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Membrane permeability and stability of liposomes made from highly fluorinated double-chain phosphocholines derived from diaminopropanol, serine or ethanolamine

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Abstract

The release of encapsulated carboxyfluorescein (CF) from liposomes made from various fluorinated amido-connected double-chain phosphocholines and their membrane permeability have been investigated at 37°C in buffer and in human serum. These fluorinated membranes and liposomes display lower permeability coefficients and are able to retain more efficiently encapsulated CF than any of their respective conventional counterparts. Several of these liposomes are as effective as the first generation of liposomes based on fluorinated phosphatidylcholines, indicating that the chemical junction (ester/amide) and nature of the unit (glycerol, diaminopropanol, serine, ethanolamine) connecting the hydrophobic chains to the phosphocholine polar head have no significant effect on permeability and CF release. Our results show further that a fluorinated intramembrane layer reduces significantly the permeability of membranes in a liquid-crystalline state, protects the liposomes from the destabilizing effects of serum, and even increases their stability (in terms of dye retention) in serum when the membranes are in the gel state. © 1997 Elsevier Science B.V.

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1. Introduction

The therapeutic potential of liposomes as drug carrier and delivery systems has stimulated numerous studies over the past decade [1–6]. Protection of the liposome's surface by gangliosides or poly(oxyethylene)-derivatized lipids with the development of 'stealth' liposomes, (i.e., liposomes that can escape premature removal from blood circulation by the mononuclear phagocytic system) has allowed significant progress in this field. Despite these improvements, several obstacles still remain that limit their applications.

We have already shown that highly fluorinated analogs of natural phosphatidylcholines (i.e., DF*n*C*m*PC compounds in Fig. 1) form bilayers and liposomes containing, inside the membrane, a highly hydrophobic and lipophobic fluorocarbon layer [7–10]. Such 'fluorinated' membranes and liposomes display remarkable and original physico-chemical and biological properties that differ substantially from those of conventional ones (lower membrane permeability, increased stability in biological media [9,10], extended in vivo blood circulation time [11]). As for

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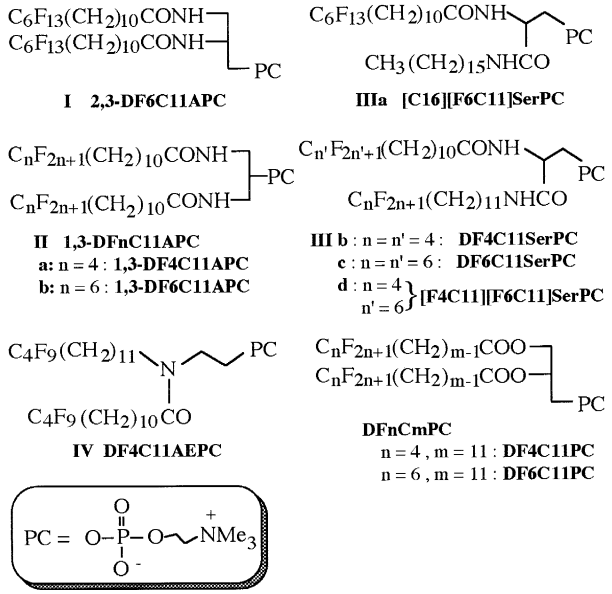

Fig. 1. Chemical structure and code name of the various fluorocarbon amido-connected phosphocholines together with those of their ester-connected glycerophosphocholine analogs used as references in this study.

membrane permeability, drug encapsulation and release, which are key issues when the use of liposomes as drug delivery systems is concerned, the intramembrane fluorocarbon 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, active loading of doxorubicin into fluorinated liposomes can also be achieved efficiently [10].

