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# Highly fluorinated amphiphiles as drug and gene carrier and delivery systems

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**Abstract**

A short review on fluorinated liposomes made from highly fluorinated double-chain phospho- or glyco-lipids as well as fluorinated lipoplexes, e.g. complexes made from highly fluorinated polycationic lipospermines and a gene, is presented. These systems display a number of interesting physico-chemical and biological properties which make them very attractive alternatives as drug or gene carrying, targeting and delivering devices. The properties of the fluorinated liposomes that are discussed include liposome formation and shelf-stability, membrane structure, thermotropism and permeability, encapsulation, release and stability in various media (serum, detergents), bioavailability and biodistribution, anti-HIV activity. The properties of the fluorinated lipoplexes that are reported include lipoplex formation, stability, in vitro cell transfection in the presence of serum or of bile. 

*Keywords:* Phospholipid; Glycolipid; (Poly)cationic lipid; Liposome; Lipoplex; Drug delivery; Gene transfer

## 1. Introduction

The aim of any drug delivery system is to increase the therapeutic efficacy of a drug while decreasing its toxicity, to take it to its site of action or to facilitate its delivery there. The association of a drug with a carrier has major effects on its pharmacokinetics which is then determined by the strength of this association: at the two extremes, the behaviour will be essentially that of the free drug if the drug is rapidly released from the carrier, or that of the carrier if the drug has a high degree of latency in the carrier.

Several powerful drug and gene delivery systems have been developed. These systems include liposomes (i.e. closed uni- or multi-lamellar vesicles made of lipids, which can carry hydrophilic drugs in their internal aqueous space, or lipophilic or amphiphilic ones associated with the bilayer) and lipoplexes (i.e. complexes resulting from mixing a gene with cationic lipids or liposomes which are used as gene delivery systems). Although these systems have demonstrated their efficiency, several obstacles limiting therapeutical applications remain and the elaboration of systems with new or meaningfully improved properties is still a challenging issue. This implies the development of components that are substantially different from those currently utilised. Polar lipids (e.g. phospholipids, glycolipids or (poly)aminolipids) with highly fluorinated hydrophobic chains are such components. Highly fluorinated amphiphiles demonstrate strongly enhanced amphiphilic character and offer some of the specific features that make up the uniqueness of fluorinated material, including their unique hydrophobic and lipophobic character.

In the recent years much effort has been focused on liposomes with increased residence in the bloodstream (e.g. ''stealth'' liposomes). Significant advances were achieved with the synthesis and assessment of phospholipids bearing hydrophilic, bulky and mobile polyoxyethylene chains which increase the hydrophilicity and hinder the access to the liposomes' surface. Specific cell and organ targeting could be further achieved by the elaboration of long circulating liposomes that are functionalized by ligands (sugars, immunoglobulins, proteins, etc.) specifically recognised by receptors present on the cell surface (for reviews, see [1–7]). Our approach, with the synthesis of highly fluorinated double-chain amphiphiles (e.g. phospholipids [8–12] and glycolipids [13,14]), consists in increasing the hydrophobic and lipophobic character of the liposomal membrane. When such fluorinated polar lipids are dispersed in an aqueous solution and form liposomes, the fluorinated tail segments flock together and create an internal highly hydrophobic and lipophobic fluorocarbon film within the

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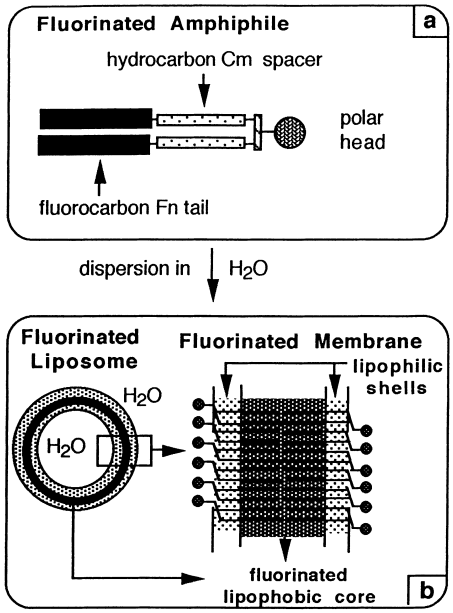



Fig. 1. Fluorinated bilayer and liposome concept.

membrane (Fig. 1) [8,15–19]. This film was found to increase the long-term shelf-stability of the liposomes, to lower the permeability of their membrane and the release of both hydrophilic and lipophilic encapsulated material, to increase their stability in biological media [20–24] and resistance to the lytic action of bile salts [23], and to prolong their in vivo blood circulation time [25], probably, by reducing their interactions with biocompounds. Liposomes co-formulated with fluorinated galactolipids were further found to exhibit anti-HIV activity [14].

Gene (or DNA) delivery or transfer has also become an increasingly important technique and powerful research tool for gene and cellular therapy. Successful gene therapy depends on the efficient delivery of genetic material to cells and its effective expression within these cells. Synthetic non-viral gene transfer vectors, which include (poly)cationic lipids, polymers, liposomes, have therefore gained wide acceptance over the last decade (for reviews, see [26–28]). Although at present, the in vivo expression levels of such "artificial viruses" are lower than for viral vectors and duration of gene expression is relatively short, these systems present several advantages for the future with respect to low-cost and large-scale production, safety, cell targeting, lower immunogenicity, and capacity to deliver large fragments of genes. Several studies have further shown that the transfer efficiency of the DNA complexes, and subsequent DNA expression, especially in vivo, vary in function, among others, of the cationic components, the stability of the complexes in biological fluids and their interactions with the cell membranes and intracellular components. Our approach, with the synthesis of lipopolyamines containing highly fluorinated hydrophobic chains, consists in increasing the hydrophobic and lipophobic character of the lipoplexes these polycationic lipids are able to form with DNA and thus to modify their physico-chemical and biological properties. Such "fluorinated" lipoplexes demonstrated improved in vitro transfection properties [29] and increased resistance to and gene expression in the presence of detergents [30], as compared with conventional lipopolyamines such as Transfectam® or LipofectAMINE®, which are among the most efficient synthetic gene transfer agents.

The scope of this review will be limited (1) to "fluorinated" liposomes made from perfluoroalkylated double-chain analogs of phospholipids and glycolipids and (2) to "fluorinated" lipoplexes made from perfluoroalkylated polyaminolipids that were developed and evaluated in our laboratory. Diverse other families of fluorinated amphiphiles have been synthesised by other teams, many of which form vesicles and other highly organised colloidal systems which may find applications in the biomedical field (for reviews, see [31–33]). These include single-chain phospholipids, double-chain glycolipids and glycophospholipids [31,33], and single- and double-chain cationic aminolipids [31,34] and will not be discussed here.

Concerning more particularly the liposome section, emphasis has been made on recent developments and the present review updates earlier overviews by some of us to which the reader is also referred [8,31]. The properties of the fluorinated liposomes that will be discussed include liposome formation and shelf-stability, membrane structure, thermotropism and permeability, encapsulation, release and stability in various media (serum, detergents), bioavailability and biodistribution, anti-HIV activity. The properties of the fluorinated lipoplexes that are reported include lipoplex formation, stability, in vitro cell transfection in the presence of serum or of bile.

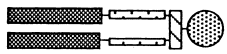
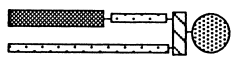
## 2. Liposomes from double-chain fluorinated amphipiles as drug carrier and delivery systems

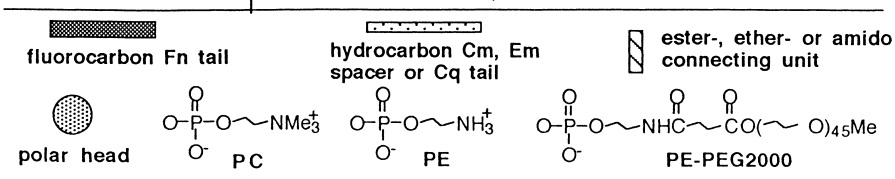
### 2.1. Liposome formation

Tables 1 and 2 collect the structure and code names of the various fluorinated phospholipids [9,11,12] and glycolipids [13,14] which form liposomes, whether the amphiphiles were used alone or in conjunction with other amphiphiles.

