ELSEVIER

Biochimica et Biophysica Acta 1285 (1996) 79–90

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Membrane permeability and stability in buffer and in human serum of fluorinated di-*O*-alkylglycerophosphocholine-based liposomes

Véronique Ravily, Catherine Santaella, Pierre Vierling *

Laboratoire de Chimie Moléculaire, URA CNRS 426 and Laboratoire de Chimie Bioorganique, EP CNRS 104, Faculté des Sciences, Université de Nice-Sophia Antipolis, B.P. 71, 06108 Nice Cedex 02, France

Received 21 May 1996; revised 17 July 1996; accepted 17 July 1996

Abstract

The stability (with respect to encapsulated carboxyfluorescein release) of liposomes made from various fluorocarbon 1,2- or 1,3-di-*O*-alkylglycerophosphocholines (ether-connected) and their membrane permeability have been investigated in buffer and in human serum. Membranes and liposomes, whether formulated with fluorocarbon/fluorocarbon or mixed fluorocarbon/hydrocarbon, 1,2- or 1,3-di-*O*-alkylglycerophospholipids, display lower permeability coefficients and are able to retain more efficiently encapsulated CF, even when incubated in human serum, than any of their conventional counterparts. These fluorinated liposomes are as stable as the first generation of liposomes formulated with their fluorocarbon ester-connected 1,2-di-*O*-acylglycerophosphocholine analogs. These results further confirm that a fluorinated intramembranar layer reduces the permeability of membranes (more significantly when they are in a fluid state), protects them from the destabilizing effects of serum components and increases even the stability of the fluorinated liposomes whose membranes are in the gel state when incubated in human serum. The impact of the modular structure of the fluorinated phospholipids (number of fluorocarbon chains, ether vs. ester bond, 1,2- vs. 1,3-isomer, etc...) and structure/permeability/stability relationships are also presented.

Keywords: Perfluoroalkylated di-*O*-alkylglycerophosphocholine; Phospholipid; Fluorinated liposome; Liposome; Vesicle; Membrane; Permeability

1. Introduction

The ability of liposomes to encapsulate a wide variety of solutes in their internal aqueous compartment has stimulated intensive efforts in view of developing their applications as drug carrier and delivery systems [1–6]. Progress in this field requires the elaboration of liposomal systems with new or significantly improved properties which in turn implies the development of components that are substantially different from those currently utilized. Highly fluorinated phospholipids (Fig. 1) are such components: they offer some of the specific features that make up the uniqueness of fluorinated material, e.g. their hydrophobic *and* lipophobic character.

We have shown recently that the fluorinated phosphatidylcholines (DF_nC_mPC compounds in Fig. 1) form stable bilayers and liposomes [7–10]. Their fluorinated F_n tails create inside the liposomal membrane a highly hydrophobic and lipophobic fluorocarbon layer inserted between two lipophilic hydrocar-

* Corresponding author (at EP CNRS 104). Fax: +33 92076151; e-mail: vierling@unice.fr.

PII S0005-2736(96)00150-2

bon shells (Fig. 2). Such fluorinated membranes and liposomes display remarkable and original physicochemical and biological properties (gel-to-fluid phase transition [8], dynamics [11], solubility of lipophilic probes [8], membrane permeability, stability in biological media [9,10], in vivo blood circulation time [12]) as compared to those of conventional ones. Concerning membrane permeability, drug encapsulation and release, the intramembranar fluorinated film constitutes an efficient barrier against the release of entrapped water soluble compounds such as carboxyfluorescein, even when the liposomes are incubated in human serum [9]. Moreover, efficient active and stable loading of doxorubicin into the fluorinated liposomes can also be achieved [10].

With the aim of extending the potential of fluorinated liposomes as drug carriers, we have developed new highly fluorinated ether-connected phospholipids [13] (Fig. 1). Their molecular structure follows a modular design, which allows incremental structural variations aimed at the establishment of structure/permability/release relationships. This design involves two hydrophobic chains, one (**IIB** and **IIIB**) or both (**IA**, **IIA** and **IIIA**) of which end in a highly fluorinated tail, in combination with saturated (**IA**, **IIA**, **IIBE** and **III**) and/or unsaturated (**IAE** and **IIBE**) aliphatic chains connected via ether bonds to a glycerophosphocholine polar head group. These

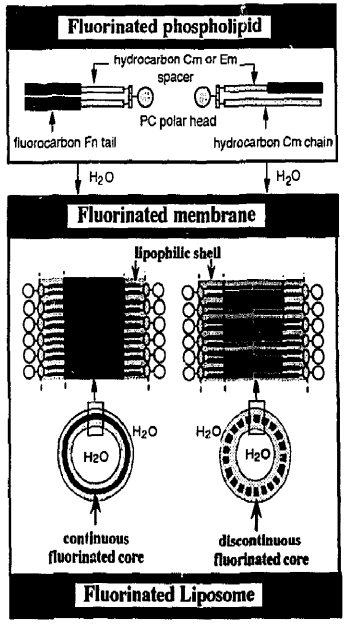

Fig. 2. Schematic representation of the fluorinated phospholipids, their membranes and liposomes with a fluorinated continuous or discontinuous bilayer core.

