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Stabilization of polymerized vesicular systems: an application of the dynamic molecular shape concept

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Abstract

A series of glycolipid surfactants derived from Tris(hydroxymethyl)acrylamidomethane (THAM) and bearing hydrocarbon or perfluorocarbon tails and an acryloyl group attached to their polar head was prepared to explore the aqueous behavior of the supramolecular systems they form. The dispersion of surfactants was achieved in water under ultrasonication conditions. Hydrocarbon compounds give heterogeneous vesicular assemblies. In the case of perfluorocarbon derivatives homogeneous vesicles were obtained. However after 1-day storage, all these systems fuse. To stabilize these vesicles, polymerization by ultra violet (UV) irradiation was carried out. During this reaction, a precipitation in water was observed for the hydrocarbon surfactants, whereas fluorocarbon structures provide stable vesicles without any alteration of their size. According to these results, the polymerization process was achieved, in the case of hydrocarbon glycolipid, in the presence of different cosurfactants bearing a single hydrocarbon tail or a polyhydroxylated head and a cholesterol terminus. In such conditions, homogeneous stable vesicles were prepared. Moreover, the THAM derived telomers bearing a cholesterol terminus were able to stabilize and reduce the size of vesicles formed with synthetic glycolipid surfactants. The drug encapsulation ability of these systems was investigated by measurement of the release kinetics of a fluorescent dye, carboxyfluorescein (CF), before and after polymerization.

Keywords: Synthetic glycolipids; Telomers, stabilized vesicles; Polymerization; Electron microscopy, molecular shape concept

1. Introduction

The use of liposomes as in vivo drug carriers has been greatly developed during the last 25 years (Ostro, 1987; Gregoriadis, 1988; Fielding, 1991; Barenholz, 2001). The principal objectives of these biocompatible carriers are (1) the increase of drug solubility and stability in vivo, (2) a reduction of the toxicity of the active compounds due to their isolation from the outer environment, (3) an increase in their effectiveness and (4) the eventual targeting of cells and diseased tissue.

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Unfortunately, their rapid elimination from the bloodstream by the reticulum endoplasmic system (RES) greatly limits their therapeutic use.

This fast blood clearance is due to the adsorption on the outer surface of the vesicles of certain blood components, the opsonins, which induce an agglutination and a rapid phagocytosis by macrophages (Scieszka and Cho, 1988; Patel, 1992). In order to limit this phenomenon and prolong their life span in the bloodstream, the structure of the liposomes must meet the following essential criteria: small size (smaller than 120 nm), no charge or zwitterionic form and a high transition phase temperature (T_c) of constitutive surfactants. Moreover, it is well-known that the presence of polyethyleneglycol on the outer surface of vesicles enhances their stability in the blood stream (Senior, 1987; Klibanov et al., 1990; Woodle and Lasic, 1992; Barenholz, 2001).

The other inherent problem linked to the use of vesicles is their unstable structure. After a period of time, they tend to merge to form tubular or spiral structures, the shape of which often depends on the molecule chirality (Kunitake et al., 1981). This behavior greatly limits their use and storage. In order to avoid these drawbacks, the vesicles can be stabilized by using polymerization. This technique which has been the subject of many studies (Gupta et al., 1979; Bader et al., 1985), requires the use of polymerizable synthetic surfactants. The polymerizable moiety may be situated around their polar head or on their hydrophobic chain(s). The polymerized vesicles thus possess a much stronger structural and chemical resistance (Hub et al., 1980). Moreover, they release the substrates trapped in their internal aqueous cavity more slowly (Regen et al., 1981).

We have already described the synthesis of polymerizable double-chain galactosylated surfactants derived from Tris(hydroxymethyl) aminomethane (Tris) and from aspartic acid. These surfactants have an acrylamide group at the end of a hydrophobic chain (Polidori et al., 1996). Without polymerization, the vesicles they form in water fuse over a period of time, forming spiral structures. This evolution can be prevented by polymerizing the vesicles immediately after formation, thus producing particularly stable supramolecular systems.

The work reported herein deals with the synthesis of a new class of double chain galactosylated surfactants derived from Tris and bearing a polymerizable moiety (an acrylamido group) on their hydrophilic head. The study of their behavior in an aqueous environment, before and after polymerization, has been carried out. We observed membrane disruption phenomena during vesicle polymerization. We assumed this behavior is closely linked to a conformational change of bilayer membrane surfactants. According to the polymorphic behavior of lipids and to the dynamic molecular shape concept proposed by Cullis et al. (Madden and Cullis, 1982; Cullis et al., 1991), we synthesized two kinds of surfactants able to stabilize the vesicles during the polymerization process. The capacity of these vesicles to encapsulate and release an active principle has been investigated by studying the diffusion kinetics of a highly hydrophilic fluorescent dye: carboxyfluorescein (CF).

2. Material and method

The progress of the reactions and the homogeneity of the compounds were monitored by thin layer chromatography (TLC Merck 254). Compound detection was achieved by exposure to ultra violet (UV) light (254 nm), by spraying a 5% sulfuric acid methanolic solution, or 5% ninhydrin ethanolic solution (in order to detect the amine-containing compound), then heating at 150 °C. Purifications were achieved by column chromatography over silica gel (Merck 60). Melting points were measured on an electrothermal machine and have not been corrected. The ^{1}H NMR and ^{13}C NMR spectra were recorded using a BRUCKER AC 250. The spectrum settings of the glycosylated compounds were taken from the acetylated compounds before deprotection (in $CDCl_3$), as this facilitates taking a reading. Chemical shifts are given in ppm relative to tetramethylsilane using the deuterium signal of the solvent ($CDCl_3$) as a heteronuclear reference for ^{1}H and ^{13}C. Mass spectra were recorded on a DX 300 JEOL apparatus. Elemental analysis were performed by the service central de microanalyses of

the CNRS in Lyon. Surface tension measurements were made with a KRUSS K12 tensiometer using Lecomte de Nouy's ring method. Sonications were performed by the pulse method power 100% with a titanium probe of 13 mm diameter on a Vibracell sonicator 72412. Reactions were carried out in anhydrous conditions under dry nitrogen. All the solvents were distilled and dried according to standard procedure.

2.1. Preparation of samples of vesicles

Type A and B monomeric glycolipids (10 mg ml^{-1}) were dispersed in deionized water (75 °C) according to conventional ultrasonication procedure. The glycolipids mixture was dissolved in methanol and the solvent was removed under vacuum. The thin film was thus dispersed in deionized water at 70 °C and hand-shaken for 5 min. Then, the dispersion was sonicated for 15 min (titanium probe of 13 mm, 1/1 pulse method). In order to characterize the particles formed, we used three different techniques and apparatus. The solutions obtained were filtered off on a 0.45 μm Dynagard filter and particle sizes were first determined at 25 °C by photon correlation spectroscopy with a Sematech F60 photon correlation granulometer (3 mW HeNe laser source with an angle of 60°). This apparatus can provide size and polydispersity (diffusion coefficient, mean diameter, dispersion and polydispersity). The mean diameter values obtained are the average of three analysis and are evaluated as a number distribution using a monomodal method (a cumulant analysis). The polydispersity index measured was always lower than 0.2. Moreover for all samples the average size of vesicles was measured by analysis and counting of electron micrographs (transmission electron microscopy (TEM) and freeze-fracture electron microscopy (FFEM) techniques).

2.2. Identification of vesicles by transmission electron microscopy and freeze-fracture electron microscopy

The formation of vesicles was observed by TEM according to the negative staining method and by FFEM as previously described (Guedj et al., 1994). The samples were examined using a Philips CM 10 microscope (TEM) or a Zeiss EM 912 electron microscope (Zeiss, Iena, Germany; FFEM).

2.3. Differential scanning calorimetry

The phase transition temperatures and the transition enthalpies were determined after hydration by a known quantity of water, using a Setaram DSC-92 by heating then cooling at different speeds using a Mettler Toledo Star System. Product **A1** (2973 mg) was hydrated with a mixture of water–ethylene glycol 60/40 (hydration rate 60%), product **A2** (2059 mg) has a 59% hydration percentage.

2.4. Polymerization of vesicular solutions

Polymerization reactions were performed in a UV polymerization quartz apparatus (HERAEUS Noblelight TQ 150 with large spectrum 200–600 nm, power 150 W). The solution (50 ml) was carefully deoxygenated by nitrogen bubbling before use. The sample was subjected to UV irradiation for 15 min until the monomer glycolipid was consumed (checked by Thin Layer Chromatography and ^{1}H NMR). A sample of the solution was taken and concentrated under vacuum, then the residue was dissolved in DMSO-*d*6 and analyzed by ^{1}H NMR (disappearance of acrylic protons between 5.5 and 6.5 ppm).

Polymerization in CF solution was performed by adding a water soluble radical initiator, 4,4′-azo bis-4-cyanovaleric acid (five equivalent in relation to monomer concentration) to the preformed vesicular suspension. The mixture was incubated for 5 h at 75 °C until the disappearance of glycolipid monomers (checked by TLC). Vesicle size was checked before and after polymerization by photon correlation spectroscopy.

2.5. Leakage assays

Preparation of encapsulated CF vesicles and leakage studies.

Tris–buffer saline containing 15 mM Tris and 150 mM NaCl was prepared and a solution of CF at a concentration of 100 mM in Tris–buffer saline (15 mM). These solutions were stored in the dark at 4 °C and used to prepare vesicles by the standard methods.

The unincorporated dye was removed by passing 500 μl of the sample over a Sephadex G-25 Column equilibrated in Tris–buffered saline. The vesicle fractions were collected and rapidly diluted with buffer to a lipid concentration of 1 μM.

The vesicles was immediately placed in glass fluorescence cuvettes. The cuvettes were transferred to a thermostated cuvettes holder at 25 °C, and at this time the clock of the actual leakage measurement was started. Measurement of CF release is a standard method for determining liposome permeability. The fluorescence of CF is self-quenched at 100 mM, and release of the dye increases the CF fluorescence because of the dilution-dependent dequenching. We monitored this change using a SPEX-Fluoromax 2 from Jobin Yvon, with the excitation wavelength set to 480 nm, and detecting the emitted light at 530 nm (slits, 0.5 nm).