These different derivatives belong to homologous series of amphiphiles or bola-amphiphiles and possess a modular design allowing incremental structural variations aimed at the establishment of structure/properties relationships. The design of the amphiphile series involves two hydrophobic chains, one or both of which are ended by a highly fluorinated tail, in combination with saturated and/or unsaturated aliphatic chains of variable lengths. The bola-amphiphiles contain one long hydrocarbon chain bridging two polar heads, and two shorter ones that are ended by a highly fluorinated tail. These chains are linked through ester, ether or amide bonds to various units (glycerol, diaminopropanol, aminoethanol or serine), these units being connected to

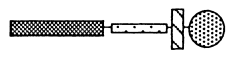
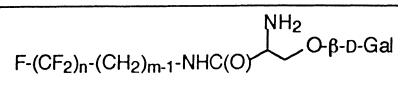
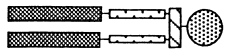
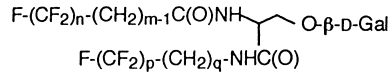
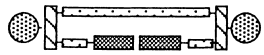
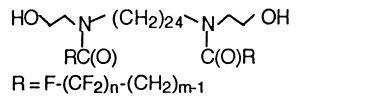
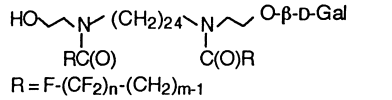
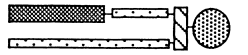
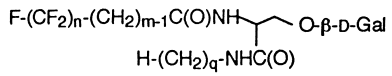
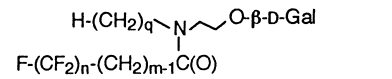
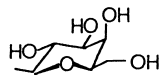
Table 1
Fluorinated double-chain glycerol-, diamidopropanol, serine and amidoethanol-derived phospholipids forming bilayers and liposomes in aqueous solutions

| Schematic Structure | Chemical Structure | Code Name |
|---|---|---|
|  | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)O$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)O$ PC | **DFnCmPC**<br>n = 4, 6, 8; m = 5, 7, 11 |
| | $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-3}C(O)O$<br>$F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-3}C(O)O$ PC | **DFnEmPC**<br>n = 6, 8; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ PC | **DFnCmOPC**<br>n = 4, 6, 8; m = 5, 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$F\text{-}(CF_2)_p\text{-}(CH_2)_q\text{-}O$ PC | **[FnCm][FpCq]OPC**<br>n = 4, 8; m = 11, 5<br>p = 8, 4; q = 11 |
| | $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}\text{-}O$<br>$F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}\text{-}O$ PC | **DFnEmOPC**<br>n = 8; m = 5 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ PC | **1,3-DFnCmOPC**<br>n = 6, 8; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ PE | **DFnCmOPE**<br>n = 4, 6, 8; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ PE-PEG2000 | **DFnCmOPE-PEG2000**<br>n = 8 ; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$ PC | **2,3-DFnCmAPC**<br>n = 6; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$ PC | **1,3-DFnCmAPC**<br>n = 4, 6; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$ PC<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}NHC(O)$ | **DFnCmSerPC**<br>n = 4, 6; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$ PC<br>$F\text{-}(CF_2)_p\text{-}(CH_2)_q\text{-}NHC(O)$ | **[FnCm][FpCq]SerPC**<br>n = 6; m = 11<br>p = 4; q = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}N$ PC<br>$F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)$ | **DFnCmAEPC**<br>n = 4; m = 11 |
|  | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$H\text{-}(CH_2)_q\text{-}O$ PC | **[FnCm][Cq]OPC**<br>n = 8; m = 11; q = 16 |
| | $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}\text{-}O$<br>$H\text{-}(CH_2)_q\text{-}O$ PC | **[FnEm][Cq]OPC**<br>n = 8; m = 5, 11<br>q = 14, 16 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$<br>$H\text{-}(CH_2)_q\text{-}O$ PC | **1,3-[FnCm][Cq]OPC**<br>n = 6, 8; m = 11<br>q = 18 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_{m-1}C(O)NH$ PC<br>$H\text{-}(CH_2)_q\text{-}NHC(O)$ | **[FnCm][Cq]SerPC**<br>n = 6; m = 11; q = 16 |




phosphocholine (PC), phosphoethanolamine (PE), *N*-polyethyleneglycol-conjugated phosphoethanolamine (PE-PEG2000) [35], hydroxyl or galactosyl head groups [14]. All these structural features were selected for acting on and modulating the properties of the fluorinated liposomes, and enlarging their potential as drug targeting devices. Our interest in the galactosyl-amphiphiles stemmed more particularly from their ability to be specifically recognised by

Table 2
Fluorinated single and double-chain serine and amidoethanol-derived hydroxy- and galacto-lipids used to form vesicles with conventional phospholipids in aqueous solutions

| Schematic Structure | Chemical Structure | Code Name |
|---|---|---|
|  |  | **FnCmSerGal** n = 6; m = 11 |
|  |  | **[FnCm][FpCq]SerGal** n = 6; m = 11 p = 4; q = 11 |
|  |  | **HO[C24][FnCm]OH** n = 6; m = 5 |
| |  | **HO[C24][FnCm]Gal** n = 6; m = 5 |
|  |  | **[FnCm][Cq]SerGal** n = 4, 6; m = 11 q = 14, 16 |
| |  | **[FnCm][Cq]AEGal** n = 8; m = 7; q = 16 |
|  polar head | β-D-Gal  | |

membrane lectins present on hepatocytes and macrophages, or by the glycoprotein gp120 of the human immunodeficiency virus (HIV), expressed at the surface of HIV-infected cells, such as macrophages which are HIV reservoirs (see Section 2.4.4). The bola-amphiphile structure was aimed at reinforcing the membrane stability as it does in the membrane of archeabacteria.

Liposomes were formed by dispersing the fluorinated phospholipids of the PC series (Table 1) in aqueous phases using sonication and/or extrusion through calibrated polycarbonate membranes [9,15–18]. Liposomes were also formed by co-dispersing the hydroxylated bola-amphiphiles or the galactosylated bola-amphiphiles (Table 2) with conventional phospholipid formulations [19]. Their formation was evidenced using usual standard techniques, including electron microscopy after freeze fracture or freeze-etching [15,17–19], and encapsulation and release experiments [20–24], and their sizes were measured by photon correlation spectroscopy [15,17–19].

The clustering of the fluorocarbon/fluorocarbon and mixed fluorocarbon/hydrocarbon double-chain PCs which results in the formation of membranes with an internal lipophobic fluorinated film, as shown in Fig. 1, was clearly attested by X-ray diffraction (see Section 2.4.1.2) [16]. Their modular structure allowed further the stepwise adjustment of the thickness of the lipophilic shells (length of the hydrocarbon spacer) and fluorinated core (length of the fluorinated tail), as also evidenced by X-ray diffraction.

### 2.2. Liposome long-term shelf-stability

The size of liposomes is known to play a determinant role on their in vivo fate. The formulation of long-term shelf-stable, size-controlled and heat-sterilizable liposomes is also needed for regulatory industrial and pharmaceutical purposes.

The vesicles based on the fluorinated phospholipids from the ester-, ether- or amide-PC series, as single components, were found to exhibit an exceptional shelf-stability with respect to particle size and particle size-distribution upon storage, which contrasts strongly with what is observed with their hydrocarbon analogs (see Table 3). The morphology and average sizes of these vesicles were most frequently unaffected by standard heat-sterilisation and upon ageing at room temperature for 10 months, while after 6–7 days an irreversible deposit formed from a DMPC, DPPC or DSPC (1,2-dimyristoyl-, 1,2-dipalmitoyl-, and 1,2-distearoylphosphatidylcholine, respectively) dispersion prepared and stored under the same conditions. The greater resistance to sterilisation and the higher shelf-stability of the fluorinated vesicles, as compared with their hydrocarbon analogs, was attributed to the increased hydrophobic character of their fluorinated membrane [10,15,17,18].

The liposomal formulations prepared from the fluorocarbon hydroxyl-bola-amphiphile HO[C24][F6C5]OH and DMPC at a 1/5 molar ratio can also be thermally sterilised and stored at room temperature for up to 2 months without

Table 3
Mean diameter and size distribution of the liposomes formed from various fluorocarbon polar lipids after heat-sterilisation and storage at 25 ± 1°C

| Liposome composition[a] | After heat-sterilisation[b] and storage at 25°C (±1°C) | | |
|---|---|---|---|
| | Diameter (±S.D.)[c] (nm) | Distribution (%) | Storage (days) |
| Fluorinated formulations | | | |
| DF4C11PC[d] | 40 (15) | 14 | 300 |
| | 120 (35) | 86 | |
| DF4C11OPC[d,e] | 60 (15) | 36 | 600 |
| | 210 (55) | 64 | |
| [F8E11][C16]OPC[f] | 120 (25) | 100 | 300 |
| 1,3-[F8C11][C18]OPC[f] | 100 (36) | 80 | 300 |
| | 750 (250) | 20 | |
| 1,3-DF4C11APC[g] | 110 (25) | 90 | 300 |
| | 560 (80) | 10 | |
| 1,3-DF6C11APC[g] | 120 (30) | 75 | 300 |
| | 950 (200) | 25 | |
| DF4C11SerPC[g] | 125 (35) | 100 | 300 |
| HO[C24][F6C5]OH/DMPC (1/5)[h] | 210 (80) | 100 | 60 |
| HO[C24][F6C5]Gal/PL/CH (0.3/2/1)[h] | 160 (40) | 100 | 180 |
| [F8C7][C16][AEGal/PL/CH (0.3/2/1)[h] | 130 (40) | 100 | 180 |
| Conventional formulations | | | |
| DMPC, DPPC[d] or DSPC[f] | Deposit after storage for 6–7 days | | |
| EPC/CH (1/1)[f] | Deposit after sterilisation | | |
| EPC[f] | 100 (25) | 100 | 300 |

[a] 10 mM of the phospholipid in a Hepes buffer. In parentheses: molar ratio.
[b] 121°C, $10^5$ Pa, 15 min.
[c] S.D. is the standard deviation; 95% of the population are corresponding to ±1.96 S.D.
[d] From [10].
[e] Not sterilised.
[f] From [17]; EPC: egg phosphatidylcholines; CH: cholesterol.
[g] From [18].
[h] From [19]; PL: phospholipon®.

any significant modification of their sizes and size distribution (Table 3). However, increasing the relative content of bola-amphiphile HO[C24][F6C5]OH led to consistently less stable formulations [19].