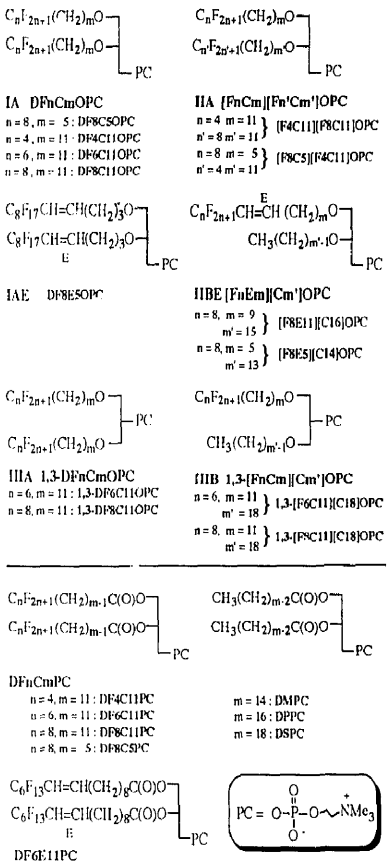

Fig. 1. Chemical structure and code name of the various fluorocarbon 1,2- and 1,3-di-*O*-alkyl(ether-connected)-glycerophosphocholines together with those of the various fluorocarbon and hydrocarbon di-*O*-acyl(ester-connected)-glycerophosphocholine analogs used as references throughout this study.

structural features are expected to play a role on the hydrophobic-lipophobic/lipophilic-hydrophilic balance, and consequently on the physico-chemical (fluidity/rigidity, permeability) and biological (stability in biological fluids, interactions with bio-compounds, in vivo fate) properties of the fluorinated membranes and liposomes. Their ether bond is more particularly intended to confer higher chemical and biological stability (in highly acidic media and more particularly towards the action of phospholipases) to these fluorinated phospholipids and to the liposomes that they form. These new fluorinated phospholipids form membranes which contain either a continuous fluorinated core, when both chains are ended by a fluorinated tail, or a discontinuous fluorinated core, when only one chain is ended by a fluorinated tail (Fig. 2). They also form long-term shelf stable liposomes (in terms of particle size and size distribution) [14] and display a rather high in vivo tolerance [13] which is a prerequisite when their use as in vivo drug delivery devices is contemplated.

We report here our detailed investigations on the ability of liposomes formulated with these new fluorinated glycerophosphocholines to retain efficiently a passively entrapped drug model, 5(6)-carboxyfluorescein (CF) [15], when these liposomes are incubated in a physiological buffer or in human serum at 37°C. The impact of the fluorocarbon lipophobic intramembranar layer, of the physical state (gel or fluid) of the liposomal membrane and of the molecular structure of the fluorinated phospholipids on the permeability of the liposomes, is also discussed. The experimental data obtained are compared to those reported for the first generation of fluorinated phosphatidylcholine (DF_nC_mPC)-based liposomes and for conventional ones made from hydrocarbon phosphatidylcholine analogs [9].

2. Materials and methods

The fluorinated ether glycerophosphocholines (Fig. 1) used here were synthesized according to Ref. [13]. Their purity (> 99%) was checked by TLC, ^{1}H- and ^{31}P-NMR. Racemic dipalmitoylphosphatidylcholine (DPPC) came from FLUKA and its purity (> 99%) was controlled by TLC before use. 5(6)-Carboxyfluorescein (CF) and sodium deoxycholate were purchased from SIGMA and used as received. Human serum was provided by the 'Centre Départemental de la Transfusion Sanguine des Alpes-Maritimes' and was a pool from several donors.

2.1. Preparation of unilamellar liposomes

Typically, the phospholipids (25 mM), as a powder, were hydrated for 30–45 min in a 100 mM CF solution (0.5 ml; pH 7.5) at a temperature 10 to 15°C above their gel-to-fluid phase transition temperature T_c (for the T_c values [14] see Table 1). These suspensions were then sonicated using a 3 mm titanium probe (Branson Sonic Power, Sonifier Cell Disrupter B300), at a temperature above T_c, until they became clear and translucent. The DPPC liposomes were prepared by extrusion through a polycarbonate membrane (Liposofast Milsch Equipment, size filter 100 nm). As most of the fluorinated phospholipids (more particularly those having a high phase transition temperature) could be extruded only with difficulty, sonication was preferred to prepare all the fluorinated formulations. Further, most of the liposomes thus obtained had a mean size in the 60–200 nm range, while sonication afforded DPPC liposomes with a mean size of 30 nm. The liposomes made from the [F8E11][C16]OPC/ DF8C11OPC (1:1, mol ratio) were prepared starting from a dried film obtained by evaporating a chloroform/methanol solution of the two lipids. To anneal membrane defects arising from sonication, all vesicle suspensions were then aged above T_c for 24 h prior to leakage measurements. Un-encapsulated CF was removed by gel-filtration through a Sephadex G-50 mini-column equilibrated with a 20 mM Hepes buffer (pH 7.5) containing 0.1 M Na_2SO_4 or 0.15 M NaCl. These separations were performed at room temperature just before the release experiments were run. The average vesicle diameter was then measured by photon correlation spectroscopy using a Coulter N4 MD submicron particle size analyzer.

2.2. CF release experiments in buffer or in human serum

The release experiments were run immediately after the separation of the un-encapsulated dye from the liposomes, as described in Ref. [9]. In a typical

Table 1
Half-leakage times, $t_{1/2}$, and permeability coefficients, P, for carboxyfluorescein (CF) leakage from unilamellar liposomes made from various fluorinated and conventional phospholipids measured at 37°C in a Hepes (20 mM)/NaCl (0.15 M) or Hepes (20 mM)/Na_2SO_4 (0.1 M) buffer and in human serum