With the aim of extending the potential of fluorinated liposomes as drug carrier and delivery devices, we have developed new highly fluorinated amido-connected phospholipids [12] (compounds **I** to **IV**, Fig. 1). The modular design of their molecular structure is expected to modulate the physico-chemical and biological properties (including permeability, stability in biological fluids, interactions with biocompounds, in vivo fate) of the fluorinated membranes and liposomes they form. This design involves two long hydrophobic chains, one (**IIIa**) or both (**I**, **II**, **IIIb–d** and **IV**) of which end in a highly fluorinated tail, connected through amide bonds to 2,3- or 1,3-diaminopropano- (**I**, **II**), serino- (**III**) or aminoethylphosphocholine (**IV**) polar head groups. The amide bonds are intended to confer higher chemical and biological stability (in acidic media and more particularly towards the action of phospholipases) to these fluorinated phospholipids and to their liposomes [13]. Furthermore, the CONH amide linkage provides an important inter- and intra-molecular hydrogen bond capability [14,15]. The formation of a hydrogen bond network within the membrane in proximity to the water interface is expected to enhance the physical and biological stability of the membrane and, consequently, to increase the in vivo blood circulation times of the liposomes formed from these amidophospholipids, as found for liposomes formulated with sphingomyelins [16], which are naturally occurring amidophospholipids.

We have already reported that the new fluorinated amidophospholipids shown in Fig. 1 form long-term shelf stable liposomes (in terms of particle size and size distribution) [17] and display a rather high in vivo tolerance [12] which are prerequisites when their use as in vivo drug delivery devices is contemplated. We report now our detailed investigations on the ability of their liposomes to retain a passively entrapped drug model, 5(6)-carboxyfluorescein (CF) [18], when these liposomes are incubated in a physiological buffer or in human serum at 37°C. The experimental data obtained are compared to those reported for the first generation of fluorinated liposomes made from fluorinated ester-connected phosphatidylcholines (i.e., DF*n*C*m*PC compounds in Fig. 1) [9] and for conventional liposomes made from hydrocarbon phosphatidylcholines alone or in combination with cholesterol (CH). The effects of the fluorocarbon lipophobic intramembrane layer and of the molecular structure of the fluorinated phospholipids on CF release and on membrane permeability are also discussed.

2. Materials and methods

The syntheses of the various fluorinated amido-connected phosphocholines (Fig. 1) used here are described in Ref. [12]. Their purity (> 99%) was periodically checked by TLC, ^{1}H- and ^{31}P-NMR. 5(6)-carboxyfluorescein (CF), cholesterol (CH; 99%) and sodium deoxycholate were purchased from Sigma and used as received. *rac*-1,2-Distearoylphosphati-

dylcholine (DSPC) came from FLUKA and its purity (> 99%) was controlled by TLC before use. The egg phosphatidylcholines (EPC) Lipoid E-100, from Lipoid KG (Ludwigshafen, Germany), contain phosphatidylcholines and lysophosphatidylcholines (98.0% w/w with lysophospholipids < 3.0%). 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) were hydrated for 30–45 min in a 100 mM CF solution (0.5 ml; pH 7.5) at 10 to 15°C above their gel to liquid-crystalline phase transition temperatures T_c listed in Table 1. These suspensions were then sonicated using a 3 mm titanium probe (Branson Sonic Power Co. Sonifier Cell Disrupter B300), at a temperature above T_c, until they became clear and translucent, or extruded through a polycarbonate membrane (Liposofast Milsch Equipment; size filter 100 nm). The liposomes made from DSPC/CH or EPC/CH (1:1, mol ratio) were prepared starting from a dried film obtained by evaporating a chloroform/methanol solution of the two lipids. These films were hydrated and the suspensions sonicated at 70 and 40°C, respectively. All vesicle suspensions were then aged above the T_c of the phospholipids for 12 h prior to release measurements. The DSPC/CH and EPC/CH vesicles were aged at 60 and 40°C, respectively. 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 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. The release of the entrapped probe from the liposomes (which increases the fluorescence of the dye because of its dilution-dependent dequenching) 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 [19], 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 and 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 incubated in buffer were in most cases mono-exponentional. In some cases, the kinetics were biphasic with an initial phase of fast release mainly related to the temperature increase (the separation of un-entrapped CF from the vesicles was indeed performed at room temperature before the release experiments were run at 37°C). The time-courses of CF release from the vesicles which had been incubated in human serum were recorded over 2-h and 24-h periods. The kinetics were then 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.