The galactosyl-based formulations as well, proved remarkably stable upon storage (Table 3) [19]. Only minor changes in terms of particle size and size distribution (particle size coarsening) were observed during 6 months of observation. Low amounts (10% molar ratio) of bolaphile HO[C24][F6C5]Gal or of single-headed [F8C7][C16]AEGal improved substantially the shelf-stability of the reference 2/1 phospholipon®/cholesterol (PL/CH) liposomes which could not be stored for a period exceeding two months. However, increasing the relative content of the galactolipid over 10% resulted in unstable preparations.

### 2.3. Biocompatibility aspects

Biocompatibility is a major concern for components of drug carriers. Their acceptable level of toxicity is related to the intended indication, route of administration and dosage. The less toxic, the larger its potential of applications. While the exploration of the polymorphism of the fluorinated lipids and of their aptitude to form membranes, liposomes and other phases is well advanced, little attention has been devoted to their biological characteristics which include their in vitro (action on cell cultures, hemolytic activity) and in vivo behaviour (acute toxicity in mice). The results of these preliminary biocompatibility tests which have been reviewed elsewhere are very promising and will not be detailed here [8,31,32]. The reader will also find more information concerning other biological aspects on fluorosurfactants which have been published recently [33]. The general trend is that the replacement of hydrocarbon chains by fluorocarbon chains in an amphiphile significantly enhanced its in vitro biological tolerance towards cell cultures and red blood cells, in spite of increasing surface activity [9,11]. There is also no indication that such a replacement increases acute toxicity [9,11]. Indeed, high acute maximum tolerated doses compatible with the survival

of all of the i.v. injected mice (from ≥800 to up to 7500 mg/kg body weight) were observed for the fluorinated DFnCmPC phosphatidylcholines and related DFnCmOPC and [FnCm][Cm′]OPC compounds, some of them being in the same range as those found for their hydrocarbon analogs.

### 2.4. Properties of fluorinated liposomes

#### 2.4.1. Properties related to the fluorinated membrane

*2.4.1.1. Thermotropic phase behaviour.* Bilayers of hydrocarbon phospholipids usually display a reversible lamellar crystal (or gel, Lβ′) to lamellar liquid–crystal (or fluid, Lα) phase transition with temperature. The temperature at which this transition occurs is a fundamental characteristic of bilayers (and consequently of liposomes) as it determines several of their properties. For instance, the physical state of the membrane has a crucial impact on permeability and drug release profiles. Bilayers formed from the fluorinated phospholipids listed in Table 1 also display such a phase transition [15–19]. Their phase behaviour has usually been investigated by optical microscopy, X-ray scattering and differential scanning calorimetry (DSC). Table 4 collects their phase transition temperatures, $T_c$, and their associated $\Delta H$ and $\Delta S$ values determined by DSC, together with those of some of their closest hydrocarbon analogs.

The gel-to-fluid phase transition of the bilayer is an energetic event which reflects a co-operative transition of the lipid chains from an ordered to a disordered state. Differences in $T_c$, $\Delta H$ and $\Delta S$ values may be assigned to differences in packing characteristics due to differences in average conformation of the chains, lateral chain/chain interactions and hydrophobic interactions.

The large collection of homologous series of fluorinated amphiphiles led to the establishment of several structure/$T_c$ relationships. Most importantly, the results show that for a similar chain length $L$ ($L = n + m$) and as compared with their closest hydrocarbon analogs, the introduction of a linear C8F17 tail in both chains, as in DF8C5PC, DF8C11PC, DF8C5OPC, DF8C11OPC, 1,3-DF8C11OPC or DF8C11OPE, caused a significant increase of $T_c$ (see Fig. 2a–c) while introducing the same tail in only one of the chains had the opposite effect (see Fig. 2b). Indeed, the mixed fluorinated/hydrocarbon compounds [F8C11][C16]OPC or 1,3-[F8C11][C18]OPC display a much lower $T_c$ as compared with their respective fluorinated double-chain or hydrocarbon analogs (Fig. 2b). Whatever the PC (Fig. 2a), OPC (1,2- or 1,3-isomer) (Fig. 2a–c), OPE (Fig. 2c), SerPC or APC (Fig. 2d) series to which the amphiphile belongs, the introduction of a linear C4F9 or C6F13 tail in both chains resulted in a decrease in $T_c$ or had almost no effect on $T_c$, respectively.

The $\Delta H$ and $\Delta S$ values measured for the phase transition for the fluorocarbon bilayers are much lower than those found for their hydrocarbon analogs (Table 4). The $T_c$, $\Delta H$ and $\Delta S$ value changes after replacing a part of the hydrocarbon tail for a fluorinated one result from a compromise between (1) increased packing disorder, which tends to lower $T_c$, $\Delta H$ and $\Delta S$ values, as a consequence, among others, of weaker lateral inter- and intramolecular interactions [36], and augmented steric repulsions between fluorinated chains as compared with hydrocarbon chains, and (2) increased order, hence higher $T_c$, related to increased hydrophobicity of the fluorocarbon chains. In the different PC and PE series, short C4F9 tails appear to be insufficient to counterbalance the former effect, while with the longer C8F17 tails the hydrophobic interactions predominate, resulting in enhanced membrane ordering and rigidity.

*2.4.1.2. Structure and interactive properties of fluorinated bilayers*. The structure and interactive properties of several fluoroalkylated glycerophosphocholine-based bilayers have been investigated by X-ray diffraction/osmotic stress and dipole potential measurements [16].

Electron density profiles (Fig. 3) showed high electron density peaks in the centre of the bilayer indicating that the fluorinated regions of the alkyl chains are localised in the bilayer centre [16]. The height and width of these peaks varied systematically depending on the number of fluorines, their position on the alkyl chains, and on whether the bilayer was in the gel or liquid crystalline phase. For gel phase, the positions and widths of the high density peaks in the centre of the profiles are consistent with the expected positions of the fluorines in the molecules (Fig. 1) in well ordered lipid bilayers with stiff alkyl chains. In particular, for DF4C11PC the extremely high and narrow density peaks in the centre of the bilayer show that the fluorines of the terminal four carbons are localised in the centre of the bilayer. The distinct terminal dip in the profile, indicative of $CF_3$ groups, is another indication of the orderly packing in the bilayer centre. In the case of 1,3-[F8C11][C18]OPC and 1,3-[F6C11][C18]OPC (not shown), the central electron density peaks due to the fluorines are wider and not as high. For these bilayers the fluorines extend farther from the bilayer centre, since the terminal 8 and six carbons of one alkyl chain are fluorinated. The presence of a large trough in the geometric centre of each electron density profile and the values of the headgroup peak separations, dpp, which are in the same range as those measured for conventional bilayers (Fig. 3 and Table 5), indicate that these fluorinated glycerophosphocholines do not form interdigitated lamellar gel phases.