Formulation (liposome mean diameter [b] / T_c [c,d])	$t_{1/2}$ in h (±S.D.) [a] in Na_2SO_4	P 10^{12} cm/s (±S.D.) [a] in Na_2SO_4	$t_{1/2}$ in h (±S.D.) [a] in NaCl (A)	$t_{1/2}$ in h (±S.D.) [a] in human serum (B)	B/A
DF8C5OPC (90 nm/60°C) [e,f]	21 (2)	13 (2)	28 (5)	17 (1)	0.6
DF8E5OPC (155 nm) [e,f]	35 (10)	14 (4)	32 (4)	4.1 (0.1)	0.1
DF4C11OPC (40 nm/17°C) [e]	38 (9)	2.8 (0.7)	24 (6)	2.4 (0.4)	0.1
DF6C11OPC (90 nm/50°C) [e]	73 (24)	3.6 (1.2)	95 (41)	43 (4)	0.4
				220 (21) [g]	2.3
DF8C11OPC (195 nm/88°C) [e] [C]	57 (35)	4.2 (1.0)	113 (41)	62 (4)	0.5
				255 (13) [g]	2.2
[F8C5][F4C11]OPC (30 nm/10°C) [e]	121 (6)	0.65 (0.03)	51 (1)	15.0 (0.1)	0.3
[F4C11][F8C11]OPC (95 nm/40°C) [e,f]	24 (4)	11 (2)	25 (4)	24 (2)	1.0
				344 (33) [g]	13.8
[F8E5][C14]OPC (40 nm/ < −25°C) [e]	21 (7)	5 (2)	15 (4)	2.9 (0.5)	0.2
[F8E11][C16]OPC (30 nm/5°C) [e] [D]	67 (26)	0.9 (0.3)	84 (15)	11.2 (0.3)	0.1
[C]/[D] (1:1) (65 nm)	62 (16)	2.7 (0.7)	57 (13)	63 (3)	1.1
				> 650 [g]	> 11
1,3-DF6C11OPC (145 nm/52°C) [e]	16 (2)	28 (4)	20 (3)	59 (7)	3
				560 (60) [g]	28
1,3-[F6C11][C18]OPC [e,f] (45 nm/39°C)	70 (20)	–	62 (18)	13 (1)	0.2
(95 nm)	3.2–38	–	14–44	1.5–18	0.03–1.3
1,3-[F8C11][C18]OPC (145 nm/46°C) [e]	23 (5)	19 (4)	23 (2)	39 (8)	1.7
				380 (60) [g]	17
DF8C5PC (60 nm/69°C) [d,f]	27 (5)	8 (1)	24 (2)	25 (3)	1
				77 (4) [g]	3
DF4C11PC (35 nm/19°C) [d,f]	83 (24)	1.2 (0.4)	83 (12)	3.6 (0.1)	0.045
DF6C11PC (75 nm/56°C) [d,f]	85 (25)	3.2 (0.8)	60 (12)	> 160	> 2.7
				> 600 [g]	> 10
DF6E11PC (160 nm/55°C) [d,g]	22 (3)	22 (3)	5 (1)	22 (3)	8.2
				41 (4) [g]	
DMPC (25 nm/23°C) [d,f]	1.1 (0.1)	53 (7)	0.33 (0.05)	< 0.017	< 0.05
DPPC (115 nm/41°C) [d]	9.0 (0.4)	37 (1)	13 (1)	6.1 (0.4)	0.5
DSPC (50 nm/58°C) [d,f]	14 (3)	9 (2)	10 (2)	19 (1)	1.9
				40 (5) [g]	4
EPC (20 nm) [f]	14 (1)	3.2 (0.3)	13 (1)	0.12 (0.02)	0.01
EPC/CH (1:1) (45 nm) [f]	30 (15)	4 (2)	33 (9)	27 (2) [g]	0.8

dye-release assay, the liposome suspension (5 to 15 μl or 30 to 50 μl) was mixed with 2 ml of buffer (20 mM Hepes buffer (pH 7.5) containing either 0.15 M NaCl or 0.1 M Na_2SO_4) or of human serum, and incubated at 37°C in a thermoregulated quartz cuvette cell (1 cm) with magnetic stirring. Release of the entrapped probe from the liposomes increases the fluorescence of the dye because of its dilution dependent de-quenching. This change was followed by continuous monitoring of the fluorescence increase, F, (excitation at 480 nm and emission at 520 nm) on a Perkin Elmer Spectrofluorometer LS 50B. The fluorescence signal, F_{max}, corresponding to 100% of CF release, was determined after lysis of the liposomes with a 10% sodium deoxycholate (20 μl) solution. In human serum, owing to serum/detergent interaction [16], F_{max} was determined in a separate experiment by adding to 2 ml of human serum the same aliquot of liposomes which have been disrupted with a 10% sodium deoxycholate solution. In the range of dye concentration used, the relation between fluorescence and dye concentration was linear both in the buffer or in human serum. F and F_{max} were corrected for dilution effects and diffusion background. Each experiment was performed at least in triplicate.

The CF leakage half-time $t_{1/2}$ (time at which 50% of CF is still encapsulated) was determined by plotting the fraction, R, of encapsulated dye ($R = (F_{max} - F)/F_{max}$) vs. time or calculated ($t_{1/2} = \ln 2/K$) from the slope K of the linear portion of the curve of $\ln R$ vs. time corresponding to the late phase of release. The profiles of CF release from the vesicles when incubated in buffer were in most cases monoexponentional and, in some cases, the kinetics were biphasic with an initial phase of fast release mainly due to the temperature increase (un-entrapped CF was indeed eliminated at room temperature before the release experiments were run at 37°C). The time-courses of CF release from vesicles of different compositions when incubated in human serum were recorded over 2 and 24 h periods. In these cases the kinetics are multiphasic, with an initial phase of fast release and a late phase of slower release, indicating that, in human serum, the slowest release phase is obtained for higher periods of incubation.

