals. In addition, helical arrays of streptavidin can act as a matrix for secondary binding reactions towards biotinylated objects. These properties are of potential interest in the field of structural biology and of protein array formation.

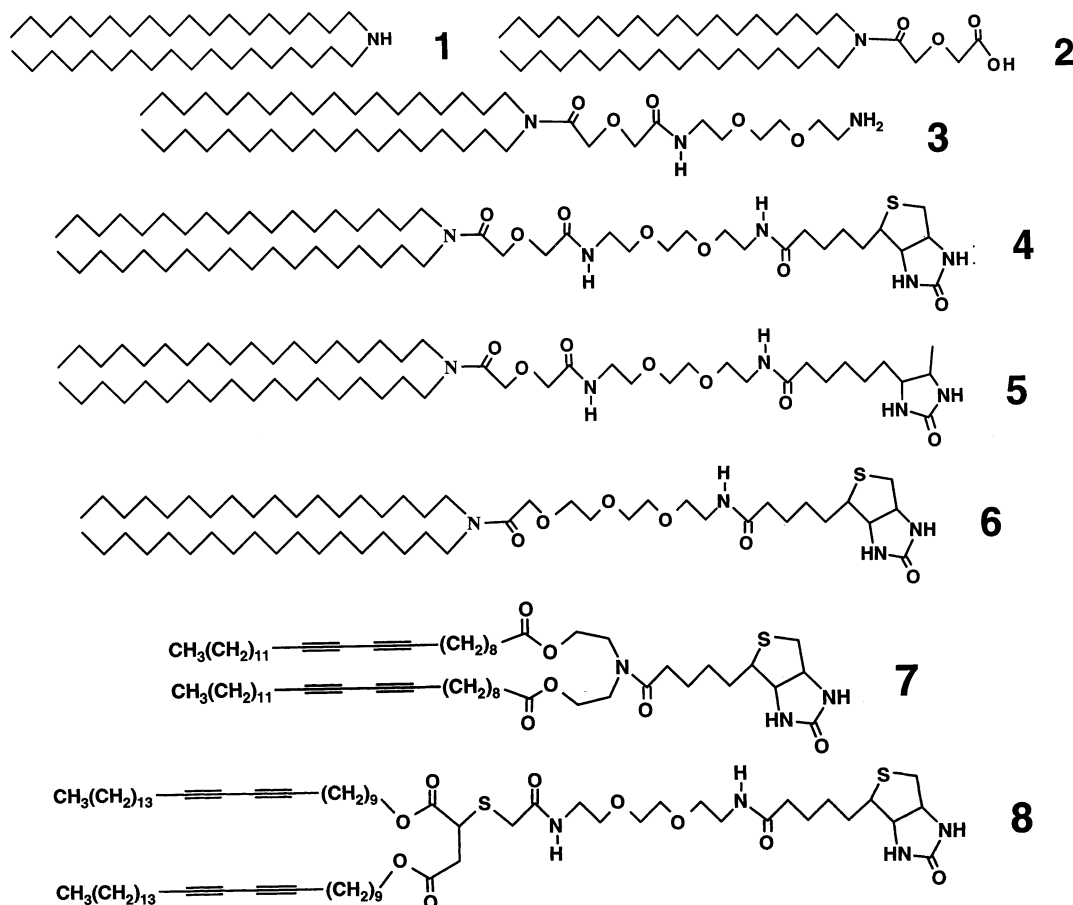
In the present paper, we investigated the structures formed in aqueous solutions by the different intermediate products isolated during the synthesis of DODA-EO₂-biotin: DODA, DODA-GSA, DODA-EO₂-NH₂ and DODA-EO₂-biotin (see Scheme 1, lipids 1–4) as well as by several lipid molecules structurally related to DODA-EO₂-biotin (lipids 5–8 in Scheme 1). Our objective was to elucidate which part of the DODA-EO₂-biotin molecule is responsible for the formation of lipid tubules and to establish a correlation between the chemical structure of these lipids and the morphology of their supramolecular self-assemblies.

2. Experimental procedures

2.1. Materials

N-octyl- β -D-glucopyranoside (β -OG) and D-biotin were purchased from Sigma. DODA, dioleoylphosphatidylcholine (DOPC) and streptavidin were purchased from Fluka, Avanti Polar Lipids and Pierce, respectively. SiO₂ gel (0.13–0.25 mm particle size; 60–120 mesh) and Al₂O₃ (neutral, activity degree I) were purchased from BDH Laboratory Supplies and Merck, respectively.

Lipids 5–8 in Scheme 1 were gifts from H. Ringsdorf. The synthesis of these lipids has been



Scheme 1. List of lipids studied in this work. Lipid 1: dioctadecylamine (DODA); lipid 2: *N,N*-dioctadecyldiglycolic acid monoamide (DODA-GSA); lipid 3: 1-*N,N*-dioctadecyl-14-amino-5-oxo-3,9,12-trioxa-6-azatetradecanoic acid amide (DODA-EO₂-NH₂); lipid 4: DODA-EO₂-biotin; lipid 5: DODA-EO₂-desthiobiotin; lipid 6: DODA-EO₃-biotin; lipid 7: diacetylene-(11,8)-biotin; lipid 8: diacetylene-(13,9)-EO₂-biotin.

described: lipid 7 (Blankenburg, 1989); lipid 6 (Erdelen, 1993); lipids 5 and 8 (Müller, 1993).

2.2. Synthesis of DODA-EO₂-biotin

The procedure used for synthesizing DODA-EO₂-biotin and isolating the intermediate compounds two and three (Scheme 1) was adapted from previous reports (Ebato et al., 1992; Ahlers, 1990) and is described briefly.

2.2.1. Synthesis of DODA-GSA

A solution of diglycolic acid anhydride (17

mmol) in dioxane was added dropwise to a solution of DODA (14 mmol) in CHCl₃/pyridine (24:1). Before mixing, both solutions were slightly heated with a heat gun for complete solubilization. A white precipitate appeared in the reaction medium after a few minutes. Upon characterization by thin layer chromatography (TLC) on silica plates with a 2% MeOH/CHCl₃ eluant and staining with iodine and ninhydrine, DODA and DODA-GSA gave spots with $R_f \approx 0.9$ and 0.1, respectively. DODA-GSA was recrystallized in methanol and a sharp melting point of 84°C was determined. DODA-GSA was obtained with a yield of nearly 100%.

2.2.2. Synthesis of DODA-EO₂-NH₂

An activated ester was formed by refluxing DODA-GSA (1.6 mmol) with an equimolar amount of carbonyldiimidazole (CDI) in dry THF (7 ml) for 2 h. As shown by NMR, the use of an equimolar quantity of CDI resulted in an incomplete transformation (60%) of the acid into the active ester, independently of the length of the reflux step and despite precautions taken for working in dry conditions. Although a complete activation of the acid was obtained with two equivalents of CDI, the formation of unwanted secondary reaction products rendered this approach unsuitable. Dropwise addition of the imidazole-activated ester to 1,8-diamino-3,6-dioxaoctane (13.5 mmol) in THF (13 ml) led to the formation of the DODA-EO₂-NH₂ molecule. This step was completed by extraction of the diamino-dioxaoctane with CHCl₃/H₂O (4:5). After evaporation of THF, DODA-EO₂-NH₂ was solubilized in CH₂Cl₂ and purified on an Al₂O₃ chromatography column with CH₂Cl₂/MeOH (5:1) as eluant, at air pressure. To prevent the presence of acid traces which may protonate the amine group, CH₂Cl₂ was used instead of CHCl₃ and the use of acid-washed sand to cover the gel was avoided. In addition, the use of Al₂O₃—instead of SiO₂—gel resulted in an improved separation of DODA-EO₂-NH₂. The compound was recrystallized in hexane. DODA-EO₂-NH₂ structure was confirmed by NMR and electrospray ionization mass spectrometry. The yield was about 50%. An $R_f \approx 0.7$ was obtained by TLC on Al₂O₃ plates with CHCl₃/MeOH (1:1). The product had a melting point of 39–40°C.