For all measurements in buffer the initial leakage (F_0) was determined within 30 s from gel filtration. The leakage was followed in each case for 4 h, measuring the fluorescence F_t at regular time intervals after the start.

The maximum intensity F_{max} of the samples was measured after lysis of the liposomes. This reaction is performed by adding a solution of Triton X-100 (10%, v/v) to the sample. The final concentration of Triton X-100 was 80 mM.

The percentage of release R was calculated from the equation:

$$\%R = \frac{F_t - F_0}{F_{max}}$$

2.6. Synthesis

2.6.1. N[(2′,3′,4′,6′-Tetra-O-acetyl-β-D-galactopyranosyl)oxymethyl bishydroxymethyl) methyl] acrylamide (2)

Compound **1** (Polidori et al., 1994) was dissolved (5 g, 8.43 mmol) in 50 ml of a mixture of acetic acid–water (8/2, v/v). The solution was stirred at 70 °C for 2 h and concentrated to dryness under reduced pressure. The residue was dissolved in toluene (40 ml) and the solvent removed under vacuum. The operation was repeated twice. The resulting oil was subjected to column chromatography on silica gel (ethyl acetate) to afford the pure compound **2** as a white powder (2.2 g, 51%), m.p. 46 °C, $[\alpha]_D = -6°$ (c, 1, $CHCl_3$); ^1H NMR (250 MHz, $CDCl_3$) δ 6.61 (1H, s, NH Tris), 6.37–5.71 (3H, m, acryl), 5.40 (1H, dd, H4), 5.18–5.11 (1H, dd, H3), 5.06–5.01 (1H, dd, H2), 4.47 (1 H, d, H1), 4.15–3.88 (4H, m, H5, H6, CH_2OGal), 3.77–3.70 (2H, dd, $C\underline{H}_2OH$), 3.51–3.46 (2H, dd, $C\underline{H}_2OH$), 2.17–1.99 (12H, m, CH_3CO); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 170.13, 170.01, 169.95, 169.76 (CH_3CO), 130.78 (CH_2=), 127.87 (=CH), 101.41 (carbon 1, β anomer), 71.21, 70.91, 69.77, 68.23, 67.22, 63.95, 62.41, 61.86, 61.02 (CH sugar, CH_2 Tris, C Tris), 20.71, 20.65, 20.51, 20.41 ($\underline{C}H_3CO$).

2.6.2. Undecyl isocyanate (3a)

Sodium azide (4.7 g, 72.9 mmol) was dissolved in 30 ml of water at 10 °C. Lauroyl chloride (15.9 g, 72.9 mmol) dissolved in 80 ml of acetone was added dropwise within 20 min. After stirring for 1 h at 15 °C cold cyclohexane (120 ml) was added at the mixture. After decantation, organic layer was dried over sodium sulfate. The solution was stirred at 70 °C for 1 h. Solvent was removed by distillation. Pure compound **3a** was obtained after distillation under reduced pressure as a colorless oil (7 g, 78%); ^1H NMR (250 MHz, $CDCl_3$) δ 3.27 (2H, t, $C\underline{H}_2NCO$), 1.57 (2H, m, $C\underline{H}_2CH_2NCO$), 1.27 (16H, m, $(CH_2)_8$), 0.88 (3H, t, CH_3); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 43.05 ($\underline{C}H_2NCO$), 32.03, 31.44, 29.80, 29.76, 29.73, 29.85, 29.47, 28.63, 22.78 (CH_2 chain), 14.14 (CH_3).

2.6.3. 3-Perfluorohexyl ethyl isocyanate (3b)

Procedure identical to that used for the synthesis of compound **3a**. From 3-perfluorohexyl propanoyl chloride, compound **3b** was obtained as a colorless oil (65%).^1H NMR (250 MHz, $CDCl_3$) δ 3.67 (2H, t, $C\underline{H}_2NCO$), 2.41 (2H, m, $C\underline{H}_2CH_2NCO$); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 35.39 (t, $\underline{C}H_2NCO$), 32.77 (t, $C\underline{H}_2CH_2NCO$); ^{19}F

NMR (235.19 MHz, $CDCl_3$) δ −81.43, −114.92, −122.16, −123.20, −124.06, −126.66.

2.6.4. Bis[(N-(3-perfluorohexyl) ethyl carboxamide)oxymethyl] O-(β-D-galactopyranosyl)oxy methyl N-acrylamidomethane (A1)

Compound **2** (0.64 g, 1.27 mmol) was dissolved in 40 ml of dry toluene. Compound **3b** (1.09 g, 2.81 mmol) and 2,2,2-diazabicyclooctane (DABCO; 100 mg) was added to the solution under an inert atmosphere. The mixture was stirred at 50 °C for 2 h and concentrated to dryness under reduced pressure. The resulting oil was subjected to column chromatography on silica gel (hexane–ethyl acetate, 6/4 v/v). The white powder obtained was dissolved in methanol with a catalytic amount of sodium methylate (100 mg). The mixture was stirred for 3 h. After treatment with an H^+-type resin (Amberlite IRC 50) followed by filtration and evaporation of the solvent, compound **A1** was isolated as a white powder (0.86 g, 61%), m.p. 159–161 °C, $[\alpha]_D = -1.7$ (c, 1, MeOH); ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.67 (1H, s, NH Tris), 6.26–5.62 (3H, m, acryl), 5.40 (1H, d, H4), 5.33 (1H, t, NH carbamate), 5.25 (1H, t, NH carbamate), 5.21–5.13 (1H, dd, H3), 5.03–4.98 (1H, dd, H2) 4.51–3.89 (9 H, m, H1, H6, CH_2 Tris), 3.54 (4H, m, C$\underline{H}_2$NH), 2.44–2.22 (4H, m, C$\underline{H}_2CF_2$), 2.16–1.98 (12H, m, CH_3CO); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 170.16, 170.11, 170.05, 169.96 (CH_3CO), 165.89 (CO carbamate), 131.28 (CH_2=), 126.80 (=CH), 126.80–110.37 (CF_2), 101.63 (carbon 1, β anomer), 71.07, 70.73, 68.97, 68.23, 67.18, 64.26, 63.75, 61.30,59.38 (CH sugar, CH_2 Tris, C Tris), 33.72 (CH_2NH), 31.17 ($\underline{C}H_2CF_2$), 20.79, 20.63, 20.54, 20.47 ($\underline{C}H_3CO$); ^{19}F NMR (235.19 MHz, $CDCl_3$) δ −81.29, −114.60, −122.40, −123.40, −124.14, −126.68; FABMS (*m*-nitrobenzyl alcohol matrix): m/z = 1138 (M + Na); $C_{31}H_{31}F_{26}N_3O_{11}\cdot 3H_2O$(1169): calcd. C, 31.80; N, 3.59; H, 2.65; found C, 31.93; N, 3.54; H, 2.70%.

2.6.5. Bis[(N-undecyl carboxamide)oxymethyl] O-(β-D-galacto pyranosyl)oxymethyl N-acrylamido methane (A2)

Procedure identical to that used for the synthesis of compound **A1**. From compound **2** (1.1 g, 2.18 mmol) and compound **3a** (1.07 g, 5.44 mmol), pure compound **A2** was obtained as a white powder (1.03 g, 65%), m.p. 135–137 °C, $[\alpha]_D = -2.6$ (c, 1, MeOH); ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.96 (1H, s, NH Tris), 6.25–5.60 (3H, m, acryl), 5.39 (1H, m, H4), 5.21–3.86 (19 H, m, CH sugar, NH carbamate, CH_2 Tris), 3.14 (4H, m, C$\underline{H}_2$NH), 2.15–1.98 (12H, m, CH_3CO), 1.49–1.26 (36H, m, $(CH_2)_9$), 0.87 (6H, t, CH_3); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 170.42, 170.23, 170.05,169.85 ($CH_3\underline{C}O$), 165.73 ($\underline{C}$ONH), 156.67 (CO urethane), 131.48 (CH_2=), 126.39 (=CH), 101.70 (carbon 1, β anomer), 70.87, 70.72, 68.81, 68.25, 67.09, 61.21, 59.64 (CH sugar, CH_2 Tris, C Tris), 41.28 (CH_2NH), 31.91–22.68 (CH_2 tail), 20.79–20.56 (CH_3CO), 14.10 (CH_3); FABMS (*m*-nitrobenzyl alcohol matrix): m/z = 754 (M + Na); $C_{37}H_{69}N_3O_{11}\cdot 1H_2O$(749.5): calcd. C, 59.23; N, 5.60; H, 9.20; found C, 59.31; N, 5.59; H, 9.34%.

2.6.6. 5-[(N-undecylcarboxamide)oxymethyl]--5-acrylamido-2,2-dimethyl-1,3-dioxane (5)

Procedure identical to that used for the synthesis of compound **A1**. From compound **4** (Polidori et al., 1994; 1.4 g, 6.51 mmol) and undecyl isocyanate **3a** (1.4 g, 7.16 mmol), pure compound **5** was obtained as a white powder (2.1 g, 78%), m.p. 89–90 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.96 (1H, s, NH Tris), 6.26–5.59 (3H, m, CH_2=CH), 4.90 (1H, t, N$\underline{H}CH_2$), 4.74, 4.69 (2H, d, CH_2O isopropyl), 4.47 (2H, s, C$\underline{H}_2$OCONH) 3.63, 3.58 (2H, d, CH_2O isopropyl), 3.18 (2H, m, C$\underline{H}_2$NH), 1.61, 1.41 (6H, 2s, CH_3 isopropyl), 1.51–1.28 (18H, m, CH_2 tail), 0.87 (3H, t, CH_3); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 165.54 (CONH), 157.53 (CO carbamate), 131.51 (CH_2=), 126.32 (=CH), 98.55 ($\underline{C}(Me)_2$), 64.73 (CH_2OCO), 60.85, 53.52 (CH_2O isopropyl), 41.33 (CH_2NH), 31.93–21.40 (CH_2 tail, CH_3 isopropyl), 14.11 (CH_3).