Assuming that the CF release occurs via a classical diffusion mechanism, the permeability coefficients, P, were calculated from the time-course of CF release from the vesicles incubated in the buffer using Fick's law. P is related to the slope K by $P = (r/3)K$, where r is the internal radius of the vesicle. The latter is related to the external diameter d (measured by photon correlation spectroscopy) by $d/2 - \Delta x$, with Δx estimated to be 3 to 3.5 nm for the membranes in the fluid state and 4 to 5 nm in the gel state [17,18] (for more details see Ref. [9]).

3. Results

The leakage half-time values, $t_{1/2}$, and the permeability coefficients of CF, P, determined or calculated from the time-course of CF release (curves not shown) from unilamellar vesicles made of the various fluorinated phospholipids displayed in Fig. 1 and incubated at 37°C in a buffer or in human serum, are listed in Table 1. The experiments, whether run in a Na_2SO_4 or in a NaCl containing Hepes buffer, afforded, in most cases, the same $t_{1/2}$ values, excepted for [F8C5][F4C11]OPC, which displays a lower $t_{1/2}$ value in the NaCl buffer (this may be due to a higher Cl^- permeability), and 1,3-[F6C11][C18]OPC, for which the results were poorly reproducible owing to its T_c (= 39°C) in the proximity of the incubation temperature. This table collects also (i) the

Notes to Table 1:

[a] The $t_{1/2}$ and P values given correspond to the mean (±S.D.) of three or four independent experiments.

[b] Liposome mean size (> 95% of the population).

[c,d] The gel-to-fluid phase transition temperatures, T_c, are taken from Refs. [14] and [8], respectively and correspond to planar membranes. For unilamellar liposomes, the T_c is generally lower by a few degrees than that indicated.

[e] No phase transition detected for this compound [8].

[f] The $t_{1/2}$ and P values are taken from Ref. [9].

[g] Values calculated from 24 h kinetic experiments (otherwise 2 h).

[*] At 37°C, these membranes are in a semi-gel semi-fluid state.

serum/buffer $t_{1/2}$ ratio values which express the serum effects on the CF release and (ii) the same parameters for the liposomes which are made from the DF_nC_mPC and hydrocarbon phosphatidylcholines, values taken from Ref. [9].

The diagrams in Fig. 3A and B illustrate the evolution of the $t_{1/2}$ values for liposomes incubated in a Hepes/NaCl (20 mM/0.15 M) or in human serum and for which the membrane is in the fluid or gel state, respectively. Fig. 4A and B represent the evolution of the permeability coefficients P of CF across the various liposomal membranes in the fluid or gel state, respectively, with the hydrophobic chain length L (L represents the number of carbon atoms constituting one chain or the mean when both chains are different). These diagrams allow a more convenient comparison between the different liposomal formulations and membranes.

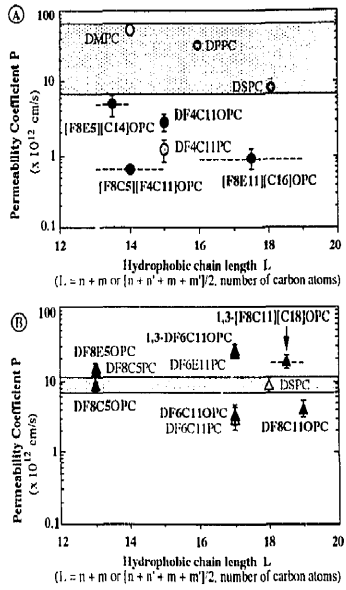

Fig. 3. Variation of the permeability coefficients, P, for (A) 'fluid' and (B) 'gel' fluorinated (filled symbols) and conventional (open symbols) unilamellar liposomes with the length L of the phospholipid's hydrophobic chains. The symbol ⊗ in (A) indicates that the P value is corresponding to liposomal membranes which are close to their phase transition (DPPC) or in the gel state (DSPC). When not indicated error bars are smaller than the signs. P is calculated from the time-course of CF release from the vesicles incubated in the Na_2SO_4 buffer (see Section 2).

3.1. CF release from liposomes and permeability for membranes in the fluid state

At 37°C, the $t_{1/2}$ values of CF leakage from the fluorinated liposomes whose membranes are in the fluid state, are found, in the Na_2SO_4 or NaCl buffer, to be in the 15 to 84 h range, while those of the hydrocarbon ones lie in the 0.33 to 13 h range (Table 1) indicating that the 'fluid' fluorinated liposomes retain their content much more efficiently (from 1 to 250 times, as expressed by the $t_{1/2}$ ratio) than their 'fluid' hydrocarbon analogs. The P values (Table 1 and Fig. 4A) calculated for the 'fluid' fluorinated vesicles are all lower than those found for the 'fluid' and even 'gel' conventional ones, indicating a lower membrane permeability of the fluorinated liposomes.

When incubated in human serum, the CF release is significantly increased but remains much slower from the fluorinated liposomes ($t_{1/2}$ in the 2.4 to 15 h range) than from conventional ones ($t_{1/2}$ in the 0.017 to 0.12 h range), indicating a greater stability of the former formulations. The effects of serum on CF release from the liposomes can be expressed by the serum $t_{1/2}$ to buffer $t_{1/2}$ (B/A) ratio: a ratio lower or higher than 1 indicates a destabilizing or a stabilizing effect of serum, respectively. Our results show that serum destabilizes the 'fluid' fluorinated liposomes as it does for the 'fluid' conventional ones. Thus, all the 'fluid' vesicles displayed $t_{1/2}$ ratios which are all below 1 (Table 1). However, the higher $t_{1/2}$ ratios found for the fluorinated liposomes (from 0.1 to 0.3), when compared to DMPC (< 0.05) or EPC (0.01), indicate that CF leakage from these fluorinated vesicles is somewhat less affected by the serum components than when encapsulated in DMPC or EPC liposomes. One can further notice that the membranes having a 'continuous' fluorinated core (both chains of the phospholipid are ended by a fluorinated tail, as in DF4C11OPC) are as destabilized by the serum than those having a 'discontinuous' fluorinated core (only one of the two chains is ended by a fluorinated tail, as in [F8E5][C14]OPC and [F8E11][C16]OPC), as expressed by similar B/A values (Table 1).