2.2.3. Synthesis of DODA-EO₂-biotin

DODA-EO₂-biotin was synthesized by adding equimolar amounts (0.27 mmol) of *N*-hydroxy-succinimido-biotin (NHS-D-biotin) and DODA-EO₂-NH₂ in dry DMF (10 ml) at room temperature. NHS-D-biotin was synthesized according to Bayer and Wilchek (Bayer and Wilchek, 1974). DODA-EO₂-biotin was purified by SiO₂ chromatography with CHCl₃/MeOH (10:1) as eluant at air pressure. Two successive steps of recrystallization were performed in hexane, at 4°C and at room temperature. About 25

mg of pure DODA-EO₂-biotin were obtained. The structure was confirmed by NMR. An $R_f \approx 0.1$ was obtained by TLC on SiO₂ plates with CHCl₃/MeOH (10:1) and spray-staining with 4-dimethylaminocinnamaldehyde (McCormick and Roth, 1970). Determination of the melting point showed a progressive clearing of the crystals from 70 to 107°C, followed by a rapid melting at 107°C over 2 or 3°. The yield of this reaction, 10%, was probably limited by the presence of amines in DMF.

2.3. Formation of lipid supramolecular assemblies by detergent dialysis

Lipids were transferred to aqueous solutions by detergent solubilization followed by detergent removal (Zumbuehl and Weder, 1981). Lipids and β -OG were solubilized in CHCl₃ at 4–10 and 100 mg/ml, respectively and mixed in a 1:10 weight ratio. After evaporation of the chloroform, the mixture was resolubilized in about 1 ml of diethyl ether and evaporated again to eliminate traces of solvent. This operation led to the rapid spreading of a white crystalline material on the tube walls. The lipid/ β -OG mixture was then resuspended to a final lipid concentration of 3–5 mg/ml by adding the adequate volume of either 20 mM Tris-HCl, 170 mM NaCl, 3 mM NaN₃, 0.25 mM EDTA, pH 7.4 (Tris buffer) or 20 mM phosphate, 100 mM NaCl, 3 mM NaN₃, 1 mM EDTA, pH 6.5 (phosphate buffer).

DODA-EO₂-NH₂ and DODA-EO₂-biotin solutions became clear almost instantaneously after addition of the aqueous solutions. On the other hand, DODA and DODA-GSA samples presented insoluble material despite the presence of detergent. After heating these suspensions above 70°C they cleared and were then left to stabilize at room temperature. The lipid/ β -OG mixtures were poured into dialysis tubings with a molecular weight cut-off of 6000–8000 Da (Spectra/Por No. 1, Medicell, London, UK). Dialysis was performed overnight at either 4°C or room temperature with a volume of either Tris or phosphate buffer 1000 times larger than the sample volume. A noticeable turbidity generally appeared after overnight dialysis.

Mixtures of DODA-EO₂-biotin and DOPC with 1:1, 1:4 or 1:10 molar ratios were prepared in the same manner, keeping the total lipid concentration at 5–6 mg/ml.

2.4. Lipids related to DODA-EO₂-biotin

The structures formed in aqueous solution by several lipids related to DODA-EO₂-biotin were also investigated: DODA-EO₂-desthiobiotin (lipid 5), DODA-EO₃-biotin (lipid 6), diacetylene-(11,8)-biotin (lipid 7) and diacetylene-(13,9)-EO₂-biotin (lipid 8) (see Scheme 1).

These lipids were treated as follows: 4 mg of lipid were dissolved in CHCl₃ and separated into two equal parts. In one part 8 mg of DOPC were added. In both, β -OG was added (β -OG/total lipid 10:1 (w/w)). Each sample was separated into two equal fractions. After evaporation of the solvent, the samples were completed to a final lipid concentration of 2 mg/ml with either phosphate buffer (see above) or Hepes buffer made of 20 mM Hepes (*N*-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid), 100 mM NaCl, 3 mM NaN₃, 1 mM CaCl₂, pH 6.5. The samples were divided into two parts for dialysis either at room temperature or at 4°C.

2.5. Characterization of lipid supramolecular structures by transmission electron microscopy (TEM)

The supramolecular assemblies formed in aqueous solution by lipid molecules 1–8 before and after detergent removal were studied by TEM. A 0.2–0.4 mg/ml lipid solution was deposited onto a formvar/carbon-coated grid, previously rendered hydrophilic by glow-discharge in air (Dubochet et al., 1982). Negative staining was carried out with 2% Na-PTA, pH 7.5, for 2 min, unless otherwise stated.

Electron microscopy observations were performed at 80 kV. Micrographs were recorded on Agfa 23D56 films and developed in Kodak HRP for 3 min.

In view of the intrinsic variability of this type of analysis, all the experiments were reproduced and representative micrographs are shown.

2.6. Binding of streptavidin to biotinylated lipid structures

The binding of streptavidin to lipid supramolecular structures formed with lipids 4–8 was carried out as previously described (Ringler et al., 1997). Negative staining was carried out with 1% uranyl acetate, pH 3.5.

3. Results

3.1. Supramolecular structures formed by DODA

Aqueous mixtures of DODA and β -OG presented a characteristic silky aspect at room temperature and 4°C. Plates of micrometer size arranged in large three-dimensional assemblies were observed by TEM (Fig. 1a). Some of these plates exhibited characteristic electron diffraction patterns, indicating that the lipids were in a crystalline gel state (data not shown).

After dialysis of β -OG the suspensions remained turbid, yet the silky aspect disappeared. Plates were observed, some of them presenting a regular geometry (Fig. 1b).

3.2. Supramolecular structures formed by DODA-GSA

Mixtures of DODA-GSA and β -OG in aqueous buffers were turbid at room temperature and 4°C, showing a sedimenting particulate material. Observed by TEM, this material consisted of plates of micrometer size, often aggregated, together with granular particles (data not shown).

After detergent dialysis, a homogeneous suspension was obtained. The predominant material consisted of thin plates with straight edges, of a few hundred nanometers in width and several micrometers in length, often radiating from thicker aggregates (Fig. 2). Vesicular structures, from 5 to 250 nm in diameter, were also present.

3.3. Supramolecular structures formed by DODA-EO₂-NH₂

Aqueous solutions of DODA-EO₂-NH₂ and β -

OG were clear both at room temperature and 4°C. They presented by TEM a very characteristic material consisting of an intricate network of rod-like structures (Fig. 3). The rods were up to several micrometers in length and had variable diameters ranging from 15 to 70 nm. Each rod exhibited a regular pattern of striations extending perpendicularly to its axis, with a repeat distance of about 5.5 nm (Fig. 3b). These repeating units may be seen as a juxtaposition of stacked disks viewed edge-on, although the presence of stain-excluding material in the central regions separating consecutive striations suggests a continuous structure (Fig. 3c). The interconnected network of rods

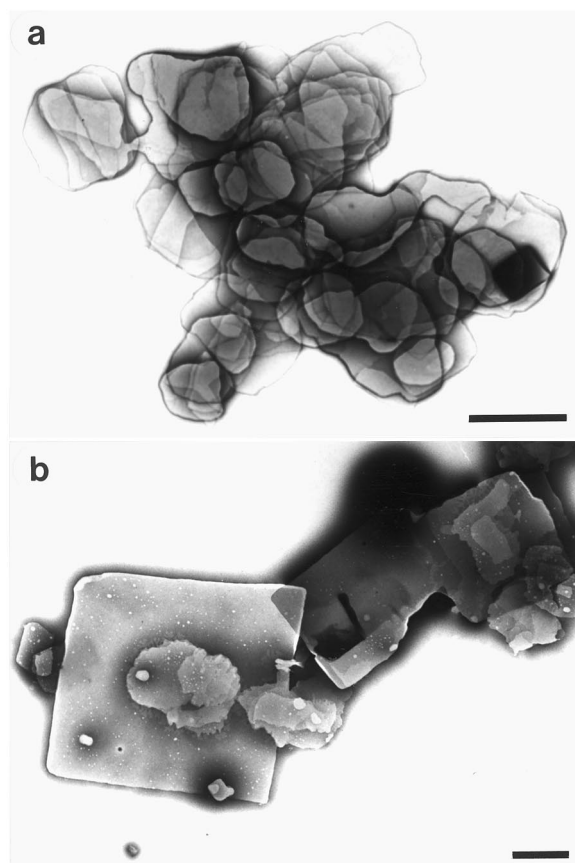


Fig. 1. Supramolecular structures formed by DODA before (a) and after (b) detergent removal. (a) Plates piling up in three-dimensional aggregates. (b) Square- and rectangular-shaped plates associated with amorphous material. Scale bar: 1 μm .

