ELSEVIER

Biochimica et Biophysica Acta 1194 (1994) 61–68

BBA Biochimica et Biophysica Acta

Fluorinated phosphatidylcholine-based liposomes: H^+/Na^+ permeability, active doxorubicin encapsulation and stability in human serum

F. Frézard, C. Santaella, M.-J. Montisci, P. Vierling *, J.G. Riess

Laboratoire de Chimie Moléculaire, URA 426 au CNRS, Faculté des Sciences, Université de Nice-Sophia Antipolis, B.P. 71, 06108 Nice Cédex 02, France

Received 14 February 1994

Abstract

The active encapsulation of doxorubicin (DOX) into fluorinated liposomes, the stability of these liposomes with respect to encapsulated DOX release in buffer and in human serum, and their H^+/Na^+ membrane permeability have been investigated and compared to those of their conventional hydrogenated analogues. These fluorinated liposomes are made from highly fluorinated phosphatidylcholines and contain a fluorinated core whithin their membrane. We found that the presence of this fluorinated core is not a barrier for the active encapsulation of DOX. Efficient (> 90%) and stable loading could be achieved using a transmembrane ammonium sulfate or even, in the absence of Na^+, a transmembrane pH gradient. The higher H^+/Na^+ permeability found for the fluorinated membranes, as compared to conventional ones, is responsible for the lower stability observed for the DOX-loaded fluorinated liposomes when incubated in a physiological buffer (PBS) or in human serum. It is also noticeable that the retention of DOX is increased in human serum and for the liposomes whose membranes are in a gel or in a semi-fluid semi-gel state at 37°C.

Key words: Perfluoroalkylated phosphatidylcholine; Fluorinated liposome; Permeability; Doxorubicin; Remote loading

1. Introduction

The use of liposomes as drug carrier and delivery systems is currently a topic of intensive investigations. Progress in liposomal formulation will require the development of new membrane and liposome-forming amphiphiles with versatile, adaptable physico-chemical and biological properties. With the aim of altering and extending the potential of liposomes as drug carriers, we have investigated the possibility of imparting to the liposomal membrane some of the unique characteristics of fluorocarbons, and in particular their low affinity for both hydrophilic and lipophilic phases [1]. The use of a liposomal membrane with an internal fluorinated lipophobic barrier was expected to reduce its permeability and its interactions with serum components and other biological compounds, hence to increase the liposomes' shelf stability and stability in biological fluids with respect to drug leakage. Hindering opsonization could also contribute to reducing liposome uptake by the mononuclear phagocytic system (MPS) and to enhancing the blood circulation time of i.v. administered liposomes. Such fluorinated liposomes were obtained from synthetic fluorinated phosphatidylcholines (Fig. 1) [2] and were found to display remarkable properties. Among others, we have already shown that the presence of a fluorinated core within the liposomal membrane constitutes an efficient barrier for the release of entrapped water soluble compounds such as carboxyfluorescein, even in human serum [3], and markedly increases the intravascular persistence of the vesicles in the bloodstream [4].

In order to further evaluate the potential of fluorinated liposomes, we have now examined the impact of the fluorinated lipophobic core on the active loading of a drug and its release from the liposomes in human serum. We selected doxorubicin (DOX), an anticancer drug which is a weak amphiphilic base (pK_a 8.25) that

* Corresponding author. Fax: +33 93 529020.

SSDI 0005-2736(94)00122-6

can be actively encapsulated into liposomes. Liposomal encapsulation of DOX increases its antitumor potency and provides significant therapeutic benefits (for review see Ref. [5]).

In this paper, we report on the H^+/Na^+ permeability of the liposomal membrane based on fluorinated phosphatidylcholines, on the active loading of DOX into fluorinated liposomes, using either a pH or an ammonium sulfate gradient, and on their stability with respect to DOX release when incubated in a physiological buffer or in human serum at 37°C. We found that the fluorinated membranes display a higher transmembrane potential-driven Na^+ permeability than classical membranes and that the presence of the fluorinated lipophobic core within the membrane does not hinder the transmembrane diffusion of lipophilic/hydrophilic compounds such as the deprotonated neutral form of DOX. We show that the stability in human serum and at 37°C of the fluorinated liposomes loaded with DOX using an ammonium sulfate gradient, although lower than that of DOX-loaded distearoylphosphatidylcholine (DSPC) and 1:1 DSPC/cholesterol (CH) liposomes, is significantly higher than that of DOX-loaded 1:1 egg phosphatidylcholines (EPC)/CH liposomes.