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

Liposome formulation [mean diameter [a] / T_c [b]]	$t_{1/2}$ (±S.D.) [c] (h) Na_2SO_4/Hepes buffer	P (±S.D.) [c] (10^{-12} cm/s) Na_2SO_4/Hepes buffer	$t_{1/2}$ (±S.D.) [c] (h) NaCl/Hepes buffer (A)	$t_{1/2}$ (±S.D.) [c] (h) in human serum (B)	B/A ratio
'Liquid-crystalline' formulation					
1,3-DF4C11APC [100 nm/14°C [d]]	22 (3)	12.4 (1.5)	19 (1)	0.80 (0.04)	0.04
DF4C11AEPC [40 nm/4°C [d]]	11 (1)	8.0 (0.6)	13 (1)	0.50 (0.04)	0.04
DF4C11SerPC [110 nm/23°C [d]]	19 (4)	17.4 (4.0)	17 (5)	2.2 (0.7)	0.2
[F4C11][F6C11]SerPC	49 (9)	8.5 (1.6)	48 (17)	3.8 (0.1)	
[140 nm/37°C [d]]				54 (8) [g]	≈ 1
[C16][F6C11]SerPC	52 (8)	6.3 (1.0)	60 (5)	1.6 (0.1)	0.02
[110 nm/34°C [d]]					
DF4C11PC [e,f] [95 nm/19°C]	16 (5)	17.6 (5.5)	17 (5)	90 (8)	
				72 (1) [g]	≈ 4
DMPC [e,f] [95 nm/23°C]	0.6 (0.1)	464 (70)	0.4 (0.1)	< 0.017 [h]	–
'Gel' formulation					
1,3-DF6C11APC [140 nm/45°C [d]]	50 (10)	8.4 (1.6)	47 (11)	16 (2)	
				> 460 [g]	> 10
2,3-DF6C11APC [140 nm/49°C [d]]	65 (16)	6.5 (1.2)	67 (8)	57 (6)	
				122 (18) [g]	≈ 2
DF6C11SerPC [200 nm/55°C [d]]	45 (4)	12.9 (1.1)	45 (6)	35 (6)	
				> 700 [g]	> 15
DF6C11PC [e,f] [75 nm/48°C]	85 (25)	3.2 (0.8)	60 (12)	> 160	
				> 600 [g]	> 10
DPPC [e,f] [115 nm/41°C]	9.0 (0.4)	37 (1)	13 (1)	6.0 (0.5)	≈ 0.5
DSPC [e,f] [50 nm/55°C]	14 (3)	9 (2)	10 (2)	19 (1)	
				40 (5) [g]	4
DSPC/CH (1:1) [95 nm/–]	56 (10)	4.9 (1.8)	56 (18)	640 (60)	
				> 1000 [g]	> 18
EPC/CH (1:1) [f] [45 nm/–]	30 (15)	4 (2)	33 (9)	27 (2) [g]	≈ 1

[a] Liposome mean size (> 95% of the population).
[b] The gel to liquid-crystalline phase transition temperatures, T_c, correspond to planar membranes. For unilamellar liposomes, the T_c is generally lower by a few degrees than that indicated.
[c] The $t_{1/2}$ and P values given correspond to the mean (±S.D.) of three or four independent experiments.
[d] The T_c data are taken from Ref. [17].
[e] The T_c data are taken from Ref. [8].
[f] The $t_{1/2}$ and P values are taken from Ref. [9]. DMPC and DPPC are *rac*-1,2-dimyristoylphosphatidylcholine and *rac*-1,2-dipalmitoylphosphatidylcholine, respectively.
[g] Values calculated from 24-h kinetic experiments (otherwise 2 h).
[h] The CF release is too fast to be measured under our conditions, indicating that the $t_{1/2}$ lies below 0.017 h.