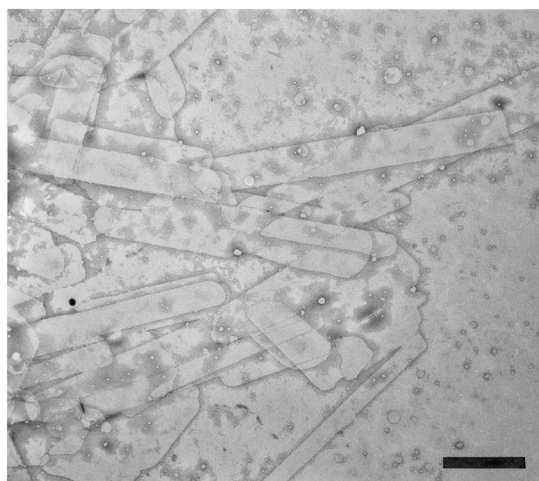


Fig. 2. Supramolecular structures formed by DODA-GSA after detergent removal. Thin elongated plates covered the grid, most of them exhibiting straight edges. Their even staining pattern suggests that they have a uniform thickness. Lipid vesicles cover the background. Scale bar: 1 μm .

extended over millimeters. This characteristic material was observed in all conditions tested, e.g. after storage at 4°C and was stable over a month period. It was observed after staining with sodium phosphotungstate (Na-PTA) as well as with 1% uranyl acetate (pH 3.5) and 2% ammonium molybdate (pH 7.9). These structures were not seen with pure β -OG prepared in the same conditions.

After dialysis of the detergent at room temperature or 4°C, solutions of DODA-EO₂-NH₂ exhibited a white precipitate. Small vesicles ranging from 20 to 100 nm in diameter, isolated or associated in small aggregates constituted the bulk of this material, together with large (μm) sheet-like structures (data not shown). No tubular structures could be detected after extensive observation.

3.4. Supramolecular structures formed by DODA-EO₂-biotin

Aqueous solutions of DODA-EO₂-biotin and β -OG were clear at room temperature, whereas a white precipitate of granulous aspect formed after cooling at 4°C. Observed by TEM, this material consisted of a mixed population of vesicles, plates

and other types of bilayer assemblies (data not shown).

After detergent dialysis at 4°C, lipid tubules were observed (Fig. 4). The lipid tubules were straight and had a constant diameter of about 27 nm and a length ranging from a few hundred nanometers up to several micrometers, as previously described (Ringler et al., 1997). The length-

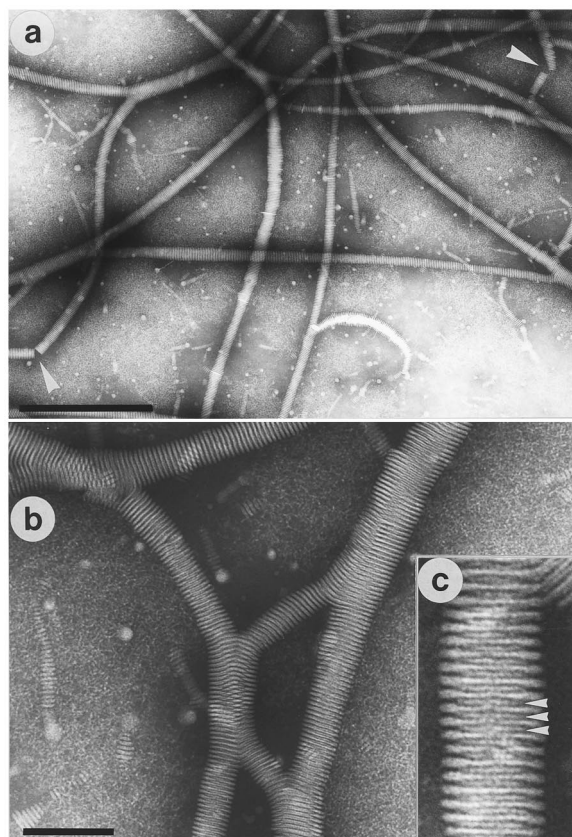


Fig. 3. Supramolecular structures formed by DODA-EO₂-NH₂ before detergent dialysis. (a) An interconnected network of rod-like structures covers the grid. The width of the rods is variable. Narrow and short rods are isolated, while wider rods of 30–70 nm form the network assembly. The presence of breaks along the rods (arrowheads) suggests that the rods are stiff structures, the breaks resulting from mechanical constraints occurring during adsorption on the support film. Scale bar: 0.5 μ m; (b) high-magnification view of connections between neighboring rods. The rods divide and join continuously. Scale bar: 100 nm; (c) thin striations (arrowheads) are observed in the interspace between the main (white) striations, giving to the assembly the aspect of a continuous helical bellows. The rod diameter is 43 nm.

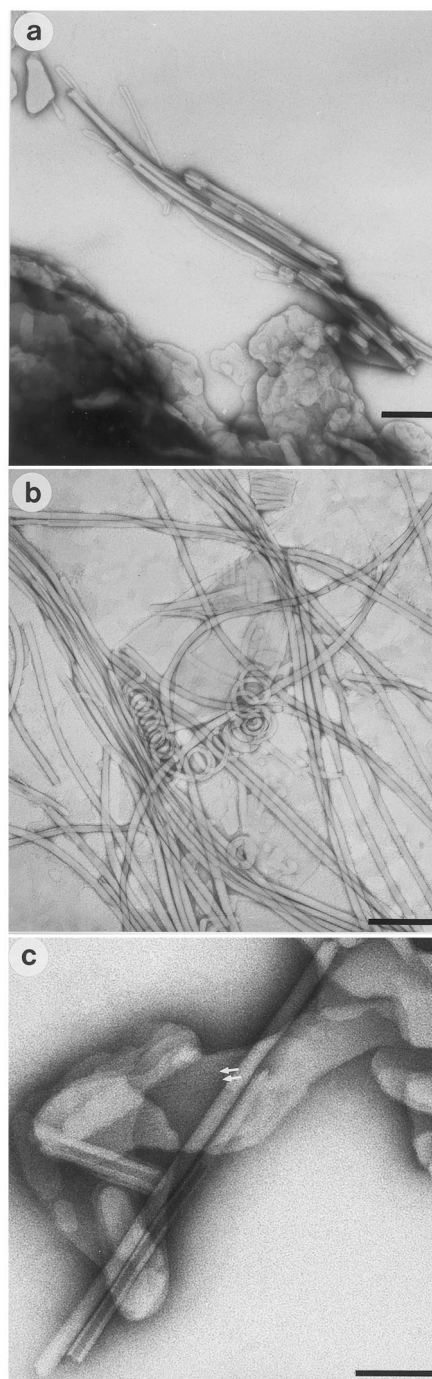


Fig. 4. Lipid tubules formed by DODA-EO₂-biotin. (a,b) Bundles of lipid tubules lining side-by-side or forming an intricate network. The staining aspect of the tubes is variable, depending on the extent of stain penetration into the hollow cylinders. (c) Plates associated with tubes often present a rippled structure (arrows). Scale bars: 200 nm in (a,b); 100 nm in (c).

to-diameter ratio of the tubular aggregates ranged from 10^2 to 10^3 . The tubes were either grouped in bundles (Fig. 4a,b), isolated or associated with lipid plates (Fig. 4c). These plates frequently exhibited faint parallel striations, with a repeat distance of about 7 nm (arrows in Fig. 4c). Tubes wound into spiral structures and multilamellar tubes with a cigar-like aspect were other rare components of these solutions (data not shown).