2. Materials and methods

The perfluoroalkylated phospholipids F6C5PC, F8C5PC, F4C11PC and F6C11PC (code in Fig. 1) were synthetized according to Ref. [2]. Their purity (> 99%) was checked by TLC, ^{1}H- and ^{31}P-NMR. Racemic dimyristoyl- (DMPC) and *sn*-distearoylphosphatidylcholine (DSPC) were purchased from Fluka. Their purity (> 99%) was checked by TLC before use. Egg-yolk phosphatidylcholines (EPC, Lipoid 100) came from Lipoid. Calf thymus deoxyribonucleic acid (DNA), sodium deoxycholate, Triton X-100 and cholesterol (CH, purity 99%) came from SIGMA. 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) was purchased from Molecular Probe. Doxorubicin chlorhydrate (DOX) was a gift from Farmitalia Carlo Erba. All these chemicals were used as received.

2.1. Preparation of small unilamellar vesicles (SUVs)

Typically, 20–50 mg of the phospholipid (20–50 mM) were hydrated for 30–60 min in the selected medium at a temperature 5–10°C above the phase transition temperature, T_c, of the phospholipid under investigation (T_c < 0°C for F6C5PC; 18°C for F4C11PC; 51°C for F6C11PC; 69°C for F8C5PC [6]). These suspensions were then sonicated at a temperature above T_c as previously described [3]. DSPC/CH, DMPC/CH and EPC/CH (1:1) liposomes were obtained using the same procedure but starting from a dried film which was made by evaporating a chloroform-methanol solution of the two lipids. The average diameters of the liposomes (which ranged from 30 to 100 nm) were then measured by photon correlation spectroscopy using a Coulter model N4 MD submicron particle size analyzer. The phospholipid concentration was determined according to Ref. [7].

2.2. H^+/Na^+ permeability measurements

H^+ diffusion into the vesicles was followed by monitoring the changes in fluorescence intensity of an entrapped fluorescent and pH-sensitive water soluble probe, pyranine [8,9]. Liposomes were prepared, as described above, in a 50 mM Na_2SO_4, 5 mM Tricine/NaOH and 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)/NaOH (pH 8.4–8.6) solution containing 2.5 mM of pyranine. The pH measured at the end of the sonication procedure was 8.2. Free (un-encapsulated) pyranine was then removed by gel chromatography through a Sephadex G-50 mini-column equilibrated with a 50 mM Na_2SO_4, 5 mM Tricine/NaOH and 5 mM Mes/NaOH pH 8.2 buffer. The liposome suspensions (20 μl) were then mixed with 2 ml of the pH 8.2 buffer and incubated at the selected

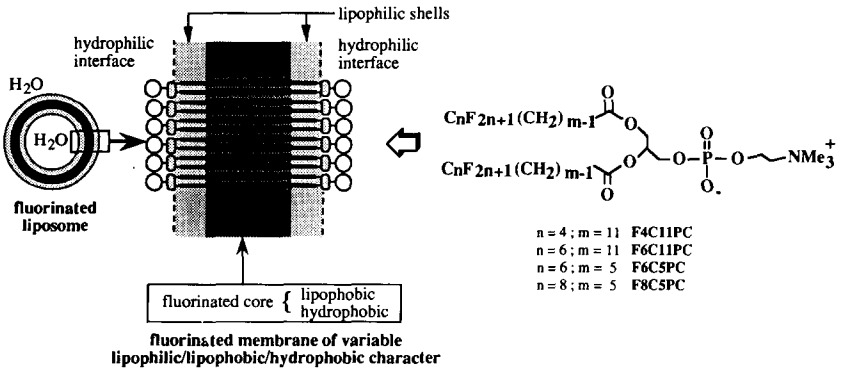

Fig. 1. Structure of the fluorinated phospholipids, membranes and liposomes.

temperature in a thermoregulated quartz cuvette cell (1 cm) with magnetic stirring. The pH of the resulting dispersions (pH_e) was then lowered to 6.7 by addition of 0.25 M H_2SO_4 (7 μl). The fluorescence intensity $F(t)$ of entrapped pyranine (excitation at 460 nm and emission at 520 nm) was recorded continuously using a Perkin Elmer Spectrofluorimeter LS 50B. During these experiments the probe remained entrapped in all the vesicles investigated: the same pyranine/phospholipid molar ratio in the case of each liposomal solution was measured before the experiments were run, at the end of the experiments and after the solution had been filtered through a Sephadex G-50 mini-column equilibrated with the pH 8.2 buffer in order to remove the probe that had been released during the experiments.

The internal pH ($pH_i(t)$) was determined from the measured fluorescence intensity $F(t)$ of the entrapped probe using the calibration curve of the fluorescence of pyranine (1 μM) in a 50 mM Na_2SO_4, 5 mM Tricine/NaOH and 5 mM Mes/NaOH solution as a function of pH. Because only the deprotonated form of pyranine is fluorescent, $pH_i(t)$ is related to the measured fluorescence $F(t)$ (using the Henderson-Hasselbach relation) by $pH_i(t) = pK_a + \log[F(t)/[F_d - F(t)]]$, where F_d and the pK_a (of pyranine) were determined from the calibration curve (F_d is the fluorescence intensity corresponding to the total amount of entrapped pyranine, in its deprotonated form).