Wide-angle diffraction showed that in both gel and liquid-crystalline bilayers the distance between adjacent alkyl chains was greater in fluoroalkylated PCs than in analogous hydrocarbon PCs [16]. This is related to the greater size of a fully extended chain of $n$$CF_2$ groups (cross-sectional area of about 30 Å$^2$) compared with that of a $n$$CH_2$ chain (cross-sectional area of about 20 Å$^2$) and to the lower fluorocarbon/fluorocarbon or fluorocarbon/hydrocarbon chain/chain

Table 4
Lamellar phase transition (Lβ′ to Lα) temperature $T_c$ (in °C), enthalpy ΔH (in kJ mol$^{-1}$) and entropy ΔS (in J mol$^{-1}$ K$^{-1}$) of fluorinated bilayers measured by DSC in water

| Fluorocarbon lipids | | | | | | Hydrocarbon lipids (Fn = H) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code name | *n/p* | *m/q* | $T_c$[a] ($\Delta T_{1/2}$) | $\Delta H$ | $\Delta S$ | Code name | *m* | $T_c$[a] ($\Delta T_{1/2}$) | $\Delta H$ | $\Delta S$ |
| DFnCmPC [15] | 4 | 5 | <−20 | – | – | DLPC [15] | 12 | −1 | 16 | 59 |
| | 4 | 11 | 19 | 7.5 | 26 | | | | | |
| | 6 | 5 | <−20 | – | – | DMPC [15,18] | 14 | 24 (0.1) | 26 | 88 |
| [8] | 6 | 7 | 4 | 6.6 | 24 | | | | | |
| | 6 | 11 | 48 | 18.4 | 57 | DPPC [15,18] | 16 | 42 (0.1) | 36.5 | 116 |
| | 8 | 5 | 69 | 10.0 | 29 | | | | | |
| | 8 | 11 | >95 | – | – | DSPC [15,18] | 18 | 56 (0.1) | 46 | 140 |
| DFnEmPC [8] | 6 | 11 | 55 | 8.7 | 26 | | | | | |
| | 8 | 11 | No transition detected between 5 and 95°C | | | | | | | |
| DFnCmOPC [16,17] | 4 | 11 | 17 (3) | 11.1 | 38.3 | DCmOPC | 14 | 27 | 30.9 | 103.7 |
| | 6 | 11 | 50 (4) | 12.6 | 39.0 | | 16 | 43–45 | 38.4 | 123.2 |
| | 8 | 5 | 60 (2) | 6.7 | 20.3 | | 18 | 55 | 41.8 | 128.2 |
| | 8 | 11 | 88 (2) | 28 | 78 | | | | | |
| DFnEmOPC [16,17] | 8 | 5 | No transition detected between −25 and 95°C | | | | | | | |
| [FnCm][FpCq]OPC [16,17] | 4/8 | 11/11 | 36 (2) | 3.3 | 10.7 | | | | | |
| | | | 40 (2) | 5.5 | 17.6 | | | | | |
| | 8/4 | 5/11 | 10 | Not mesurable (large) | | | | | | |
| [FnCm][Cq]OPC [16,17] | 8 | 11/16 | 44 (4) | 8.2 | 25.9 | | | | | |
| [FnEm][Cq]OPC [16,17] | 8 | 5/14 | <−25 | – | – | | | | | |
| | 8 | 11/16 | 5 (3) | 7.7 | 27.7 | | | | | |
| 1,3-DFnCmOPC [16,17] | 6 | 11 | 52 (2) | 11.3 | 34.8 | 1,3-DCmOPC [17] | 14 | 29 | 25.9 | 85.9 |
| | 8 | 11 | 87 (2) | 18.1 | 50.1 | | 16 | 33 | 25.1 | 82.0 |
| 1,3-[FnCm][Cq]OPC [16,17] | 6 | 11/18 | 39 (2) | 28.9 | 92.8 | | 18 | 59 (1) | 43.0 | 129.7 |
| | 8 | 11/18 | 46 (3) | 17.7 | 55.4 | | | | | |
| 2,3-DFnCmAPC [18] | 6 | 11 | 49 (3) | 8.3 | 25.6 | 2,3-DCmAPC [18] | 14 | 18 (1) | 10.6 | 36.4 |
| | | | | | | | | 23 (5–6) | 12.7 | 42.9 |
| 1,3-DFnCmAPC [18] | 4 | 11 | 14 (5) | 12.6 | 43.7 | | | | | |
| | 6 | 11 | 45 (6) | 10.1 | 31.8 | | | | | |
| DFnCmSerPC [18] | 4 | 11 | 23 (2) | 19.3 | 65.3 | | | | | |
| | 6 | 11 | 55 (3) | 16.4 | 49.9 | | | | | |
| [FnCm][FpCq]SerPC [18] | 6/4 | 11/11 | 37 (3) | 14.8 | 47.6 | | | | | |
| [FnCm][Cq]SerPC [18] | 6 | 11/16 | 34 (1) | 9.4 | 30.6 | | | | | |
| DFnCmAEPC [18] | 4 | 11 | 4 (3) | 9.6 | 34.7 | | | | | |
| DFnCmOPE[b] [17] | 4 | 11 | 32 (3) | 20.2 | 66.2 | DCmOPE [17] | 14 | 56 | 24 | 73 |
| | 6 | 11 | 63 (5) | 20.3 | 60.4 | | 16 | 69 | 33.1 | 97 |
| | 8 | 11 | 94 (5) | 15.7 | 43.0 | | 18 | 77 | 39.1 | 112 |

[a] $\Delta T_{1/2}$ is the transition width at half-maximal excess specific heat capacity.
[b] The transition can also be a Lβ′ to $H_{II}$ transition [17].

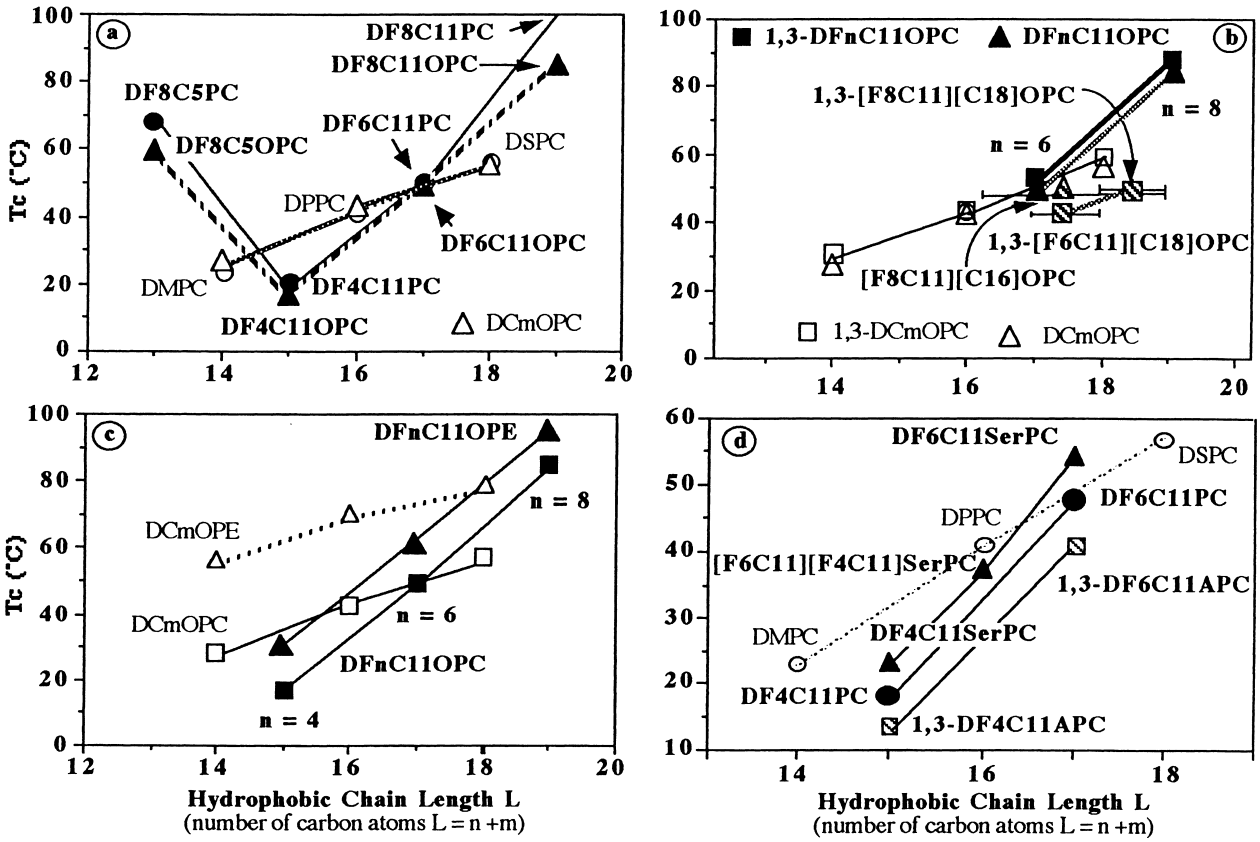



Fig. 2. Phase transition temperature vs. hydrophobic chain length for various fluorinated and hydrocarbon phospholipids: (a) ester and ether-PC series; (b) fluorinated double-chain and mixed-chain 1,3- and 1,2-ether-PC series; (c) ether-PC and PE series; (d) ester- and amide-(serine or amidoethanol)-PC series (data from [15–18]).