One can further observe that the mixed fluorocar-

bon/hydrocarbon phospholipids (e.g. [F8E5][C14]OPC and [F8E11][C16]OPC) form liposomes which are at least as stable, with respect to CF leakage, as those made from their fluorocarbon/fluorocarbon analogs and more stable than conventional ones. Thus, and for similar chain lengths, this is the case of [F8E5][C14]OPC as compared to DF4C11OPC, both formulations displaying similar $t_{1/2}$ values ($\approx$ 30 h in buffer and 2.5 h in serum) or to DMPC which displays much lower $t_{1/2}$ values (1 h in buffer and 0.017 h in serum). This is also the case of the [F8E11][C16]OPC liposomes ($t_{1/2}$ of 84 h) which are more efficient than those of DF4C11OPC and as stable as those of [F8C5][F4C11]OPC ($t_{1/2}$ of 50 h). The CF release from the 'fluid' [F8E11][C16]OPC liposomes is furthermore comparable to that from some 'gel' formulations such as those made from DF6C11OPC ($t_{1/2}$ of 95 h) or DF8C11OPC ($t_{1/2}$ of 113 h) or even lower than from the [F4C11][F8C11]OPC ones ($t_{1/2}$ of 25 h). This tendency is further confirmed by the P coefficient of the [F8E11][C16]OPC-based membranes which is among the lowest P values listed in Table 1, indicating that these membranes are among the less permeable ones, irrespective of their physical state.

Fluorinated liposomes, whether made from the ester DF4C11PC or ether DF4C11OPC phospholipids display comparable CF release rates and permeability

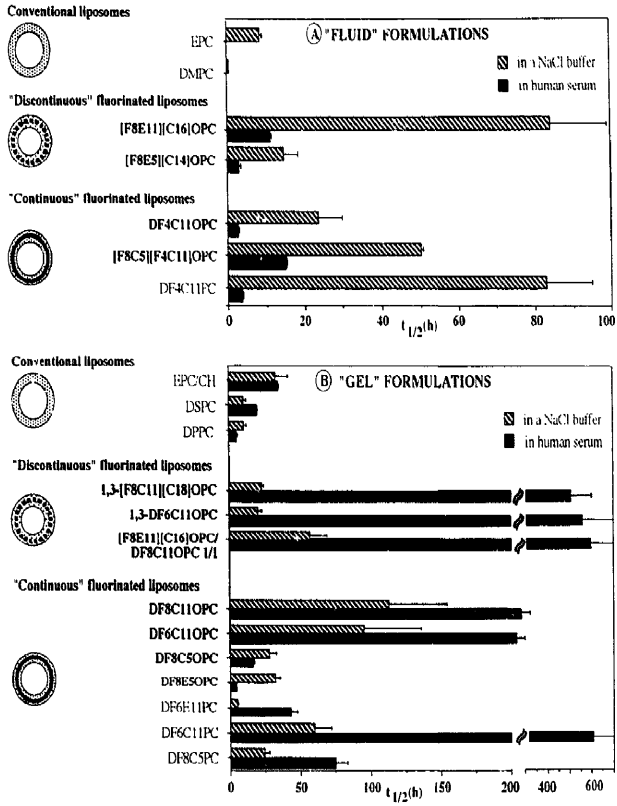

Fig. 4. Comparison of the carboxyfluorescein (CF) release from conventional, 'discontinuous' or 'continuous' fluorinated unilamellar liposomes when incubated in a buffer or in human serum at 37°C. The liposomal membrane is, at 37°C, (A) in the fluid state, (B) in the gel state.

coefficients when incubated in a buffer or in human serum (Table 1, Fig. 3 and Fig. 4). This indicates that the chemical nature (ester or ether) of the connecting group between the hydrophobic chains and the glycerol backbone has almost no impact on membrane permeability to CF.

3.2. CF release from liposomes and permeability of membranes in the gel phase

The 'gel' fluorinated liposomes display high $t_{1/2}$ values which are from 1 to 10 times higher than those of the 'gel' DSPC conventional ones. Some of them are also more stable than those made from an equimolar EPC/CH mixture. However, we found that both types of membranes exhibit close P values (Fig. 4B), indicating that in the gel state no significant difference in permeability exists between fluorocarbon and conventional membranes.

All 'gel' fluorinated liposomes (excepting those of DF8C5OPC and F8E5OPC) are more stable when incubated in human serum rather than in buffer, as shown by the B/A values which are much larger than 1 (Table 1). While these fluorinated formulations are significantly stabilized by the human serum components, those of DSPC or EPC/CH are almost not perturbed by these components. It is noticeable that the 'discontinuous' fluorinated liposomes, e.g. 1,3-[F8C11][C18]OPC, are, in human serum, as stable as the 'continuous' fluorinated ones, e.g. 1,3-DF6C11OPC or DF8C11OPC (see $t_{1/2}$ values in Table 1 and Fig. 4B).