2.6.7. (N-undecyl carboxamide)oxymethyl bishydroxymethyl N-acrylamidomethane (B1)

Procedure identical to that used for the synthesis of compound **2**. From compound **5** (2.1 g, 0.509 mmol), pure compound **B1** was obtained as a white powder (1.7 g, 90%), m.p. 57–58 °C, ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.71 (1H, s, NH Tris), 6.34–5.69 (3H, m, CH_2=CH), 5.28 (1H, t,

NHCH$_2$), 4.76 (2H, s, OH), 4.31 (2H, s, CH$_2$OCONH) 3.71, 3.51 (4H, dd, CH$_2$OH), 3.18, 3.15 (2H, m, CH$_2$NH), 1.49–1.26 (18H, m, CH$_2$ chain), 0.88 (3H, t, CH$_3$); ^{13}C NMR (62.86 MHz, CDCl$_3$) δ 166.86 (CONH), 157.65 (CO carbamate), 130.60 (CH$_2$=), 127.76 (=CH), 62.50, 62.37 (CH$_2$OH, CH$_2$OCO), 61.95 (C Tris), 41.34 (CH$_2$NH), 31.85–22.66 (CH$_2$ chain), 14.09 (CH$_3$); FABMS (*m*-nitrobenzyl alcohol matrix): $m/z = 367$ (M + Na); $C_{17}H_{32}N_2O_5$(344): calcd. C, 59.28; N, 8.13; H, 9.36; found C, 59.31; N, 8.10; H, 9.33%.

2.6.8. Hydroxymethyl (N-undecyl carboxamide)-oxymethyl O-(β-D-galactopyranosyl)oxymethyl N-acrylamidomethane (**B2**)

Procedure identical to that used for the synthesis of compound **A1**. From compound **2** (1.35 g, 2.68 mmol) and compound **3a** (0.62 g, 2.95 mmol), pure compound **B2** was obtained as a white powder (1.12 g, 70%), m.p. 109–110 °C, $[\alpha]_D$ = (c, 1, MeOH); ^{1}H NMR (250 MHz, CDCl$_3$) δ 6.70 (1H, s, NH Tris), 6.25–5.59 (3H, m, acryl), 5.33 (1H, m, H4), 5.10–4.98 (3 H, m, H3, H2, NH carbamate) 4.45–3.64 (10H, m, H1, H5, H6, CH$_2$ Tris), 3.10–3.07 (4H, m, CH$_2$NH), 2.09–1.91 (12H, m, CH$_3$CO), 1.42–1.26 (18H, m, (CH$_2$)$_9$), 0.80 (3H, t, CH$_3$); ^{13}C NMR (62.86 MHz, CDCl$_3$) δ 170.37, 169.85, 169.16, 168.96 (CH$_3$CO), 165.56 (CONH), 155.87 (CO carbamate), 129.93 (CH$_2$=), 126.28 (=CH), 101.64 (carbon 1, anomer β), 69.78, 69.53, 67.85, 67.06, 66.02, 62.83, 62.51, 60.16, 60.00 (CH sugar, CH$_2$ Tris, C Tris), 40.27 (CH$_2$NH), 30.87–21.65 (CH$_2$ chain), 19.78–19.51 (CH$_3$CO), 13.08 (CH$_3$); FABMS (*m*-nitrobenzyl alcohol matrix): $m/z =$ 557 (M + Na); $C_{25}H_{46}N_2O_{10}\cdot 2H_2O$ (570.6): calcd. C, 52.57; N, 4.90; H, 8.06; found C, 53.04; N, 4.82; H, 8.29%.

2.6.9. Bis[O-(β-D-galactopyranosyl)oxymethyl] (N-undecylcarboxa mide) oxymethyl N-acrylamidomethane (**B3**)

Compound **B1** (1.2 g, 3.1 mmol) and mercuric cyanide (2.35 g, 9.3 mmol) were dissolved in dry acetonitrile (60 ml). After stirring for 15 min with drierite (2 g), acetobromogalactose (3.83 g, 9.3 mmol) was added and the mixture sonicated under a nitrogen atmosphere for 15 min at room temperature. The precipitate was filtered off and the solvent removed under vacuum. The syrupy residue was dissolved in ethyl acetate and successively washed with saturated sodium hydrogenocarbonate (60 ml), 10% sodium iodide (30 ml), saturated sodium thiosulfate (60 ml) and water (100 ml). The organic layer was dried over sodium sulfate and then concentrated to dryness under reduced pressure. The crude product was purified by column chromatography over silica gel (ethyl acetate–hexane, 6/4). The white powder obtained was dissolved in methanol with a catalytic amount of sodium methylate (100 mg). The mixture was stirred for 3 h. After treatment with an H$^+$-type resin (Amberlite IRC 50) followed by filtration and evaporation of the solvent, compound **B3** was isolated as a white powder (1.74 g, 67%), m.p. 92–93 °C, $[\alpha]_D = -2.4$ (c, 1, MeOH); ^{1}H NMR (250 MHz, CDCl$_3$) δ 6.80 (1H, s, NH Tris), 6.26–5.62 (3H, m, acryl), 5.39 (1H, m, H4), 5.20–5.13 (2 H, m, H3), 5.08 (1H, t, NH carbamate), 5.03–4.97 (2H, m, H2), 4.50–3.87 (14H, m, H1, H5, H6, CH$_2$ Tris), 3.17–3.14 (2H, m, CH$_2$NH), 2.07–1.98 (24H, m, CH$_3$CO), 1.49–1.26 (18H, m, (CH$_2$)$_9$), 0.87 (3H, t, CH$_3$); ^{13}C NMR (62.86 MHz, CDCl$_3$) δ 170.42, 170.35, 170.21, 170.05, 169.69, 169.54 (CH$_3$CO), 165.66 (CONH), 157.06 (CO carbamate), 131.41 (CH$_2$=), 126.50 (=CH), 101.69, 101.66 (carbon 1, anomer β), 70.86, 70.81, 70.69, 70.62, 68.85, 68.81, 68.58, 68.35, 67.03, 66.99, 64.53, 61.21, 61.16, 59.54 (CH sugar, CH$_2$ Tris, C Tris), 41.26, 40.72 (CH$_2$NH), 31.85–22.66 (CH$_2$ chain), 20.80–20.56 (CH$_3$CO), 14.09 (CH$_3$); FABMS (*m*-nitrobenzyl alcohol matrix): $m/z =$ 719 (M + Na); $C_{31}H_{56}N_2O_{15}\cdot 3H_2O$ (732.7): calcd. C, 49.55; N, 3.73; H, 7.45; found C, 49.71; N, 3.87; H, 7.89%.

2.6.10. 3-(Tritylmercapto) propanoic acid (**6**)

3-Mercaptopropanoic acid (6 g, 56.6 mmol) was dissolved in methylene chloride (50 ml). Triphenyl methyl chloride (17.34 g, 62.3 mmol) previously dissolved in methylene chloride (30 ml) was added dropwise and the mixture stirred for 16 h at 25 °C. The precipitate was filtered off and washed with diethyl ether. Pure compound **6** was isolated as a white powder (18.5 g, 94%), m.p.

203–204 °C, ^{1}H NMR (250 MHz, DMSO-*d*6) δ 7.3 (15H, m, phenyl), 2.2 (4H, m, CH_2S, CH_2CO), ^{13}C NMR (62.86 MHz, DMSO-*d*6) δ 173 (COOH), 144.25, 129–126.4 (phenyl), 66 ($Ph_3\subseteq S$), 33 (CH_2S), 126.50 (=CH), 26.5 (CH_2CO).

2.6.11. Cholesteryl 3-(tritylmercapto) propanoate (7)

Compound **6** (5 g, 14.3 mmol), *N*,*N*-dimethylaminopyridine (1.74 g, 14.3 mmol) and dicyclohexylcarbodiimide (3.57 g, 17.16 mmol) was dissolved in methylene chloride (50 ml). Cholesterol was added to the mixture and the solution stirred at room temperature for 2 h. The precipitate of dicyclohexylurea was eliminated by filtration. After concentration under vacuum the crude product was purified by recrystallization in diethyl ether–methanol mixture. Pure compound **7** was obtained as a white powder (10.1 g, 97.4%) m.p. 132–133 °C, $[\alpha]_D = -14.9$ (c, 1, CH_2Cl_2); ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.2 (15H, m, phenyl), 5.3 (1H, m, H_6 cholesterol), 4.5 (1H, m, H_3 cholesterol), 2.35 (2H, m, CH_2S), 2.2 (2H, m, CH_2CO), 2.18 (2H, m, H_4 cholesterol), 0.93 (3H, s, H_{19} cholesterol), 0.84 (3H, m, H_{21} cholesterol), 0.79 (6H, m, H_{26}–H_{27} cholesterol), 0.60 (3H, s, H_{18}), ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 170.8 (CO ester), 144.3 (C5 cholesterol), 139.2, 129.2–126.2 (phenyl), 122.9 (C6 cholesterol), 74.5 (C3 cholesterol), 64.2 ($Ph_3\subseteq S$), 56.7 (C14 cholesterol), 56.2–11.9 (cholesterol, CH_2S).

2.6.12. Cholesterol mercaptopropanoate (8)

Compound **7** (5 g, 6.98 mmol) and triethylsilane (0.8 g, 6.98 mmol) were dissolved in methylene chloride at 0 °C. Trifluoroacetic acid (22 ml) dissolved in methylene chloride (200 ml) was added dropwise to the cold mixture. After stirring for 1 h, the mixture was concentrated under vacuum. The crude product was dissolved in diethyl ether, and washed with $NaHCO_3$ saturated solution. After drying and concentration under vacuum the crude product was recrystallized in diethyl ether–methanol mixture. Pure compound **8** was obtained as a white powder (3.15 g, 95%) m.p. 69–70 °C, $[\alpha]_D = -20.5$ (c, 1, CH_2Cl_2); ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.3 (1H, m, H_6 cholesterol), 4.6 (1H, m, H_3 cholesterol), 2.67 (2H, m, CH_2S), 2.53 (2H, m, CH_2CO), 2.27 (2H, d, H_4 cholesterol), 1.54 (1H, t, SH), 0.94 (3H, m, H_{19} cholesterol), 0.80 (3H, m, H_{21} cholesterol), 0.77 (6H, m, H_{26}–H_{27} cholesterol), 0.60 (3H, s, H_{18}), ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 171 (CO ester), 143.9 (C5 cholesterol), 139.6, 129.5–126.4 (phenyl), 122.9 (C6 cholesterol), 74.5 (C3 cholesterol), 56.8 (C14 cholesterol), 56.2–11.9 (cholesterol, CH_2S).

