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Vesicles made of glycophospholipids with homogeneous (two fluorocarbon or two hydrocarbon) or heterogeneous (one fluorocarbon and one hydrocarbon) hydrophobic double chains

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Abstract

The vesicle-forming ability of the new anionic double chain glycophospholipids **1–4**, with either two hydrocarbon or two perfluorocarbon chains, or a mixed double chain (one fluorinated, one hydrogenated), was investigated. When dispersed in water, **1a–c,e**, **2b,c** and **4b,c** readily gave heat-sterilizable vesicles, 30–70 nm in diameter. The galactose and mannose-based fluorinated vesicles were also highly stable on aging. The 6-substituted glucose derivatives **3** formed tubules that reversibly interconverted into vesicles, depending on temperature. The leakage rate in buffer of carboxyfluorescein or calcein from vesicles made from **1a–c,e**, **2b,c** and **4b,c** depended on the sugar ($t_{1/2}$ galactose > mannose > glucose). It decreased significantly with increasing fluorination and length of the hydrophobic tails. The mixed perfluorocarbon/hydrocarbon-tailed amphiphiles were found to be miscible with both the two fluorocarbon chains and the two hydrocarbon chains derivatives. Such admixing tended, however, to increase the small unilamellar vesicles' permeability. In buffered serum, all the vesicles investigated were highly permeable, but incorporation of cholesterol or DSPC in vesicles made of **1e** significantly reduced their permeability in serum. The new vesicle and membrane components have i.v. maximum tolerated doses as high as 500 mg/kg body weight in mice; hemolytic activity sharply decreases with increasing degree of fluorination.

Keywords: Perfluoroalkylated sugar; D-Glycose (alkyl phosphate); Glycophospholipid; Fluorinated surfactant; Liposome; Drug delivery; Membrane permeability

1. Introduction

Amphiphiles with appropriate structures, when dispersed in excess water above their crystal-to-liquid crystal transition temperature, tend to self-organize into supramolecular assemblies and, in particular, into vesicles [1–7]. These structures are of interest as models of biological membranes and for the understanding of membrane properties. They can also be loaded with diverse molecules, and constitute promising drug carrier and delivery systems [2–7].

However, liposomes made from monodisperse phospholipids often display chemical and physical instability. Polydisperse phospholipids such as lecithins from egg yolk or soybean are often used, but they are sensitive to hydrolysis and oxidation, giving degradation products that can be toxic [8]. Liposomes, when oxidized phospholipids are present, can also lose their encapsulation properties [5]. Incorporation of cholesterol is usually needed to achieve stable liposomes with effective potential as drugs carriers [9].

These drawbacks and limitations have prompted the preparation of synthetic amphiphiles that are less fragile and form more stable membranes [1,10,11]. Recent reports indicate that amphiphiles with perfluoroalkyl chains in their hydrophobic tails confer some unique characteristics to the supramolecular self-assemblies they form [11–25]. Thus, fluorinated vesicles, i.e., vesicles with a hydrophobic and lipophobic fluorinated film within their bilayer membrane, showed increased stability and reduced permeability to drugs and probes compared to conventional amphiphile-based vesicles. Prolonging the circulation time of vesicles in the blood stream [26–28], and proper targeting the carrier to specific tissues, are among the present challenges in the field. The latter objective could be addressed by incorporating recognition markers such as sac-

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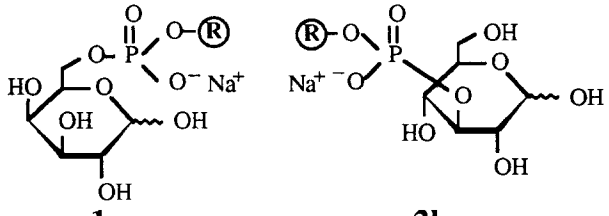
charides, polysaccharides, glycoproteins, or glycolipids onto the surface of liposomes [7,28–31].

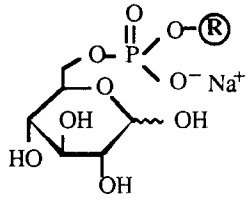
These reasons led us to synthesize a new family of amphiphilic glycophospholipids with various sugar-derived polar heads and various hydrophobic tails (Table 1) and to investigate their self-aggregation capability. Fluorocarbon chains have been shown to provide a powerful driving force for self-aggregation, to improve the stability of bilayer membranes, and to increase their hydrophobicity [11–23,32]. The totally hydrogenated analogs **1d**, **3d** and **4d** were prepared to assess the impact of the fluorinated chains on vesicle properties. The mixed hydrophobic tails, with both a fluorocarbon and a hydrocarbon chain present, were expected to facilitate the homogeneous dispersion of these anionic, sugar-labelled amphiphiles within membranes made from other lipids, including both standard phospholipids and fluorinated ones [11]. The association of these mixed tail glycophospholipids with homologs having two hydrocarbon chains, or with cholesterol, or with DSPC were therefore also investigated. The new amphiphiles' hydrophobic tails are linked to the hydrophilic head group either directly or through a spacer group which facilitates the adjustment of the amphiphile's total length. The anionic phosphate linkage was of interest since certain biolipids contain this function in their hydrophilic head group [33–35] and because it allows the introduction of a relatively hidden negative charge into the bilayer structure. As glycophospholipids are known to play a role in various biological recognition processes [28–31], various sugar moieties, i.e., galactose, glucose and mannose, were utilized as hydrophilic head groups to allow an assessment of their influence on aggregation behavior and vesicle properties.