The latter is related to the external diameter d (measured by photon correlation spectroscopy) by $r = (d/2) - \Delta x$, with Δx estimated to be 3.6 nm for the membranes in the liquid-crystalline state and 4.8 nm in the gel state [20–22] (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. This table collects also (i) the serum/buffer $t_{1/2}$ ratio values which express the serum effects on CF release and (ii) the same parameters for selected fluorinated liposomes made from DFnCmPC phosphatidylcholines (Fig. 1), and for conventional liposomes made from hydrocarbon phosphatidylcholines used either alone or in combination with cholesterol.

The diagrams in Fig. 2 A and B illustrate the evolution of the $t_{1/2}$ values for liposomes incubated in a Hepes/NaCl (20 mM/0.15 M) buffer or in human serum and for which the membranes are in the liquid-crystalline (Fig. 2A) or gel state (Fig. 2B). Fig. 3 shows the dependence of the permeability coefficients P of CF across the various liposomal membranes on 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.

3.1. CF release from liposomes and permeability of membranes in the liquid-crystalline state

In a Na_2SO_4 or NaCl buffer and at 37°C, the $t_{1/2}$ values of CF leakage from the fluorinated amido-based liposomes whose membranes are in the liquid-crystalline state, are in the 11–60 h range, while that of the 'liquid-crystalline' hydrocarbon *rac*-1,2-dimyristoylphosphatidylcholine (DMPC) is of 0.4 h (Table 1). In addition, the P coefficients for the 'liquid-crystalline' fluorinated membranes (Table 1 and Fig. 3) are all significantly much lower than that

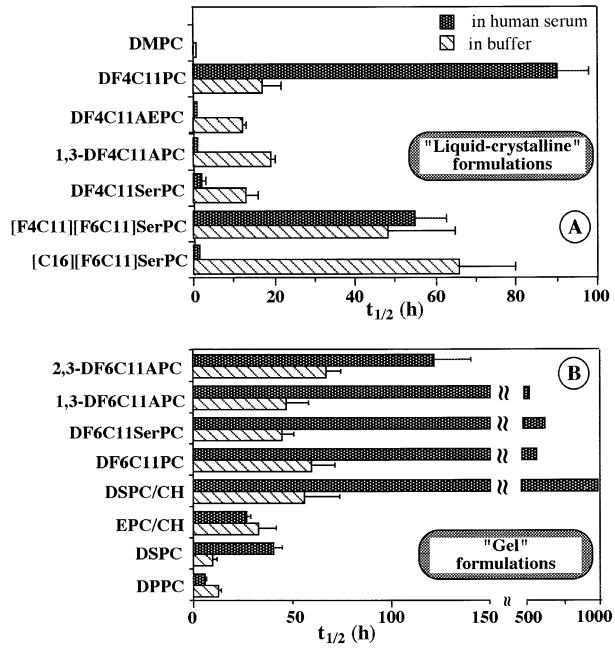

Fig. 2. Comparison of the carboxyfluorescein (CF) release from conventional or fluorinated unilamellar liposomes when incubated in a buffer (hatched bars) or in human serum at 37°C. The liposomal membrane is, at 37°C, (A) in the liquid-crystalline state, (B) in the gel state.

calculated for DMPC, some being even lower than that of 'gel' *rac*-1,2-dipalmitoylphosphatidylcholine (DPPC) membranes. These results indicate that the 'liquid-crystalline' fluorinated liposomes retain their content much more efficiently and display a significantly lower membrane permeability than their 'liquid-crystalline' conventional DMPC analogs. This is already the case for the mixed-chain hydrocarbon/fluorocarbon [C16][F6C11]SerPC phospholipid which forms liposomes and membranes that are even less leaky and less permeable than those made from double-chain fluorocarbon/fluorocarbon analogs (Table 1).

