ELSEVIER

Biochimica et Biophysica Acta 1192 (1994) 61–70

BBA Biochimica et Biophysica Acta

Permeability and stability in buffer and in human serum of fluorinated phospholipid-based liposomes

Frédéric Frézard, Catherine Santaella, Pierre Vierling *, Jean G. Riess

Laboratoire de Chimie Moléculaire, URA 426 au CNRS, Faculté des Sciences, Université de Nice-Sophia Antipolis, 06108 Nice Cédex 02, France

(Received 16 November 1993)

Abstract

The stability (with respect to encapsulated carboxyfluorescein release) of fluorinated liposomes and their membrane permeability have been investigated in buffer and in human serum as compared to conventional hydrogenated analogues. These fluorinated liposomes are made from highly fluorinated phosphatidylcholines and contain a fluorinated core whithin their membrane. In buffer and in their fluid state, the fluorinated liposomes retain much more efficiently their entrapped content and display lower membrane permeability coefficients than any of their hydrogenated counterparts. This indicates that the fluorinated core acts as a very efficient barrier to permeation. In terms of molecular structure/permeability relationships, the thicker the fluorinated lipophobic core, the more efficient the barrier to permeation. In their gel state, the fluorinated core has, however, almost no effect on permeation. Interestingly, some of the 'fluid' fluorinated liposomes were even less permeable than 'gel' or 'gel-like' ones, including egg phosphatidylcholines/cholesterol liposomes. Human serum destabilizes the 'fluid' fluorinated liposomes but to a lesser extent than the 'fluid' hydrogenated ones, indicating that the fluorinated lipophobic core inside the liposomal membrane protects the vesicles, possibly by reducing their interactions with serum components. 'Gel' or 'gel-like' fluorinated liposomes are significantly more stable in serum than in buffer. They are also more stable than conventional 'gel' or 'gel-like' liposomes.

Key words: Perfluoroalkylated phosphatidylcholine; Phospholipid; Fluorinated liposome; Vesicle; Membrane; Permeability

1. Introduction

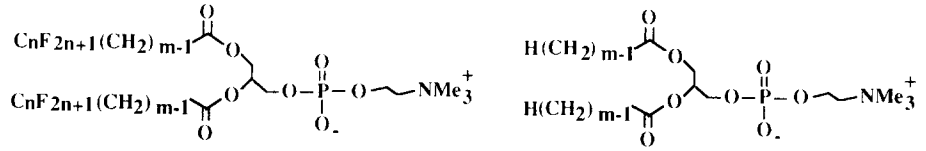
The ability of liposomes to encapsulate a wide variety of solutes in their internal aqueous compartment has stimulated intensive efforts in view of developing their applications as drug carrier and delivery systems [1,2]. Several liposomal drug formulations are presently in clinical trials. However, additional research is needed to improve our ability to control and manipulate the liposomes' permeability, long-term shelf-stability and stability in biological environments (stability is defined here as the extent to which the carrier retains its structure and drug content), in vivo recognition, intravascular persistence and biodistribution.

The elaboration of a stable membrane allowing the encapsulation and retention of a variety of drugs is a challenging objective. It has been achieved to some extent by increasing the rigidity or order of the membrane either by including cholesterol (CH) into the membrane or by using phospholipids which tend to self-organize into a rigid gel lamellar phase [3,4]. Such liposomal formulations have, however, limited potential: (i) cholesterol is unsuitable for certain pathologies; (ii) a hydrocarbon membrane constitutes only a tenuous barrier especially for sustaining water-soluble lipophilic drugs; (iii) vesicles with rigid membranes display slow drug release but do not allow for modular drug release.

With the aim of extending the potential of liposomes as drug carriers, we have investigated the possibility of endowing the liposomal membrane with some of the unique properties of fluorocarbons, i.e., high hydrophobicity and high lipophobicity. We have therefore developed synthetic highly fluorinated phospholipids (Fig. 1) which were expected to form fluorinated membranes and liposomes, i.e., liposomes that possess

* Corresponding author. Fax: +33 93 529020.

SSDI 0005-2736(94)00028-N



n = 4 ; m = 11 F4C11PC n = 6 ; m = 5 F6C5PC
n = 6 ; m = 11 F6C11PC n = 8 ; m = 5 F8C5PC
n = 6 ; m = 7 F6C7PC

m = 12 DLPC m = 16 DPPC
m = 14 DMPC m = 18 DSPC

Fig. 1. Structure and nomenclature of the perfluoroalkylated and hydrogenated phospholipids used.

within their membrane a fluorinated lipophobic core as illustrated in Fig. 2. The modular molecular structure of these fluorine-modified phosphatidylcholines (variable fluorinated tail and hydrogenated spacer lengths) allows stepwise adjustement of the thickness of both the lipophilic shells and internal lipophobic fluorinated core (hence of the fluorophilicity/lipophilicity/hydrophilicity balance) and, consequently, of the physicochemical properties (stability, gel to fluid phase transition temperature, fluidity/rigidity, permeability) of the liposomes they will form. We have already shown that these phospholipids form highly stable (in terms of size and size distribution evolution with time) fluorinated liposomes as a result of enhanced hydrophobic interactions [5]. The use of a liposomal membrane with an internal highly hydrophobic and lipophobic core was also expected to act on its permeability characteristics and its interactions with serum constituents and other biological compounds, hence to modify the stability of the liposomes in biological fluids with respect to drug leakage. It was also intended to modify their in vivo behavior such as recognition by the mononuclear phagocytic system, intravascular persistence and biodistribution. We found indeed that the fluorinated liposomes display remarkably higher in vivo blood circulation times than conventional ones [6].

We report here our detailed investigations on the ability of liposomes formulated with fluorinated phosphatidylcholines to retain efficiently an entrapped drug model, 5(6)-carboxyfluorescein (CF) [7], when these liposomes are incubated in a buffer at various temperatures, or in human serum at 37°C. The impact of the fluorinated lipophobic core, 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. In all cases, the experimental data obtained are compared with those obtained for conventional liposomes made from their hydrogenated phosphatidylcholine analogs.