Table 1
Structure of glycophospholipids **1–4**

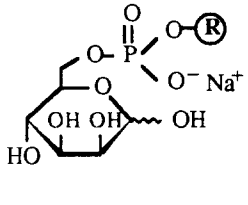


1a-e
6-galactose derivatives

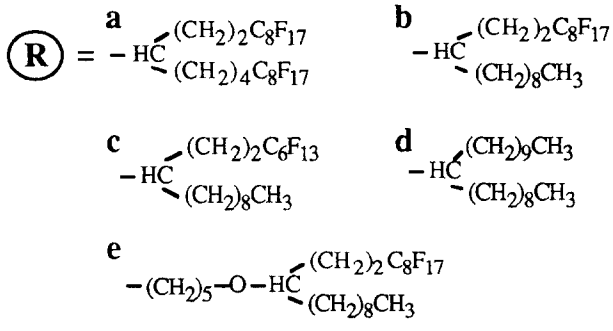
2b-c
3-glucose derivatives



3b-d
6-glucose derivatives



4b-d
6-mannose derivatives



2. Materials and methods

2.1. Materials

The anionic perfluoroalkylated double chain glycosyl phosphodiesters and their hydrocarbon analogs **1–4** were prepared using a three-step hydrogen-phosphonate route [36,37]. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) came from Fluka (Mulhouse, France). Carboxyfluorescein (CF), calcein (CA), deoxycholic acid, Hepes buffer (*N*-2-hydroxyethyl-1-piperazine-*N'*-ethanesulfonic acid), cholesterol (CH) and Sephadex G-50 were purchased from Sigma Chimie (St. Quentin Fallavier, France). Water for injectable preparations (Laboratoire Fandre, Ludres, France) was employed for all surfactant dispersion preparations.

Vesicles were prepared using a Branson model B-30 sonicator, 350 W, at power dial 2 (scale 1–10), equipped with a 13-mm diameter titanium probe. Transmission electron microscopy was effected using a Philips microscope (model CM 12, 80 kV). Vesicle sizes and size distributions were measured at 25°C by laser light scattering (LLS) spectroscopy with a Coulter N4MD submicron particle analyzer. Distributions were considered as homogeneous when the suspensions contained more than 95% of the vesicles in one single population. Fluorescence measurements were achieved on a Perkin-Elmer LS50B spectrofluorimeter equipped with a temperature-controlled cell holder and a magnetic stirring device. Differential scanning calorimetry (DSC) measurements were performed on a Setaram DSC 92 apparatus, and turbidity values with a Philips PU 8650 spectrometer equipped with a thermoregulated cell.

2.2. Liposome preparations with a single component

Vesicle preparation involved the hydration of the phosphodiester (50 mM) in water for 30 min at 50°C, followed by sonication for 2 min at the same temperature. Homogeneous populations of small unilamellar vesicles (SUV) were obtained. Storage at room temperature led to a gel in

the case of **1d**, **3b**, **3c** and **4d** after 2 days, 24 h, 1 min and 12 h, respectively.

2.3. Liposome preparations from mixtures of components

*(a) From **1a** and **1b**, or **4d** and **4b***: a mixture of amphiphiles **1a** and **1b**, or **4d** and **4b** (1:2 molar ratio, 50 mM) was dissolved in methanol (0.5–1 ml). A thin dry film of the mixture was produced on the walls of a round-bottomed flask by rotoevaporation. The film was then hydrated with 0.5 ml of water for 30 min at 50°C, and dispersed by sonication for 2 min at the same temperature. Homogeneous distributions of SUVs were obtained.

*(b) From **1a** and **1d**:* the same procedure as described in (a) was used to prepare a dispersion of **1a**/**1d** (1:1 molar ratio, 50 mM).

*(c) From **1b** and cholesterol, or **1e** and cholesterol*: amphiphile **1b** or **1e**, and CH (2:1 molar ratio, 50 mM) were dissolved in a chloroform-methanol (1:1, v/v) mixture. A thin dry film of the mixture was obtained by rotoevaporation. The film of **1b**/CH was then hydrated with 0.5 ml of water for 1 h at 65°C and dispersed by sonication for 1 h at the same temperature. The film of **1e**/CH was hydrated with 0.5 ml of water for 30 min at 50°C and sonicated (25 min, power dial 2) at the same temperature. Homogeneous populations of SUVs were obtained in both cases.

*(d) From **1e** and DSPC*: the same procedure as described in (c) for **1e**/CH was used to prepare a dispersion of **1e**/DSPC (1:4 molar ratio, 50 mM) (3 min at power dial 2, then 6 min at power dial 4).

2.4. Phase transition temperature determinations

(a) By DSC: ~7 mg of powdered amphiphile and ~10 μl of deionized water were directly weighted into the sample pan in which glycol was added as a cryoprotector in a 1:1 (w/w) ratio. After sealing, the pan was placed in the DSC cell compartment and cooled with liquid nitrogen. Measurements were effected between −30 and 90°C with a heating rate of 2°C per min.