Strikingly, no tubes could be detected when dialysis of β -OG containing samples was performed at room temperature. These solutions showed mainly vesicles, superimposed plates and short rod-like structures (data not shown).

To investigate whether DODA-EO₂-biotin tubes also formed in the presence of extra lipids, mixtures of DODA-EO₂-biotin and DOPC were prepared, with 1:1, 1:4 and 1:10 (mol/mol) ratios. After overnight dialysis of β -OG at 4°C, long lipid tubules were found in all three samples (Fig. 5). These tubes had the same diameter of 27 nm but distinct morphologies. With 1:10 DODA-EO₂-biotin/DOPC solutions, many tubes were curved (Fig. 5b). The number of tubes decreased from 1:1 to 1:10 lipid solutions and a concomitant increase in the number of accompanying vesicles was noticed. These vesicles were similar to those obtained with pure DOPC. The fact that vesicles were often found associated with the tubes certainly explains the decreased tendency of the tubes to aggregate into bundles, because of steric effect. No tubes were obtained when dialysis was performed at room temperature.

Many tubes presented areas where tubular portions and sheet-like structures juxtaposed in a continuous manner (Fig. 6). Such areas were mainly found at tube endings. These images suggest that a conversion can occur between tube and sheet structures.

3.5. Supramolecular structures formed by lipids related to DODA-EO₂-biotin

DODA-EO₂-desthiobiotin (lipid 5) solutions presented, after β -OG dialysis, vesicular structures in all conditions tested: room temperature and 4°C, with or without DOPC (1:4 molar ratio), with Ca²⁺ or with EDTA (data not shown). Not

a single tube was observed with this lipid, which differs from DODA-EO₂-biotin by the absence of the thio moiety in the biotin group.

Solutions of DODA-EO₃-biotin (lipid 6) presented, after β -OG dialysis, a mixed population composed of vesicles and elongated sheet-like structures (Fig. 7). Striations extending along the

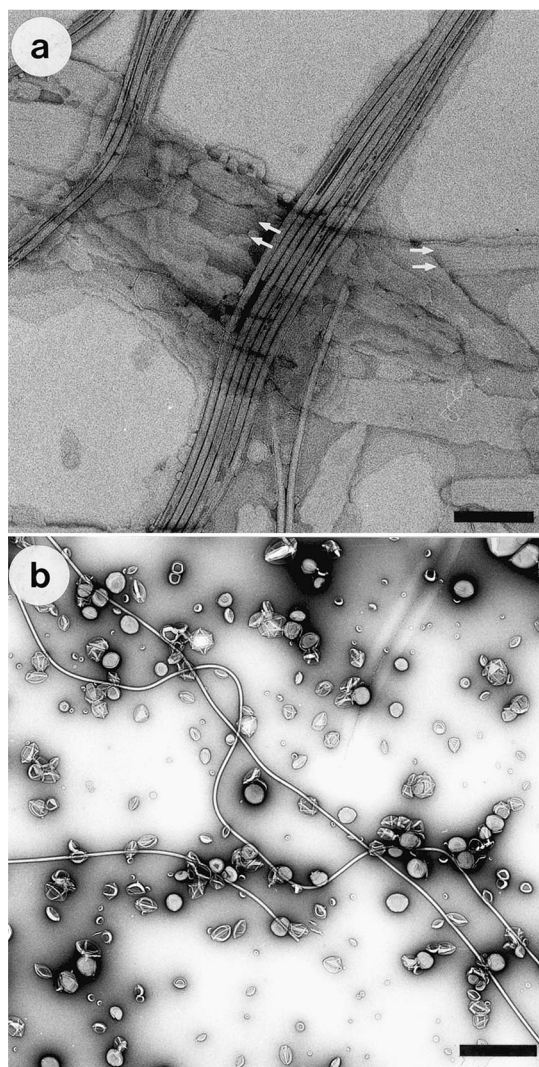


Fig. 5. Lipid tubules in mixed DODA-EO₂-biotin/DOPC solutions. (a) 1:1 ratio (mol/mol). Bundles of juxtaposed tubes coexist with plate-like structures. Some of these plates present parallel striations (arrows) similar to those seen in Fig. 4c. Scale bar: 200 nm. (b) 1:10 ratio (mol/mol). Tubes with a wavy morphology coexist with lipid vesicles. Scale bar: 100 nm.

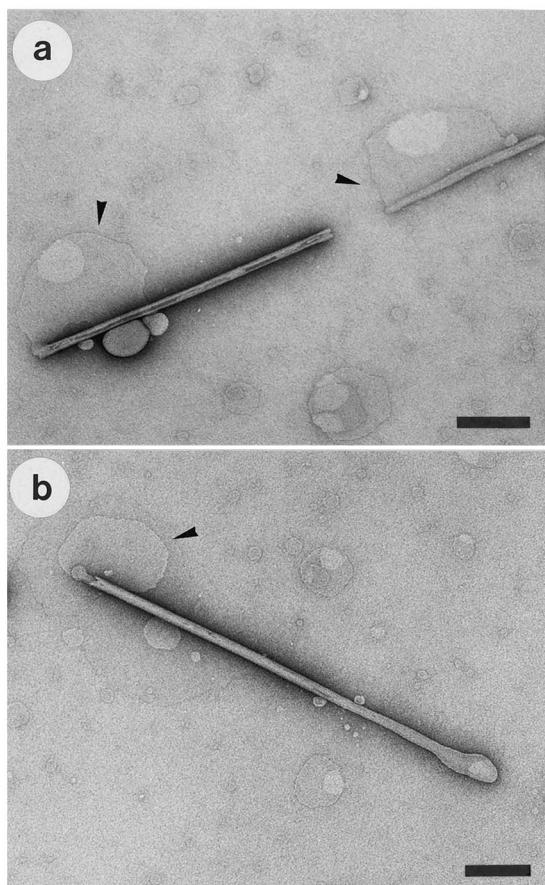


Fig. 6. (a,b) Images of tube endings showing sheet-like structures extending on only one side of the tubes (arrowheads), as if they resulted from an unwrapping of the tubes in a cigar-like manner. Many sheet-like structures present white, less dense, areas of unknown nature. Scale bar: 200 nm.

length of these sheets were often visible, with a repeat distance of about 5 nm (Fig. 7 inset). This striated pattern was similar to that previously described with DODA-EO₂-biotin. In the presence of DOPC, DODA-EO₃-biotin solutions presented only vesicles (data not shown).

The supramolecular structures formed by diacetylene-(11,8)-biotin (lipid 7) and diacetylene-(13,9)-EO₂-biotin (lipid 8) were similar, with a marked difference observed before and after β -OG dialysis. Before dialysis, ribbon-like structures covered the grids entirely (Fig. 8a,c). Ribbons formed by lipid 7 presented a wavy aspect and consisted of the juxtaposition of filamentous

structures. Ribbons formed by lipid 8 had a width of 50–60 nm and a thickness of about 10 nm, as could be estimated at turns where ribbons changed direction. Some of these ribbons presented internal striations. After detergent dialysis no ribbons were detected, but a mixed population composed of vesicular material and of elongated rod-like structures (Fig. 8b,d). These rod-like structures looked like wrapped membranes with geometrical characteristics distinct from the tubes obtained with DODA-EO₂-biotin. After polymerization of lipids 7 and 8 by exposure to UV light, the wrapped rods predominated (data not shown).