It was shown previously that membranes dissipate transmembrane pH gradients in a biphasic manner [8,9]. The initial phase, which is very fast, corresponds to an electrically uncompensated diffusion of protons into the vesicles upon addition of an acid in the external water phase. This creates a transmembrane potential that stops further entry of protons and drives the ensuing step which, in our case, is the outward diffusion of Na^+. As the transmembrane potential is maintained, the inward proton diffusion is thus controlled by the outward flow of Na^+. Since under our conditions outward Na^+ flux is a first-order process, plots of $\ln[pH_i(t) - pH_e]$ against time are linear. When possible, the half-times of dissipation of the pH gradient ($t_{1/2}$) were calculated from these plots: $t_{1/2}$ is related to the slope, K, of these curves by the relation of $t_{1/2} = \ln(2)/K$.

2.3. Entrapment of DOX using a transmembrane pH gradient / stability of the transmembrane pH gradient

The active encapsulation of DOX into the liposomes using a transmembrane pH gradient was performed according to Ref. [10]. Liposomes were prepared, as described above, in a 0.3 M sodium citrate pH 4 buffer. These dispersions were then incubated at 37°C and their external pH was adjusted to 7.5 with a 0.325 M NaOH, 0.278 M sucrose and 0.0325 M Hepes solution (the internal and external osmolarity and sodium ion concentration were equal). DOX was added to the liposome suspensions (0.5 mM DOX for a 1:5 DOX/lipid molar ratio) at 37°C either before or at selected times (0.5, 10 and 20 min) after the external pH jump, in order to evaluate the stability of the transmembrane pH gradient. In the case of F6C11PC liposomes, the external aqueous medium was also exchanged, at 25°C, for a 0.3 M sucrose 20 mM Hepes pH 7 buffer on a Sephadex G-50 column equilibrated with this buffer. DOX was then added to the liposomes (0.5 mM DOX for a 1:30 DOX/lipid molar ratio). The resulting solution was maintained at 25°C and examined for DOX entrapment.

In all cases, the kinetics of DOX encapsulation into the liposomes and of its release were determined using the spectrophotometric method described in Ref. [11]. This method relies on the measurement of DOX concentration in its deprotonated form, which absorbs at 600 nm. Briefly, at the selected time t, 200 μl of the dispersion was mixed with 800 μl of an ice-cold 0.15 M NaCl 20 mM Hepes pH 7.3 buffer. This solution was then rapidly transferred to a cuvette and used as a blank for the spectrophotometric measurement at 600 nm. The pH was adjusted to approx. 10.5 by addition of 1 M NaOH and the absorbance $A(t)$ was measured. The liposomes were then lysed using a 20% (w/v) solution of Triton X-100 (10 μl) and the absorbance, Ao, against a blank containing the liposomes and Triton X-100 at the same concentration, was measured. The percentage of encapsulated DOX was calculated as % Encapsulation = $100 \times [A\text{o} - A(t)/A\text{o}]$.

2.4. Entrapment of DOX using a transmembrane ammonium sulfate gradient

The active entrapment of DOX using a transmembrane ammonium sulfate gradient was performed according to Ref. [12]. Liposomes were prepared in a 155 mM ammonium sulfate pH 5.5 solution. The external medium was exchanged for 0.3 M sucrose pH 5.5 on a Sephadex G-50 mini-column equilibrated with this solution. DOX in 0.3 M sucrose pH 5.5 was then added to the liposomes (2 mM DOX for a 1:5 DOX/lipid molar ratio). After 1 h of incubation at 60°C, the samples were cooled and assayed for encapsulation efficiency and stability as described below.

2.5. Stability of DOX-loaded liposomes in buffer and in human serum

For these studies, liposomes were loaded with DOX using a transmembrane pH or ammonium gradient (see above). In the former case, the liposomes were prepared, as described above, in 0.15 M sodium citrate (pH 3.8). The external medium was then exchanged at

25°C for a 0.3 M sucrose 20 mM Hepes pH 7 medium by filtration on a Sephadex G-50 gel equilibrated with the latter buffer. DOX was then added to yield solutions containing 0.5 mM DOX at a 1:30 DOX/lipid molar ratio. The preparations were then incubated at 25°C for at least 1 h, after which more than 85% DOX was loaded.