(b) By optical density: turbidity changes of the dispersion of liposomes (20 mM) were recorded at 550 nm. The temperature was raised gradually from 3 to 65°C and then lowered gradually to 3°C; the time interval between two measurements was of ca 15 min. Each determination has been repeated twice.

2.5. Sterilization and long-term stability studies

Aqueous dispersions of vesicles (5 ml, 50 mM), prepared as indicated above and bottled in 6 ml sealed flasks sparged with nitrogen, were sterilized in an autoclave at 121°C for 15 min under 10^5 N m^{-2}. These suspensions were transferred into ampoules which were sealed and stored at 25°C. At regular intervals of time, an ampoule

Table 2
Particle size of dispersions of vesicles of glycophospholipids

Compounds	After preparation		After sterilization		After aging at 25°C		
	Size [nm] (S.D.)	Amount [%]	Size [nm] (S.D.)	Amount [%]	Age	Size [nm] (S.D.)	Amount [%]
Galactose							
1a	45 (15)	95	40(12)	95	10 months	28 (5)	95
1b	30 (9)	100	40 (11)	100	10 months	40 (8)	98
1c	35 (8)	97	42 (6)	97	10 months	35 (12)	96
1d	30 (9)	100	32 (4)	96	2 days [b]	33 (10)	38
						90 (22)	34
						900 (240)	28
1e	65 (23)	97	60 (15)	97	10 months	70 (25)	97
Glucose 3-substituted							
2b	30 (8)	100	47 (13)	98	20 days [a]	32 (10)	97
2c	30 (6)	99	35 (8)	99	10 days [a]	40 (12)	98
Glucose 6-substituted							
3b	30 (6)	100	[c]		1 day [b,d]	30 (6)	100
3d	50 (28)	17	[c]		8 days [d]	150 (48)	46
	190 (70)	23				1000 (150)	53
	3100 (460)	60					
Mannose							
4b	30 (7)	100	40 (12)	100	10 months	40 (11)	96
4c	32 (9)	100	47 (10)	96	10 months	47 (17)	96
4d	40 (12)	100	50 (15)	100	12 h [b]	40 (12)	100

S.D., standard deviation [nm] in parentheses; [a] then the dispersions precipitated; [b] then the dispersions turned into a gel; [c] not attempted; [d] aging measured on a non-sterilized dispersion.

1: 6-substituted galactophospholipid; **2**: 3-substituted glucophospholipid; **3**: 6-substituted glucophospholipid; **4**: 6-substituted mannophospholipid.

a: $[C_8F_{17}(CH_2)_4][C_8F_{17}(CH_2)_2]CH$-; **b**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CH$-; **c**: $[C_6F_{13}(CH_2)_2][CH_3(CH_2)_8]CH$-; **d**: $[CH_3(CH_2)_9][CH_3(CH_2)_8]CH$-; **e**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CHO(CH_2)_5$-.

Table 3
Particle size of dispersions of mixed vesicles made of glycophospholipids

Compounds	After preparation		After sterilization		After aging at 25°C		
	Size [nm] (S.D.)	Amount [%]	Size [nm] (S.D.)	Amount [%]	Age	Size [nm] (S.D.)	Amount [%]
1a/1b (1:2) [b]	25 (8)	99	20 (6)	99	10 months	27 (9)	99
4b/4d (2:1) [b]	40 (10)	100	38 (12)	98	15 days [a]	70 (18)	90
						700 (130)	10
1a/1d (1:1) [b]	25 (6)	76			30 days	32 (4)	63
	60 (10)	15	[c]			55 (7)	22
	130 (22)	5				170 (31)	7
	4300 (640)	4				5300 (530)	8
1b/CH [e] (2:1) [b]	20 (6)	98	30 (7)	97	10 months	35 (5)	96
1e/CH [e] (2:1) [b]	50 (15)	95	28 (13)	95	10 months	40 (12)	99
DSPC [e]	43 (13)	98	45 (10)	96	7 days [d]	135 (43)	67
						1500 (400)	33
1e/DSPC [e] (1:4) [b]	20 (5)	91	27 (8)	91	15 days	30 (8)	88
	100 (15)	9	100 (36)	9		100 (22)	12

S.D., standard deviation [nm] in parentheses; [a] then the dispersions precipitated; [b] molar ratio; [c] not attempted; [d] aging measured on a non-sterilized dispersion; [e] 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol (CH).
1: 6-substituted galactophospholipid; **4**: 6-substituted mannophospholipid.
a: $[C_8F_{17}(CH_2)_4][C_8F_{17}(CH_2)_2]CH$-; **b**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CH$-; **d**: $[CH_3(CH_2)_9][CH_3(CH_2)_8]CH$-; **e**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CHO(CH_2)_5$-.

was opened and vesicle size and size distribution were measured by LLS spectroscopy.