3.6. Binding of streptavidin to supramolecular assemblies of biotinylated lipids

The binding of streptavidin to the supramolecular structures formed by DODA-EO₂-biotin/

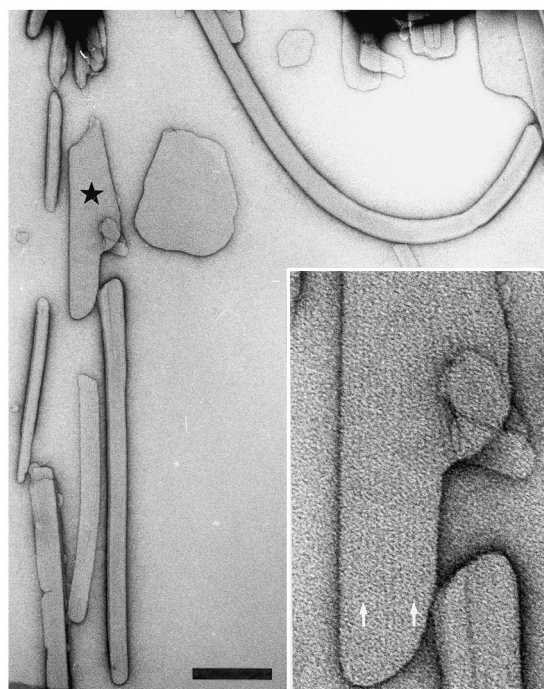


Fig. 7. Supramolecular structures formed by DODA-EO₃-biotin after β -OG dialysis. Long sheet-like structures with striations covered EM grids (Inset): Enlarged view of the structure (*), showing striations aligned along the direction of the arrows. These striations have a similar aspect to those shown by DODA-EO₂-biotin (see Fig. 4c, Fig. 5a). Scale bar: 200 nm.

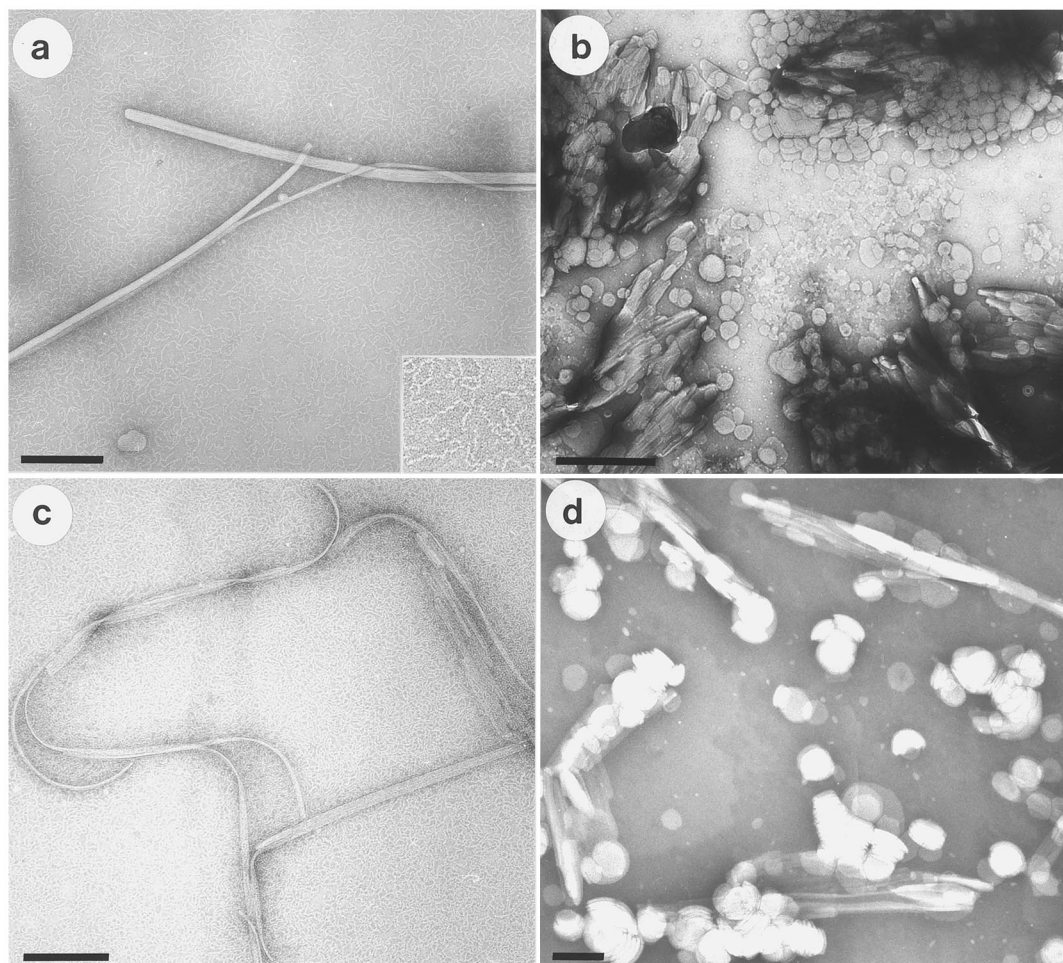


Fig. 8. Supramolecular structures formed by diacetylene-(11,8)-biotin (a,b) and diacetylene-(13,9)-EO₂-biotin (c,d). (a,c) Before dialysis, wavy ribbon-like structures coexist with a background made of thin and elongated structures. An enlarged view of the background structures is presented in the inset in (a). They correspond to micelles of DOPC/ β -OG, while the micelles observed in the absence of DOPC present a dotted appearance (A. Mukhopadhyay and A. Brisson, unpublished observations). Scale bars: 200 nm. (b,d) After dialysis, three-dimensional aggregates or tubular-like structures coexist with small vesicles, often piling up. Scale bars: (b) 500 nm; (d) 100 nm.

DOPC mixtures and by lipids 5–8 was studied by TEM. When streptavidin was added in saturating conditions to tubes obtained at 4°C with 1:1, 1:4 and 1:10 DODA-EO₂-biotin/DOPC solutions, ordered helical arrays of streptavidin were obtained, exhibiting the same structural characteristics as previously described (Ringler et al., 1997) (Fig. 9).

Streptavidin also bound to supramolecular assemblies made by lipid 5, as expected from the high affinity between desthiobiotin and strep-

tavidin ($K_a = 5 \times 10^{13} \text{ mol}^{-1}$) (Green, 1975). However, no ordered arrays were observed (data not shown). In the case of lipids 6–8, no binding of streptavidin could be detected by TEM. This result could be explained by a lack of accessibility of biotin groups, as complementary experiments using a migration shift assay (Arnold et al., 1995) showed that no binding of streptavidin occurred with liposomes made by lipids 6–8, although binding was detected with lipid micelles in the case of lipids 6 and 8 (data not shown).

4. Discussion

The goal of the present study was to elucidate which part of the DODA-EO₂-biotin molecule is responsible for the formation of the lipid tubules previously described (Ringler et al., 1997). In order to answer this question and to establish a correlation between the molecular structure of DODA-EO₂-biotin and its supramolecular assemblies, we investigated the structures formed in aqueous solutions by lipid intermediates isolated during the synthesis of DODA-EO₂-biotin as well as by several other structurally related lipids.

The main result is that, from the eight lipid molecules studied in this work, lipid tubes are only obtained with DODA-EO₂-biotin. Our study of the four molecules obtained from DODA to DODA-EO₂-biotin by progressive elongation of the polar head group indicates that the necessary structural information required to form the tubes of fixed geometry—straight unilamellar structures of 27 nm diameter and of micrometer length—is only present when the complete molecule is as-

sembled. The absence of tubes with lipids 3 and 5–8 enables to draw some conclusions on the structural elements required to induce the tubular morphology. The fact that both DODA-EO₂-NH₂ and DODA-EO₂-desthiobiotin molecules lack the molecular characteristics responsible for the tube formation further indicates that the presence and integrity of the biotin moiety, which presents three asymmetric carbon atoms in a *cis-cis* configuration (Traub, 1958; DeTitta et al., 1976), constitutes a crucial requirement. DODA-EO₂-desthiobiotin differs from DODA-EO₂-biotin by the lack of the thio moiety in the biotin group, resulting in a more flexible head group lacking the asymmetric carbon position defining the D-enantiomery of the biotin. The presence of a chiral center defining the spatial orientation of the biotin group and the correlated possibility of stacking interactions between biotins through hydrogen bonding or other types of intermolecular interaction are likely to be essential features in the formation of tubes exhibited by DODA-EO₂-biotin. This is in keeping with the concept that chirality can be a key element in the formation of lipid tubular structures (Schnur, 1993; Nakashima et al., 1984; Singh et al., 1988; Fuhrhop et al., 1988).