One can further notice that some of the fluorinated 'liquid-crystalline' liposomes and membranes (e.g., [F4C11][F6C11]SerPC or [C16][F6C11]SerPC) display CF release rates and P coefficients (Table 1) which are, respectively, comparable to those of the fluorinated 'gel' ones (see Section 3.2). Indeed, the $t_{1/2}$ and P values corresponding to these liposomes and membranes lie in similar range values (45–85 h and $(3.2–17.6) \times 10^{-12}$ cm/s, respectively). This differs considerably from what is found for the con-

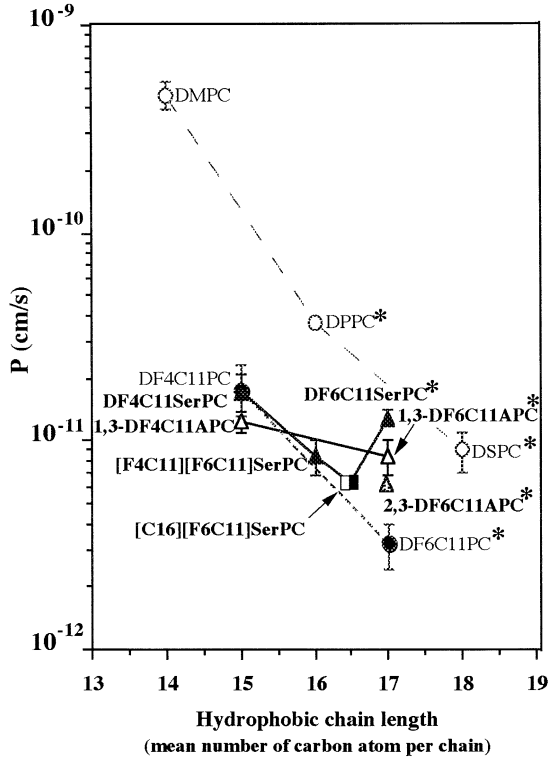

Fig. 3. Variation of the permeability coefficients, P, for fluorinated and conventional unilamellar liposomes with the length L of the phospholipid's hydrophobic chains ($L = n + m$ or $[n + n' + m + m']/2$ when both chains are of different length; n (n') = number of carbon atoms of the fluorinated tail; m (m') = number of carbon atoms of the hydrocarbon spacer or chain, see Fig. 1). The symbol * indicates that the P value is corresponding to liposomal membranes which are close to their phase transition (DPPC) or in the gel state. 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).

ventional liposomes and membranes for which the impact of the physical state on $t_{1/2}$ and P is much more important: thus, $t_{1/2}$ increases from 0.6 h for liquid-crystalline DMPC to 9 and 14 h for gel DPPC or DSPC, and, concomitantly, P decreases from 464 $\times 10^{-12}$ cm/s for DMPC to 37(or 9) $\times 10^{-12}$ cm/s for DPPC or DSPC, respectively (see Fig. 3). These results most likely indicate that the fluorocarbon intramembrane layer acts as an efficient barrier to permeation when the membrane is in a liquid-crystalline state.

When incubated in human serum, the CF release from the fluorinated amido-based liposomes ($t_{1/2}$ in the 0.4 to 54 h range) is also much slower than from conventional DMPC ones ($t_{1/2} < 0.017$ h), indicating a greater stability of the former formulations (Table 1, Fig. 2A). However, as shown by the serum/buffer $t_{1/2}$ ratios (Table 1) which, in most cases, are far below 1, serum destabilizes most of these 'liquid-crystalline' fluorinated liposomes as it is also the case for the 'liquid-crystalline' conventional DMPC ones. By contrast, the [F4C11][F6C11]SerPC liposomes are almost unaffected by the serum components ($t_{1/2}$ ratio close to 1). Among the fluorinated formulations, the liposomes made from the mixed-chain fluorocarbon/hydrocarbon [C16][F6C11]SerPC phospholipid, e.g., the less fluorinated one of the series investigated (Fig. 1), appear to be most destabilized.