2.6. Probe entrapment and release studies

Homogeneous sonicated vesicle dispersions were prepared in the presence of carboxyfluorescein or calcein (100 mM, pH 7.4) according to a previously described procedure [38]. Prior to measurements, annealing of the vesicles was allowed at 25°C for 15 h to reduce probe leakage through membrane imperfections present after sonication. Non-entrapped material was then removed. Therefore, 100 μl of the dispersion were submitted to gel filtration chromatography (Sephadex G-50 equilibrated overnight with a solution containing 20 mM Hepes buffer and 0.15 M of Na_2SO_4 or 0.15 M NaCl), using a Hepes/NaCl buffer or Hepes/Na_2SO_4 buffer solution as the eluent.

The release of the entrapped probes was monitored using Weinstein's method which takes advantage of the self-quenching of the fluorescence of fluorescent dyes

Table 4
Probe leakage half-times, $t_{1/2}$, for calcein (CA) and carboxyfluorescein (CF) at 37°C from SUVs prepared from perfluoroalkylated glycophospholipids

Compounds	CA (buffer)		CF (buffer)		Probe (serum)	
	$t_{1/2}$ (h) [a]	S.D. (h) [b]	$t_{1/2}$ (h)	S.D. (h)	$t_{1/2}$ (h)	S.D. (h)
1a	[e]		82	13	CF, 0.3	0.1
1b	16	4.5	0.7	0.1	CA, [f]	
1c	1.6	0.2	0.3	0.1	CA, [f]	
1e	[e]		18.2	0.4	CF, [f]	
2b	0.8	0.1	0.3	0.1	CA, 0.20	0.05
2c	0.6	0.1	0.10	0.05	CA, [f]	
4b	3.8	0.3	0.8	0.1	CA, [f]	
4c	1.4	0.2	0.70	0.01	CA, [f]	
1a/1b (1:2) [c]	[e]		1.1	0.1	CA, [f]	
4b/4d (2:1) [c]	0.3		0.1	[e]	[e]	
1b/CH (2:1) [c,d]	1.9		0.6	[e]	[e]	
1e/CH (2:1) [c,d]	[e]		22.4	0.8	CF, 0.60	0.05
DSPC [d]	[e]		14	2	CF, 19	2.3
1e/DSPC (1:4) [c,d]	[e]		5.3	0.4	CF, 0.7	0.2

[a] The $t_{1/2}$ given correspond to the mean of at least four independent measurements in a 0.15 M Na_2SO_4, 20 mM Hepes buffer; [b] standard deviation; [c] molar ratio; [d] 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol (CH); [e] not determined; [f] $t_{1/2} < 1$ min.
1: 6-substituted galactophospholipid; **2**: 3-substituted glucophospholipid; **4**: 6-substituted mannophospholipid.
a: $[C_8F_{17}(CH_2)_4][C_8F_{17}(CH_2)_2]CH$-; **b**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CH$-; **c**: $[C_6F_{13}(CH_2)_2][CH_3(CH_2)_8]CH$-; **d**: $[CH_3(CH_2)_9][CH_3(CH_2)_8]CH$-; **e**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CHO(CH_2)_5$-.

when concentrated in vesicles [39]. Release of the probe results in its dilution and build-up of fluorescence. Just after removing the non-entrapped dye, 5 μl of the effluent of the Sephadex column were placed in a cuvette containing 2 ml of saline buffer or human serum. For release kinetic studies in the buffer, the solution was stirred for 30 s prior to starting the kinetic, whereas in serum the solution was stirred throughout the study. Fluorescence was measured at 37°C with an emission wavelength of 520 nm, and an excitation wavelength of 480 nm for CF and 490 nm for CA. Maximum fluorescence was determined after lysis of the vesicles by a solution of deoxycholic acid [40]. The probe leakage half-time, $t_{1/2}$ (time at which 50% of the probe is still encapsulated), was directly determined on the curve when possible, or calculated using the linear portion of the logarithm of the entrapped dye fraction vs. time; this slope (K) is related to $t_{1/2}$ by the relation: $t_{1/2} = \ln(2/K)$. Each experiment was performed at least in quadruplicate.

3. Results and discussion

The main objectives of this study were to investigate the aggregation behavior in water of the new potential membrane components and to determine the effect of both the polar sugar part, and hydrophobic part (fluorinated or not) on the stability (Tables 2 and 3) and permeability (Table 4) of the liposomes they were seen to form. In order to evaluate the potential of the compounds as recognition