2. Materials and methods

The perfluoroalkylated phospholipids F6C5PC, F8C5PC, F6C7PC, F4C11PC and F6C11PC (Fig. 1) were synthetized according to Ref. 8. Their purity (> 99%) was checked by TLC, ^{1}H- and ^{31}P-NMR. Racemic dilauroyl- (DLPC), dimyristoyl- (DMPC), dipalmitoylphosphatidylcholine (DPPC), and *sn*-distearoylphosphatidylcholine (DSPC) were purchased from FLUKA and their purity (> 99%) was controlled by TLC before use. Egg-yolk phosphatidylcholines, EPC (Lipoïd 100), came from LIPOID and were used as received. Cholesterol (CH), 5(6)-carboxyfluorescein (CF) and sodium deoxycholate were purchased from SIGMA and used as received. Human serum was pro-

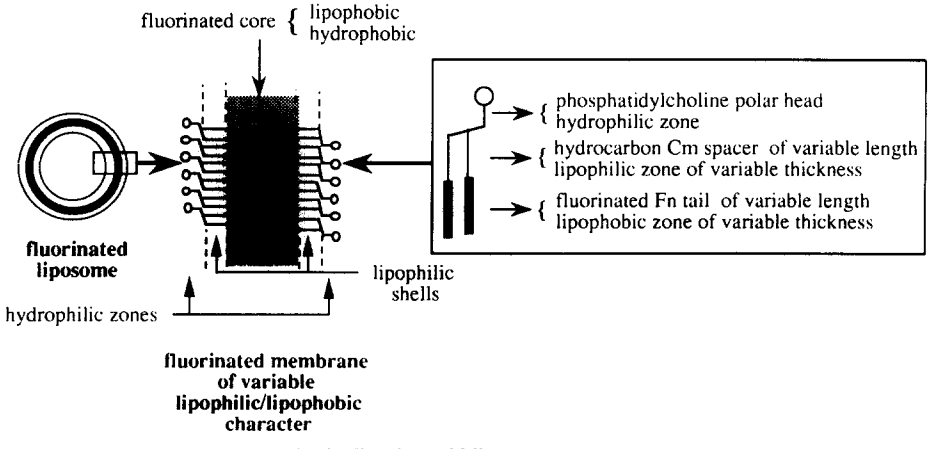


Fig. 2. Fluorinated bilayer core concept.

Table 1
Half-leakage times $t_{1/2}$, permeability coefficients P, and activation energies E_a, for carboxyfluorescein (CF) leakage from SUVs made with various fluorinated and hydrogenated phospholipids measured at different temperatures [a,b]

	F6C5PC (35 nm) [c]	F8C5PC (60 nm) [c]	F6C7PC (55 nm) [c]	F4C11PC (35 nm) [c]	F6C11PC (75 nm) [c]	DLPC	DMPC (25 nm) [c]	DPPC (30 nm) [c]	DSPC (50 nm) [c]	EPC (20 nm) [c]	EPC/CH (45 nm) [b]
7°C [d]											
$t_{1/2}$ in h (±)	*253 (160)*	160 (20)		203 (51)	405 (85)	*< 0.017* [g]	130 (30)	110 (3)	35 (8)		
P (10^{12}) cm/s (±)	*0.49 (0.23)*	1.24 (0.17)		0.44 (0.14)	0.57 (0.13)		0.42 (0.10)	0.74 (0.31)	3.3 (0.7)		
37°C [d]											
$t_{1/2}$ in h (±	*53 (10)*	27 (5)	*140 (20)*	*83 (24)*	85 (25)	*< 0.017* [g]	*1.1 (0.1)*	21 (9)	14 (3)		
P (10^{12}) cm/s (±)	*1.75 (0.15)*	8 (1)	*1.1 (0.2)*	*1.2 (0.4)*	3.2 (0.8)		*53 (7)*	4 (2)	9 (2)	*3.2 (0.3)* [f]	4 (2) [f]
75°C [d]											
$t_{1/2}$ in h (±)	*1.8 (0.1)*	*204 (33)*	*9 (2)*	*1.9 (0.2)*	*31 (5)*		*0.027 (0.003)*	*0.19 (0.03)*	*1.4 (0.3)*		
P (10^{12}) cm/s (±)	*56 (1)*	*0.84 (0.13)*	*17 (3)*	*48 (4)*	*7.1 (1.2)*		*2150 (300)*	*305 (34)*	*85 (3)*		
Ea kJ/mol (±)	*84.2 (5.5)*	*137 (57)*	*129 (19)*	*120.7 (6.5)*	*85.4 (3.3)*		*84.5 (3.8)*	*108.2 (10.8)*	*117.2 (5.1)*		
37°C											
a, $t_{1/2}$ in h (±)	*56 (5)*	24 (4)	*74 (20)*	*83 (12)*	60 (12)		*0.33 (0.05)*		10 (2)	*13 (1)*	33 (9)
in a NaCl buffer					66 (10) [e]						
b, $t_{1/2}$ in h (±)	*0.66 (0.06)*	25 (3)	*14 (1)*	*3.6 (0.1)*	> 160		*< 0.017* [g]		19 (1)	*0.12(0.02)*	35 (1)
in serum		77 (4) [e]			> 600 [e]				40 (5) [e]		27 (2) [e]
b/a	*0.012*	3	*0.2*	*0.045*	> 10				4	*0.01*	0.8

[a] Values in italics indicate that the liposomal membranes are in the fluid state at the given temperature.
[b] The $t_{1/2}$ and P values given correspond to the mean (±S.D.) for 3 to 6 independent experiments.
[c] Liposome mean size (> 95% of the population).
[d] Experiments run in the Na_2SO_4 containing buffer.
[e] Values calculated from 24 h kinetic experiments (otherwise 2 h).
[f] Values calculated from experiments run in the NaCl containing buffer.
[g] The CF release is too fast to be measured under our conditions, indicating that the $t_{1/2}$ lie below 0.017 h.

vided by the 'Centre Départemental de la Transfusion Sanguine des Alpes-Maritimes' and was a pool from several donors.