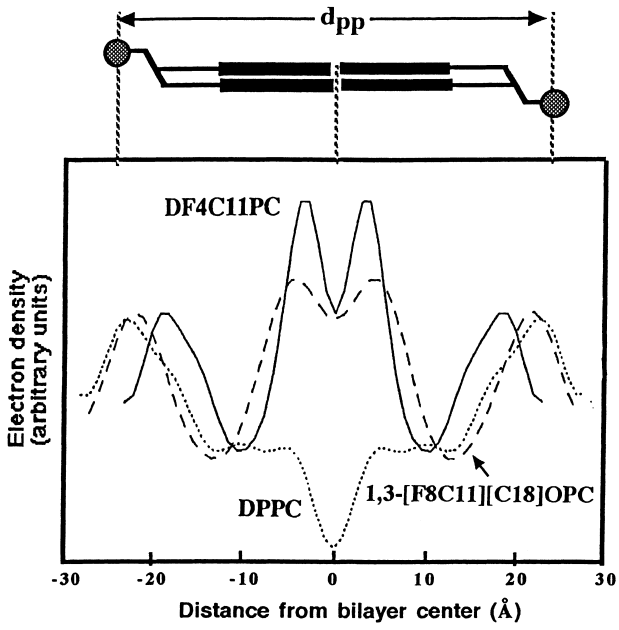



Fig. 3. Electron density profile of bilayers made from fluorinated phospholipids, as compared with that of conventional DPPC (data from [16]).

interactions compared with hydrocarbon/hydrocarbon ones [36].

Large negative dipole potentials (from −495 to −680 mV) were measured for the fluorinated PCs with terminal $–CF_3$ moieties in both chains whereas DPPC monolayers with terminal $–CH_3$ groups in both chains have large positive dipole potentials (+575 mV) (Table 5). This result is in line with dipole potential measurements on monolayers which have shown that for stearic acid the substitution of the terminal $CH_3$ with $CF_3$ changed the dipole potential from +275 to −915 mV. PCs with one fluoroalkylated and one hydrocarbon chain have relatively smaller negative dipole potentials (from −184 to −485 mV). The dipole potential is less sensitive to the number of $CF_2$ groups in the alkyl chain(s), differing by about 40 mV with the addition of two $CF_2$s in one chain, from −184 for 1,3-[F6C11][C18]OPC to −220 mV for 1,3-[F8C11][C18]OPC. Thus, these data indicate that the terminal $CF_3$ group has a larger influence on dipole potential than does the symmetric $CF_2$ groups and that the large $CF_3$ dipole markedly contributes to the dipole potential of monolayers at air–water interfaces. This is further confirmed by the larger magnitude of the dipole potential found for

Table 5
Distance between headgroup across the bilayer (dpp) from X-ray diffraction and dipole potential data measured on monolayers (see Fig. 3) [16]

| Lipid | Temperature (°C) | Bilayer phase | dpp (Å) | Dipole potential (mV) |
|---|---|---|---|---|
| DF8C5PC | 23 | Gel | 37.7 | −680 |
| DF4C11PC | 12 | Gel | 35.1 | – |
| DF4C11PC | 23 | l.-c | – | −576 |
| 1,3-DF6C11OPC | 23 | Gel | 39.5 | −495 |
| [F8C11][C16]OPC | 23 | Gel | 46.5 | −445 |
| [F8E11][C16]OPC | 23 | l.-c | 45.4 | −485 |
| 1,3-[F8C11][C18]OPC | 23 | Gel | 45.4 | −220 |
| 1,3-[F6C11][C18]OPC | 23 | Gel | 42.7 | −184 |
| EPC | 20 | l.-c | 37.8 | +415 |
| DPPC | 20 | Gel | 41.9 | +575 |

DF4C11PC compared with that of [F8C11][C16]OPC. These two compounds possess the same number of fluorinated carbons (8), but the former has two $CF_3$ groups whereas the latter contains one $CF_3$ group. Altering the position of the chains on the glycerol backbone from a 1,2-isomer to a 1,3-isomer changes the dipole potential by about 200 mV (−445 mV for [F8C11][C16]OPC and −220 mV for 1,3-[F8C11][C18]OPC). However, changing the chain linkage from ester to ether seems to have relatively little effect on dipole potential in the fluorinated series (the increase of about 200 mV from DF8C5PC to 1,3-DF6C11OPC corresponds to the increase found when going from a 1,2- to a 1,3-isomer). The dipole potential is larger in magnitude for [F8E11][C16]OPC than for [F8C11][C16]OPC. In this case the *trans* double bond in [F8E11][C16]OPC could modify the orientation of the fluorine dipoles.

*2.4.1.3. Lipophobicity of fluorinated bilayers*. The presence of the fluorinated core within the membrane was found to decrease the solubility of lipophilic compounds within this membrane, hence to increase its lipophobicity. The partitioning of a lipophilic/hydrophilic paramagnetic probe between the aqueous and lipidic phases of dispersions of the fluorinated amphiphiles showed indeed a very low solubility of this probe in the fluorinated membranes [15].

The fluorinated core within the membrane also modified the binding of hydrophobic anions to the membrane. This was established by measuring the change in dipole potential, as a function of subphase concentration of the tetraphenylboron anion. This change was found to be much less important for fluorinated PC monolayers than for egg PC [16].

As a consequence, one can expect that the interactions between fluorinated liposomes and biological compounds, such as hydrophobic enzymes, proteins (including opsonins, lipoproteins), peptides and cells, will be affected and that the penetration of lipophilic residues into the fluorinated membrane will be hindered. This may have interesting potential not only in drug delivery systems but also in membrane/liposome-supported reactions.

*2.4.2. Encapsulation in and release from fluorinated liposomes (membrane permeability)*

Stable encapsulation of a variety of materials during storage and incubation in biological compatible media, and sustained drug release at or near the site of action are essential prerequisites for liposomes to be used as drug carrier and delivery systems. Increasing the rigidity of the membrane, either by including cholesterol (CH) in the membrane or by using phospholipids which form a rigid gel lamellar phase, improved the retention of entrapped drugs [37]. Such liposomal formulations have, however, limited potential, especially for sustaining water-soluble lipophilic drugs and for modular release.

Endowing the liposomal membrane with the high hydrophobicity and high lipophobicity of fluorocarbons appeared as an attractive way of generating original membrane permeability and liposome stability profiles with respect to release of both hydrophilic or lipophilic encapsulated materials.

*2.4.2.1. Permeability to and release of entrapped carboxyfluorescein in buffer.* Release experiments from fluorinated liposomes of entrapped fluorescent dyes (such as 5(6)-carboxyfluorescein, CF, or calcein, CA, which are hydrophilic drug models) provided evidence for the strong impact of the fluorinated lipophobic core on membrane permeability and on dye release kinetics [20–23].

In the fluid state of the membrane, the fluorinated core acts indeed as a very efficient barrier to the transmembrane diffusion of CF: the ''fluid'' fluorinated vesicles exhibited much lower CF membrane permeability coefficients and retained entrapped CF much more efficiently than any of their ''fluid'' hydrocarbon counterparts (see Fig. 4A). It is noticeable that even fluorinated liposomes formed from mixed fluorocarbon/hydrocarbon-tailed phospholipids already showed significantly lower CF permeation. Thus, for example, ''fluid'' liposomes made from [F8E11][C16]OPC or [F6C11][C16]SerPC displayed a CF release rate much slower than conventional ''fluid'' liposomes and comparable to that of ''fluid'' liposomes made from double fluorocarbon-tailed PCs (see Fig. 4A).