Fluorinated liposomes made from phospholipids possessing the same hydrophobic chains (e.g. F4C11) belonging either to the amide series deriving from serine (DF4C11SerPC), diaminopropanol (1,3-DF4C11APC) or aminoethanol (DF4C11AEPC), or to the ester series deriving from glycerol (DF4C11PC), display comparable CF release rates and permeability coefficients when incubated in a buffer (Table 1, Fig. 2A and Fig. 3). However, this is no more the case when the liposomes are incubated in human serum: all the amide-based formulations are much more leaky than the vesicles made from the ester DF4C11PC. These results indicate that the chemical nature of the junction (amide, ester) and/or the connecting group (serine, diaminopropanol, aminoethanol, glycerol) between the hydrophobic chains and the PC polar head has almost no effect on membrane permeability to CF but does have a significant contribution on CF release when the liposomes are placed in the presence of serum components.

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

When incubated in a buffer, the 'gel' fluorinated amido-based liposomes display $t_{1/2}$ values which are higher than those of the conventional 'gel' DSPC ones and comparable to those of CH-containing ones (Table 1, Fig. 2B). However, both types of membranes exhibit similar P values (ranges (6.5–12.9) $\times 10^{-12}$ cm/s and (4–9) $\times 10^{-12}$ cm/s, respectively; Table 1, Fig. 3), indicating that in the gel state no significant difference in permeability exists between fluorocarbon and conventional membranes.

As for the 'liquid-crystalline' formulations, it was also found that the 'gel' amido-based (2,3- or 1,3-DF6C11APC or DF6C11SerPC) and 'gel' DF6C11PC ester-based liposomes exhibit comparable CF release rates and P coefficients (Table 1 and Fig. 3), indicating that the chemical nature of the connecting group (diaminopropanol, serine or glycerol) has almost no influence on membrane permeability.

When incubated in human serum, all 'gel' fluorinated amido-based liposomes display very high $t_{1/2}$ values which are larger than those of DSPC or of EPC/CH liposomes. The 1,3-DF6C11APC and DF6C11SerPC liposomes display CF release rates similar to those of the fluorinated ester-based DF6C11PC or of conventional DSPC/CH ones (Table 1 and Fig. 2B). It should be noticed that the 'gel' 1,3-DF6C11APC, DF6C11SerPC or DF6C11PC liposomes and the conventional 'gel' DSPC/CH ones are much more stable when incubated in human serum rather than in buffer, as shown by the large serum/buffer $t_{1/2}$ values (>10; Table 1). This is also the case for 'gel-like' EPC/CH ones, but to a lesser extent ($t_{1/2}$ ratio of 4). On the other hand, the 'gel' conventional DSPC vesicles are almost not perturbed by the human serum components ($t_{1/2}$ ratio of 1). These results indicate that the presence of a fluorocarbon film within a membrane improves the stability of the liposomes, in terms of dye retention, in a similar way to that resulting from the addition of cholesterol to DSPC or EPC membranes.

As far as the impact of the 2,3- or 1,3-isomerism is concerned, both 2,3- and 1,3-DF6C11APC form liposomes that efficiently retain encapsulated CF and possess comparable *P* coefficients when incubated in buffer (Table 1). However, when incubated in human serum, liposomes formed from the 1,3-isomer are significantly more stable (4-fold) than those from isomer 2,3.

4. Discussion

The main objective of this study was to determine the potential of liposomes made from highly fluorinated amidophosphocholines (Fig. 1) as alternative drug carrier and delivery systems, comparatively to that of liposomes formed from fluorinated (such as DF*n*C*m*PC in Fig. 1) or conventional phosphatidylcholines [9,10]. In order to further evaluate the impact of the modular structure of the fluorinated phospholipids (amide vs. ester bond, serine, diaminopropanol or ethanolamine vs. glycerol connecting backbone) 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 [23]. Our following discussion takes also into account that, at the incubation temperature, the liposomal membranes are either in the gel or liquid-crystalline state (which is well-known to influence membrane permeability [4,24]).