To determine the kinetics of DOX release from the liposomes in PBS and in human serum at 37°C, the DOX-loaded liposomes were incubated in these solutions to which DNA was added, as described in Ref. [13]. The outward-released DOX intercalates between the base pairs of DNA present in the external phase, therefore quenching the intrinsic fluorescence of DOX [14]. In a typical drug-release assay, the DOX-loaded liposome suspension (16 μl) was diluted with 2 ml of PBS or with 2 ml of 50% (v/v) human serum (1 ml PBS + 1 ml human serum) and incubated at 37°C in a thermoregulated quartz cuvette cell (1 cm) with magnetic stirring. For each sample, two cuvettes were prepared and DNA was added to one of them at a high concentration. Fluorescence was then continuously monitored on each cuvette (excitation at 505 nm; emission at 600 nm) with a Perkin Elmer spectrofluorometer LS 50B. In the absence of DNA, the fluorescence signal measured at a given time t, $F1(t)$, is the sum $[F1(t) = F_i(t) + F_e(t)]$ of the contribution of the entrapped species, $F_i(t)$, and of the extravesicular species, $F_e(t)$. In the presence of DNA, the extravesicular contribution to the fluorescence of DOX can be neglected and the measured fluorescence intensity at time t, $F2(t)$, is therefore equal to $F_i(t)$. Consequently, the difference between the fluorescence signals measured in the absence and in the presence of DNA, i.e., $F1(t) - F2(t)$, is equal to $F_e(t)$, which is directly related to the extravesicular DOX concentration. Finally, the fraction of DOX that remains inside the liposomes is equal to $1 - F_e(t)/F_{total}$, where F_{total} is determined after lysis of the liposomes by a sodium deoxycholate solution (in the concentration range of DOX used, its fluorescence was shown in both media to depent linearly on its concentration).

Additional experiments have been performed in order to show that the presence of DNA does not alter DOX release: the fluorescence intensities $F2(t)$ measured in the continuous presence of DNA were indeed identical to those measured after DNA has been added at time t.

3. Results

3.1. Active encapsulation of DOX and H^+/Na^+ permeability

Fig. 2 shows the time-course of DOX encapsulation into different liposomes at 37°C under the influence of a transmembrane pH gradient and the stability of the resulting DOX-loaded liposomes with respect to DOX release. These experiments were performed at 37°C in order to increase the rate of encapsulation [10,11].

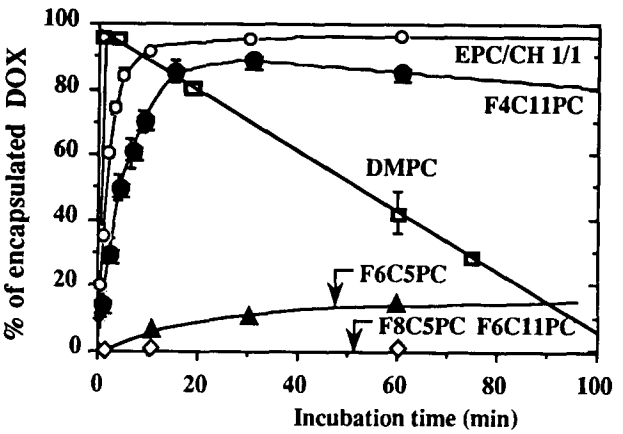

Fig. 2. pH gradient-mediated encapsulation of doxorubicin into fluorinated and conventional liposomes at 37°C. DOX was added to the liposome suspensions (0.5 mM DOX for a 1:5 DOX/lipid molar ratio) before the external pH jump. The transmembrane pH gradient was generated by forming the liposomes in 0.3 M citrate buffer (pH 4) and subsequent adjustment of the external pH to 7.5. (for details see Materials and methods).

Among the four fluorinated vesicles investigated, only those made from F4C11PC were found to entrap a high level of DOX (90% after 30 min). This value is slightly lower than that obtained (95%) for DMPC or EPC/CH 1:1 liposomes. It is also interesting to note that the initial rate of DOX entrapment is slower for F4C11PC vesicles than for DMPC and EPC/CH ones.

These results, and more particularly the low level of DOX encapsulation into the liposomes made from the fluorinated F6C5PC, F6C11PC and F8C5PC phospholipids (Fig. 2), are most likely related to the instability of the transmembrane pH gradient in the presence of Na^+ in the outer aqueous phase (hence to a high transmembrane potential-driven Na^+ permeability) [1] rather than to a low DOX permeability of the membranes based on these fluorinated phospholipids. Indeed, as illustated in Fig. 3, the loading efficiency into F4C11PC liposomes (and into the other fluorinated and DMPC ones) decreased significantly when DOX was added at increasing intervals of time after the pH jump. By contrast, 1:1 DMPC/CH liposomes (as well as those made from EPC, EPC/CH(1:1) or DSPC, data not shown) were found to incorporate the same level of DOX, even when DOX was added 1 h after the pH rise. On the other hand, the absence of sodium

[1] It has been shown that dissipation of transmembrane pH gradients depends on the permeability of the membrane to the counterion that will exchange with the proton [7]. Under our conditions, only sodium ions were available for exchange with protons.