2.1. Preparation of small unilamellar vesicles (SUVs)

Typically, 25–50 mg of the phospholipid (30–50 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 5–10°C above the phase transition temperature, Tc, of the phospholipid under investigation (Tc < 0°C for F6C5PC; 4°C for F6C7PC; 18°C for F4C11PC; 48°C for F6C11PC; 69°C for F8C5PC [9]). These suspensions were then sonicated using a 3 mm titanium probe (Branson Sonic Power Co. Sonifier Cell Disrupter B300), at a temperature above Tc, until they became clear and translucent. EPC/CH (1/1) SUVs were obtained using this procedure but starting from the dried film which was obtained by evaporating a chloroform-methanol solution of the two lipids. To avoid marker leakage through membrane defects arising from sonication, all vesicle suspensions were then aged at room temperature for 24 h prior to measurements. Un-encapsulated CF was removed by gel-filtration through a Sephadex G50 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. In the case of the experiments performed with DMPC vesicles in the gel state, the separation of un-encapsulated from encapsulated CF was made at 7°C. This separation could not be performed in the case of DLPC liposomes because of fast CF release, even at 7°C. The average vesicle diameter was then measured by photon correlation spectroscopy using a Coulter model N4 MD submicron particle size analyzer.

2.2. Carboxyfluorescein (CF) release experiments in buffer or in human serum

The release experiments were run immediatly after the separation of the un-encapsulated dye from the liposomes. In a typical dye-release assay, the liposome suspension (5–20 μl or 30–50 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 various temperatures in a thermoregulated quartz cuvette cell (1 cm) with magnetic stirring. Release of the entrapped probe from the liposomes increases fluorescence of the dye because of the 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. When the CF release was followed for long periods of time (over 2–3 h), F was measured using classical published methods. 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 [10], 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. When possible, 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. When the CF release rate was too slow to allow direct determination of $t_{1/2}$, the linear portion of the curve of ln R vs. time

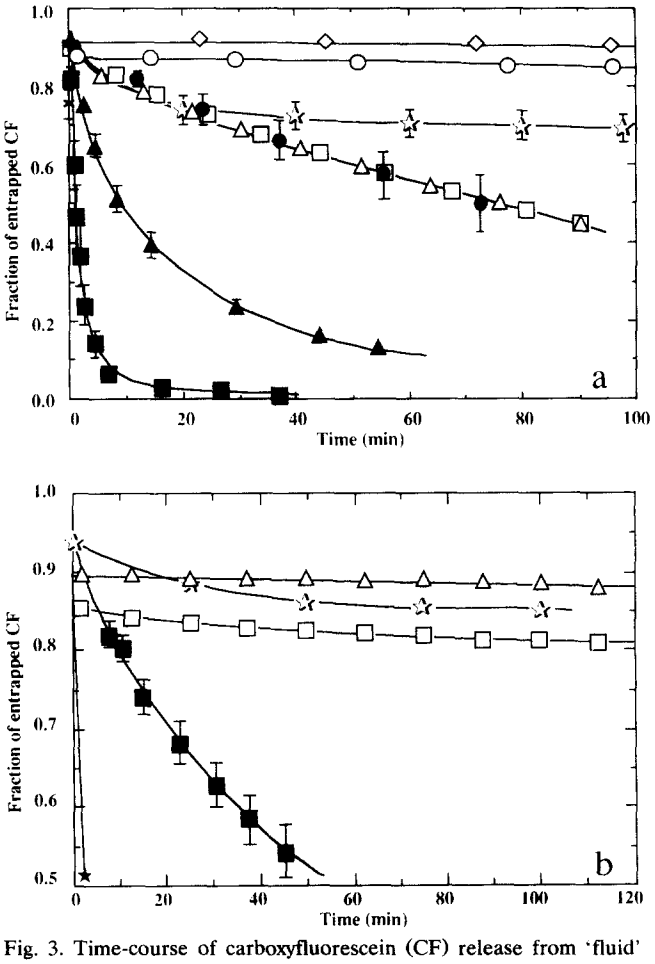


Fig. 3. Time-course of carboxyfluorescein (CF) release from 'fluid' fluorinated (open symbols) and hydrogenated (filled symbols) liposomes, incubated in a 0.1 M Na_2SO_4 buffer at 75°C (3a) or at 37°C (3b); □, F6C5PC; ☆, F6C7PC; ◇, F8C5PC; △, F4C11PC; ○, F6C11PC; ★, DLPC; ■, DMPC; ▲, DPPC; ●, DSPC. When not indicated error bars are smaller than the signs.

was used. The slope, K, of this curve is indeed related to $t_{1/2}$ by the relation: $t_{1/2} = \ln 2/K$ ($R = R_0/2$ in the equation below). Each experiment was performed at least in triplicate.

Assuming that the release of CF occurs via a classical diffusion mechanism, Fick's law can be used to estimate the permeability coefficient, P, of CF across the various membranes. Indeed, the theorical expression of R as a function of time is:

$$\ln R = (S/V)(Dk/\Delta x)t + \ln R_0 = (S/V)Pt + \ln R_0 = Kt + \ln R_0$$

where D is the diffusion coefficient, k the partition coefficient of CF between the membrane and the aqueous phase, Δx the membrane thickness, S the internal surface of the liposome, V the internal volume of the liposome and R_0 the fraction of entrapped CF at $t = 0$.

The permeability coefficient, $P = Dk/\Delta x$, is thus related to the slope K by $P = (V/S)K$ or $= (r/3)K$, 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 3–3.5 nm for the membranes in the fluid state and 4–5 nm in the gel state [11,12].

The permeability coefficients P were also determined at several temperatures. These are also related to the activation energy, E_a, for CF permeation by the relation $\ln P = E_a/RT$. The straight $\ln P$ vs. $1/T$ lines were therefore used to calculate the activation energies listed in Table 1.