When comparing the gel 'ether' to the gel 'ester' liposomes (DF6C11OPC vs. DF6C11PC and DF8C5OPC vs. DF8C5PC), it appears that the ester ones display, in human serum, a higher stability although the chemical ester/ether nature has almost no influence on the CF release rates nor on the P coefficients in a buffer.

Where the impact of the 1,2/1,3-isomerism is concerned, it appears that the 1,2-DF6C11OPC isomer forms liposomes and membranes that are less leaky and permeable than those of 1,3-DF6C11OPC (Table 1; Fig. 3B and Fig. 4B) when incubated in a buffer. However, in human serum, no significant differences are found between these two formulations.

One should also notice that, for the membranes whose phase transition temperature is close to the incubation temperature (37°C), e.g. [F4C11][F8C11]OPC (T_c = 40°C) and DPPC (T_c = 41°C), [but excepted 1,3-[F6C11][C18]OPC (T_c = 39°C) which forms an unstable lamellar phase], the corresponding fluorinated liposomes display lower CF release rates than the DPPC ones, whether incubated in the buffer or in human serum. Although much caution must be exercised in the interpretation of these results, it appears that they are in line with the greater stability found here and elsewhere [9] for the fluorinated liposomes.

4. Discussion

The main objectives of this study were to determine the potential of the liposomes made from the new fluorinated di-*O*-alkylglycerophosphocholines (Fig. 1) as alternative drug carrier and delivery systems, comparatively to that of the liposomes formed from their fluorinated DF_nC_mPC phosphatidylcholines or conventional ones [9,10]. In order to evaluate further the impact of the modular structure of the intramembranar fluorinated core (discontinuous vs. continuous as illustrated in Fig. 2) and of the fluorinated phospholipids (ether vs. ester bond, 1,2- vs. 1,3-isomer, etc...) and to gain some additional structure/permeability/stability (with respect to drug release) relationships, we have therefore investigated and compared the ability of the various new fluorinated vesicles to retain encapsulated carboxyfluorescein (CF). These liposomes were incubated at 37°C in a buffer and in human serum which, among the biological fluids, is known to have most pronounced effects on membrane permeability and liposome stability [19]. Our following discussion takes also into account that, at the incubation temperature, the membrane of these liposomes was in the gel or fluid state (which is well-known to influence membrane permeability [4,20]).

4.1. Liposome stability and membrane permeability in a buffer

Our results clearly demonstrate that the presence of a fluorinated core, whether continuous or discon-

tinuous, within the liposomal membrane in its fluid state constitutes a very efficient barrier for the permeation of the hydrophilic CF. Indeed and regardless of the number of fluorocarbon chains and length of both the hydrophobic chains and fluorinated tails, the vesicles made from the fluorinated di-*O*-alkyl phospholipids, when incubated in a buffer at 37°C, retain entrapped CF much more efficiently and exhibit much lower membrane permeability than any of their 'fluid' conventional counterparts. However, in the gel state, it appears that the fluorinated core has almost no effect on CF permeation: both fluorinated and conventional liposomal membranes display indeed comparable permeability coefficients. These results are in line with our previous study concerning the first generation of fluorinated phosphatidylcholines [9] and may be further rationalized in terms of hydrophobic/lipophobic properties and rigidifying/ordering effects arising from the presence of fluorinated alkyl chains.

Permeation of a dye across a membrane is controlled, among others, by its solubility and diffusion in the hydrophobic phase of this membrane. To explain the decrease in permeability induced by the fluorinated tails, one can assume that the probe's solubility (or its penetration) and/or diffusion across the highly hydrophobic and lipophobic fluorinated core created by these tails, are strongly reduced (the much lower solubility of hydrophilic and lipophilic compounds in fluorinated membranes has been established elsewhere [8]). This core constitutes then, with respect to a conventional lipid membrane, a supplementary solubility and diffusion barrier limiting permeation. The formation of a highly organized fluid phase for the fluorinated membranes might also contribute in reducing the solubility of the dye and/or its diffusion across such membranes. The lipophobic fluorinated tails have indeed been found to impose a highly rigid and ordered arrangement to the hydrocarbon spacer, hence to the lipophilic shells (see Fig. 2), when the membrane is in the fluid state while they do not affect the order within fluorinated 'gel' membranes [11]. These arguments account for (i) the higher efficiency of the DF4C11OPC liposomes to retain CF as compared to the conventional DMPC ones, (ii) the low impact of the fluorinated intramembranar layer on CF release in the case of 'gel' fluorinated membranes and (iii) the small gaps in CF release between 'fluid' and 'gel' fluorinated liposomes while the differences are more important for the conventional ones.

The remarkable efficiency of the fluorinated tails to reduce membrane permeability and drug release is best illustrated by the lower CF permeability P coefficients and the higher $t_{1/2}$ values of the 'discontinuous' and 'fluid' fluorinated [F8E11][C16]OPC or [F8E5][C14]OPC membranes and liposomes as compared to those of (i) the 'fluid' conventional DMPC or EPC ones, (ii) the 'fluid' 'continuous' fluorinated DF4C11OPC or [F8C5][F4C11]OPC ones and (iii) even as compared to those of some 'gel' fluorinated ones. It is indeed noteworthy that the 'fluid' discontinuous fluorinated membranes of [F8E11][C16]OPC and [F8E5][C14]OPC display the lowest P coefficients among all the membranes investigated here. The same tendencies are also found for 'gel' formulations. Thus, 'discontinuous' membranes and liposomes of 1,3-[F8C11][C18]OPC display (i) a lower P coefficient and higher $t_{1/2}$ values than DSPC ones and (ii) similar P and $t_{1/2}$ values than the 'continuous' ones formulated from several glycerophosphocholines having two fluorocarbon chains (e.g. DF8C5OPC, DF8C5PC, [F4C11][F8C11]OPC and 1,3-DF6C11OPC).