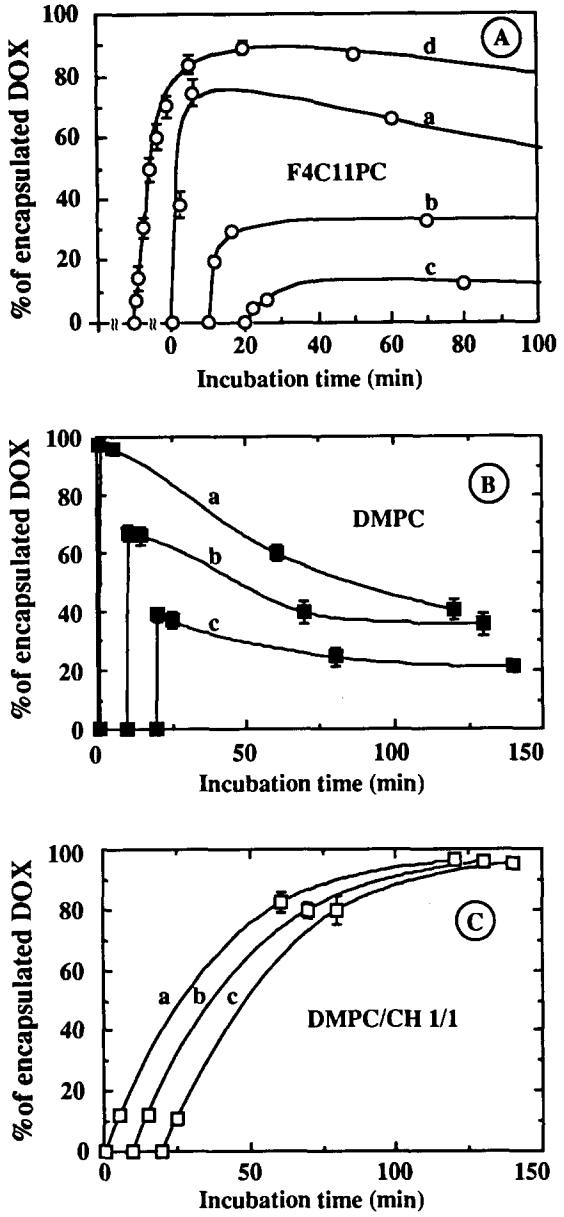

Fig. 3. Active encapsulation of doxorubicin at 37°C in (A) F4C11PC, (B) DMPC and (C) DMPC/CH liposomes for different time intervals between the establishment of the pH gradient (pH_i 4 and pH_e 7.5) and the addition of DOX to the liposome suspensions. The transmembrane pH gradient was allowed to stay at 37°C for (a) 0.5, (b) 10, and (c) 20 min prior to the addition of DOX (0.5 mM for a 1:5 DOX/lipid molar ratio) to the liposomal solutions. In (d), the pH gradient was generated in the presence of DOX (positioning of curve d in A is arbitrary).

salts in the external medium was found to improve DOX encapsulation efficiency into the fluorinated liposomes, hence the transmembrane pH gradient stability. F6C11PC liposomes were found to incorporate 86% of DOX for a 1:30 drug/lipid molar ratio when the external medium had been replaced, prior to the addition of DOX, by a sucrose solution.

The particularly low stability of the pH gradient in

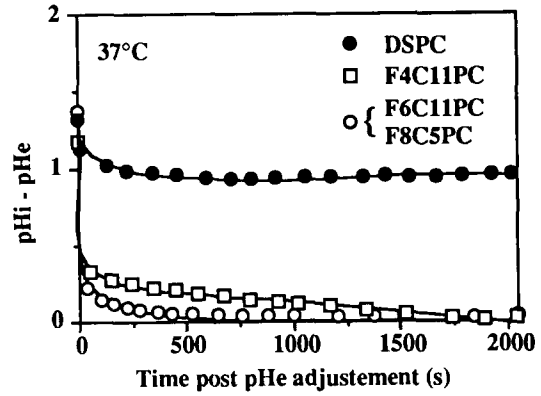

Fig. 4. Stability of the transmembrane pH gradient for F4C11PC, F6C11PC and DSPC liposomes prepared in a 2.5 mM pyranine solution (pH 8.2). After removal of un-encapsulated pyranine, the liposomes were incubated at 37°C and pH_e was then lowered to 6.7. The dissipation of the pH gradient ($pH_i(t)$–pH_e) as a function of time was followed by monitoring of the fluorescence decrease of entrapped pyranine (for details see Materials and methods).

the presence of Na^+ observed for the fluorinated liposomes was further confirmed by the rapid decrease of the fluorescence of entrapped pyranine, used as a pH indicator of the intraliposomal aqueous phase. As illustrated in Fig. 4, the dissipation of the pH gradient (pH_i-pH_e) at 37°C in the case of F8C5PC, F6C11PC liposomes was very fast, too fast to be measured in our conditions. It was significantly faster than for F4C11PC ($t_{1/2} = 11 \pm 4$ min) liposomes and much faster than for DSPC ones. Furthermore, the stability of the pH gradient for the fluorinated liposomes appeared to be strongly increased by decreasing the temperature of incubation: as shown in Fig. 5, the half-time of the pH gradient dissipation for the liposomes made from F4C11PC (and, to a lesser extent, for those made from F6C11PC) increased when the incubation temperature was lowered.