3. Results

3.1. CF release from liposomes and permeability in a buffer

3.1.1. In the fluid phase

Fig. 3 shows the time-course of CF release from small unilamellar vesicles (SUVs) made from different fluorinated and hydrogenated phospholipids in the fluid phase at various temperatures. These experiments were run (i) at 75°C (Fig. 3a) where all the fluorinated and hydrogenated liposomal membranes were in the fluid state, (ii) at 37°C (Fig. 3b) where only the F6C5PC, F6C7PC, F4C11PC, DLPC and DMPC membranes were in the fluid state and (iii) at 7°C (not shown) where the F6C5PC and DLPC membranes were in the fluid phase. The leakage half-time values, $t_{1/2}$, determined from these curves, are listed in italics in Table 1. The experiments, whether in a Na_2SO_4 or in a NaCl containing buffer, afforded, in most cases, the same $t_{1/2}$ values. Concerning the permeability coefficients of CF, P, Fig. 4 shows that for the fluorinated as for the hydrogenated membranes, $\ln P$ varies, as expected,

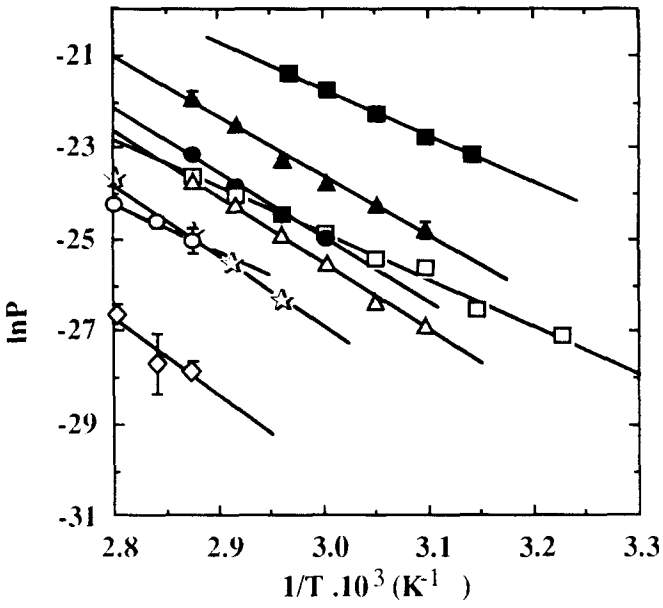


Fig. 4. Arrhenius plots for carboxyfluorescein (CF) permeation across various 'fluid' fluorinated (open symbols) and hydrogenated (filled symbols) membranes of unilamellar vesicles. Vesicles were suspended in a 0.1 M Na_2SO_4 buffer; □, F6C5PC; ☆, F6C7PC; ◇, F8C5PC; △, F4C11PC; ○, F6C11PC; ■, DMPC; ▲, DPPC; ●, DSPC. When not indicated error bars are smaller than the signs.

linearly as a function of $1/T$ in the temperature range where the bilayers are in the fluid state. The P values calculated at 75°C are also listed in Table 1.

At 75°C, the $t_{1/2}$ values of CF leakage from the fluorinated liposomes were found to be in the 1.8–204 h range, while those for the hydrogenated ones lay in the 0.027–1.4 h range (Table 1) indicating that the 'fluid' fluorinated liposomes retain their content much more efficiently (from 1 to at least more than 7500 times, as expressed by the $t_{1/2}$ ratio) than their 'fluid' hydrogenated analogs. The P values (Table 1) calculated for the fluorinated vesicles are all lower than those found for the hydrogenated ones, indicating a lower membrane permeability of the fluorinated liposomes. They decrease along the following sequence DMPC > DPPC > DSPC > F6C5PC > F4C11PC > F6C7PC > F6C11PC > F8C5PC. It is noticeable that the most efficient among the fluorinated vesicles, i.e., F8C5PC, displays $t_{1/2}$ and P values which are, respectively, almost 150 times higher and 100 times lower than those found for DSPC, which forms among the least permeable 'fluid' hydrogenated liposomes. The same trend was obtained (i) at 37°C where the fluid F6C7PC, F6C5PC and F4C11PC liposomes displayed significantly higher $t_{1/2}$ values (50–100 times) and concomitantly lower P values (at least 30 times) than DMPC ones and (ii) at 7°C where the fluid fluorinated F6C5PC liposomes were found to retain CF more than at least 15 000 times more efficiently than liposomes made from DLPC.

Table 1 also displays the values of the activation energy, E_a, for CF diffusion across hydrogenated and fluorinated membranes; E_a was calculated from the slopes of the ln P vs. $1/T$ straight lines (Fig. 4). It appears that the E_a values (80–137 kJ/mole) found for the fluorinated phospholipids are in the same range than those measured for the hydrogenated ones.

3.1.2. *In the gel phase*

Figs. 5a and 5b display the time-course of CF release from fluorinated and hydrogenated SUVs in the gel state as measured respectively at 7°C (F4C11PC, F6C11PC, F8C5PC, DMPC, DPPC and DSPC) and 37°C (F6C11PC and DSPC). The $t_{1/2}$ values calculated from these curves are listed in Table 1.

Most of the 'gel' fluorinated liposomes displayed high $t_{1/2}$ values and the highest values were found for the fluorinated liposomes based on F6C11PC. Although the 'gel' fluorinated liposomes showed significantly higher $t_{1/2}$ values than the 'gel' hydrogenated ones, we found that both types of membranes exhibited comparable P values, indicating that in the gel state no significant difference in permeability exists between those. Surprisingly, a higher permeability of DSPC membranes was found, which, most probably arises from the presence of packing defects due to the high curvature of DSPC SUVs [13].