The fact that lipid 8 does not form tubes, although it possesses almost the same hydrophilic terminal portion as DODA-EO₂-biotin (amide bond-EO₂-amide bond-biotin), reflects the importance of either the connector domain close to the lipid tails and/or of the saturated nature of the hydrophobic chains in stabilizing tubular structures. It should be remembered however, that the formation of tubules by lipids with diacetylenic chains is well documented (Georger et al., 1987; Frankel and O'Brien, 1994). The strength of hydrophobic interactions in the DODA-based molecules is supported by several experimental observations: (i) both DODA and DODA-GSA form extremely stable aggregates, even in the presence of β -OG; (ii) the striations observed in plate structures with both DODA-EO₂-biotin and lipid 5 are likely to correspond to ripple phases formed by lipids below their main phase transition. Such ripple phases have been demonstrated to exist both in multilamellar bilayers (Rand et al., 1975;

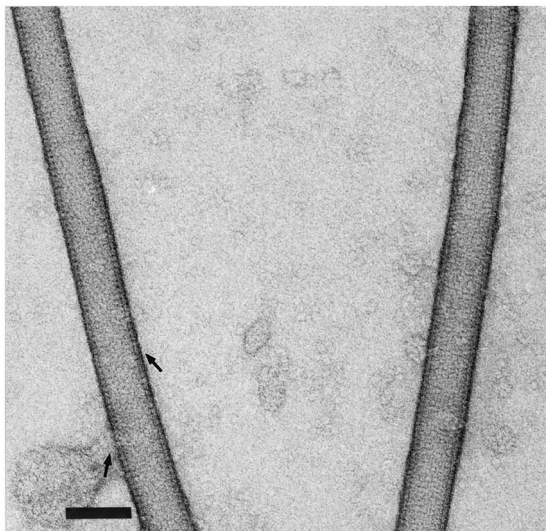


Fig. 9. TEM image of helical arrays of streptavidin. The surface of the lipid tubes is covered with streptavidin molecules arranged in a regular manner (alignments of particles are visible by viewing the image at glancing angle along the arrows). The tube diameter increases from 27 nm to about 38 nm, an increase which corresponds to about twice the thickness of streptavidin molecules. Scale bar: 50 nm.

Zasadzinski et al., 1988) and in single bilayers (Mou et al., 1994); and (iii) the molecular area of DODA-EO₂-biotin, measured by surface-pressure area isotherms, is close to 40 Å² (data not shown), a standard value for double-chain lipids in a crystalline state (Chapman et al., 1966). Several studies have already pointed out the importance of the highly ordered conformation of the acyl chains in the formation of lipid tubules (Nakashima et al., 1985; Burke et al., 1988; Chapell and Yager, 1991).

Lipid 6 possesses a complete biotin moiety and the same hydrophobic structure as DODA-EO₂-biotin, yet does not form tubes. Lipid 6 mainly differs from DODA-EO₂-biotin by the structure of its spacer domain, which is shorter (by an equivalent of three single bonds) and lacks an amide bond close to the amino-group of the DODA moiety. The possibility of formation of an intramolecular hydrogen bond between the H-atom of this secondary amide group and the O-atom of the ether bond close to the DODA part was suggested by molecular model building. These properties might be of importance in providing a favorable orientation of the biotin molecules for stacking interactions. Several additional elements support the idea that biotin groups have a tendency to form stacked assemblies: (i) their molecular packing observed in the crystal structure reveals a stacking of the biotin rings along one of the crystal axes (DeTitta et al., 1976); and (ii) the concentration of biotin molecules used in our experiments is close to the limit of solubility of biotin in aqueous solutions (0.9 mM). Although interactions between biotin moieties are likely to stabilize the lipid tubules, some of the biotin groups must have a minimal degree of freedom necessary for binding streptavidin molecules. These biotin-streptavidin interactions are not sufficient to destabilize the tubule organization because only a small number of biotin groups participate in these interactions, as one streptavidin molecule covers a surface equivalent to about seventy DODA-EO₂-biotin molecules (Hendrickson et al., 1989).

The stabilization of tubular morphologies via the formation of an extended network of hydrogen bonds has already been stressed by several

authors (Archibald and Mann, 1993b), particularly in the case of amide bonds (Pffannemüller and Welte, 1985; Fuhrhop et al., 1987; Frankel and O'Brien, 1994). The formation of amide–amide hydrogen bonds has recently been shown to be favorable in aqueous solvents, the process being largely entropy driven by the release of bound water molecules (Doig and Williams, 1992). The formation of a β -sheet like structure by hydrogen bonding between amide groups could constitute a higher state of intermolecular association.

In summary, the lipid tubules formed by DODA-EO₂-biotin are highly ordered structures, which are stabilized by a number of interactions involving both the hydrophobic and hydrophilic portions of the molecule. The saturated nature of the lipid chains, the presence of the chiral position defining the D-enantiomery of the biotin moiety and the presence of a spacer domain of appropriate structure are all elements which have to be present for inducing the formation of tubes.

Our observations indicate that experimental conditions, in particular the temperature at which the detergent dialysis is performed, play an important role in the formation of DODA-EO₂-biotin tubules, as is the case for other tube-forming lipids (Ratna et al., 1992). This is in keeping with the notion that lipids have a tendency to form thermodynamically metastable supramolecular structures and that the system can be trapped in a given state depending on its history (Giulieri and Krafft, 1996; Lasic, 1990, 1993). The absence of tubes at room temperature certainly reflects the influence of the physical state of the lipid chains on tube formation. Although complementary experiments of differential scanning calorimetry indicated that the main transition temperatures of the aggregates were above 38°C (data not shown), it is possible that the optimal arrangement of the molecules existing in a tube is only attained at a lower temperature. Several reports on other tube-forming lipids have indicated that metastability can extend well below the chain melting temperature (Burke et al., 1988) or have stressed the influence of thermal cycling or cooling rate on the degree of conversion of lipids into helices or tubules, further suggesting that these structures are stabilized by an optimal arrangement of the

molecules (Archibald and Yager, 1992; Thomas et al., 1995). Another important observation is that lipid tubules are not the only structures formed by DODA-EO₂-biotin in aqueous solutions. Plates constitute the other major supramolecular aggregate formed by DODA-EO₂-biotin. The relative amount of tubes and plates varied when the products from two different syntheses were compared, although their NMR spectra were almost identical. Tubes and plates seem to constitute two metastable states and the possibility of conversion from tube to plate is suggested by some images of tube endings (Fig. 7). The formation of multilamellar tubes by rolling-up of liposomes has already been proposed in the case of diacetylenic phospholipids (Yager et al., 1988). In the case of DODA-EO₂-biotin tubules, neither lipid tubules observed by cryo-EM (data not shown) nor lipid tubules covered with streptavidin presented images of continuity between tube and plate areas, suggesting that this tube-plate conversion results from severe conditions, such as drying, during negative staining. We also found that tubes disappeared from some suspensions after several months of storage, while tubes still exist in other suspensions after more than 18 months at 4°C. In the former case, tubes could be regenerated after a new step of solubilization with β -OG followed by detergent dialysis at 4°C.

The fact that the tube morphology was influenced by the presence of DOPC (Fig. 6b) indicated that extra lipids could be incorporated into the tubes although the exact level of incorporation is not known. This aspect can be of particular interest to design tubes with other properties or specificities.