Finally, the use of an ammonium sulfate gradient to actively encapsulate DOX in place of a pH gradient

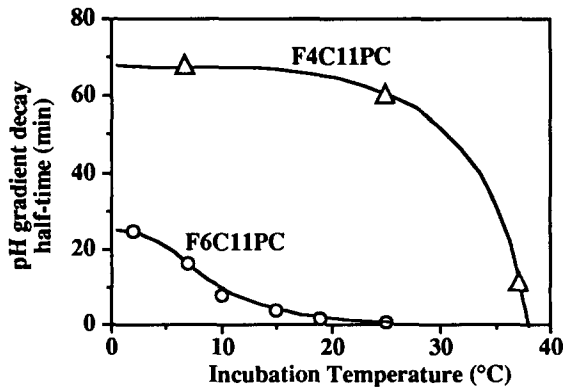

Fig. 5. Half-times ($t_{1/2}$) of transmembrane pH gradient decay as a function of the incubation temperature for F4C11PC and F6C11PC liposomes. For $t_{1/2}$ determination see Materials and methods.

proved to be even more efficient since higher levels of DOX (90–95% for 1:5 drug/lipid molar ratio) could be incorporated into the F4C11PC, F6C11PC and F8C5PC vesicles.

Following its encapsulation, DOX was slowly released from F4C11PC liposomes when these liposomes had been loaded using a pH gradient (see Fig. 2). After 5 h of incubation at 37°C in the loading medium, 74% of the total DOX amount added for loading was still inside the F4C11PC liposomes, while DMPC vesicles had lost almost all of their content. The DOX release from the F4C11PC vesicles was, however, greater than from EPC/CH ones, which contained almost the same amount of DOX (95%) after 5 h of incubation than immediately after loading (data not shown). The stability of the loading into the F4C11PC liposomes could be improved by the exchange of the extraliposomal sodium salt containing-aqueous phase for a sucrose solution prior to the addition of DOX.

On the other hand, no significant DOX release could be detected, even after one week of storage at room temperature, when the liposomes had been loaded with DOX using an ammonium sulfate gradient (results not shown). This high shelf-stability is also related to the fact that DOX, in its protonated form, associated with the sulfate anion is present in the intraliposomal aqueous phase in an aggregated gel-like state which contributes to preserving the integrity of the liposomes with respect to liposome fusion and aggregation [12].

3.2. Stability of DOX-loaded liposomes in physiological buffer (PBS) and in human serum at 37°C

Fig. 6 displays the time-course of DOX release at 37°C from F4C11PC, F8C5PC, F6C11PC, DSPC, DSPC/CH 1:1 and EPC/CH 1:1 liposomes, loaded using an ammonium sulfate gradient and then incubated in PBS (Fig. 6A) or in 50% (v/v) human serum (Fig. 6B). As shown in Fig. 6A, the release of DOX from the fluorinated liposomes in PBS (from 60 to 80% after 1 h of incubation) was found to be higher than from the conventional ones (less than 30% after 1 h). This is most likely related to the fact that the former liposomes display a higher membrane permeability to Na^+ than the latter ones. Indeed, Na^+ is present in PBS and absent in the intraliposomal aqueous phase. This most likely generates, for the fluorinated liposomes, an inward flux of Na^+ facilitated by an outward flux of H^+, which will result in an increase of the internal pH, hence in an increase of deprotonated DOX, thus facilitating the flow of DOX outwards of the liposomes.

We found that F8C5PC and F6C11PC vesicles were strongly stabilized in the presence of serum. As shown in Fig. 6B, these liposomes released, respectively, only

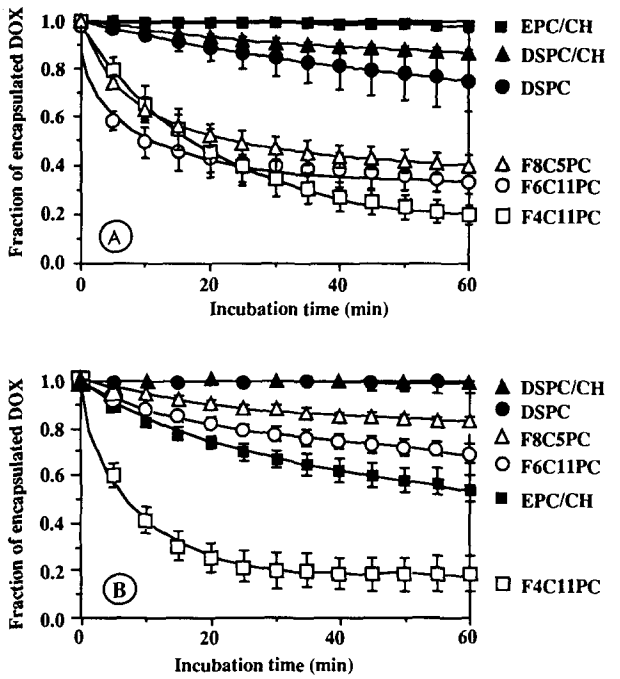

Fig. 6. Time-course of doxorubicin release at 37°C from fluorinated and conventional liposomes incubated (a) in PBS and (B) in 50% (v/v) human serum/PBS solution. The encapsulation of DOX (2 mM for a 1:5 DOX/lipid molar ratio) into the liposomes was performed using an ammonium sulfate gradient as described in the Materials and methods.