2.6.13. Typical synthesis of type C-telomer cosurfactants by radical telomerization: compound C

To a solution of Tris(hydroxymethyl)acrylamidomethane (THAM) (3.38 g, 19.3 mmol) in anhydrous methanol (50 ml) warmed to 40–50 °C under a nitrogen atmosphere, were added compound **8** (0.611 g, 1.29 mmol) and freshly recrystallyzed α,α′azo-bis(isobutyronitrile) (42 mg, 0.26 mmol) dissolved in anhydrous tetrahydrofuran (5 ml). The mixture was refluxed under stirring and the progress of the reaction monitored by TLC (hexane–ethyle acetate, 9:1 v/v). After refluxing for 3 h, THAM was entirely consumed. The mixture was concentrated under reduced pressure and the resulting oil was precipitated by adding diethyl ether under vigorous stirring. The solution was filtered off. The precipitate was dissolved in water and freeze-dried. Compound **C** was obtained as a white powder (3.9 g, 60%). The DPn (20) was determined by comparison of the area of the cholesterol methyl 19 signal (singlet $\delta = 0.66$ ppm, 3H) with the area of the signals ascribed to THAM hydroxyl protons ($\delta = 5$ ppm, 3H).

3. Results and discussion

The hydrophobic moiety of structure **A** surfactants is made up of two chains of eight or 11 hydrocarbon or perfluorocarbon atoms (Fig. 1).

These chains are grafted to the THAM hydroxyl groups by carbamate bonding. The galactose residue is linked by its anomeric carbon to the third hydroxyl group of THAM. This sugar must supply the hydrophilicity required for the hydrosolubility of the amphiphilic agents and furthermore allows the recognition of polymerized vesicles by the galactose specific lectins of cells

such as, for example, kupffer cells, *Kluyveromyces bulgaricus* yeasts or melanome B16 cells (Coulon et al., 1998). The galactopyranose is just an example, other glycosides (such as mannopyranose) could be fixed by their anomeric carbon to the THAM hydroxyl.

3.1. Synthesis of double chain surfactants *A1–A2*

During the synthesis of the components, carried out under optimal conditions, we draw attention to the following (Scheme 1).

(a) After hydrolysis of the acetal group of compound **1**, the carbon chains were introduced to both THAM hydroxyl groups, using carbamate bonding.
(b) The condensation of hydro and perfluocarbon isocyanates was carried out in an inert atmosphere using toluene as a solvent at 50 °C. Reaction was performed in the presence of DABCO yielding 75% of peracetylated **A1** or **A2** derivatives. The pure components were isolated by chromatography on a silica gel column (eluent, AcOEt–hexane).
(c) The perfluorcarbon-isocyanate was prepared beforehand in three steps from the 2H, 2H, 3H, 3H perfluorononanoic acid as follows.
  (i) Formation of the acyl chloride with an excess of thionyl chloride.
  (ii) Conversion of the acyl chloride into azido derivative using sodium azide in water and acetone.
  (iii) Finally, heating the mixture in dry cyclohexane produces an isocyanate group by the well-known Curtius rearrangement with an overall yield of 65%.

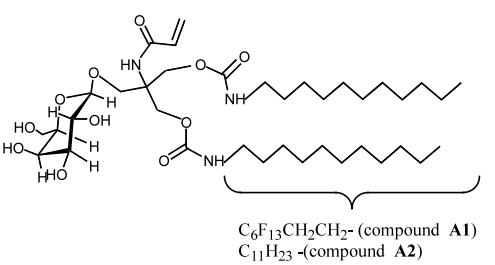
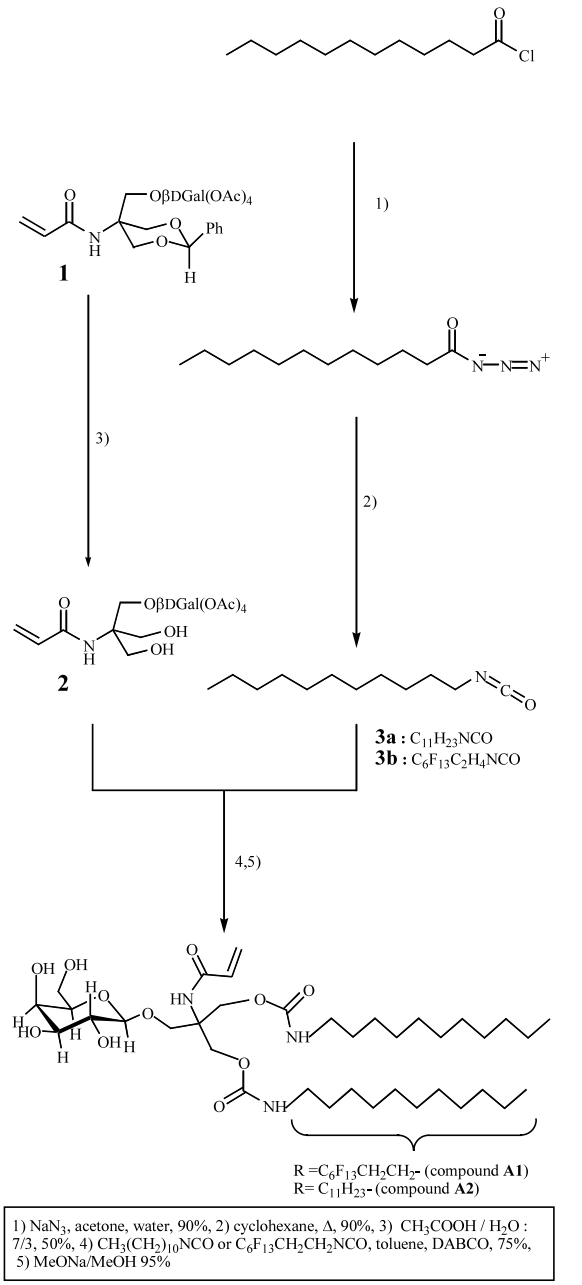


Fig. 1. Structure of type-**A** compounds.



Scheme 1. Synthesis of hydro and perfluoroglycolipids **A1** and **A2**.

After hydrolysis of the acetyl groups by transesterification with a catalytic amount of sodium methylate in the methanol, the pure glycolipids **A1** and **A2** were isolated as white amorphous powders.

3.2. Auto-aggregation behavior and polymerization of compounds *A1–A2*

Differential scanning calorimetry (DSC) measurements were performed on compounds **A1**–**A2**. They have low phase transition temperatures (17 °C for **A1** and 30 °C for **A2**). The compounds **A1** or **A2** showed poor solubility in water at 70 °C. Dispersion in distilled water of these products could be achieved by the classic sonication procedure at this temperature for 15 min (titanium probe 13 mm, power 100%, pulse method 1/1). A clear bluish solution was obtained (Tyndall effect). The analysis of the dispersion by photon correlation spectroscopy indicated the presence of particles, the sizes of which varied from 100 to 150 nm. We noted that the dispersion of these surfactants in a phosphate buffer solution (0.1 M, pH 7.5) did not modify their aqueous behavior, as we obtained similar sized particles. The study by TEM after negative staining by phosphotungstic acid, carried out few hours after storage at 25 °C, confirmed the vesicles formation (Fig. 2a). However the beginning of a coalescence phenomenon was noticed. Beside small particles, we observed the formation of large vesicles (until 500 nm).

The formation of these aggregates showed the instability and the quick fusion of the initial vesicular system. At room temperature, after 1-week storage for compound **A1** and 1-day for compound **A2**, the vesicles fused to form tubular systems (Fig. 2b) and a precipitate. The quick fusion of the vesicles is probably due to the fact that the storage and the manipulation of the vesicles solution were carried out at 25 °C, i.e. within the temperature zone of the phase transition of the surfactants. This was particularly the case of the **A2** compound having a 30 °C T_c. This problem of stability underlined the need for preparing polymerized vesicular structures.

Thus, we tried to polymerize these double chain surfactants in water. After ultrasonication as reported above, and deoxygenation of the solution, the vesicles were submitted to UV irradiation. The progress of the reaction was monitored by thin layer chromatography and by ^{1}H NMR. Whichever surfactant was used, hydro or fluorocarbon type, within 10 min of irradiation, all the monomer was depleted. While the polymerization of the fluorocarbon amphiphiles (compound **A1**) was absolutely conclusive and allowed us to obtain some particularly stable aggregates (Fig. 3), the hydrocarbon surfactants (compound **A2**) precipitated after 5 min of irradiation.

This precipitation could be due to the transition from a phase L_α bilayer to an inverted hexagonal phase HII which is both hydrophobic and thoroughly insoluble in the water. This transition from one phase to the other may be interpreted as an evolution from a cone-shape to an inverted cone-shape and could be due to a dehydration of

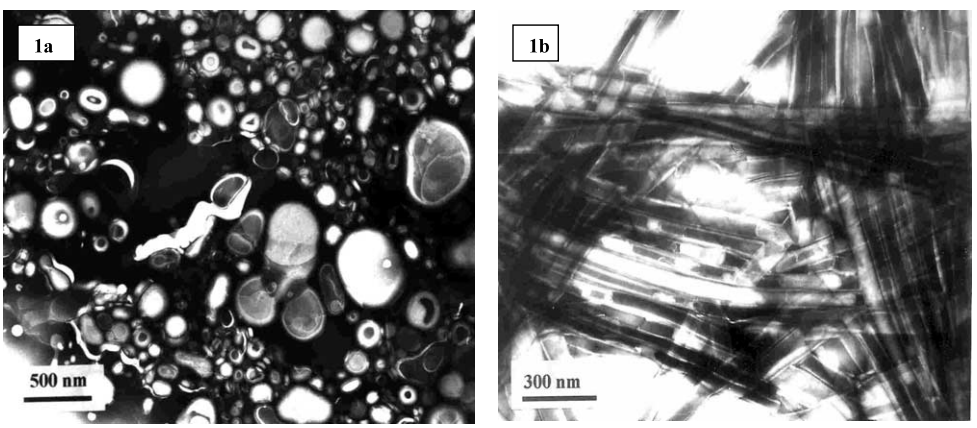


Fig. 2. Electron micrograph of aqueous dispersion of **A2** after sonication (1a) and after 1 week storage (1b). Negative staining by phosphotungstic acid.