One common view of the formation of tubes from chiral lipid molecules is that ribbons wind into open helices due to edge activity and evolve into continuous cylinders (Helfrich, 1986; Helfrich and Prost, 1988; Selinger et al., 1996). Although open helical structures were not observed with DODA-EO₂-biotin, it should be noted that the dialysis method used here takes place over hours and structures formed at earlier stages of the assembly were not characterized in this study. The presence of sheet-like structures and the continuity observed between tube and plate areas suggest

that the formation of DODA-EO₂-biotin tubes is likely to follow a process similar to that mentioned above.

The characteristic supramolecular assemblies formed by other lipids studied here deserve some comments. The extended network of rods formed by DODA-EO₂-NH₂ is very similar to structures obtained with different types of amphiphile or lipid-detergent mixtures (Pffannemüller and Welte, 1985; Fromherz et al., 1986; Kunitake et al., 1981). The fact that these structures were observed in a wide range of experimental conditions and with various staining agents contrasts with our unsuccessful attempts to image them by cryo-TEM and suggests that they could be due to a stacking of discoidal micelles due to a drying effect. A similar interpretation was given by Fromherz et al. (1986) in the case of egg lecithin/cholate mixtures. Another interpretation of such structures is that a rod-shaped micelle winds into a tightly coiled helical strand (Köning et al., 1993).

Both diacetylenic lipids 7 and 8 form twisted ribbons and rod-like structures in the presence or absence of detergent. Although this could reflect a tendency to assemble into helical bilayer structures, none of these lipids form tubular structures similar to DODA-EO₂-biotin. The supramolecular structures formed by DODA-EO₂-biotin and lipids 7 and 8 also differ markedly in the way they interact with streptavidin. Both micelles and liposomes of DODA-EO₂-biotin bind streptavidin in a quantitative manner (Ringler et al., 1997), whereas no binding of streptavidin could be measured with liposomes made of lipids 7 or 8, nor with micelles of lipid 7. This indicates a marked difference in accessibility of the biotin groups in these different structures and constitutes another indication of the difference in the molecular arrangement of the structures formed by these lipids and the tubes of DODA-EO₂-biotin. Most important for their application in crystallization of proteins, ordered arrays of streptavidin were only found with DODA-EO₂-biotin.

In order to further elucidate the relationship between molecular structure and supramolecular morphology, X-ray diffraction and/or solid state NMR analysis of DODA-EO₂-biotin tubes would

be required, as this has already been done with several tube-forming lipids (Thomas et al., 1992; Svenson et al., 1994). The design of new lipid tubes based on principles identified in this study constitutes a challenging objective for developing a general method of helical crystallization of proteins.

Acknowledgements

We are extremely grateful to Professor H. Ringsdorf for the gift of the lipids 5–8. We thank Dr A.P. Bruins and C.M. Jeronimus-Stratingh for help with mass spectrometry measurements and K. Gilissen for his expert technical assistance with the photographic work. This work was supported by the Netherlands Foundation for Chemical Research (SON), the Institut National de la Santé et de la Recherche Médicale and Human Frontier Science Program Organization (RG-430/93 to A.B.). P.R. thanks the Association Française contre les Myopathies for support. P.H. and F.K. were recipients of post-doctoral fellowships from the European Communities and Human Frontier, respectively.

References

- Ahlers, M., 1990. PhD dissertation. Mainz University, Germany.
- Archibald, D.D., Mann, S., 1993a. Template mineralization of self-assembled anisotropic lipid microstructures. *Nature* 364, 430–433.
- Archibald, D.D., Mann, S., 1993b. Structural studies of lipid fibers formed by sphingosine. *Biochim. Biophys. Acta* 1166, 154–162.
- Archibald, D.D., Yager, P., 1992. Microstructural polymorphism in bovine brain galactocerebroside and its two major subfractions. *Biochemistry* 31, 9045–9055.
- Arnold, M., Ringler, P., Brisson, A., 1995. A quantitative electrophoretic migration shift assay for analyzing the specific binding of proteins to lipid ligands in vesicles or micelles. *Biochim. Biophys. Acta* 1233, 198–204.
- Bayer, E., Wilchek, M., 1974. Insolubilized biotin for the purification of avidin. *Methods Enzymol.* 34, 265–267.
- Birault, V., Pozzi, G., Plobeck, N., Eifler, S., Schmutz, M., Palanche, T., Raya, J., Brisson, A., Nakatani, Y., Ourisson, G., 1996. Di(polyprenyl) phosphates as models for primitive membrane constituents: synthesis and phase properties. *Chem. Eur. J.* 2, 789–799.
- Blankenburg, R., 1989. PhD Dissertation. Mainz University, Germany.
- Burke, T.G., Rudolph, A.S., Price, R.R., Sheridan, J.P., Dalziel, A.W., Singh, A., Schoen, P.E., 1988. Differential scanning calorimetric study of the thermotropic phase behavior of a polymerizable, tubule-forming lipid. *Chem. Phys. Lipids* 48, 215–230.
- Chapman, D., Owens, N.F., Walker, D.A., 1966. Physical studies of phospholipids. II. Monolayer studies of some synthetic 2,3-diacyl-DL-phosphatidylethanolamines and phosphatidylcholines containing *trans* double bonds. *Biochim. Biophys. Acta* 120, 148–155.
- Chappell, J.S., Yager, P., 1991. Electrolyte effects on bilayer tubule formation by a diacetylenic phospholipid. *Biophys. J.* 60, 952–965.
- DeTitta, G.T., Edmonds, J.W., Stallings, W., Donohue, J., 1976. Molecular structure of biotin. Results of two independent crystal structure investigations. *J. Am. Chem. Soc.* 98, 1920–1926.
- Doig, A.J., Williams, D.H., 1992. Binding energy of an amide–amide hydrogen bond in aqueous and nonpolar solvents. *J. Am. Chem. Soc.* 114, 338–343.
- Dubochet, J., Groom, M., Müller-Neuteboom, S., 1982. Advances in Optical and Electron Microscopy. In: Cosslett, V.E., Barer, R. (Eds.), Academic Press, London, pp. 107–135.
- Ebato, H., Herron, J.N., Müller, W., Okahata, Y., Ringsdorf, H., Suci, P., 1992. Docking of a second functional protein layer to a streptavidin matrix on a solid support: studies with a quartz crystal microbalance. *Angew. Chemie* 31, 1087–1090.
- Erdelen, C., 1993. PhD Dissertation. Mainz University, Germany.
- Frankel, D.A., O'Brien, D.F., 1994. Supramolecular assemblies of diacetylenic aldonamides. *J. Am. Chem. Soc.* 116, 10057–10069.
- Fromherz, P., Röcker, C., Rüppl, D., 1986. From discoid micelles to spherical vesicles—the concept of edge activity. *Faraday Discuss. Chem. Soc.* 81, 39–48.
- Fuhrhop, J.-H., Köning, J., 1994. Membranes and Molecular Assemblies: The Synkinetic Approach, In: Stoddart, F.F., (Ed.), Cambridge University Press, Cambridge, pp. 99–148.
- Fuhrhop, J.-H., Schnieder, P., Boekema, E., Helfrich, W., 1988. Lipid bilayer fibers from diastereomeric and enantiomeric *N*-octylaldonamides. *J. Am. Chem. Soc.* 110, 2861–2867.
- Fuhrhop, J.-H., Schnieder, P., Rosenberg, J., Boekema, E., 1987. The chiral bilayer effect stabilizes micellar fibers. *J. Am. Chem. Soc.* 109, 3387–3390.
- Georger, J.H., Singh, A., Price, R.R., Schnur, J.M., Yager, P., Schoen, P.E., 1987. Helical and tubular microstructures formed by polymerizable phosphatidylcholines. *J. Am. Chem. Soc.* 109, 6169–6175.
- Giulieri, F., Guillod, F., Greiner, J., Krafft, M.-P., Riess, J.G., 1996. Anionic glucophospholipids—A new family of tubule-forming amphiphiles. *Chem. Eur. J.* 2, 1335–1339.