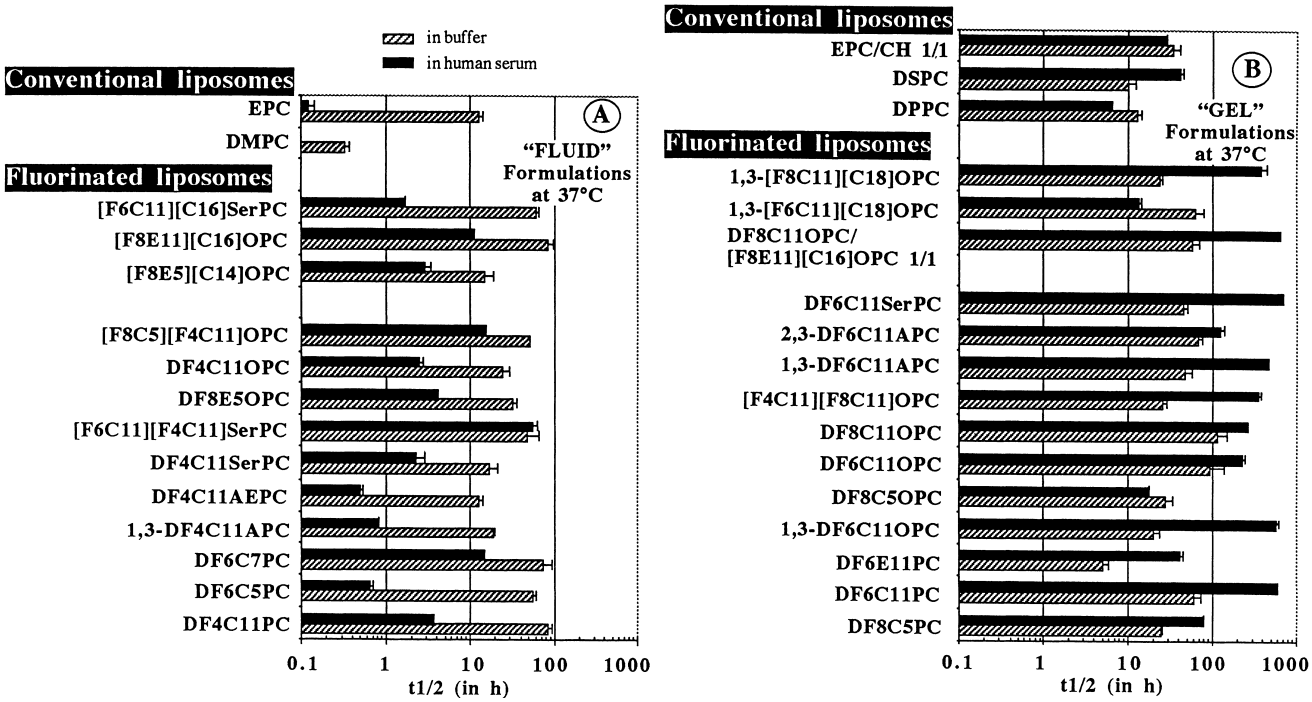



Fig. 4. Comparison of the carboxyfluorescein (CF) release from liposomes made from conventional, mixed-chain and double-chain fluorinated phospholipids and incubated in a physiological buffer (hatched bars) or in human serum (full bars) at 37°C. The liposomal membrane is, at 37°C, in (A) the liquid-crystalline or "fluid" state; (B) the crystal or "gel" state. $t_{1/2}$ is the leakage half-time corresponding to the time at which 50% of CF is still encapsulated within the liposomes (data from [20–22]).

In terms of molecular structure versus CF permeability (or CF leakage) relationships, permeability of the "fluid" fluorinated membranes decreased significantly when the degree of fluorination increased, indicating that the thicker the fluorinated lipophobic core, the more efficient the barrier to CF permeation [20–23]. That several of the "fluid" fluorinated liposomes were even less leaky and permeable than some "gel-like" fluorinated or conventional ones (see Fig. 4A, as compared with Fig. 4B) illustrates further the remarkable efficiency of the fluorinated core as a diffusion barrier.

These results are attributable to the high hydrophobicity and high lipophobicity of the fluorinated membrane which increases the order [38] and substantially decreases the solubility of the permeant dye within the membrane in its fluid state. These effects compensate largely for the weaker chain/chain interactions existing between fluorocarbon chains or between a fluorocarbon and hydrocarbon chain, hence for the lower cohesion of a fluorinated membrane in a liquid-crystalline state. This is also supported by the fact that in the gel state, the differences between fluorinated and conventional membranes or liposomes with respect to CF permeation coefficients and release profiles are less important [20–22] (Fig. 4B), the solubility of CF even within conventional membranes in their crystal state being extremely low.

*2.4.2.2. Remote loading of doxorubicin.* Efficient drug encapsulation is another important parameter to consider for liposomes being developed as drug carriers, more particularly if the drug is expensive and/or if the presence of "free" drug outside the liposomes, even in very low amount, has toxic side effects. Encapsulation into liposomes of ionizable drugs, such as doxorubicin (DOX) which is an efficient anticancer drug used in clinics, can be efficiently mediated using transmembrane ammonium sulfate or pH gradients. DOX can be considered both as a model of lipophilic/hydrophilic (amphipathic) drugs and as a model for remote loading. Its encapsulation into long-circulating liposomes increases its antitumor potency and provides substantial therapeutic benefits, a liposomal-doxorubicin formulation has been recently approved by the FDA for the treatment of Kaposi's sarcoma [39,40].

Remote loading of DOX into fluorinated liposomes was investigated and remained effective in spite of the high lipophobic character of the fluorinated membrane and the high efficiency of the fluorinated core as a barrier to transmembrane diffusion [24]. Efficient remote loading (>90%) and sustaining (less than 5% release after 1 week at room temperature in an isotonic sucrose solution) of DOX into liposomes made from DFnCmPCs could indeed be achieved using a transmembrane ammonium sulfate or even a transmembrane pH gradient, provided $Na^+$ was absent in the outer aqueous space (see next section).

*2.4.2.3. Permeability to $H^+$/$Na^+$.* Membrane permeability to ions, such as $H^+$, $Na^+$, is an important parameter to control for liposomes, more particularly if they are destined

to protect pH-sensitive drugs, or to facilitate the transmembrane gradient-driven encapsulation or release of ionizable drugs (see Section 2.4.3.2) [24]. The permeability to the $H^+/Na^+$ exchange of various fluorinated phospholipid-based membranes was evaluated by measuring the dissipation rate of a liposomal transmembrane pH gradient in the presence of $Na^+$ [40]. The fluorinated liposomes were made from fluorocarbon/hydrocarbon or fluorocarbon/fluorocarbon double-chain ether-connected glycero-PCs or amido-connected PCs deriving from diaminopropanol or serine (Table 1). The fluorocarbon/hydrocarbon mixed-chain PCs, as compared with the fluorocarbon/fluorocarbon ones, formed membranes that are substantially more able to maintain a transmembrane pH gradient in the presence of $Na^+$ and display a lower $Na^+$ permeability (Fig. 5). However, these membranes were more permeable to the $H^+/Na^+$ exchange than conventional DSPC ones. A detrimental impact of the membrane fluorination degree on $H^+/Na^+$ permeability could be detected: the lower the fluorination degree of the membrane, the higher the half-time of pH gradient dissipation, hence the lower its $H^+/Na^+$ permeability (Fig. 5b). Concerning structure/permability relationships, it appeared that the replacement of the ester connecting bond in the fluorinated phosphatidylcholines for an ether or amide one lowers the transmembrane $H^+/Na^+$ exchange [41].

The higher $Na^+$ permeability of the fluorinated membranes as compared with conventional formulations contrast with their lower CF permeability. Several reasons may account for the higher $Na^+$ versus CF permeability of the fluorinated membranes. First, $Na^+$ is a much smaller ion than CF which is, at neutral pH, an anion. Small ions, such as $Na^+$, travel preferably along the transient kinks formed by the C–C *trans/gauche* conformational rotations in the hydrophobic chains. Their permeation is also more sensitive to the presence of membrane defects than permeation of the larger CF anion. Furthermore, the phase transition of the fluorinated bilayers is progressive and occurs over a large temperature range while that of conventional membranes is highly co-operative. This results most likely in increasing the formation and propagation of kinks and packing defects and, consequently, in favouring, to a larger extent, the diffusion of small ions across fluorinated membranes. These arguments seem to be supported by the surprising lower $H^+/Na^+$ permeability found for some fluorinated "liquid–crystal" membranes ([F8E5][C14]OPC, [F8E11][C16]OPC, 1,3-[F6C11][C18]OPC and DF4C11PC) as compared with the "gel" fluorinated bilayers. Secondly, the fluorinated phospholipids in the bilayer are less tightly packed than their hydrocarbon analogs, a perfluorocarbon chain being much stiffer and bulkier, and the interactions between fluorocarbon chains (or between a fluorocarbon and hydrocarbon chain) being much lower than between hydrocarbon ones [36]. This could also play a significant role in facilitating the diffusion, especially of small ions such as $Na^+$ across fluorinated membranes.

With respect to the controlled $Na^+$-inducible release of ionizable drugs, fluorinated liposomes could constitute an attractive alternative to the conventional DSPC liposomes from which release cannot be triggered through a transmembrane $H^+/Na^+$, exchange (see Section 2.4.3.2).