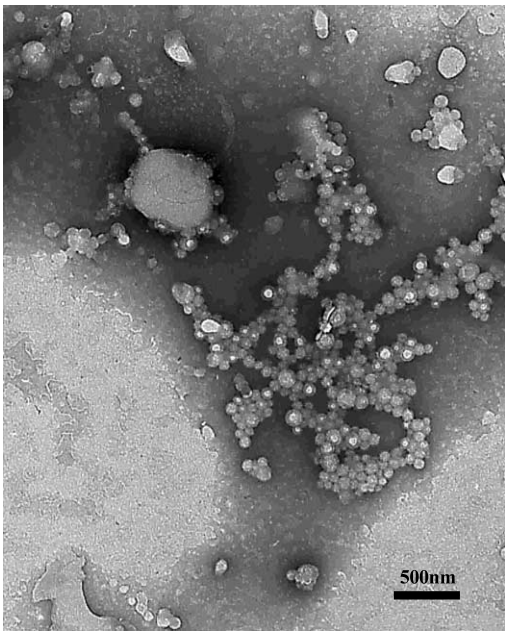


Fig. 3. Electron micrograph of aqueous dispersion of **A1** after UV polymerization (negative staining by phosphotungstic acid).

the glycosidic polar heads of the membrane surfactants.

We suspected that the reticulation of the polar heads during the polymerization of the compound **A2** created an imbalance in the volumes between the hydrophilic and hydrophobic parts. This could be explained by an increase of the lateral constraints between surfactants, due to the reticulation of the hydrophilic heads. By bringing together the hydrophobic parts and by increasing considerably these steric interactions, the polymerization disrupts the fragile equilibrium of the bilayer. This induced the precipitation of the polymer which has become too hydrophobic. In the vesicles prepared from the fluorocarbon surfactants **A1**, the lateral tensions are compensated by the great organization and the rigidity of the hydrophobic layer. In fact, it is well-known that fluorophilic interactions between the perfluocarbon chains in a polar environment are greatly favored (Kunitake, 1992). In this environment, the interactions between perfluorocarbon chains are strong enough to avoid the disorganization during the polymerization. They probably reduce the bilayer shape modification induced by the polymerization process and thus they should limit the steric interactions suggested above. In such conditions, we can assume they limit the polymerization reactions of the polar heads and decrease the average degree of polymerization (DPn) of the macromolecules created by the UV irradiation (the DPn is equal to the amount of repeating units of surfactants).

Following these previous considerations, in order to avoid any precipitation during the polymerization process, two possible modifications may be considered,

(i) separating the polymerizable group from the polar head by using a variable length spacer arm;
(ii) introducing cosurfactants, which can be seen as cone-shaped molecules in the bilayer. Such compounds would counterbalance the lateral constraints brought about by the reticulation of the hydrophilic heads.

Israelachvili was the first to take into account the geometric considerations of surfactants in order to explain the nature of the aggregates they form in water (Israelachvili, 1985). Many authors already have demonstrated that the driving force which leads to the formation of membranes is due to the asymmetric sharing of different cone-shaped surfactants between the internal and outer monolayers. Thus, Yamano et al. (1993) measured the fluidity of a membrane composed of a phosphatidylethanolamine (PE)–phosphatidylcholine (PC) mixture (thanks to the ESR technique) and showed that the PE was preferentially distributed in the internal layer so as to increase the piling up and the stability of the membranes. This dynamic molecular shape concept was the subject of a major study by Cullis et al. (1991), Madden and Cullis (1982). The later demonstrated that the addition of non-ionic detergents (octylglucoside, Triton X100) in appropriate concentrations to a phospholipid mixture, which usually resulted in phases HII, enabled the formation of a lamellar phase L_{α}. These results were interpreted in terms of molecular shape where the 'inverted cone shaped' detergents combine in a complementary fashion with 'cone shaped' phospholipid to result

in a stable bilayer structure. More recently, Cullis and Madden (1996) showed that the utilization of appropriate concentrations of phospholipids endowed with a 2000–5000 Da PEG head in a dioctadecylphosphatidylethanolamine (DOPE)–cholesterol mixture enabled the transition from a phase HII to a lamellar phase L_α. Subsequently, the PEG moieties led to the curvature of the bilayers by steric obstructions on the polar heads and the formation of small vesicles. Actually, the utilization of lipids with a voluminous and hydrated hydrophilic part seems to offset or at least, to limit the transition L_α-HII.

Finally, we showed in previous work that in the presence of various THAM derived telomer cosurfactants stable vesicles can be prepared from lactobionolactone derived glycolipids (Guedj et al., 1994). The glycolipids aggregation behavior was altered by the addition of the cosurfactant. Instead of multilayered vesicles of high polydispersity along with non-closed membranes, we observed in the presence of the cosurfactant the formation of unilamellar vesicles with a small average diameter. Based upon these last results, we chose to synthesize two kinds of cosurfactants having a possible complementary shape to compound **A2**.

- A substrate having a structure similar to **A**: the hydrophilic lipophilic balance (HLB) and the volumetric ratio of the hydrophilic and hydrophobic parts should be modulated by glycosylation of one or two Tris hydroxyl groups (Fig. 4). The length of the hydrophobic chain should be limited to 11 carbon atoms.
- A cholesterol derived cosurfactant (Fig. 5) with a hydrophilic head formed by a polyTris telomer. The control of the surfactant shape should be afforded by the average DPn of the polar head.

3.3. Synthesis of surfactants *B1*, *B2* and *B3*

The synthesis of these cosurfactants was carried out under the same conditions as those previously described for double chain amphiphilic compounds **A1**–**A2** (Scheme 2). The three compounds were synthesized from THAM derivatives. **B1** was synthesized by grafting undecyl isocyanate on isopropylidene THAM, followed by an acidic hydrolysis of the isopropylidene group (overall yield, 70%). **B2** was prepared with an overall yield of 29% by grafting one equivalent of undecyl isocyanate in the presence of DABCO. Finally **B3** was obtained by glycosylation of **B1** according to the Helferich reaction in a 75% yield.

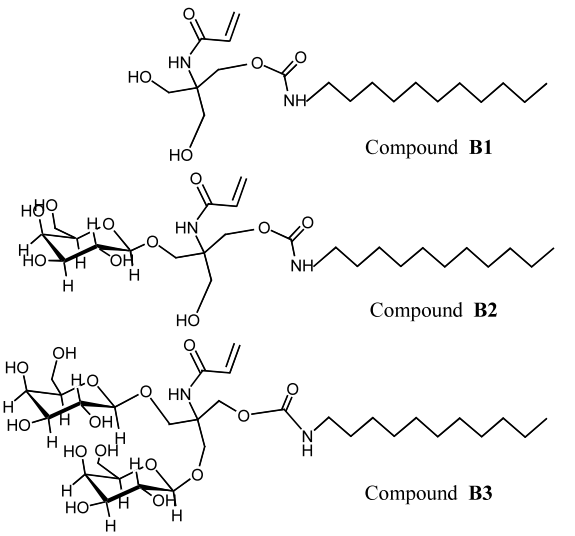


Fig. 4. Structure of type-**B** compounds.

The three compounds were purified by chromatography on a silica gel column (eluent ethyl acetate–hexane). The glycosyl compounds **B2** and **B3** were deacetylated by transesterification in methanol with a catalytic amount of sodium methylate.

The surfactant **B1** showed a poor solubility in water. So, it has not been possible to carry out

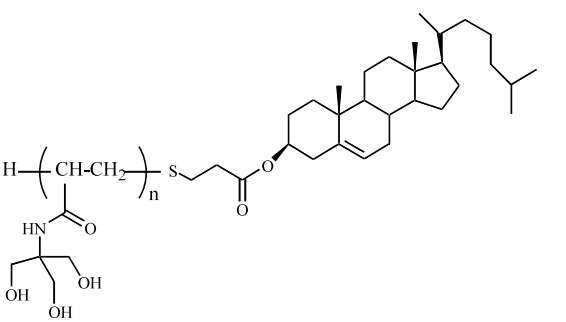
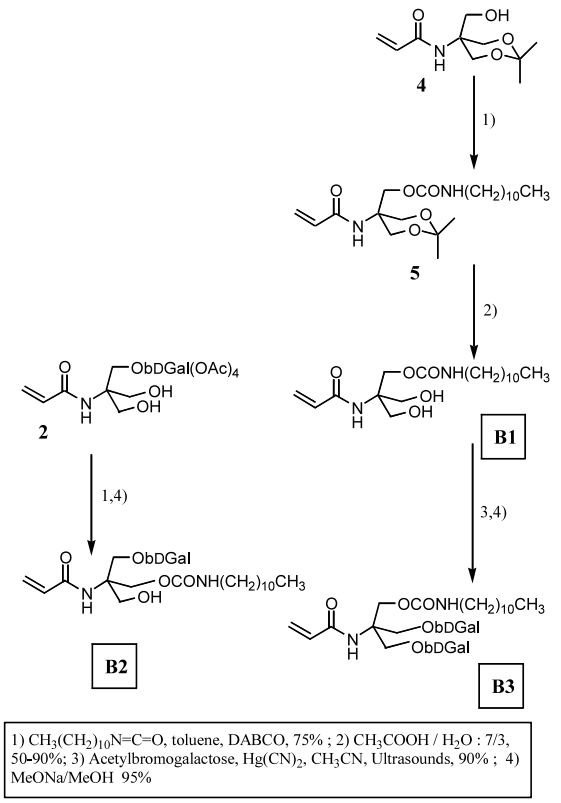


Fig. 5. Structure of type-**C** compound.



Scheme 2. Synthesis of single-chain cosurfactants **B1**, **B2** and **B3**.

any study of its water behavior. On the other hand, the two glycosylated products **B2** and **B3** displayed a sufficient solubility in water. We measured their surface tension by using Lecomte de Noüy's ring method (Fig. 6). The results indicated the formation of aggregates (probably a micellar system) at a concentration of 0.8 mM for the mono-galactosylated derivative **B2** and 2.4 mM for the digalactosylated derivative **B3**. The evolution of these CMC could be correlated with the increase of the surfactants hydrophilic part. Some similar observations were recorded in respect of partially protected glycosylated surfactants (Goueth et al., 1995).