These results may be tentatively rationalized in terms of fluorination degree (df) of the membrane, which can be defined as the ratio of the number of F-carbons on the total number of carbon atoms constituting the hydrophobic chains (df is equal to $n/n+m$ or $n+n'/n+n'+m+m'$ for the mixed derivatives and is ranging from 0.22 for 1,3-[F8C11][C18]OPC to 0.62 for DF8C5OPC). They show that a df value of 0.23, as for the 'fluid' [F8E11][C16]OPC-based membranes and liposomes, which is among the lowest df value of the fluorinated membranes and liposomes investigated here, is sufficient to reduce efficiently membrane permeability and drug release from liposomes to a level lower or similar to those found for 'fluid' or 'gel' membranes and liposomes displaying a higher fluorination degree. The surprising lower or similar efficiency of gel fluorinated membranes as compared to some fluid fluorinated ones could also arise from the presence of packing defects which are more important for gel membranes and/or for gel-like membranes whose phase transitions are large and occur at a temperature

close to the incubation temperature, thus facilitating transmembranar diffusion.

4.2. Liposome stability in human serum

Serum is among the biological fluids known to induce strong destabilizing effects on membranes in their fluid state [19]. The new fluorinated liposomes investigated here whose membranes are in the fluid state at 37°C are also destabilized by the serum components but to a considerably lesser extent than 'fluid' conventional ones. These results confirm those found for liposomes formulated with the DF_nC_mPCs [9]. They indicate further that the fluorinated intramembranar core with its lipophobic, hydrophobic and ordering effects definitely protects the vesicles, possibly by reducing their interactions with the lipophilic and/or hydrophilic serum components limiting their adsorption at the surface and/or hindering their penetration deeper into the fluorinated bilayer. This protection may also result from a reduction of the phospholipid exchange between the fluorinated liposomes and the high density lipoproteins present in serum, owing to the very low miscibility between endogenic and fluorinated phospholipids (even for weakly fluorinated ones, such as DF4C11OPC; J.P. Rolland et al., unpublished data). However, the latter phenomenon is most probably of minor importance as the 'fluid' and 'discontinuous' liposomes made from the mixed fluorocarbon/hydrocarbon phospholipids (e.g. [F8E11][C16]OPC or [F8E5][C14]OPC), which are more miscible with fully hydrocarbon ones (J.-P. Rolland et al., unpublished data), are found to be as stable in human serum as some made from fluorocarbon/fluorocarbon phospholipids. One would have expected in fact a lower stability of the liposomes made from these mixed phospholipids with membranes consisting in a juxtaposition of lipophilic and lipophobic regions (Fig. 2) as a result of increased protein adsorption into the lipophilic regions.

On the other hand, our results show, in line with our previous study [9], that the fluorinated core further improves the retention of the dye, in serum and for the fluorinated liposomes whose membranes are in the gel state. Indeed, these 'gel' fluorinated liposomes display not only a significantly greater stability in serum than in the physiological buffer, but they are noticeably even more stabilized in serum than conventional 'gel' (DSPC) or 'gel'-like (EPC/CH) liposomes. These results most likely indicate that serum constituents participate in increasing the order or packing of the phospholipids within the membrane, this effect being more pronounced for the fluorinated ones and/or in suppressing packing defects which arise frequently for liposomes with membranes in a gel state.

4.3. Structure/permeability/CF release relationships

One of the objectives of this study was to evaluate the potential of the liposomes formulated with these new fluorinated ether glycerophosphocholines as compared to those reported for the first generation of fluorinated ester phosphatidylcholine-based liposomes, hence to evaluate the impact of the ester/ether bond on permeability and release. We found that membranes prepared either from the DF4C11PC or DF6C11PC esters or from their DF4C11OPC or DF6C11OPC ether homologs, respectively, behave very similarly in terms of permeability and CF release (in buffer or in human serum). Surprisingly, this tendency is not found for the CF release from DF8C5OPC or DF8C5PC liposomes which is accelerated by the serum components for the ether ones while the opposite result is observed for its ester homolog. Two reasons may account for these differences, (i) one lies in the semi-gel semi-fluid state of these membranes which is known to generate packing defects and consequently to increase permeability (while the two former couples of compounds are in the fluid and gel state, respectively) and (ii) the other, in the very low cooperativity of the phase transition of DF8C5OPC and DF8C5PC which occurs over a large temperature range (almost 40°C) and the closer proximity of the phase transition temperature of DF8C5OPC (60°C) with the incubation temperature, as compared to that of its DF8C5PC ester analog (T_c = 69°C), thus enhancing the formation of packing defects in the case of DF8C5OPC membranes.

Owing to the modular structure of the di-*O*-alkylglycerophosphocholines investigated, our study has allowed to deduce some other structure/permeability/CF release relationships in addition to the number of fluorocarbon chains, hence the

discontinuous/continuous nature of the fluorinated core, which has already been commented above.

We found that the use of 1,2-isomers of the fluorinated glycerophospholipids in place of their 1,3-isomers leads to membranes and liposomes displaying, to some extent and depending on the incubation milieu, reduced permeability and CF release rates. However, it is surprising that (i) DF6C11OPC forms liposomes which are less permeable and more stable than its 1,3-isomer when incubated in a buffer and (ii) the 1,3-DF6C11OPC liposomes are stabilized by the serum components to a larger extent than those formulated with the 1,2-isomer. The differences in behavior observed for these isomers are most likely related to the phase behavior rather than to membrane permeability properties. Indeed, we have shown elsewhere that DF6C11OPC and its 1,3-isomer form a metastable lamellar phase which slowly converts into a ribbon like phase. This phase transition may be accelerated for one or slowed down for the other in the presence of encapsulated CF and its evolution may further depend upon the nature of the incubation milieu. Our results show clearly that this evolution is slowed down for the 1,3-DF6C11OPC isomer ($t_{1/2}$ serum/buffer ratio of 28 vs. ≈ 3 for its 1,2-isomer).