4.1. Liposome stability in a buffer and membrane permeability

Our results clearly demonstrate that the presence of a fluorinated core within the liposomal membrane in the liquid-crystalline 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 amidophospholipids (e.g., 1,3-DF4C11APC, DF4C11AEPC, DF4C11SerPC, [F4C11][F6C11]SerPC or [C16][F6C11]SerPC), when incubated in a buffer at 37°C, retain entrapped CF much more efficiently and exhibit much lower membrane permeability than the 'liquid-crystalline' conventional DMPC counterparts (Table 1 and Figs. 2 and 3). The remarkable efficiency of the fluorinated core to reduce membrane permeability and drug release is best illustrated by the characteristics of the membranes and liposomes of the mixed hydrocarbon/fluorocarbon double-chain [C16][F6C11]SerPC which is the less fluorinated among the fluorinated phospholipids investigated here. It is indeed noteworthy that the [C16][F6C11]SerPC-based membrane displays a *P* coefficient which is (i) not only much lower than that of the DMPC-based membrane, but also comparable (ii) to that of the 'liquid-crystalline' fluorocarbon/fluorocarbon double-chain

[F4C11][F6C11]SerPC-based one and, even more remarkable, (iii) to that of the 'gel' fluorocarbon/fluorocarbon double-chain DF6C11SerPC one. Similar results have been evidenced for mixed double-chain fluorocarbon/hydrocarbon glycerophosphocholines as compared to homogeneous fluorocarbon double-chain ones [25].

However, for membranes in the gel state, the fluorinated intramembrane film has almost no effect on CF permeation: both the 'gel' fluorinated amidophosphocholine-based and conventional DSPC-based membranes display indeed comparable permeability *P* coefficients (Table 1 and Fig. 3). This result is also in line with our previous studies concerning 'gel' fluorinated membranes and liposomes made from fluorinated diacyl DF*n*C*m*PC [9] or dialkyl glycerophosphocholines [25].

The effects on membrane permeability arising from the presence of a fluorinated intramembrane film can be attributed to its high hydrophobic and lipophobic character. Such a character is expected to reduce (i) the probe's solubility (or its penetration) in the hydrophobic phase of fluorinated membranes (the much lower solubility of hydrophilic and lipophilic compounds in fluorinated membranes has indeed been established [8]), and/or (ii) its diffusion more particularly across a fluorinated layer. Both these factors, along with others, control permeation of a dye across a membrane. This analysis accounts for (i) the higher efficiency of any of the fluorinated 'liquid-crystalline' liposomes to retain CF as compared to the conventional 'liquid-crystalline' DMPC ones, (ii) the very low impact of the fluorinated intramembrane layer on CF release in the case of 'gel' fluorinated membranes and (iii) the small gaps in CF release rates and permeability *P* coefficients between 'liquid-crystalline' and 'gel' fluorinated liposomes while the differences are much more important for the conventional ones. The surprising larger or similar membrane permeability and dye release ability of 'gel' fluorinated liposomes as compared to some 'liquid-crystalline' fluorinated ones (e.g., [F4C11][F6C11]SerPC or [C16][F6C11]SerPC) could also arise from the presence of packing defects which are more important for 'gel' membranes and also for membranes whose phase transitions are large and occur over a temperature range including the incubation temperature (which is the case for the fluorinated 'gel' membranes [17]), thus facilitating transmembrane diffusion.