*3.4. Synthesis of cosurfactant **C***

Taking into account the recent work of Madden and Cullis (1996) on the phospholipidic PEG derivatives and the results we obtained (Guedj et al., 1994), we chose to prepare a THAM derived telomer surfactant, in fact a cholesterol derived cosurfactant **C** bearing a polyTris polar head. This kind of surfactant is endowed with a voluminous hydrophilic poly-alcoholic head and should enhance the stability of vesicles formed in water. The ability of the cholesterol moiety to get and fix into the membrane bilayer justified their use as the hydrophobic part of the surfactant.

The synthesis of the hydrophilic telomer part is achieved easily through a radical polymerisation of THAM in the presence of a mercaptan derived cholesterol as a transfer reagent (Scheme 3).

Radical telomerization is a technique similar to radical polymerization. It differs by the fact that the growth of the macroradical is stopped by a transfer reaction to a molecule called the telogen. The telomers which are obtained have much lower weights since one can check the DPn of the macromolecule. Moreover, the telogen agent allows the functionalization of the extremity of the polymeric backbone. By grafting a cholesterol residue or a hydrophobic chain on the telogen, one can modify the lipophilic hydrophilic balance of the macromolecule.

The transfer agent is synthesized very easily from cholesterol in three steps.

– First we achieved the protection of the mercaptopropanoic acid thiol group by condensing with triphenylmethyl chloride in dichloromethane. The tritylmercaptopropionic

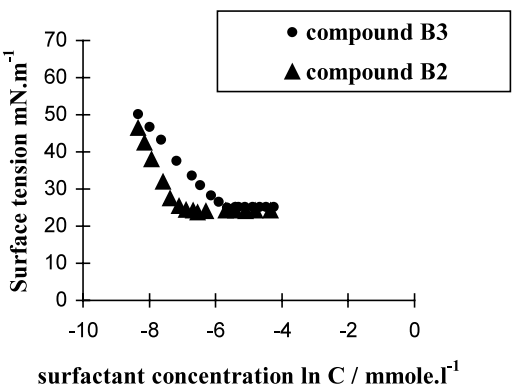
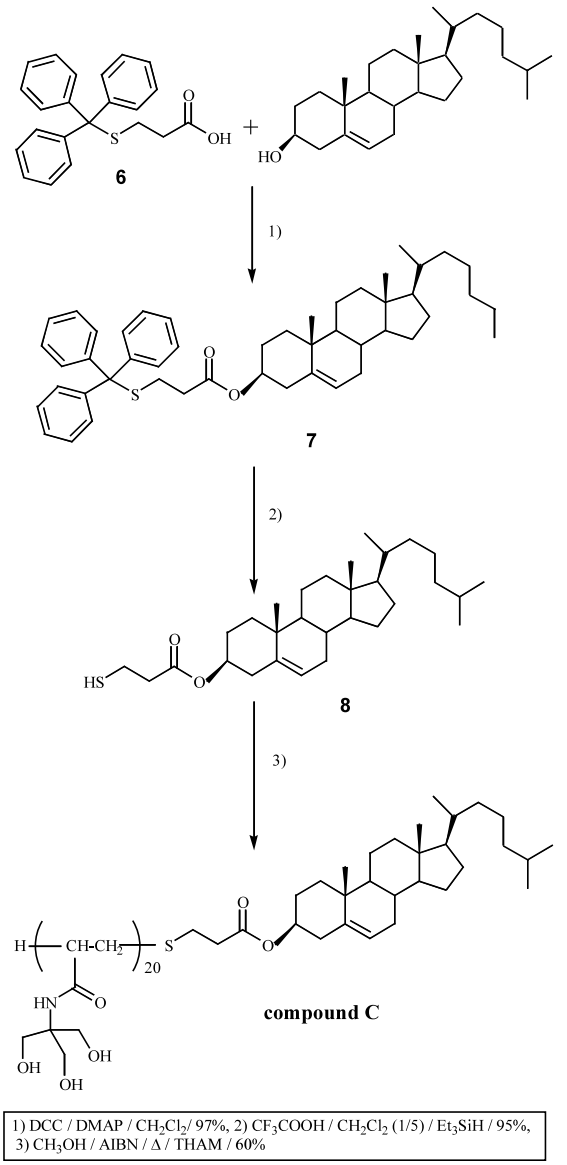


Fig. 6. Plots of surface tension at 25 °C in water vs. ln concentration of cosurfactants **B2** and **B3**.

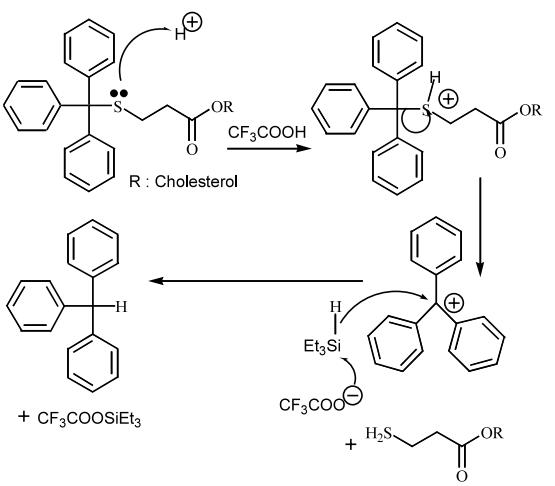


Scheme 3. Synthesis of the cholesterol derived THAM telomer **C**.

acid, so obtained, is grafted on the cholesterol hydroxyl function in the presence of dicyclohexylcarbodimide (DCC) and dimethyl aminopyridine (DMAP). The trityl group hydrolysis was carried out in dichloromethane using a scavenger such as triethylsilane (Pearson et al., 1989) and trifluoroactetic acid (Scheme 4). The cholesterol-derived telogen was obtained with a 87% overall yield.

– Telomerization experiments were performed in methanol at 65 °C under a nitrogen atmosphere, using α,α′-azobisisobutyronitrile (AIBN) as radical initiator. The AIBN concentration in the reaction mixture was roughly ten times lower than the telogen's (Pucci et al., 1988). The initial ratio of THAM and cholesterol-derived telogen **8** concentrations used are $R_0 = 14$. These proportions were chosen taking into account previous results obtained with THAM telomerization (Pucci et al., 1988, 1991; Pavia et al., 1992). Each experiment was pursued until the complete disappearance of the monomers (checked by TLC and ^{1}H NMR spectroscopy).

The DPn of the macromolecule was determined by ^{1}H NMR (DMSO-*d*6 as solvent) by comparing the area of typical signals of cholesterol and THAM monomer (19-methyl signal of cholesterol at 0.65 ppm singlet and the signal of the hydroxylic protons of the THAM adducts at about 4.5–5 ppm, multiplet). Since the area of these two signals each corresponds to three protons, the ratio of their respective areas gives the DPn of the telomers. An initial concentration ratio [THAM]–[telogen] of 15 resulted in a DPn of 20. The telomer is well-soluble in water. The determination of the CMC by using Lecomte de Noüy's ring method at 25 °C was difficult to carry out on



Scheme 4. Deprotection of trityl group.

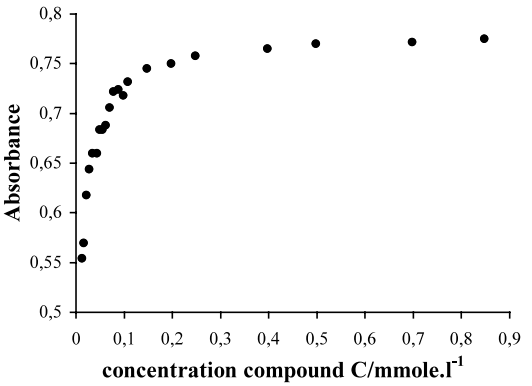


Fig. 7. CMC determination of compound **C** by Menger and Portnoy method, absorbance measurement at 610 nm and 25 °C of an 1.10^{-6} M aqueous pinacyanol chloride solution with variable surfactant concentrations.

this amphiphile telomer because the rate of the surfactant migration onto the surface of the solution was very slow. The equilibrium of the sample being difficult to reach, the CMC value of this compound was measured (Fig. 7) using Menger and Portnoy (1967) method. The absorbance of an aqueous solution of a dye, pinacyanol chloride, was measured at 610 nm and 25 °C in the presence of different amounts of surfactant. After successive dilutions of a surfactant mother solution, the CMC is reached when a change of the aqueous solution absorbance incline is observed. The cholesterol derived telomer surfactant shows a CMC of 0.20 ± 0.05 mM.

*3.5. Aqueous dispersion and polymerization of a mixture of single-chain cosurfactants **B1**–**B3** or **C** and double chain surfactant **A2***

Dispersion tests on the double-chain compound **A2** in the presence of increasing amounts (molar ratio from 10 to 50%) of cosurfactants **B1**–**B3** have been performed (Table 1). The dispersions were prepared to an aqueous overall concentration of 10 mg ml^{-1}. These mixtures were submitted to ultrasound under the conditions previously described. At this stage, in order to check the vesicles formation, samples were examined by TEM after negative staining with phosphotungstic acid (TEM). After filtration through a 0.45 μm filter, the dispersions were analyzed by photon correlation spectroscopy and electron microcopy (FFEM and TEM) to determine the size of the particles obtained.

We can underline that the dispersion tests carried out in Tris–buffer solution at pH 7 again did not show any significant evolution of the aggregates size and shape. This can be explained by the non-ionic nature of the polar heads.

The samples were then polymerized by UV irradiation. The progress of the polymerization reaction was monitored by TLC and by ^{1}H NMR spectroscopy. The analysis of the dispersion by TEM and by light scattering allowed us to determine the nature and the size of the particles formed. The results obtained with various ratios of surfactants **A2** and **B** are reported in Table 1.

The results prompt the following remarks.