20 and 26% of their initial DOX content after 1 h of incubation in human serum, whereas almost 60% of DOX had been released when they were placed in PBS for the same length of time. A similar behavior is also observed for the conventional DSPC and DSPC/CH 1:1 liposomes. On the other hand, the DOX-loaded F4C11PC and EPC/CH 1:1 liposomes displayed the opposite behavior: the release of DOX increased significantly from buffer to serum.

4. Discussion

The main objectives of our work were to evaluate the ability of fluorinated liposomes to actively entrap and retain DOX. This study led us to investigate two different methods of DOX encapsulation, as well as to address more fundamental questions such as the impact of the fluorinated core on membrane permeability. This work is the continuation of our previous study which was concerned with the encapsulation of hydrophilic compounds such as carboxyfluorescein (CF) into fluorinated liposomes, with the impact of the fluorinated core on the diffusion of CF across the liposomal membrane, and with the stability of the liposomes with respect to encapsulated CF release in human serum [3]. It was found that (i) the fluorinated core within the membrane (Fig. 1) constituted a very

efficient barrier for CF permeation and (ii) the fluorinated liposomes were, with respect to CF release, less destabilized than conventional liposomes and in some cases were even found to be stabilized by the serum components.

Most importantly, we found that the presence of a fluorinated core within the membrane (which imparts a lipophobic character to the membrane) is not a barrier for the active encapsulation or remote loading into the fluorinated liposomes of weak lipophilic bases such as DOX. Efficient (> 90% for a drug/phospholipid molar ratio as high as 0.2) and stable loading into the fluorinated liposomes' inner aqueous phase could indeed be achieved using a transmembrane ammonium sulfate gradient, or even, in the absence of Na^+, a transmembrane pH gradient (see below). The former method, which led to encapsulated DOX in an aggregated gel-like form, proved to be more efficient and led to more stable DOX-loaded formulations than the latter one, as it has already been shown for conventional or sterically-stabilized liposomes of a broad spectrum of types, sizes and compositions [12,15].

The active encapsulation of DOX into the fluorinated liposomes using a transmembrane pH gradient could be achieved efficiently only when the extraliposomal aqueous phase did not contain Na^+. The low level of DOX entrapment into the fluorinated liposomes and its reduced stability following DOX encapsulation were due to rapid dissipation of the transmembrane pH gradient in the presence of Na^+, and revealed a higher Na^+ permeability for the fluorinated membranes as compared to conventional ones [2]. Taken together our data concerning the transmembrane pH gradient stability using entrapped pyranine and the DOX-loading and release experiments indicate that the membrane permeability to Na^+ increases along the sequence DSPC, EPC/CH (1:1), DMPC/CH (1:1) $\ll$ F4C11PC < DMPC $\ll$ F6C11PC, F8C5PC. These results differ significantly from those obtained in our previous study where we showed that F8C5PC and F6C11PC membranes exhibited the lowest CF permeability.

Several reasons may account for the higher Na^+ permeability of the latter two fluorinated membranes. First, CF is, at neutral pH, an anion and is much bulkier than Na^+, and their respective mechanisms of permeation across membranes will therefore differ substantially [16]; permeation of the small Na^+ cation is expected to be more sensitive to the presence of membrane defects than permeation of the larger CF anion. Secondly, compared to its hydrocarbon counterpart, a perfluorocarbon chain is much stiffer and bulkier. Furthermore, its most favorable conformation is a helix which has a cross-sectional area (~ 0.3 nm^2) significantly larger than that (~ 0.2 nm^2) of all-trans hydrocarbon chains [17,18]. Therefore, hydrophobic chains composed of a perfluorocarbon tail connected to a hydrocarbon spacer (as in the fluorinated phospholipids investigated here) are less tightly packed than all hydrocarbon chains; this is further reflected by the lower enthalpy of the main phase transition measured for fluorinated phosphatidylcholines [6]. All these properties could play a significant role in facilitating the diffusion especially of small ions such as Na^+ across fluorinated membranes. Most importantly, the F6C11PC or F8C5PC membranes are, at 37°C, in a semi-fluid semi-gel like state owing to partial fusion of the fluorinated tails [6]; as compared to a fluid or gel state, this is more likely to generate packing defects in the membrane, hence to increase permeation [16]. This seems to be further supported by the lower DOX release level observed from the 'gel' F6C11PC liposomes, which is of 50% after 1 h at 25°C in PBS (results not shown), while it is of 50% after 10 min at 37°C (Fig. 6A).