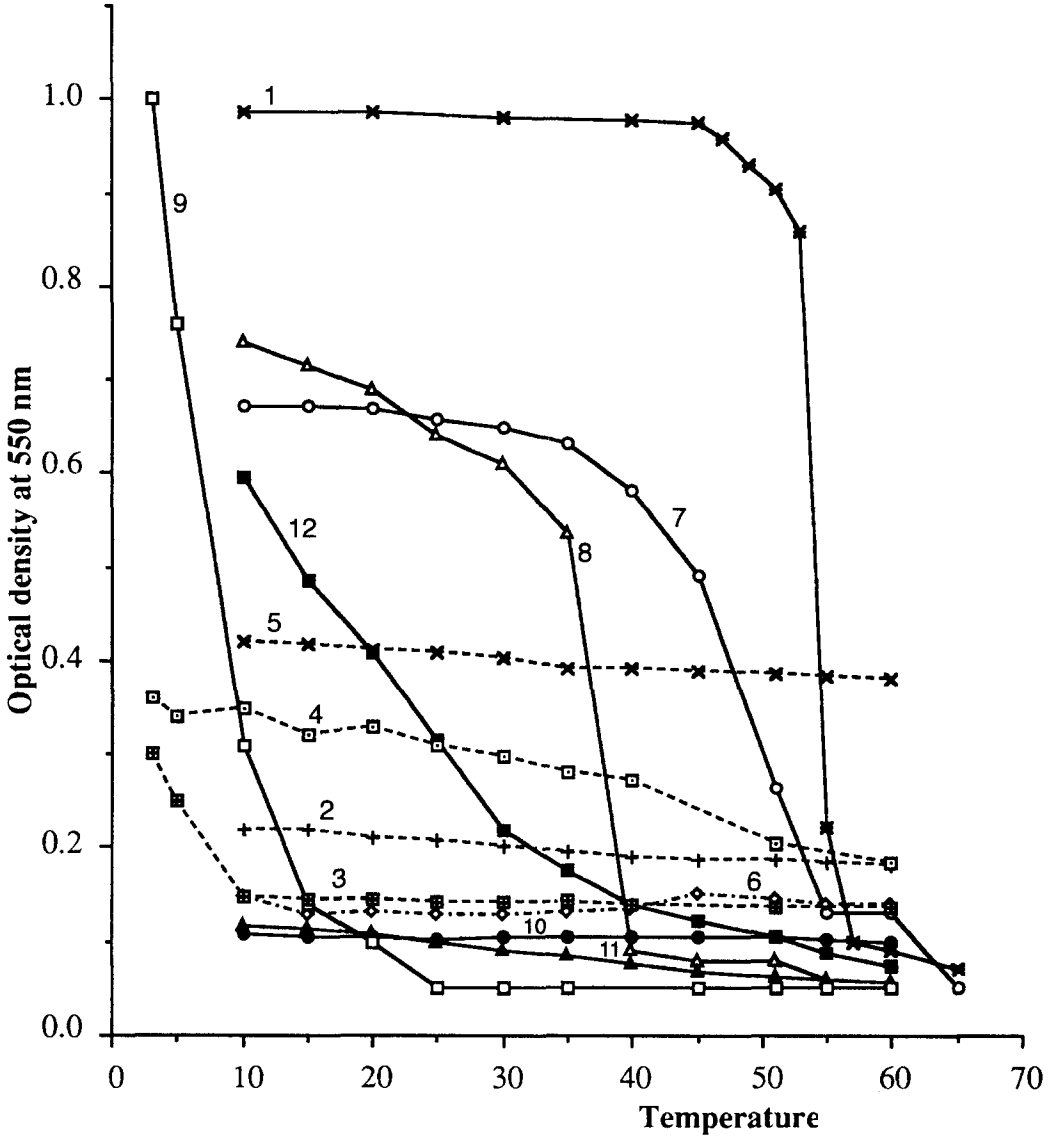


Fig. 1. Optical density at 550 nm vs. temperature. Curves: 1, DSPC; 2, **1b**; 3, **1d**; 4, **1e**; 5, **1a**; 6, **2b**; 7, **3b**; 8, **3c**; 9, **3d**; 10, **4b**; 11, **4c**; 12, **4d**. **1**: 6-substituted galactophospholipid; **2**: 3-substituted glucophospholipid; **3**: 6-substituted glucophospholipid; **4**: 6-substituted mannophospholipid **a**: $[C_8F_{17}(CH_2)_4][C_8F_{17}(CH_2)_2]CH$-; **b**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CH$-; **c**: $[C_6F_{13}(CH_2)_2][CH_3(CH_2)_8]CH$-; **d**: $[CH_3(CH_2)_9][CH_3(CH_2)_8]CH$-; **e**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CHO(CH_2)_5$-.

marker, it was also essential to determine their ability to mix together or with standard compounds used in conventional carrier.

3.1. Self-aggregation behavior and phase transitions

All amphiphiles tested but **3d**, when sonicated in water, readily produced clear dispersions in which homogeneous populations of small unilamellar vesicles (SUV) were seen by LLS spectroscopy. The morphology of all the vesicles, as observed by electron microscopy (negative staining), was fairly similar. But for those made of **1e**, these vesicles had an average diameter of 30–40 nm (Table 2), which is the range of diameters observed for standard liposomes [41]. The SUVs obtained from **1e** had a larger average diameter of 60 nm. The pentenyloxy spacer group in **1e** was intended to increase the thickness of the lipophilic domain in the membrane, and was indeed expected to also affect its curvature.

Phase transition from a crystalline gel to a less-ordered liquid crystalline state, corresponding to the 'melting' of the alkyl chains, is an important characteristic of membranes and vesicles. Both differential scanning calorimetry [42], and turbidity measurements [43,44] were employed to investigate this transition. No phase transition was, however, detected by DSC between −30 and 90°C for any of the amphiphiles **1–4**. It is doubtful that the transition occurred at higher temperature. This is likely to mean that the energy (ΔH) required for transition is very low. It is indeed commonly found that fluorocarbon bilayers exhibit significantly smaller ΔH values than their hydrocarbon counterparts [11,16]; the transition was, however, not detected for the hydrocarbon mannose derivative **4d** either.