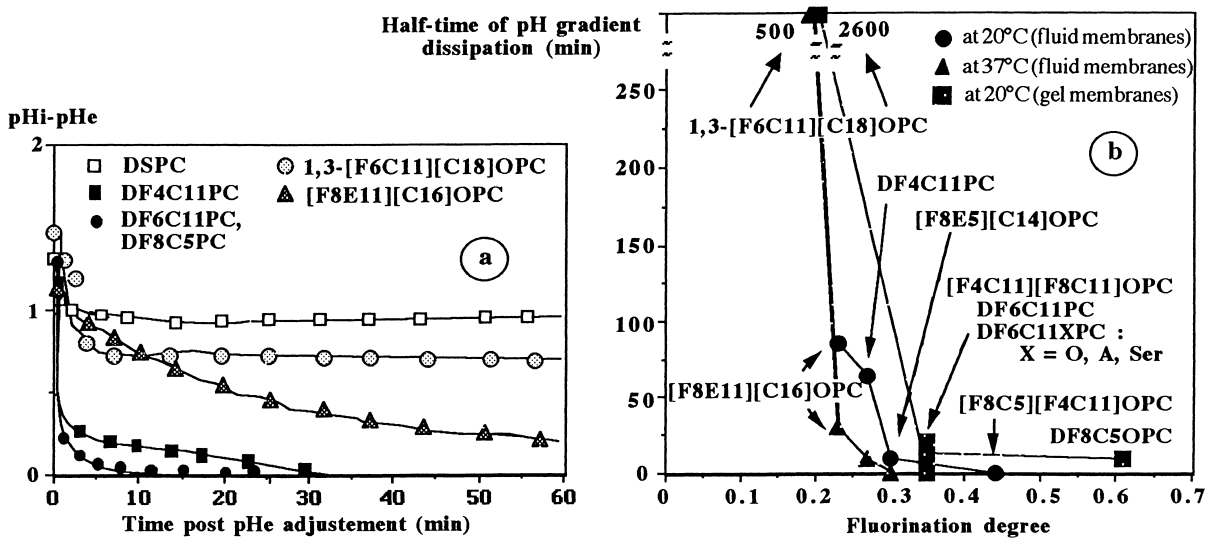



Fig. 5. (a) Stability of the transmembrane pH gradient for various fluorinated liposomes, as compared with DSPC liposomes (data from [24,41]). The liposomes were prepared in a 2.5 mM pyranine solution (pH 8.2). After removal of un-encapsulated pyranine, the liposomes were incubated at 37°C and the external pHe was then lowered to 6.7. The dissipation of the pH gradient (pHi(t)-pHe) as a function of time was followed by monitoring the fluorescence decrease of entrapped pyranine. (b) Half-times ($t_{1/2}$) of transmembrane pH gradient decay as a function of the fluorination degree of the liposomes and for various incubation temperatures (data from [41]). The fluorination degree is defined as the ratio of the number of F-carbons to the total number of carbons which constitutes the two hydrophobic chains.

*2.4.3. Fluorinated liposomes in biological environments*

The high hydrophobic and lipophobic character of a fluorinated liposomal membrane was expected (1) to have an impact on its interactions with serum components and other biological compounds, and (2) consequently, to modify the stability of such liposomes in biological fluids with respect to entrapped-drug leakage as well as their in vivo recognition and uptake by the mononuclear phagocytic system (MPS), hence their blood circulation time and biodistribution.

*2.4.3.1. Stability in biological fluids*. When incubated in human serum (100%) at 37°C, most of the fluorinated PC-based liposomes whose membranes were in the fluid state released CF more rapidly than in a physiological buffer, but to a considerably lesser extent than "fluid" conventional ones, as shown in Fig. 4A. Four "fluid" fluorinated formulations, e.g. [F8E5][C14]OPC, [F8C5][F4C11]OPC [21], [F6C11][F4C11]SerPC [22] and DF6C7PC [20] (with CF half-leakage times greater than 10 h), were however found very stable in human serum. These results indicate that the interactions of the fluorinated liposomes with the serum components are reduced, their adsorption at the liposome's surface being limited and/or their penetration into the bilayer being hindered. This can also be due to a much lower phospholipid exchange between the fluorinated liposomes and the high-density lipoproteins present in serum, owing to the very low miscibility between fluorinated and hydrocarbon components [42].

On the other hand, most of the fluorinated liposomes whose membranes are in the gel-like state, displayed a significantly greater stability in human serum than in a physiological buffer, and were noticeably even more stabilised in serum than conventional gel-like (i.e. DSPC or 1/1 EPC (egg phosphatidylcholine)/CH) liposomes (see Fig. 4B). These results were attributed to the reduction or suppression, in the presence of serum components, of packing defects generally present in gel-like membranes [20]. These observations correlate also with the increased stability of the doxorubicin-loaded "gel" fluorinated liposomes when in human serum rather than in a buffer [24].

*2.4.3.2. Release of encapsulated doxorubicin*. The release of encapsulated DOX from the liposomes is critical for its therapeutic activity. The kinetics of this release should be tailored with the lipsome's circulation half-live to achieve the best therapeutic benefit expected from the use of a carrier system.

When incubated in human serum at 37°C (Fig. 6B), the "gel" fluorinated formulations displayed interestingly release kinetics (20–26% release after 1 h) lying between those of DSPC/CH and EPC/CH (~0% and 40% release after 1 h, respectively), which are wide-used conventional liposomal formulations for drug encapsulation.

When incubated in a physiological i.v. injectable buffer at 37°C (Fig. 6A), the DOX-loaded fluorinated liposomes

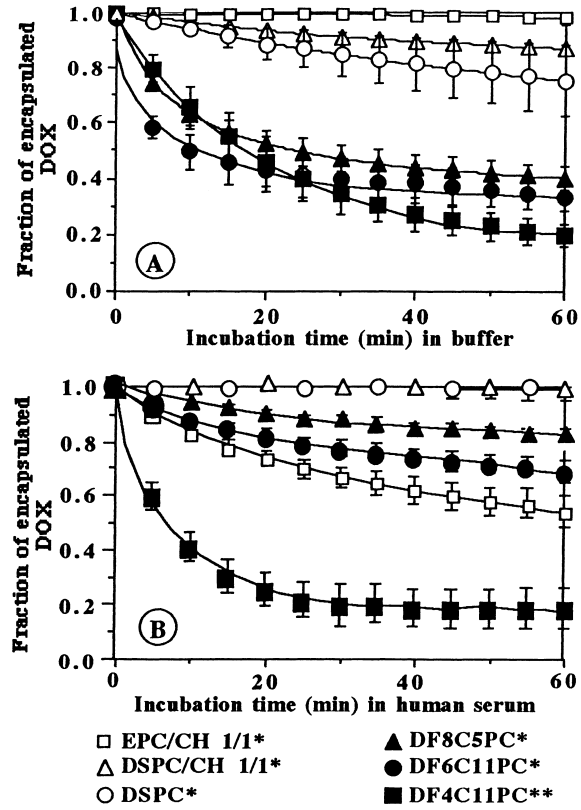



Fig. 6. Doxorubicin release kinetic at 37°C from fluorinated and conventional liposomes incubated in (A) a phosphate buffer saline and (B) in a 50% v/v human serum/phosphate buffer saline solution (data from [24]). *: "gel" or "gel-like" formulations at 37°C; **: "fluid" formulations at 37°C.

released their entrapped content more rapidly (from 60 to 80% after 1 h of incubation) than the conventional DOX-loaded liposomes (less than 30% after 1 h) [24]. This was assigned to the higher permeability of the fluorinated membranes to $Na^+$ (see Section 2.4.2.3), thus facilitating a liposomal inward $H^+$ — outward $Na^+$ flow. This results in an increase of the internal pH, hence in an increase of deprotonated DOX which diffuses more readily outwards of the liposomes.

These results indicate that the lipophobic character of fluorinated membranes, although it strongly reduces the solubility of lipophilic/hydrophilic compounds within the membrane [15], does not hinder the diffusion of a lipophilic/hydrophilic drug across these membranes when driven by its concentration gradient or when triggered through a transmembrane $H^+/Na^+$ exchange. They indicate also that the drugs are not sequestered but remain still available when encapsulated into fluorinated liposomes, and if the drug is ionizable, its release is inducible.

*2.4.3.3. Fluorinated liposomes in the presence of bile salts*. Drug carrier systems are particularly attractive for the oral administration of chemical- and/or biological-sensitive drugs or vaccines [43]. For liposomes, progress in these

fields requires liposomal components which confer to the vesicles significantly improved properties in terms of integrity and drug or antigen release in the presence of gastro-intestinal fluids, i.e. stability in acidic media, resistance towards large pH-variations and towards the action of pancreatic lipases or of bile salts, the latter playing a key role in the liposome's membrane destabilisation process [44].

With the aim of extending the potential of fluorinated liposomes as drug carrier and delivery systems for oral administration, their stability in terms of detergent-induced release of encapsulated carboxyfluorescein was evaluated as compared with conventional liposomes. The fluorinated 1,3-DF6C11OPC or 1,2-DF8C11OPC liposomes proved substantially more resistant towards the lytic action of sodium taurocholate than conventional DSPC or even 1/1 DSPC/CH liposomes [23] (cholesterol increases consistently the resistance of the conventional formulations to their disruption by the bile salts [43–45]). Although the CF release increased when raising the lipid/detergent molar ratio $R$ for all formulations (Fig. 7), the release of CF from the fluorinated 1,3-DF6C11OPC or 1,2-DF8C11OPC liposomes was significantly reduced relatively to DSPC ones (5 and 30-folds decrease of the $R_{50}$ parameter of DSPC, respectively). This effect was even larger than that resulting from the incorporation of cholesterol into the DSPC membrane which, for a 1/1 DSPC/CH molar ratio, provoked only a three fold decrease of the $R_{50}$ of DSPC [23].