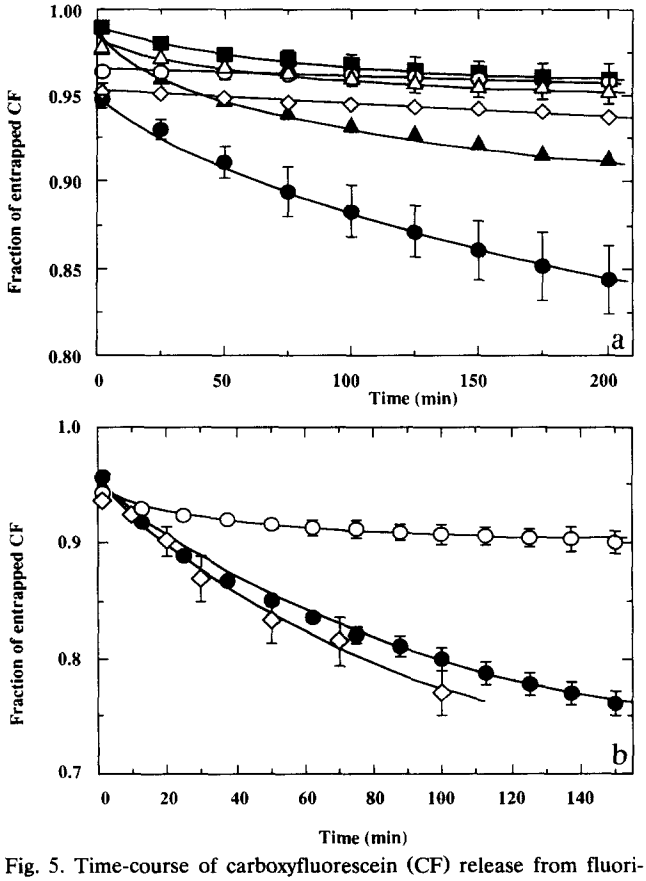


Fig. 5. Time-course of carboxyfluorescein (CF) release from fluorinated (open symbols) and hydrogenated (filled symbols) liposomes in the gel state, incubated in a 0.1 M Na_2SO_4 buffer at 7°C (5a) or at 37°C (5b); ◇, F8C5PC; △, F4C11PC; ○, F6C11PC; ■, DMPC; ▲, DPPC; ●, DSPC. When not indicated error bars are smaller than the signs.

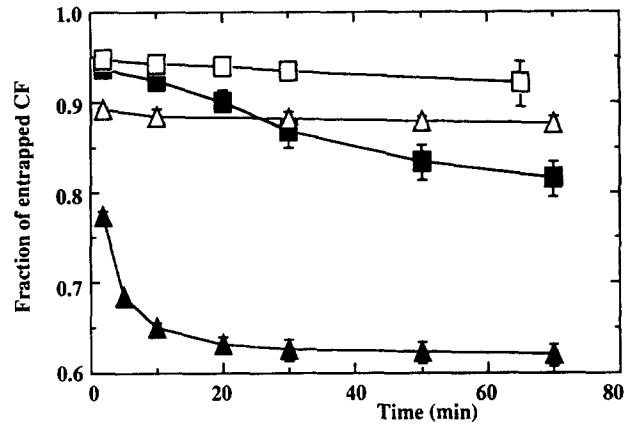


Fig. 6. Kinetic profiles at various temperatures of carboxyfluorescein (CF) release from F8C5PC liposomes suspended in a 0.1 M Na_2SO_4 buffer; □, from 7 to 30°C; ■, 37°C; ▲, 50°C; △, 75°C. When not indicated error bars are smaller than the signs.

All 'gel' liposomes, except those formulated with F8C5PC, exhibited, as expected, higher $t_{1/2}$ values than when their membranes are in the fluid state. Furthermore, the fluid to gel phase transition is, as expected, accompanied by a significant decrease in P, indicating that the permeability of their membrane is decreased. By contrast, F8C5PC liposomes were found to be less leaky and permeable at 75°C, i.e., above the T_c (69°C) of its membrane, rather than at 37°C, i.e., below T_c. Furthermore, in the gel state of F8C5PC, the profiles of the CF release kinetics were strongly temperature dependent, as shown in Fig. 6. In the 7–30°C temperature range (and above T_c) the profiles did not vary with temperature and were monoexponential while, at 37 and 50°C, the profiles were markedly biphasic, comprising a first phase of fast CF release followed by a phase of slower release. This surprising behavior of the F8C5PC vesicles probably arises from the fact that the main phase transition of its membrane occurs over a large temperature range (from 30°C to 69°C): at 37 and 50°C, the fluorinated core of this membrane is, as shown elsewhere, in a semi-fluid to fluid state while the hydrocarbon spacers are still rigid and strained [9]. This is likely to generate packing defects in the membrane, hence to facilitate permeation across such membranes [14].

3.2. *CF release from liposomes in human serum*

Figs. 7a and 7b show, respectively, the time-courses of CF release from SUVs of different compositions incubated at 37°C in human serum over 2 and 24 h

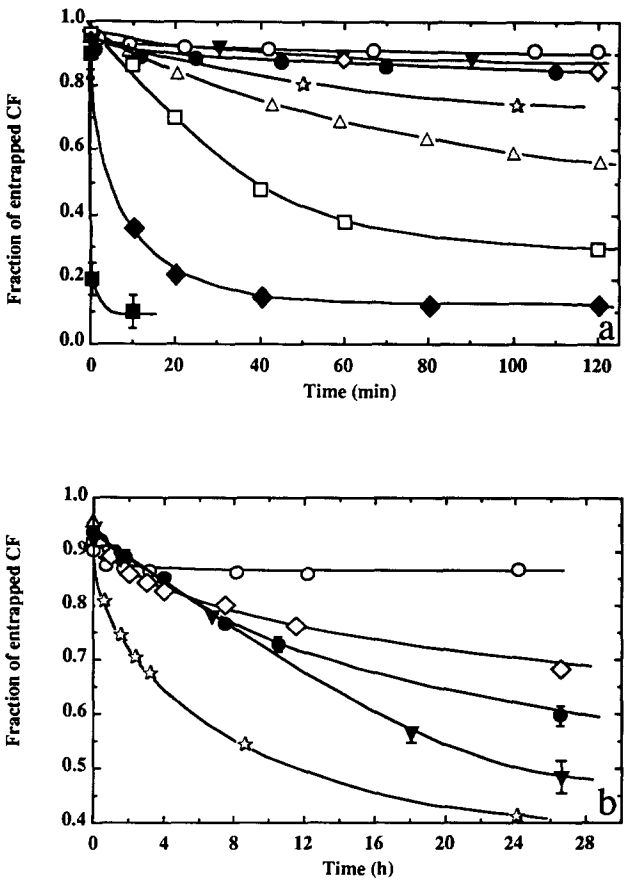


Fig. 7. Time-course over 2 h (7a) or 24 h (7b) of carboxyfluorescein (CF) release from fluorinated (open symbols) and hydrogenated (filled symbols) liposomes incubated in human serum at 37°C; □, F6C5PC; ☆, F6C7PC; ◇, F8C5PC; △, F4C11PC; ○, F6C11PC; ■, DMPC; ●, DSPC; ◆, EPC; ▼, EPC/CH. When not indicated error bars are smaller than the signs.