– When we disperse double-chain amphiphile **A2** with the cosurfactant **B1** (non-glycosylated surfactant) in a molar ratio of 45%, we observe the formation of vesicles. In these conditions, the polymerization can be carried out and produces a clear bluish translucent solution of polymerized vesicles (Fig. 8f). Outside of this strict margin of concentration the precipitation

Table 1
UV polymerization and dispersion tests of compound **A2** with variable concentration of surfactants **B1**–**B3**

	Molar ratio (%)	Before polymerization (nm)[a]	After UV polymerization (nm)[b]
Compound **B1**	25	Precipitate	–
	35	98 nm ± 40	Precipitate
	45	98 nm ± 53	70 ± 36
	55	Precipitate	–
Compound **B2**	20	68 ± 44	Precipitate
	25	70 ± 47	67 ± 36
	30	120 ± 61	128 ± 50
	35	81 ± 42	109 ± 70
	45	101 ± 56	Precipitate
Compound **B3**	25	104 ± 58	Precipitate
	35	154 ± 84	90 ± 53
	45	104 ± 58	80 ± 62
	55	120 ± 73	Precipitate

[a] Size of particles determined by photon correlation spectroscopy and checked by TEM.

[b] Total polymerization after 15 min of UV irradiation.

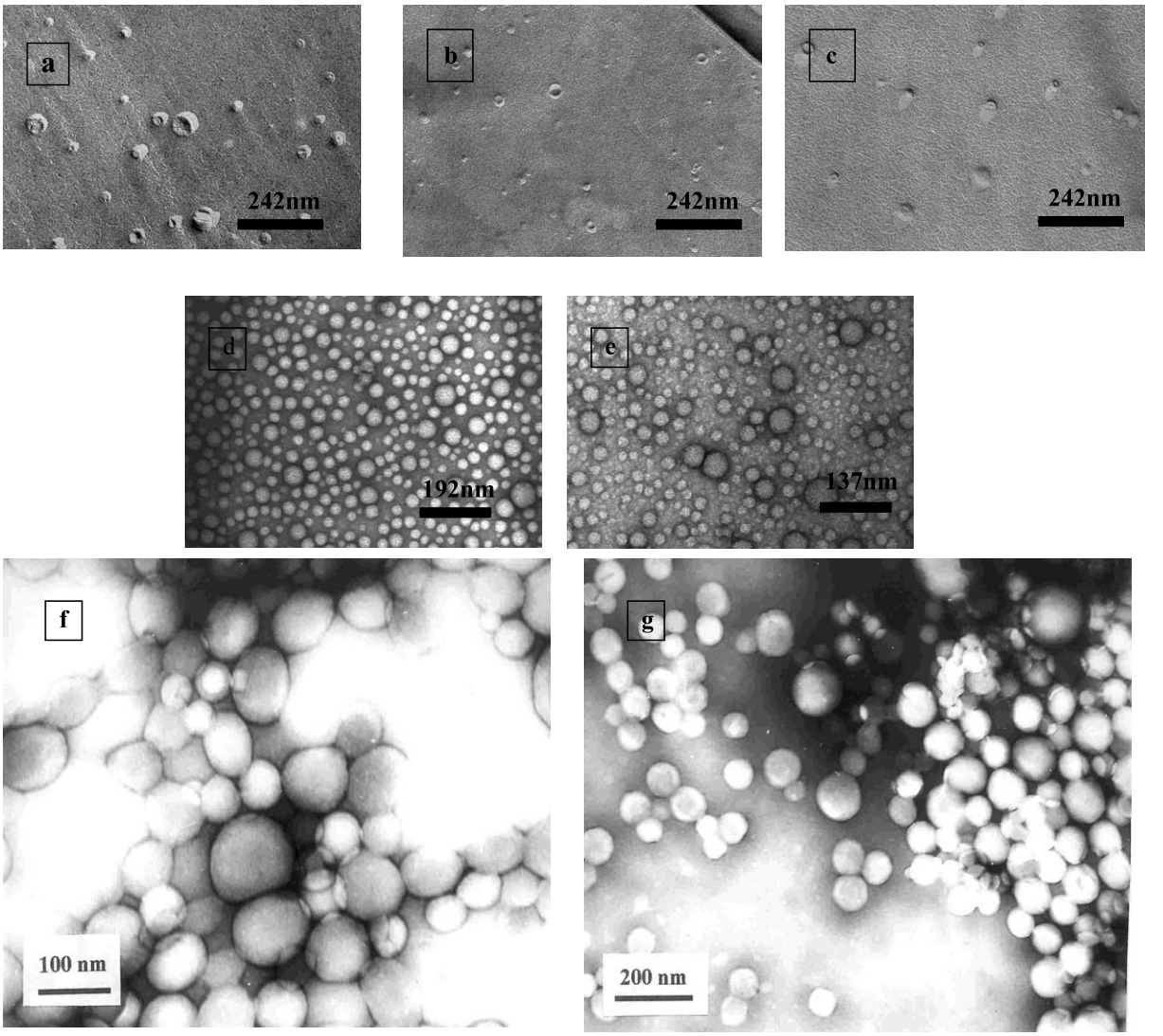


Fig. 8. Electron micrographs of polymerized surfactant **A2** vesicles (a) in the presence of a molar ratio of 10% **C**, (b) 20% **C** and (c) 30% **C** (cryofracture microscopy). TEM (negative staining by phosphotungstic acid) of polymerized surfactant **A2** vesicles (d) with a molar ratio of 10% **C** (e) with a molar ratio of 20% **C** (f) with a molar ratio of 45% **B1** and (g) with a molar ratio of 45% **B3**.

of the polymer occurs. The stabilization of the lamellar phase L_α during the copolymerisation can only be explained by the piling up of complementary cone-shaped surfactants which enables, by compensation of shape, the reduction of reticulation constraints of the polar heads, and inhibits the transition to the inverted phase HII.

– The cosurfactants **B2** and **B3** are soluble in water. Whatever their concentration used during the dispersion of compound **A2**, we can observe the production of vesicles. Their size measured by photon correlation spectroscopy is in the 70–150 nm range. The vesicles sizes were also checked by TEM and FFEM. These techniques showed that the photon correlation

spectroscopy induces a 10–20% average overestimation of the particle diameters (Table 1 and Fig. 9). For well-defined ratios of the single to double chain surfactant concentrations, the polymerization by UV irradiation checked by ^{1}H NMR leads to a solution of polymerized vesicles with a low polydispersity (Fig. 8f and g and Fig. 9).

– In order to stabilize the membrane during polymerization and to avoid any precipitation, a particular molar ratio between the single and double chain surfactants is required. It depends entirely on the volumes of the hydrophilic compounds **B2** and **B3**. Molar ratios must range from 25 to 35% for the monogalactosylated compound **B2** and 35–45% for the digalactosylated compound **B3**. These proportions do not conflict with the theory of the compensation and equilibrium stated before if one takes into account the values of the CMC of both products. The monogalactosylated derivative **B2** actually presents a CMC nearly three times lower than its digalactosylated homologue. Their solubility in the form of monomers in water shows the same ratio. During the ultrasonic dispersion of the surfactant mixtures, a state of equilibrium is set up for the cosurfactants **B2** or **B3** between the vesicular system (the hydrophobic membrane) and the solution. In any case, the total concentration of cosurfactants **B2** or **B3** is, at the most, equal to 0.6 mM, therefore less than their CMC, which are, respectively, equal to 0.8 and 2.4 mM. One can admit that this state of equilibrium between the monomer form and the form associated with the vesicle depends, in those conditions, on their aqueous solubility. Thus, with the same initial proportions, the concentration in the membrane of the monogalactosylated derivative **B2** (the most 'hydrophobic' compound) should be higher than that of its digalactosylated equivalent **B3**. Hence, in order to stabilize the vesicle during the radical polymerization, it seems reasonable to use the derivative **B3** in greater proportion than the derivative **B2**.

Ueno (1989) showed that below its CMC, octyl glycoside is divided between the membrane phase (phospholipidic bilayer) and the water phase. The partition coefficient of this non-ionic surfactant is independent of the detergent concentration. These observations are in good agreement with our results. Moreover, our observations can be correlated with the work already reported by Inoue (1996) about the partition behavior of surfactant between a phospholipid bilayer and the aqueous phase. This author proposed that the membrane-water partition coefficient of surfactant, expressed by $Kx = x_s^l/x_s^w$ (where x_s^l and x_s^w refer to the mole fractions of surfactants in lipid phase and in aqueous phase, respectively), is connected to their CMC. The results obtained with different ionic or non-ionic surfactants and egg-yolk phosphatidyl

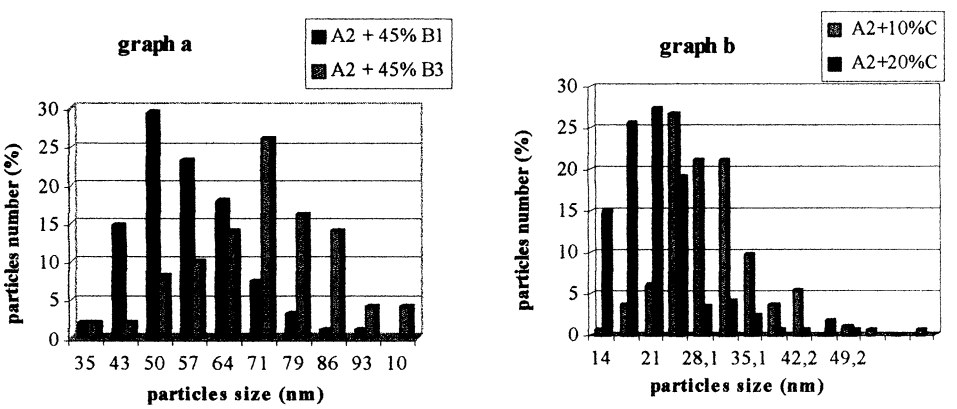


Fig. 9. Distribution of observed polymerized liposomes diameter as determined by analysis of electron micrographs, mixture of compounds **A2** and **B** (graph a). Mixture of compounds **A2** and **C** (graph b).

choline allowed him to draw up a linear correlation between ln *Kx* and ln CMC. We can assume that this relation could be extended to our synthetic glycolipids bilayers. Thus, according to this hypothesis the **B3** partition coefficient should be three times lower than that of **B2**, and the molar ratio of this surfactant necessary to stabilize the membrane during the polymerization process should be three time lower than observed. Under this condition, we can assume that the effective molar ratio of **B3** effectively included in the bilayer membrane and actually necessary to avoid any membrane precipitation is significantly lower (at less three times) than the 35–45% added to the solution.