Changes in turbidity vs. time, which reflect an evolution of the bilayer structure, were also investigated. When dispersed in water, the 6-substituted glucose derivatives **3b**, **3c** and **3d** gave turbid gels below 45°C, 35°C and 15°C, respectively. When examined by transmission electron microscopy (negative staining or freeze fracture), these dispersions showed the presence of cylindrical aggregates (tubules), ca. 500 nm in average diameter, made of rolled-up bilayers and possessing an internal aqueous core (Guillod, F., Giulieri, F., Greiner, J. and Riess, J.G., unpublished data). Optical microscopy showed that these tubules readily convert into vesicles upon warming above 45°C, 35°C and 15°C for **3b**, **3c** and **3d**, respectively; a strong decrease in turbidity, giving slightly opalescent dispersions, was observed concomitantly (Fig. 1). The vesicles slowly converted back again into tubules when the temperature was decreased below 15°C. The reversible transformation from tubules into vesicles is likely to occur at the gel-fluid phase transition [45–49] and a similar behavior has recently been reported for various other perfluoroalkylated amphiphiles [50,51]. The assumed Tc appear to increase with the fluorophilic character of the hydrophobe, indicating a decrease in membrane fluidity resulting from an increase in hydrophobic character.

No obvious change in turbidity was observed for dispersions of the other perfluoroalkylated amphiphiles, which is likely to mean that the bilayers were already in the fluid state at these temperatures, in contrast to those made of **3b** and **3c**. If this is the case, it may be concluded that the sugar residue is to a large extent responsible for the differences in behavior observed. As previously shown for other amphiphilic sugar derivatives, such changes probably result from differences in hydration of the amphiphiles [52,53]. Water molecules, by penetrating the network of hydrogen bonds between sugar headgroups, reduce the cohesion between these sugar units, which, in turn, results in lower melting temperature of the alkyl chains. For the hydrocarbon analog **4d** (and to a lesser extent **1d**), a smooth but definite decrease in turbidity was observed when temperature increased but a precise Tc could not be identified [54].

3.2. Thermal and shelf stability of the vesicles

SUVs made from **1a–e**, **2b,c**, and **4b–d** were found to withstand heat sterilization without major alteration of their average particle size (Table 2). Chemical stability of compounds **1b**, **2b**, **3c**, **4b,c** was ascertained in the same conditions; no degradation products were detected by thin-layer chromatography or by ^{1}H and ^{31}P-NMR analysis.

The heat-sterilizable vesicles were subsequently investigated for shelf stability. Neither sedimentation nor any other visible change were observed for the fluorinated SUVs made of **1a–c,e** and **4b,c** after 10 months at 25°C: mean particle sizes were still in the 30–70 nm range (Table 2) and particle size distributions remained essentially unaffected, indicating exceptional stability for single component liposomes. By contrast, the initially clear dispersions of SUVs made of the hydrocarbon analogs **1d** and **4d** turned into a gel after two days and 12 h, respectively, at room temperature; these gels consisted primarily of large lamellae and some multiwalled vesicles, as shown by electron microscopy (negative staining). The higher stability of the fluorinated SUVs is assigned to the strong hydrophobic effect induced by the fluorinated tails which complements the hydrogen-bonding interactions that exist between the polar heads.

The fluorinated vesicles produced from the 3- and 6-substituted glucose derivatives **2b,c** and **3b,c** were considerably less stable than those made from the galactose and mannose analogs **1a–c,e** and **4b,c**. Those made from **2b,c** precipitated after 20 and 10 days, respectively (Table 2), while the clear dispersion obtained from **3b** turned into a gel after only 1 day at room temperature, and **3c** gave a gel immediately after sonication. LLS measurements on dispersions of **3d** indicated a broad multimodal distribution whose profile evolved over time. This illustrates again the strong influence of the sugar on stability and even on the

type of aggregate formed. Packing of the 6-substituted glucose derivatives **3** may be tighter than for the 3-substituted glucose, or for the mannose or galactose ones [52,53], which would result in lesser ability for water to penetrate into the surfactant film, and would thus favor the more crystalline tubule arrangement.

3.3. Encapsulation and release studies in a buffer and in human serum

The existence of closed vesicles in the above preparations was further confirmed by entrapment and release experiments. Such experiments were aimed at assessing the potential of these vesicles as drug delivery systems. They were performed with two fluorescent hydrophilic probes, calcein and carboxyfluorescein, which were entrapped in SUVs formed from **1a–c,e**, **2b,c** and **4b,c**, as well as from diverse mixtures and reference compounds. The leakage half-times, $t_{1/2}$, when the SUVs were incubated at 37°C either in a Hepes buffer or in human serum are collected in Table 4.

Permeation of a dye across a membrane is controlled, among others, by its solubility and diffusion in the hydrophobic phase of this membrane. To permeate the membrane, the dye is in a neutral form. CA, with 4 carboxylic groups, is more hydrophilic than CF (2 carboxylic groups), hence has more difficulty to cross the hydrophobic membrane. Consequently, the use of CA in release studies allows, when necessary, the enhancement of differences between membranes of high permeability. Thus, in buffer, the values of $t_{1/2}$ calculated for CA clearly reflect an influence of the sugar on membrane permeability (Table 4) and yield the following order of impermeability: galactose **1b** > mannose **4b** > 3-substituted glucose **2b**. The same order was found when the perfluorohexyl termini was used instead of the perfluorooctyl termini. This order is consistent with the same rationale as for the Tc: the more important the hydration, the weaker the interactions between head groups, and the looser the membrane [40,52,53]. Hydrogen-bonding between galactosyl groups appears to be stronger than between mannosyl and 3-glucosyl groups, resulting in tighter packing and lesser permeability of the membranes.