periods. It is noticeable that in these cases the kinetics are multiphasic, with an initial phase of fast release and a late phase of slower release. This indicates that, in human serum, the slowest release phase is obtained for higher periods of incubation.

Among the fluorinated liposomes, those formulated with F6C11PC and F8C5PC, which, at 37°C, are, respectively, in the gel and semi-gel state, exhibited much slower CF release than the 'fluid' F6C5PC, F6C7PC and F4C11PC liposomes. Furthermore our results indicate clearly that, in human serum, 'fluid' and 'gel' fluorinated liposomes are significantly more stable than, respectively, 'fluid' and 'gel' conventional liposomes. Indeed, the 'gel' F6C11PC vesicles (and even the 'semi-gel' F8C5PC ones) showed a much lower CF release rate (from 2 to 30 times, as expressed by the $t_{1/2}$ ratio) than 'gel' DSPC or than 'gel-like' 1/1 EPC/CH liposomes (Table 1). Similarly, 'fluid' liposomes made from the F4C11PC, F6C5PC and, most significantly, F6C7PC phospholipids, released CF considerably more slowly – from 5 [$= t_{1/2}$(F6C5PC)/ $t_{1/2}$(EPC)] to more than 800 times [$= t_{1/2}$(F6C7PC)/ $t_{1/2}$(DMPC)] – than the 'fluid' DMPC or EPC vesicles. It is also noticeable that the CF leakage from the 'fluid' F6C7PC liposomes was comparable to that observed from the 'gel' DSPC ones.

The effects of serum on CF release from the liposomes can be expressed by the serum $t_{1/2}$ to buffer $t_{1/2}$ ratio: a ratio below 1 or higher than 1 is taken to indicate that serum has a destabilizing or a stabilizing effect, respectively. Our results show that serum destabilizes the fluorinated liposomes whose membranes are in the fluid state at 37°C as it does for the 'fluid' hydrogenated ones. Thus, all the 'fluid' F6C5PC, F6C7PC, F4C11PC, DMPC, and EPC based vesicles displayed $t_{1/2}$ ratios which are all well below 1 (Table 1). Important enough, the higher $t_{1/2}$ ratios found for F6C7PC and F4C11PC, when compared to EPC, indicate that CF leakage from these fluorinated vesicles is somewhat less affected by the serum components than when encapsulated in EPC liposomes.

On the other hand, our results indicate that the 'gel' F6C11PC and DSPC liposomes are significantly more stable in serum than in the physiological buffer, as expressed by the $t_{1/2}$ ratios larger than 1. Surprisingly, this is also the case, although to a lesser extent, for the 'semi-fluid semi-gel' F8C5PC liposomes. It is noteworthy that the F6C11PC and F8C5PC vesicles are even more stable in serum (at least 20 and 3 times, respectively) and more stabilized by serum (at least 10 and 3 times, respectively) than EPC/CH liposomes, which are almost not affected by this change in medium.

As a consequence of the stabilizing effects of serum on 'gel' or 'gel-like' vesicles and of its destabilizing effects on 'fluid' ones, the F8C5PC liposomes were found to be more effective (respectively 100, 20 and 5 times) in retaining CF in serum than the F6C5PC, F4C11PC and F6C7PC ones while the opposite was found in buffer at 37°C.

4. Discussion

The main objectives of this study were to determine the effect of fluorinated phosphatidylcholines (Fig. 1) on the permeability of the membranes and on the stability (with respect to drug release) of the liposomes they form. In order to evaluate the impact of the fluorinated core within the liposomal membrane of modular structure (as illustrated in Fig. 2) and to gain some structure/permeability/stability relationships, we have therefore investigated and compared the ability of various fluorinated vesicles vs. conventional hydrogenated liposomes to retain encapsulated carboxyfluorescein (CF). These liposomes were incubated in buffer at different temperatures, so that their membrane shells were either in the gel or in the fluid state. The physical

state (gel or fluid) of the membrane and its thickness are known, among others, to influence its permeability. CF, which is often utilized to study membrane permeability, was chosen as a water-soluble drug model [7].

In order to evaluate the potential of the fluorinated liposomes as alternative drug carrier and delivery systems, we also investigated and compared their ability to retain encapsulated CF when they are incubated in human serum at 37°C, as compared to conventional liposomes. Among the biological fluids, human serum is known to have the most pronounced effects on membrane permeability and liposome stability.

4.1. Liposome stability and membrane permeability in a buffer

Our results clearly demonstrate that the presence of a fluorinated core 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 length of both the hydrophobic chains and fluorinated tails, the fluorinated vesicles, when incubated in buffer at a temperature where their membranes are in the fluid state, retain entrapped CF much more efficiently (from 1 to at least more than 7500 times, as expressed by the $t_{1/2}$ ratio) and exhibit much lower membrane permeability (from 1.5 to more than 2500 times, as expressed by the P ratio) than any of their 'fluid' hydrogenated counterparts.