– The polymerization of the vesicles affects their size very little. The solutions obtained are very stable. We do not observe any aggregation after 1-year storage in sterile conditions.Thus, our assumption which involves a bilayer disorganization during the polymerization due to a deformation of the cone-shape surfactants, seems quite true. Moreover, it is possible to avoid this drawback by adding to the aqueous dispersion a cosurfactant (such as **B2** or **B3**) having a complementary shape to glycolipid **A2**. In order to verify the validity of this concept, we used an another kind of cosurfactant: the compound **C** bearing a cholesterol moiety as hydrophobic part and a THAM derived polyhydroxylated head.The dispersion and polymerization experiments of glycolipid **A2** in the presence of cosurfactant **C** were carried out under the conditions previously described. Results obtained are reported in Table 2.Analysis of these dispersions through photon correlation spectroscopy and electronic microscopy pictures deserve several comments. The addition of increasing amounts of cholesterol telomer derivative causes an important decrease of the vesicular system size (from 120 to 20–27 nm). Even with low concentrations (2.5%) the dispersion of the **A2** compound is easy and induces the formation of small unilamellar vesicles (77 nm). The enhancement of the molar ratio of telomer cosurfactant provokes an important decrease of the vesicles size until the level of 20 nm (Fig. 8a–c) which is reached with a concentration of 20%. For higher concentrations of cosurfactant (30%), because size does not decrease, we can assume that the additional telomer cosurfactant does not mix with the membrane, and forms micelles. However at this stage, cosurfactant **C** could act as a membrane detergent. Such membrane dissolutions have already been reported by Edwards et al. (1997) with pegylated phospholipids. They showed, by using the cryoTEM technique, that molar ratios higher than 10% of this compound caused a membrane dissolution of EPC liposomes. They observed a progressive transition towards open vesicular structures, flats disks, and then the formation of mixed micelles. These characteristic structures can also be shown by cryofracture (Zemb et al., 1999). The analysis by this last technique of **A2**–**C** mixtures have not shown such aggregates even with cosurfactant molar ratios higher than 30% (Fig. 8). By using TEM or cryofracture techniques, we observed very small vesicular systems. The TEM pictures, allowed us to count and measure the size of the vesicles, which were in good agreement with the results obtained by photon correlation spectroscopy (Fig. 9, graph b). These compounds have thus very interesting spreading properties close to these already reported for the pegylated phospholipids.

– The **C** compound is also able to stabilize the vesicles during polymerisation. From a molar ratio of 10%, the effects of shape compensation

Table 2
UV polymerization and dispersion tests of compound **A2** with variable concentration of surfactant **C**

	Molar ratio (%)	Before polymerization[a]	After polymerization[b]
Compound **C**	2.5	77 ± 32	Precipitate
	5	66 ± 33	Precipitate
	10	28 ± 1	25 ± 5
	20	20 ± 3	21 ± 1
	30	20 ± 2	16 ± 1

[a] Size of particles determined by photon correlation spectroscopy and checked by TEM.
[b] Total polymerization after15 min of UV irradiation.

induced by this compound eliminates the membrane destabilization phenomenon during polymerization of the **A2** compound polar heads. The vesicle polymerization takes place without any noticeable modification of their size. However, a cosurfactant molar ratio lower than 10% can not stabilize the vesicles. This phenomenon was already observed with the **B** type derivatives.

– After lyophilization, whatever the experimental conditions used (ultrasound and/or very high temperatures), none of the samples of polymerized vesicles can be dispersed again in solution. Whatever the mixtures used (**A2**/**B** or **A2**/**C**), it seems that the polymerization of membrane components form only short polymers with low DPn. After lyophilization, rehydration of the samples cannot put these oligomers back into vesicular shape.The very small size of the particles obtained from the **A2**–**C** mixtures caused us to wonder if these aggregates, in spite of the electronic microscopy pictures, are really vesicular systems containing an internal aqueous cavity. Therefore, in order to check their structures, we studied their ability to encapsulate and to release a hydrophilic fluorescent dye, CF.

3.6. Permeability of A2–C type liposomes formulations measured by CF release

In order to specify the drug carrier ability of supramolecular systems derived from compounds **A2**–**B** or **A2**–**C** mixtures, we carried out various encapsulation tests and release kinetics of a fluorescent dye, CF, before and after vesicle polymerization.

Measurement of CF release is a spectrophotometric standard method for determining liposome permeability (Weinstein et al., 1984). CF is less lipophilic than its parent compound fluorescein, and has a much lower tendency to associate with phospholipid membranes. Consequently, in order to mark the aqueous compartment of liposomes, the use of this compound in place of fluorescein is recommended. The property of CF which lends itself to in situ determinations of liposome contents, is its ability to self-quench. Indeed, at high concentrations, the fluorescence of CF is very much reduced compared with diluted samples, probably because of intermolecular interactions. Thus, the basic approach when using this compound is to prepare liposomes containing a high quenching concentration of CF (usually about 100 mM in 15 mM Tris at pH 7.4).

First, it is noteworthy that, because of the very high CF concentration used to reach the autoquenching phenomenon, it was impossible to disperse the fluorocarbon surfactant **A1** in this aqueous solution whatever the temperature or the ultrasonication conditions. In fact, **A1** shows a lower solubility than **A2** because of their higher hydrophobic perfluorocarbon chains. Thus, we could not achieve any measurement with the **A1** compound.

With the **A2** surfactant, the vesicular systems could be obtained easily in the aqueous CF solution. The CF release kinetics (Fig. 10) show a very high permeability of these vesicular systems.

Within 15 min all the CF entrapped in the vesicles is released. This phenomenon can easily be explained by the instability and the quick fusion of these particles. This behavior has been previously displayed through photon correlation spectroscopy and by electron microscopy. Moreover, the release kinetics are carried out at 25 °C. In this temperature range close to the T_c of the **A2**

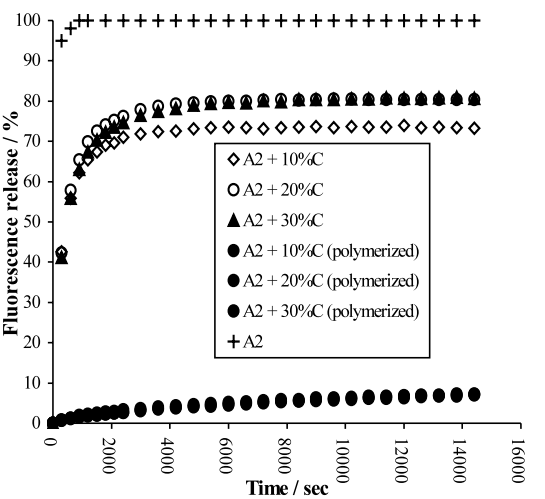


Fig. 10. Time course of CF release from **A2** to **C** liposomal formulations before and after polymerization.

compound (T_c, 30 °C), the transition from a solid state of the membrane to a fluid one occurs with local disorders due to the coexistence of gel and liquid crystal states. These disorders favor the vesicles fusion but also the transmembrane crossing of the encapsulated substrates. The vesicles formed with various molar ratios of **A2** and **C** surfactants release the CF less quickly than the vesicles made of the single **A2** amphiphile. After 4 h, 73–80% of the CF is released. It is well-known that increasing amounts of cholesterol within the phospholipid bilayer induce a decrease of its permeability coefficient. However, in this particular case, the **C** compound molar ratio inside the vesicle seems to have a negligible impact on the CF release kinetic. No conclusion can be drawn from this observation though, because the vesicles made of the **A2** compound show too rapid kinetic diffusion of the CF. In order to check the impact of the amount of **C** cosurfactant on the membrane permeability, it will be necessary to use a less permeable membrane made, for instance, from egg yolk phospholipids.

The opacity of the CF solution prevents the polymerization of the vesicles by UV irradiation. Thus, we performed this reaction by chemical means at a temperature of 70 °C through a radical initiator, ACVA (4,4′-azobis-4-cyanovaleric acid). After 5 h, the whole monomer had disappeared (checked by TLC). The **A2**–**C** vesicle size checked by photon correlation spectroscopy before and after polymerization did not change during the reaction.

In these conditions, the polymerization of preformed **A2**–**C** vesicles dramatically decreases the CF release kinetics. Whatever the concentration of **C** compound in the vesicles, after 4 h, only 7–8% of the encapsulated CF has diffused through the membrane. This last result is in good agreement with the studies already reported in this field by Regen et al. (1981). Whatever the **C** compound concentration incorporated into the membrane, the vesicles exhibit a noticeable encapsulation ability. This last result show that the very small aggregates previously obtained by using high concentrations of cholesterol derived amphiphile telomer, show a classical vesicle behavior.

4. Conclusion

The polar head reticulation (by UV irradiation) of vesicles prepared from hydrocarbon surfactants disorganizes the bilayer membrane and leads to a precipitate. This phenomenon is not observed in the case of fluorocarbon surfactants because of the favored interactions which are set up between the perfluorocarbon tails in an aqueous environment. However, the introduction, during the aqueous dispersion of compound **A2**, of complementary cone-shaped single chain surfactants induces the formation of unilamellar vesicles. Finally, polymerization leads to homogeneous and very stable vesicles.

The work reported herein confirms the dynamic molecular shape concept already proposed by Cullis and Madden (1982) for a supramolecular polymerized system. The curvature modulation by suitable single chain polymerizable cosurfactants can favor the vesicles stabilization during polymerization.

We have shown that this shape compensation model could be extended in this particular case to another type of non-polymerizable cosurfactant: a cholesterol derived polyhydroxylated telomer. Its non-ionic polar head volume can be easily adjusted. Indeed, non-polymerizable cone-shape surfactants derived from cholesterol allow the stabilization of the vesicle bilayer membrane, notably during the polymerization process. Moreover, this new telomer surfactant family shows a remarkable ability to form very small unilamellar vesicles. Their characteristics could be compared with those of the pegylated phospholipids already studied by the scientific community.

The grafting of these polar heads derived from polyhydroxylated telomers onto hydrophobic double chains enables the creation of a new surfactant family. Their physicochemical characteristics in relation to the volume head variation (which can be modulated by the average DPn) is being studied in our laboratory.

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