The most important effect on membrane permeability is, however, related to the hydrophobic tail. Permeability can be decreased either by increasing the total length of the amphiphile or by replacing part of a hydrocarbon tail by a perfluoroalkyl one [16,38], hence by enhancing the amphiphilic character. The impact of the fluorinated tails is considerable since in the absence of such a tail, as in **1d** and **4d**, release studies could not be done due to too low encapsulation stability. For vesicles based on **1a**, which has two fluorinated chains, the values of $t_{1/2}$ for the release of CF in buffer, were about 120 times larger than for those made from **1b** which has only one fluorinated chain (Table 4). The introduction of fluorinated segments significantly increases the hydrophobic character of the tail and creates a highly ordered, hydrophobic and lipophobic fluorinated core within the bilayer membrane [14,15].

The leakage half-time of CF (Table 4) for liposomes made of **1e** was significantly larger than for those of **1b**, (18.2 ± 0.4 h vs. 0.7 ± 0.1 h). This effect of the pentenyloxy spacer is likely to result primarily from increased thickness of the lipophobic barrier; by moving the hydrophilic head away from the hydrophobic double tail, the spacer may also facilitate bilayer packing [55].

We also investigated the capacity of fluorinated vesicles to retain CA or CF in human serum which, among biological fluids, is known to induce strong destabilization of liposomes [9]. Interactions can, for example, take place with lipoproteins, in particular high density lipoproteins, leading to the formation of pores in the bilayer structure and to its eventual destruction and the release of the entrapped solutes [4,56]. The results (Table 4) show that leakage of the probes from these liposomes was considerably faster in serum than in the buffer. 80% of the initial CA content was released in less than 1 min from liposomes based on **1b,c,e**, **2c** and **4b,c**, whereas $t_{1/2}$ for vesicles based on **2b** was 0.2 h. Since in buffer, liposomes based on the latter compound were the most permeable with respect to CA among those made from compounds of the series **b**, this difference may mean that proteins interact more specifically with 6-substituted galactose and mannose derivatives than with this 3-substituted glucose which has a non-natural phosphate substitution.

Most noteworthy is the dramatic increase in permeability to CF that occurs with vesicles made of **1a** when changing from buffer to serum. The estimated $t_{1/2}$ drops then from 82 ± 13 h, a value 6 times larger than for DSPC, to 0.3 ± 0.1 h, a value 60 times lower than for the latter. Earlier results on liposomes made from fluorinated phosphatidylcholines have also shown destabilization by serum but to a lesser extent than for those made from **1a** [38]. Since the amphiphiles from the two families have quite similar fluorinated hydrophobic double chains, it is clear that the difference in behavior originates here principally from the presence of the sugar moiety.

3.4. Mixed membranes

As a consequence of the above observations, amphiphiles **1–4** may act as recognition markers in drug delivery systems. These glycophospholipids were therefore investigated from the standpoint of their incorporation in standard lipid bilayers. It was expected that besides the marker function of the sugar, some of the properties of fluorinated compounds would be conferred to the liposomes.

First we investigated the mutual miscibility of compounds **1** to **4**. Fluorocarbon and hydrocarbon amphiphiles show limited miscibility and tend to form separate domains within membranes [1,11]. The results obtained here

with glycophospholipids follow the same trend. Thus, a broad multimodal particle size distribution was observed by LLS when equimolar amounts of the symmetrically-tailed amphiphiles **1a** (two fluorocarbon chains) and **1d** (two hydrocarbon chains) were co-dispersed by sonication (Table 3), while each one of these compounds taken separately gives sharp, stable distributions.

Sizes and proportions within the **1a/1d** mixture changed over time, indicating an unstable system. On the contrary, the mixed amphiphiles, with one fluorocarbon and one hydrocarbon chain, were expected to be more easily and more uniformly dispersible in other amphiphiles, whether hydrogenated or fluorinated [57,58]. A 2:1 (mixed/symmetrical) molar ratio was adopted in order to favor homogeneous distribution. Homogeneous populations of small vesicles were indeed obtained by combining the non-fluorinated mannose amphiphile **4d** with the mixed fluorocarbon/hydrocarbon analog **4b**, while **4d** taken alone gave only unstable dispersions. Similarly, admixing and dispersing the bis(perfluoroalkyl) chain-containing amphiphile **1a** with the mixed fluorocarbon/hydrocarbon analog **1b**, yielded homogeneous populations of SUVs which were heat-sterilizable and stable for at least 10 months at room temperature.