These results may be further rationalized in terms of molecular structure/permeability (or CF leakage) relationships. It has been shown for hydrogenated phosphatidylcholines that increasing the length of their hydrophobic chains (e.g., from DLPC to DSPC), hence increasing membrane thickness, resulted in a decrease in permeability of their membranes when taken in the fluid state [16]. In contradiction with this model, we found that the membranes formed from F6C5PC, which, among all the phospholipids investigated, possesses the shortest chains (11 carbon atoms), are less permeable than the DSPC-based ones (which, among the fluid hydrogenated membranes, exhibit the lowest permeability coefficient), although DSPC has much longer hydrophobic chains (18 C). This result not only illustrates the high efficiency of the fluorinated barrier to permeation but further indicates that the presence of a fluorinated core does more than compensate the increase in leakage which is expected from reducing membrane thickness, hence, for example, when going from DSPC to F6C5PC.

Nevertheless, the correlation, found for the fluid hydrogenated membranes, between membrane thickness and permeability still exists within the fluorinated series. As illustrated in Fig. 8, permeability is decreased when the hydrophobic chain length of the fluorinated phospholipid is increased by lengthening

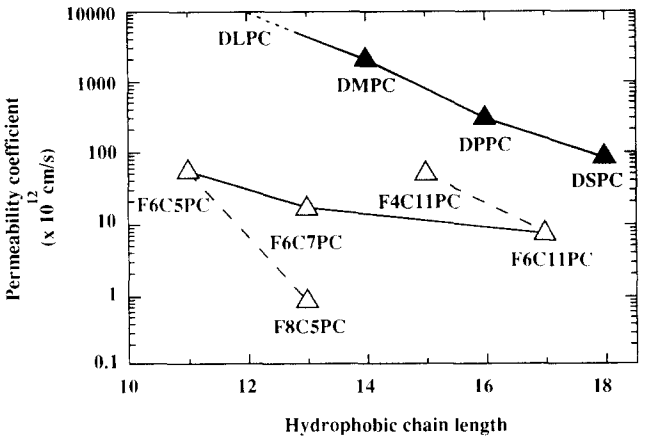


Fig. 8. Permeability coefficient of carboxyfluorescein (CF) at 75°C as a function of the length of the phospholipid hydrophobic chain (number of carbon atoms); △, fluorinated membranes; ▲, hydrogenated membranes. Error bars are smaller than the signs.

either its fluorinated tail or its hydrogenated spacer. The fact that permeation across the 'fluid' fluorinated membranes either remains unmodified from F6C5PC to F4C11PC, or increases from F8C5PC to F6C11PC or to F4C11PC shows that the loss of two CF_2 equals or outweights the gain of six CH_2. These results indicate that the thicker the fluorinated lipophobic core, the more efficient the barrier to CF permeation. This is further illustrated with compounds F6C7PC and F8C5PC which possess hydrophobic chains of identical length (13 C) but differ by their degree of fluorination: in the fluid state, the F8C5PC liposomes are almost 20 times less leaky and less permeable than the F6C7PC ones.

However, it appears that, in the gel state, the fluorinated core has almost no effect on CF permeation: both fluorinated and hydrogenated liposomes display indeed comparable permeability coefficients. It is well known that, in their gel state, hydrogenated membranes are considerably less leaky and permeable than in their fluid state [15,16]. Interestingly, we found that the physical state – gel or fluid – of the membrane is much less important for the fluorinated phosphatidylcholine-based membranes. Indeed, the increase in $t_{1/2}$ and decrease in P values observed, when going from the fluid to the gel state, is always lower for the fluorinated liposomes than for the hydrogenated ones. This behavior may indicate that the differences in the order and packing of the lipids, which exist between the fluid and gel states, are more tenuous for the fluorinated membranes than for the hydrogenated ones. This could arise from a rigidifying and ordering effect of the fluorinated tails on the interfacial hydrocarbon region (see also below). This may also account for the remarkable behavior of some of the 'fluid' fluorinated liposomes which were found to be no more leaky and permeable, or even less leaky and permeable, than 'gel'

or 'gel-like' ones. This is more particularly the case, at 37°C, for the 'fluid' F6C7PC liposomes which are less permeable than the 'gel' F6C11PC or DSPC liposomes or than the EPC/CH ones which are in a highly ordered fluid state.

Permeation of a dye across a membrane is controlled, among others, by its solubility and diffusion in the hydrophobic phase of this membrane. It has also been shown that dehydration of the permeant polar species is required for it to cross the hydrophilic/hydrophobic interface of phospholipid membranes. It was suggested that this step determines the activation energy of the process [15,16]. Thus, for a given species, almost identical activation energies were generally found for its permeation across various membranes. This result was obtained in spite of the widely different permeability coefficients displayed by these membranes, indicating further that the selectivity of membrane transport is entropy-controlled. This seems to be also the case for diffusion across fluorinated membranes: the activation energies calculated for the diffusion of CF across these latter membranes are indeed in the same range as those found for its diffusion across hydrogenated ones (Table 1). However, the fact that a change in phospholipid or in the physical state of the bilayer is accompanied by significant changes in permeability, indicates the importance of the hydrophobic and lipophobic regions of the membrane in the permeation process which largely determines the magnitude of the entropy of activation. Since a membrane is more structured than the surrounding water, the transfer of CF into this hydrophobic barrier would be accompanied by a unfavorable entropy change. To explain the decrease in permeability induced by the fluorinated tails, one can assume that the probe's solubility (or its penetration) and/or its diffusion across the fluorinated core, owing to the highly hydrophobic and lipophobic character of this core, are strongly reduced. This core constitutes then, with respect to conventional lipid membrane, a supplementary solubility and diffusion barrier which limits membrane permeation. The lipophobic fluorinated tails can, furthermore, impose a more rigid and more ordered arrangement to the hydrocarbon spacer, hence to the lipophilic shells (see Fig. 2), which might also reduce the solubility and/or the diffusion of the dye in the membrane. These differences would result indeed in a change in permeability without change in activation energy, as found here for both fluorinated and hydrogenated membranes in their fluid state.