When compared to the conventional DSPC and DSPC/CH membranes and liposomes, the higher permeability to Na^+ of the fluorinated membranes may also account for the lower stability of the fluorinated liposomes loaded with DOX using an ammonium sulfate gradient and then incubated in PBS or in human serum. The presence of Na^+ in the outer-liposomal aqueous phases results in an increase of the internal pH (after H^+/Na^+ exchange) hence in an increase in deprotonated neutral DOX which can diffuse more easily across membranes than protonated (charged) DOX. These results indicate furthermore that the lipophobic character of the fluorinated membranes, although it reduces strongly the solubility of lipophilic/hydrophilic compounds inside such membranes [6], does not hinder significantly the diffusion of a lipophilic/hydrophilic drug across these membranes, when driven by its concentration gradient. They also indicate that such drugs are not sequestered but remain still available when encapsulated into fluorinated liposomes.

It is also noticeable that in human serum, and for the liposomes whose membranes are at 37°C in a gel or in a semi-fluid semi-gel state (i.e., DSPC, DSPC/CH, F6C11PC and F8C5PC), the retention of DOX is increased. Indeed, these liposomes display a significantly greater stability in serum than in PBS while the opposite result is observed for the 'fluid' F4C11PC liposomes or for the highly-ordered fluid EPC/CH liposomes. Except for EPC/CH liposomes, this correlates well with our previous study where CF was used as a probe of liposome stability [3]. The decrease of DOX release when going from buffer to serum most likely

[2] See footnote [1] on p. 64.

indicates that serum constituents participate in increasing the order or the packing of the phospholipids whithin gel or semi-fluid semi-gel membranes or in suppressing packing defects which are very often present in such membranes.

Although DOX release from F6C11PC and F8C5PC liposomes is, in human serum, higher than from DSPC or DSPC/CH ones, the fluorinated DOX formulations are more stable than those prepared from EPC/CH. Furthermore, the most promising biocompatibility (i.v. LD_{50} in mice higher than 8 g/kg body weight [19]) and the higher in vivo blood circulation times measured for these fluorinated liposomes than for conventional ones [4], indicate that fluorinated liposomes have potential as drug carrier and delivery systems.

Acknowledgments

We thank the CNRS and ATTA for financial support.

References

[1] Mukerjee, P. and Handa, I. (1981) J. Phys. Chem. 85, 2298–2303.
[2] Santaella, C., Vierling, P. and Riess, J.G. (1991) New J. Chem. 15, 685–692.
[3] Frézard, F., Santaella, C., Vierling, P. and Riess, J.G. (1994) Biochim. Biophys. Acta 1192, 61–70.
[4] Santaella, C., Frézard, F., Vierling, P. and Riess, J.G. (1993) FEBS Lett. 336, 481–484.
[5] Mayer, L.D., Madden, T.D., Bally, M.B. and Cullis, P.R. (1993) in Liposome Technology (Gregoriadis, G., ed.), Vol. 2, pp. 27–44.
[6] Santaella, C., Vierling, P., Riess, J.G., Gulik-Krzywicki, T., Gulik, A. and Monasse, B. (1994) Biochim. Biophys. Acta 1190, 25–39.
[7] Stewart, J.C.M. (1980) Anal. Biochem. 104, 10–14.
[8] Clement, N.R. and Gould, J.M. (1981) Biochemistry 20, 1534–1538.
[9] Biegel, C.M. and Gould, J.M. (1981) Biochemistry 20, 3474–3479.
[10] Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985) Biochim. Biophys. Acta 817, 67–74.
[11] Mayer, L.D., Tai, L.C.L., Bally, M.B., Mitelenes, G.N., Ginsberg, R.S. and Cullis, P.R. (1990) Biochim. Biophys. Acta 1025, 143–151.
[12] Lasic, D.D., Frederick, P.M., Stuart, M.C.A., Barenholz, Y. and McIntosh (1990) FEBS Lett. 312, 255–258.
[13] Frézard, F. (1994) J. Liposome Res. 4, in press.
[14] Frézard, F. and Garnier-Suillerot, A. (1990) Biochim. Biophys. Acta 1036, 121–127.
[15] Haran, G., Cohen, R., Bar, L.K. and Barenholz, Y. (1993) Biochim. Biophys. Acta 1151, 201–215.
[16] Lasic, D.D. (1993) Liposomes: from Physics to Applications, pp. 56–61, Elsevier, Amsterdam.
[17] Hsiung, H., Rodriguez-Parada, J. and Bekerbauer, R. (1991) Chem. Phys. Lett. 182, 88–92.
[18] Eaton, D.F. and Smart, B.E. (1990) J. Am. Chem. Soc. 112, 2821–2823 and references therein.
[19] Greiner, J., Riess, J.G. and Vierling, P. (1993) in Organofluorine Compounds in Medicinal Chemistry and Biomedical Applications (Filler, R., Kobayashi, Y. and Yagupolskii, L.M., eds.), pp. 339–380, Elsevier, Amsterdam.