However, although similar or higher shelf stability was obtained by combining compounds having mixed alkyl/perfluoroalkyl chains with compounds bearing two alkyl or two perfluoroalkyl chains, this resulted in higher membrane permeability in both buffer and serum (Table 4). In buffer, CA was released significantly faster from the heterogeneous **4b/4d** vesicles than from those composed from the fluorinated compound **4b** alone. Likewise, vesicles made from a mixture of **1a/1b** released their entrapped CF content much faster than vesicles made from **1a** alone, and the gain with respect to **1b** alone was only marginal. In serum the $t_{1/2}$ of CF for the liposomes formulated with the **1a/1b** mixture could not be measured because of too fast release of the probe.

Another approach to obtaining stable and impermeable liposomes with sugar markers at their surface consisted in studying the co-dispersion of **1e** with DSPC or with cholesterol. DSPC is known to yield membranes with low permeability, although their shelf stability is limited. In general, membranes based on phosphatidylcholine-derived amphiphiles become more stable with regard to leakage of solutes when a high molar ratio of cholesterol is introduced [9].

The fluorinated phosphogalactose **1e** was chosen among the sugar-labelled glycophospholipids because its hydrophobic chain length is closest to that of DSPC. Compound **1e** did indeed mix with DSPC. When **1e** was incorporated into DSPC in a 1:4 molar ratio, the crystal-to-liquid crystal phase transition temperature Tc, as determined by DSC, decreased from 57.6°C to 51.7°C and the transition enthalpy ΔH was reduced from about 34 kJ mol^{-1} [59] to 27 kJ mol^{-1}. The variation of Tc is fairly similar to that obtained when incorporating cholesterol [60]. However, the release of CF from SUVs prepared from **1e** and DSPC/**1e** (4:1), and incubated at 37°C (i.e., below Tc) in buffer or serum (Table 4), show that CF is released more rapidly from the DSPC/**1e** liposomes than from those made from pure DSPC. It may be that the incorporation of amphiphile **1e** generated packing defects that led to higher permeability. It can, however, be noticed that in serum the DSPC/**1e** preparation released 50% of the CF during the first 40 min of incubation, whereas liposomes made of **1e** alone lost their content within 1 min.

Incorporation of cholesterol (**1e**/CH, molar ratio 2:1) led to marginal membrane stabilization in buffer (Table 4). The difference was more marked in serum, $t_{1/2}$ for the **1e**/CH mixture being about 0.6 h (CA, 37°C), while leakage from liposomes made of **1e** alone was too rapid to allow the determination of $t_{1/2}$. On the contrary, the insertion of the steroid in vesicles based on **1b** increased membrane permeability (Table 4). It is likely that the aliphatic chain of **1b** is too short; the absence of carbonyl group and the fact that cholesterol may have its hydroxyl group level to the galactosyl head groups, may hinder hydrogen-bonding between galactosyl head groups, which could result in higher disorder, hence increased membrane permeability.

3.5. Biological tests

Since the compounds investigated are primarily destined for biomedical applications, some preliminary in vivo and in vitro tolerance tests, including hemolytic activity and acute toxicity, were performed. In vitro hemolytic activity on human red blood cell suspensions appears to be essentially independent of the polar head, and strongly decreases with increasing fluorinated character of the hydrophobic tail (Table 5). Thus amphiphile **1a**, with two fluorinated termini, is ca. 10^5 times (in terms of concentration) less hemolytic than **4d** which has two hydrocarbon chains, and ca. 10^3 times less hemolytic than the semi-fluorinated amphiphile **1b**, in agreement with data previously published for other fluorinated amphiphiles [14,15,61].

Acute intravascular toxicity tests indicated that the maximum tolerated dose (MTD) in mice (tail vein) is as high as ca. 500 mg/kg body weight for **1e** and **1a**, but of only 25 mg/kg for compound **3c** (Table 5). The low MTD of **3c** may, however, be due to poorer solubility and dispersibility in water.

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Table 5
Acute toxicity in mice (intravenous injection, tail vein) and hemolytic activity of glycophospholipids

Compounds	Acute toxicity			Hemolytic activity [a]	
	Injected dose (mg/kg)	Concn. (g/l)	Survival ratio	Concn. (g/l)	Hemolysis ratio (%)
1a	500	20	10/10	40	39
				20	8
				10	0
1b	125	5	10/10	0.1	100
				0.01	0
1c				0.1	100
				0.01	0
1e	500	20	9/9	15	13
				10	9
				5	3
2b	125	5	9/10	0.1	100
				0.01	0
3c	25	1	9/10		
4b				0.1	100
				0.01	0
4c	125	5	10/10	0.1	100
				0.01	0
4d				0.001	100
				0.0001	0

[a] References: 0% hemolysis for saline (0.9% NaCl); 100% for distilled water (55% hemolysis).

1: 6-substituted galactophospholipid; **2**: 3-substituted glucophospholipid; **3**: 6-substituted glucophospholipid; **4**: 6-substituted mannophospholipid. **a**: $[C_8F_{17}(CH_2)_4][C_8F_{17}(CH_2)_2]CH$-; **b**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]$-CH-; **c**: $[C_6F_{13}(CH_2)_2][CH_3(CH_2)_8]CH$-; **d**: $[CH_3(CH_2)_9]$-$[CH_3(CH_2)_8]CH$-; **e**: $[C_8F_{17}(CH_2)_2][CH_3(CH_2)_8]CHO(CH_2)_5$-.

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