4.2. Liposome stability in human serum

Serum is among the biological fluids known to induce strong destabilizing effects on membranes in their liquid-crystalline state [23]. Most of the new fluorinated herein investigated liposomes, whose membranes are in the liquid-crystalline state at 37°C, are also destabilized by the serum components but to a considerably lesser extent than the 'liquid-crystalline' DMPC conventional ones (Table 1 and Fig. 2A). On the other hand, the fluorinated core improves dye retention for the fluorinated liposomes in serum, when their membranes are in the gel state (Fig. 2B). Indeed, the 'gel' fluorinated liposomes display not only a significantly greater stability in serum than in buffer, but they are noticeably even more stabilized in serum than conventional 'gel' DSPC liposomes. A similar behavior was found for conventional DSPC or EPC membranes but only upon addition of cholesterol to these membranes [16] [1].

These results are very similar to those already found for 'liquid-crystalline' or 'gel' liposomes formulated with fluorinated glycerophosphocholine analogs [9,25]. They further indicate that the fluorinated intramembrane core with its lipophobic/hydrophobic characteristics 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. As the stability of liposomes in serum involves the interaction of serum proteins (usually negatively charged) with the bilayer [26], the extended stability of the fluorinated liposomes in serum may also stem from the large negative dipole potential of fluorinated membranes (while that of conventional ones is positive) [22]. This has been shown to modify substantially the binding of negatively charged lipophilic/hydrophobic ions [22], thus strongly supporting a reduction of the binding of the negatively

[1] In the absence of cholesterol, EPC liposomes are, at 37°C, much more leaky in human serum ($t_{1/2}$ of CF release is 0.12 h) than in buffer ($t_{1/2}$ = 13 h) [8].

charged serum proteins that partition into the interfacial region of the bilayer. In addition, the greater stability of the 'gel' fluorinated vesicles in serum than in buffer could indicate that some serum constituents participate in increasing the order or packing of the phospholipids within the membrane, and/or in suppressing packing defects which frequently arise for liposomes with membranes in the gel state.

4.3. Structure / permeability / CF release relationships

Another objective of this study was to compare the potential of the liposomes formulated with these new fluorinated double-chain amidophosphocholines to that reported for the first generation of fluorinated ester phosphatidylcholine-based liposomes, hence to evaluate the impact of the ester/amide bond or of the connecting backbone (ethanolamine, diaminopropanol, serine vs. glycerol) on permeability to CF and release. We found that these structural variations have in most cases almost no effect: indeed, membranes and liposomes prepared either from the amido-connected DF4C11AEPC, 1,3-DF4C11APC, DF4C11SerPC, DF6C11APC or DF6C11SerPC or from their DF4C11PC or DF6C11PC ester homologs, respectively, behave very similarly in terms of permeability and CF release. Significant differences of behavior in human serum are only observed between the 'liquid-crystalline' [F4C11][F6C11]SerPC or DF4C11PC liposomes and the other 'liquid-crystalline' fluorinated amido-based formulations. However, the two former formulations seem to constitute exceptions since several 'liquid-crystalline' fluorinated formulations made from other fluorinated phosphatidylcholines were found to be less stable in human serum than in buffer [9].

The differences in permeability and CF release rates observed for the 2,3- and 1,3-DF6C11APC isomer-based liposomes, when incubated in human serum, are most likely related to phase behavior rather than to membrane permeability properties. Indeed, we have shown elsewhere that 2,3-DF6C11APC, when dispersed in an aqueous phase, forms a metastable lamellar phase which slowly converts into a ribbon-like phase [17]. This phase transition may be accelarated by the serum components, thus increasing CF release.

5. Conclusion

We have shown that membranes and liposomes whether formulated with double-chain fluorocarbon/fluorocarbon or mixed fluorocarbon/hydrocarbon amido-based phosphocholines 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 as these new compounds bring an easy access to drug carrier system components, together with improved chemical and biochemical stability. They further confirm that a fluorinated intramembrane layer reduces the permeability of membranes (more significantly when they are in a liquid-crystalline state), protects them from the destabilizing effects of serum and increases even the stability (in terms of dye retention) of the fluorinated liposomes whose membranes are in the gel state, when incubated in serum. This effect is similar to that resulting from the addition of cholesterol to conventional membranes.

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