- Giulieri, F., Krafft, M.-P., 1996. Self-organization of single-chain fluorinated amphiphiles with fluorinated alcohols. *Thin Solid Films* 284–285, 195–199.
- Giulieri, F., Krafft, M.-P., Riess, J.G., 1994. Stable flexible fibers and rigid tubules made from single-chain perfluoroalkylated amphiphiles. *Angew. Chem. Int. Ed. Engl.* 33, 1514–1515.
- Green, N.M., 1975. Avidin. *Adv. Protein Chem.* 29, 85–133.
- Helfrich, W., 1986. Helical bilayer structures due to spontaneous torsion of the edges. *J. Chem. Phys.* 85, 1085–1087.
- Helfrich, W., Prost, J., 1988. Intrinsic bending force in anisotropic membranes made of chiral molecules. *Phys. Rev.* A38, 3065–3068.
- Hendrickson, W.A., Pähler, A., Smith, J.L., Satow, Y., Merritt, E.A., Phizackerley, R.P., 1989. Crystal structure of core streptavidin determined from multiwavelength anomalous diffraction of synchrotron radiation. *Proc. Natl. Acad. Sci. USA* 86, 2190–2194.
- Imae, T., Takahashi, Y., Muramatsu, H., 1992. Formation of fibrous molecular assemblies by amino acid surfactants in water. *J. Am. Chem. Soc.* 114, 3414–3419.
- Kaplun, A., Talmon, Y., Konikoff, F.M., Rubin, M., Eitan, A., Tadmor, M., Lichtenberg, D., 1994. Direct visualization of lipid aggregates in native human bile by light- and cryo-transmission electron-microscopy. *FEBS Lett.* 340, 78–82.
- Köning, J., Boettcher, C., Winkler, H., Zeitler, E., Talmon, Y., Fuhrhop, J.-H., 1993. Magic angle (54.7°) gradient and minimal surfaces in quadruple micellar helices. *J. Am. Chem. Soc.* 115, 693–700.
- Kulkarni, V.S., Anderson, W.H., Brown, R.E., 1995. Bilayer nanotubes and helical ribbons formed by hydrated galactosylceramides: acyl chain and headgroup effects. *Biophys. J.* 69, 1976–1986.
- Kunitake, T., Okahata, Y., Shimomura, M., Yasunami, S., Takarabe, K., 1981. Formation of stable bilayer assemblies in water from single-chain amphiphiles. Relationship between the amphiphile structure and the aggregate morphology. *J. Am. Chem. Soc.* 103, 5401–5413.
- Lasic, D.D., 1990. On the thermodynamic stability of liposomes. *J. Coll. Interface Sci.* 140, 302–304.
- Lasic, D.D., 1993. Liposomes, From Physics to Applications. In: Lasic, D.D. (Ed.), Elsevier, Amsterdam.
- Lubensky, T.C., Prost, J., 1992. Orientational order and vesicle shape. *J. Phys. II* 2, 371–382.
- McCormick, D.B., Roth, J.A., 1970. Colorimetric determination of biotin and analogs. *Methods Enzymol.* 18A, 383–385.
- Mou, J., Yang, J., Shao, Z., 1994. Tris(hydroxymethyl)aminomethane (C₄H₁₁NO₃) induced a ripple phase in supported unilamellar phospholipid bilayers. *Biochemistry* 33, 4439–4443.
- Müller, W., 1993. PhD Dissertation. Mainz University, Germany.
- Nakashima, N., Asakuma, S., Kim, J.-M., Kunitake, T., 1984. Helical superstructures are formed from chiral ammonium bilayers. *Chem. Lett.* 1709–1712.
- Nakashima, N., Asakuma, S., Kunitake, T., 1985. Optical microscopic study of helical superstructures of chiral bilayer membranes. *J. Am. Chem. Soc.* 107, 509–510.
- Papahadjopoulos, D., Vail, W.J., Jacobson, K., Poste, G., 1975. Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. *Biochim. Biophys. Acta* 394, 483–491.
- Pfannemüller, B., Welte, W., 1985. Amphiphilic properties of synthetic glycolipids based on amide linkages. I. Electron microscopic studies on aqueous gels. *Chem. Phys. Lipids* 37, 227–240.
- Rand, R.P., Chapman, D., Larsson, K., 1975. Tilted hydrocarbon chains of dipalmitoyl lecithin become perpendicular to the bilayer before melting. *Biophys. J.* 15, 1117–1124.
- Ratna, B.R., Baral-Tosh, S., Kahn, B., Schnur, J.M., Rudolph, A.S., 1992. Effect of alcohol chain length on tubule formation in 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine. *Chem. Phys. Lipids* 63, 47–53.
- Ringler, P., Müller, W., Ringsdorf, H., Brisson, A., 1997. Functionalized lipid tubules as tools for helical crystallization of proteins. *Chem. Eur. J.* 3, 620–625.
- Schnur, J.M., 1993. Lipid tubules: a paradigm for molecularly engineered structures. *Science* 262, 1669–1676.
- Selinger, J.V., MacKintosh, F.C., Schnur, J.M., 1996. Theory of cylindrical tubules and helical ribbons of chiral lipid membranes. *Phys. Rev. E* 53, 3804–3818.
- Singh, A., Burke, T.G., Calvert, J.M., Georger, J.H., Herendeen, V., Price, R.R., Schoen, P.E., Yager, P., 1988. Lateral phase separation based on chirality in a polymerizable lipid and its influence on formation of tubular microstructures. *Chem. Phys. Lipids* 47, 135–148.
- Svenson, S., Kirste, B., Fuhrhop, J.-H., 1994. A CPMAS ¹³C NMR study of molecular conformations and disorder of *N*-octylhexanamides in microcrystals and supramolecular assemblies. *J. Am. Chem. Soc.* 116, 11969–11975.
- Thomas, B.N., Safinya, C.R., Plano, R.J., Clark, N.A., 1995. Lipid tubule self-assembly: length dependence on cooling rate through a first-order phase transition. *Science* 267, 1635–1638.
- Thomas, B.N., Safinya, C.R., Plano, R.J., Clark, N.A., Ratna, B.R., Shashidar, R., 1992. X-ray diffraction studies of tubules formed from a diacetylenic phosphocholine lipid. *Mat. Res. Soc. Symp. Proc.* 248, 83–88.
- Traub, W., 1958. Possible biochemical implications of the crystal structure of biotin. *Science* 129, 210.
- Uchegbu, I.F., Florence, A.T., 1995. Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry. *Adv. Colloid Interface Sci.* 58, 1–55.
- van Nunen, J.L.M., Stevens, R.S.A., Picken, S.J., Nolte, R.J.M., 1994. Tunable supramolecular structures from clips and baskets derived from glycoluril. *J. Am. Chem. Soc.* 116, 8825–8826.
- Yager, P., Price, R.R., Schnur, J.M., Schoen, P.E., Singh, A., Rhodes, D.G., 1988. The mechanism of formation of lipid tubules from liposomes. *Chem. Phys. Lipids* 46, 171–179.
- Yager, P., Schoen, P.E., 1984. Formation of tubules by a polymerizable surfactant. *Mol. Cryst. Liq. Cryst.* 106, 371–381.

- Yamada, K., Ihara, H., Ide, T., Fukumoto, T., Hirayama, C., 1984. Formation of helical super structure from single-walled bilayers by amphiphiles with oligo-L-glutamic acid-head group. *Chem. Lett.* 1713–1716.
- Zasadzinski, J.A.N., Schneir, J., Gurley, J., Elings, V., Hansma, P.K., 1988. Scanning tunneling microscopy of freeze-fracture replicas of biomembranes. *Science* 239, 1013–1015.
- Zumbuehl, O., Weder, H.G., 1981. Liposomes of controllable size in the range of 40–180 nm by defined dialysis of lipid/detergent mixed micelles. *Biochim. Biophys. Acta* 640, 252–262.