Finally, where the impact of the double bond (which is, for the compounds investigated here, mainly of *trans* configuration, *cis*/*trans* ratio $\leq$ 20:80) is concerned, our results show as expected that the CF release from liposomes incubated in human serum is more important when they are made from the unsaturated DF8E5OPC or DF6E11PC rather than from their saturated DF8C5OPC or DF6C11PC analogs, respectively. This trend is also found in a buffer for DF6E11PC vs. DF6C11PC. This may be attributed to the well-known membrane perturbations (decrease of chain order) generated by the presence of an unsaturation in the hydrophobic chains. By contrast, we found that the unsaturated DF8E5OPC and saturated DF8C5OPC membranes and liposomes display, in buffer, similar membrane permeabilities and CF release rates. This particular behavior in buffer as compared to that in serum may be due to the fact that, although both these two membranes are at 37°C in a semi-fluid semi-gel state, the additional decrease of order induced by the presence of double bonds facilitates the insertion of serum components and, consequently, CF release. Furthermore, one can observe that when going from buffer to human serum, DF8E5OPC and DF8C5OPC are destabilized while DF6E11PC and DF6C11PC are stabilized. Again several reasons may account for this difference in behavior. One lies in the physical state of the membranes, serum stabilizing gel membranes (DF6E11PC, DF6C11PC) rather than semi-fluid semi-gel ones (DF8E5OPC, DF8C5OPC), another one lies in the much broader phase transition of DF8E5OPC and DF8C5OPC.

5. Conclusion

We have shown that membranes and liposomes whether formulated with fluorocarbon/fluorocarbon (continuous fluorinated core) or with mixed fluorocarbon/hydrocarbon (discontinuous fluorinated core), 1,2- or 1,3-di-*O*-alkylglycerophospholipids are able to retain efficiently encapsulated CF, even when incubated in human serum. These liposomes are as stable, with respect to CF release, as the first generation of liposomes formulated with the fluorinated phosphatidylcholines [9]. These results are most important if an easy access to the drug carrier systems or its chemical and biochemical stability are wanted. They further confirm that a fluorinated intramembranar layer reduces the permeability of membranes (more significantly when they are in a fluid state), protects them from the destabilizing effects of serum components and increases even the stability of the fluorinated liposomes whose membranes are in the gel state when incubated in human serum.

References

[1] Gregoriadis, G. (1988) Liposomes as Drug Carriers. Recent Trends and Progress, J. Wiley and Sons, New York.
[2] Lopez-Berenstein, G. and Fidler, I.J. (1988) Liposomes: The Therapy of Infectious Diseases and Cancer, Alan R. Liss, New York.
[3] Gregoriadis, G. (1993) Liposome Technology, Vol. 1, 2, 3, 2nd Edn., CRC Press, Boca Raton.
[4] Lasic, D.D. (1993) Liposomes: From Physics to Applications, Elsevier, Amsterdam.
[5] Lasic, D.D. (1994) Angew. Chem. Int. Edn. Engl. 33, 1685–1698.
[6] Puisieux, F., Couvreur, P., Delattre, J. and Devissaguet, J.P.

(1995) Liposomes: New Systems and New Trends in their Applications, Edition de Santé, Paris.
[7] Vierling, P., Santaella, C. and Riess, J.G. (1995) in Liposomes: New Systems and New Trends in their Applications, (Puisieux, F., Couvreu, P., Delattie, J., and Devissaguet, J.P., eds.), pp. 293–318, Edition de Santé, Paris.
[8] Santaella, C., Vierling, P., Riess, J.G., Gulik-Krzywicki, T., Gulik, A. and Monasse, B. (1994) Biochim. Biophys. Acta 1190, 25–39.
[9] Frézard, F., Santaella, C., Vierling, P. and Riess, J.G. (1994) Biochim. Biophys. Acta 1192, 61–70.
[10] Frézard, F., Santaella, C., Montisci, M.-J., Vierling, P. and Riess, J.G. (1994) Biochim. Biophys. Acta 1194, 61–68.
[11] Santaella, C. and Vierling, P. (1995) Chem. Phys. Lipids 77, 173–177.
[12] Santaella, C., Frézard, F., Vierling, P. and Riess, J.G. (1993) FEBS Lett. 336, 481–483.
[13] Ravily, V., Gaentzler, S., Santaella, C. and Vierling, P. (1996) Helv. Chim. Acta 79, 405–425.
[14] Ravily, V., Santaella, C., Vierling, P., Gulik-Krzywicki, T., Gulik, A. (1996) Biochim. Biophys. Acta, in press.
[15] Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489–491.
[16] Lelkes, P.I. and Tandeter, H.B. (1982) Biochim. Biophys. Acta 716, 410–419.
[17] Janiak, M.J., Small, D.M. and Shipley, G.G. (1977) Biochemistry 15, 4575–4580.
[18] Tardieu, A., Luzzati, V. and Renan, F.C. (1973) J. Mol. Biol. 75, 711–733.
[19] Senior, J. and Gregoriadis, G. (1982) Life Sci. 30, 2123–2136.
[20] New, R.R.C. (1990) in Liposomes, a Practical Approach (New, R.R.C., ed.), pp. 1–32, Oxford University Press, Oxford.