Studies concerning the partition of a lipophilic/hydrophilic paramagnetic probe (i.e., 2,2,6,6-tetramethylpiperidine-1-oxyl, Tempo) in aqueous dispersions of the fluorinated phosphatidylcholines displayed in Fig. 1 have shown that this probe was located both in the aqueous phase and in the hydrophobic regions in the case (and only in the case) of 'fluid' F4C11PC membranes [8]. The solubility of this probe in this membrane, which is among the least lipophobic fluorinated ones (shortest fluorinated tail and longest hydrogenated spacer), was higher than in 'fluid' DPPC membranes but lower than in 'fluid' DMPC ones. We take these results on the paramagnetic probe to indicate that the solubility of CF in the lipophilic region of the fluorinated F4C11PC bilayer should not be negligeable. F4C11PC membranes were nevertheless found to be less permeable to CF than DMPC and DPPC membranes. This strongly suggests that the permeation of CF across the fluorinated membranes is mainly controlled by its solubility in and/or its diffusion across their fluorinated core. The efficiency of the fluorinated core in reducing permeation is therefore expected to increase with the thickness of this core, hence with increasing the Fn tail length. This is indeed what we have observed. It may account in particular for the surprisingly low permeability of the membranes based on F6C5PC, F6C7PC and F8C5PC, which all possess shorter hydrophobic chains but longer fluorinated tails than F4C11PC. However, for all the fluorinated membranes investigated besides the F4C11PC ones, the solubility of the paramagnetic probe in the lipophilic regions was extremely low, even in their fluid state, much lower than, for instance, in DPPC membranes [9]. This indicates that the fluorinated core enhances the lipophobic character of the whole membrane. The rigidifying and ordering effects induced more particularly by the C_8F_{17} tails, as in F8C5PC (with its high T_c of 69°C) are also likely to contribute to the remarkably low permeability found for the F8C5PC membrane in its fluid state.

In the gel state, the small differences in permeability between fluorinated and hydrogenated membranes, indicate that the penetration into the fluorinated bilayers is almost as unfavored as in rigid hydrogenated ones and that the decrease in entropy which accompanies the permeation of CF across the membranes is comparable for both types of membranes.

4.2. Liposome stability in human serum

Serum is among the biological fluids known to induce the strongest destabilization effects on membranes in their fluid state [3]. Fluorinated liposomes 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. Interestingly, this indicates that the fluorinated lipophobic and hydrophobic core inside the liposomal membrane protects the vesicles to some degree, possibly by reducing their interactions with the lipophobic and/or hydrophilic serum components limiting their adsorption at the surface and/or hindering their pene-

tration into the fluorinated bilayer, or by reducing the phospholipid exchange between the fluorinated liposomes and the HDL present in serum.

On the other hand, our results show that in serum and for the fluorinated liposomes whose membranes are in the gel state (i.e., F6C11PC) and, surprisingly, in a semi-fluid semi-gel state (i.e., F8C5PC), the fluorinated core within their membrane further improves the retention of the dye. Indeed, these 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' or 'gel'-like liposomes: this is the case (i) for the F6C11PC liposomes as compared to DSPC ones (which are also more stable in serum than in buffer) and (ii) for the F6C11PC and F8C5PC vesicles as compared to EPC/CH liposomes (which are almost not affected by this change in medium). These results most likely indicate that serum constituents participate in increasing the order or the packing of the phospholipids whithin the membrane, this effect being more pronounced for the fluorinated ones. The ordering and/or compacting effect of serum seems to be supported by the unexpected behavior of the F8C5PC vesicles which were found in serum to retain entrapped CF more efficiently than the F6C5PC, F4C11PC and F6C7PC ones while the opposite result was observed in buffer. Packing defects arising from the semi-fluid semi-gel state of the liposomal F8C5PC membranes were most probably responsible for the higher CF release level observed for these liposomes in buffer. The stability enhancement of the F8C5PC vesicles when going from buffer to serum may therefore be attributed to the suppression of these defects by the serum components.

Acknowledgements

We thank the CNRS and ATTA for financial support.

References

[1] Gregoriadis, G. (1988) in Liposomes as Drug Carriers, Recent Trends and Progress, J. Wiley, New York.
[2] Lopez-Berenstein, G. and Fidler, I.J. (1988) in Liposomes: The Therapy of Infectious Diseases and Cancer, Alan R. Liss, New York.
[3] Senior, J. and Gregoriadis, G. (1982) Life Sci. 30, 2123–2136.
[4] Kirby, C., Clark, J. and Gregoriadis, G. (1980) Biochim. J. 186, 591–598.
[5] Santaella, C., Vierling P. and Riess, J.G. (1991) Angew. Chem. Int. Ed. Engl. 30, 567–568.
[6] Santaella, C., Frézard, F., Vierling P. and Riess, J.G. (1993) FEBS Lett. 336, 481–484.
[7] Weinstein, J.N., Yoshikami S., Henkart P., Blumenthal R. and Hagins, W.A. (1977) Science 195, 489–491.
[8] Santaella, C., Vierling P. and Riess, J.G. (1991) New J. Chem. 15, 685–692.
[9] Santaella, C., Vierling P., Riess J. G, Gulik-Krzywicki, T., Gulik A. and Monasse B. (1994) Biochim. Biophys. Acta 1190, 25–39.
[10] Lelkes, P.I. and Tandeter, H.B. (1982) Biochim. Biophys. Acta 716, 410–419.
[11] Janiak, M.J., Small, D.M. and Shipley, G.G. (1977) Biochemistry 15, 4575–4580.
[12] Tardieu, A., Luzzati, V. and Renan, F.C. (1973) J. Mol. Biol. 75, 711–733.
[13] Spiker, R.C. and Levin, I.W. (1976) Biochim. Biophys. Acta 433, 457–468.
[14] Doniack, S. (1978) J. Chem. Phys. 68, 4912–4916.
[15] Papahadjopoulos, D., Nir, S. and Ohki, S. (1971) Biochim. Biophys Acta 266, 561–583.
[16] Bresselers, J.M., Goderis, H.L. and Tobback, P.P. (1984) Biochim. Biophys. Acta 772, 374–382.
[17] De Gier, J., Mandersloot, J.G. and Van Deemer, L.L.M. (1968) Biochim. Biophys. Acta 150, 666–675.