The high resistance of the fluorinated liposomes to their disintegration by the detergent likely stems from the reduction of the detergent solubility into their membrane, owing to the very low affinity of hydrocarbon detergents for highly fluorinated surfactants [42].

These results show that fluorinated liposomes have a promising potential as drug carrier and delivery systems for oral administration.

*2.4.3.4. Blood clearance and biodistribution.* In the early 1990s, the development of vesicles which evade the mononuclear phagocytic system (MPS) tissues (particularly, liver and spleen), hence exhibit prolonged circulation times in the blood stream, has been achieved with the conjugation onto the liposome's surface of hydrophilic, bulky and mobile polyethylene glycol (PEG) groups [3–7]. Such sterically stabilised PEG-liposomes (or "stealth" liposomes) showed prolonged circulating times in blood, irrespective of the dose administered, surface charge density, bilayer composition and fluidity [3–7].

The presence of a highly hydrophobic and lipophobic fluorinated core inside the liposome's membrane was also expected to have pronounced effects on the pharmacokinetics profiles of the liposomes. This fluorinated film was indeed found to strongly retard the liposome's blood clearance [25]. Fluorinated vesicles showed circulation half-lives attaining 8.6–9.8 h, i.e. ~20 and six times larger than those of similarly sized conventional DSPC and DSPC/CH liposomes, respectively (Fig. 8). Furthermore, their blood clearance was dose-independent as for the PEG-labelled "stealth" liposomes.

The co-formulation of fluorinated liposomes with a fluorinated PE conjugated to PEG improves significantly their blood circulation half-times in mice [46]. DF6C11PC liposomes and those containing 5 mol% DF8C11OPE-PEG2000 loaded with $^{125}$I-tyraminylinulin (a radiolabelled marker of the interior aqueous space of the liposome) showed circulation half-lives of 9.8 and 12.8 h, respectively. However, these "fluorinated and sterically stabilised PEG-liposomes" do not exhibit circulation half-lives that are larger than those of"conventional stealth" PEG-liposomes (Fig. 8).

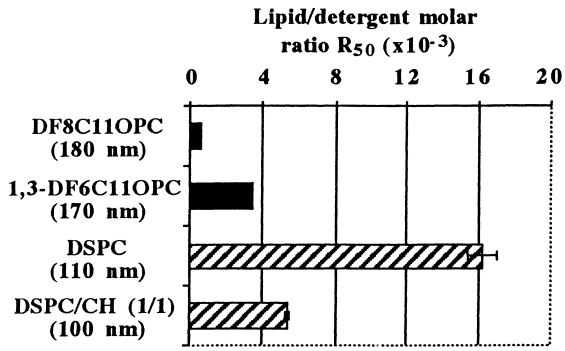



Fig. 7. Lipid/sodium taurocholate molar ratio $R_{50}$ coefficients corresponding to 50% of CF release from liposomes (size in nanometer) incubated in a Hepes (20 mM)/NaCl (0.15 M) buffer at 37°C. For all formulations, the membrane is, at 37°C, in the "gel" state. The smaller the $R_{50}$ value, the higher the resistance of the liposomes towards their disruption by sodium taurocholate (data from [23]).

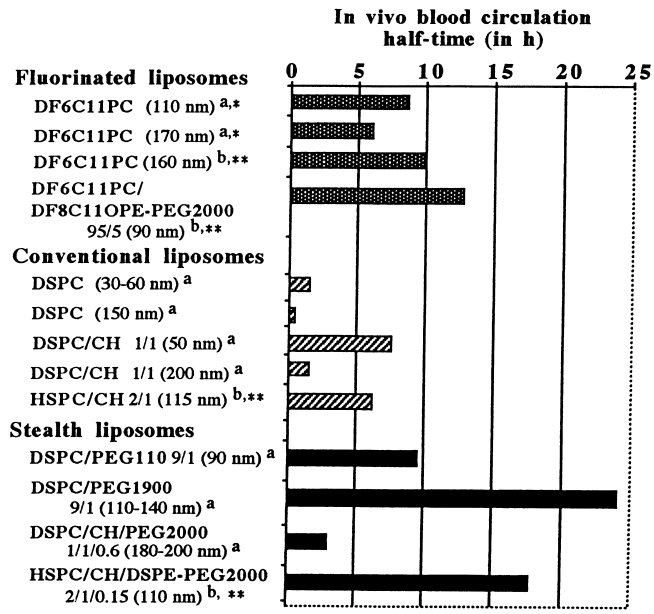



Fig. 8. Effect of lipid composition (molar ratio) and liposome size (in parentheses) on in vivo blood circulation half-times for fluorinated, conventional and PEG-liposomes. Encapsulated CF (∗) or $^{125}$I-tyraminylinulin (∗∗) were used as markers of the internal liposomal aqueous space: (a) data from [25], and references therein; (b) data from [46].

Liposome clearance from the blood stream occurs mainly as a result of interactions with blood components, leading to liposome opsonization followed by macrophage uptake, and/or to liposome disintegration by lipid exchange and depletion. In the case of fluorinated liposomes, their lipophobic character most likely hinder the adsorption and anchoring of lipophilic plasma proteins onto their surface, thus inhibiting their recognition and uptake [16]. This seems to be supported by in vitro experiments which have shown that protein adsorption on and phagocytosis of microspheres was reduced when coated with fluorinated amphiphiles [47]. The enhanced hydrophobic and lipophobic character of the fluorinated membranes and their very low miscibility with endogenous polar lipids [42] may also hinder their disintegration and depletion through lipid exchange induced by plasma proteins.

By contrast to its most important impact on liposome's blood clearance, the intramembranar fluorinated film was found to have only minor effects on their in vivo biodistibution [46]. In order to show the impact of this fluorinated film on biodistibution, formulations that display similar blood clearance kinetics were compared. For this study, an appropriate label of the liposome interior aqueous space, such as $^{125}$I-tyraminylinulin, was used (lipophilic markers that are associated to the liposome bilayer are not recommended as such a marker would not mix with the fluorinated lipids and/or would rapidly dissociate from the fluorinated liposomes, owing to the extremely low miscibility between fluorinated and hydrocarbon lipids).

Thus, using $^{125}$I-tyraminylinulin labelled liposomes, higher (∼15 versus ∼7% of the dose remaining in vivo) and lower amounts (31 versus 38%) of the fluorinated DF6C11PC liposomes were detected 48 h post injection in the spleen and in the carcass, respectively, as compared with conventional (2/1) HSPC (hydrogenated soya phosphatidylcholine)/CH liposomes [46]. Furthermore, both types of liposomes were mainly localised in the liver and at comparable levels (∼50% of the dose remaining in vivo). Concerning the stealth PEG-formulations, no significant differences in biodistribution 48 h post injection could be evidenced for the fluorinated DF6C11PC/DF8C11OPE-PEG2000 (5 mol%) and conventional (2/1/0.1) HSPC/CH/DSPE-PEG2000 liposomes, both types of liposomes being mainly localised in the carcass (∼50% of the dose remaining in vivo), in the liver (∼30%), and a lower amount (∼6–8%) being detected in the spleen [46].

*2.4.4. Biological activity of fluorinated galacto-liposomes*

One of the main objectives of using liposomes as drug delivery systems is to provide targeted drug delivery at the site of action. Carbohydrate-derived amphiphiles are particularly attractive for such targeting purposes [48,49]. Specific recognition of carbohydrates by membrane lectins expressed by different cells (such as hepatocytes, macrophages) is indeed known to be involved in numerous biological events. HIV, which mainly infects cells expressing CD4, was also found to infect, in vitro, cells which express galactosylceramide (GalCer) at their surface. Their infection occurs through binding of the HIV envelope glycoprotein gp120 to GalCer [50]. It was further suggested that this interaction involves GalCer-rich domains [51,52]. These findings, together with the potential of galactosphingolipids to inhibit HIV uptake and infection, have instigated the syntheses of various galactolipids and the examination of their biological (anti-influenza virus and anti-HIV) activities, and, more particularly, of the highly fluorinated galactosylated (bola)amphiphiles listed in Table 2 ([14], and references therein). Such compounds, owing to their lipophobic character and their expected low miscibility with classical polar lipids [19,42], should exhibit a stronger tendency to form galactosyl-rich clusters and domains or patches when incorporated within conventional membranes, thus increasing their interactions with gp120 hence their HIV inhibition [53,54]. They may further serve as components for the formulation of liposomes targeting anti-HIV drugs to HIV-infected cells, such cells expressing also gp120.

The fluorinated double-chain [FnCm][FpCq]SerGal, [FnCm][Cq]SerGal, [F8C7][C16]AEGal, and HO[C24][F6C5]Gal when incorporated into conventional liposomes display an anti-HIV activity on the GalCer(+)HT-29 cells which was significantly higher than that of the single chain F6C11SerGal analog [14]. By contrast, hydrocarbon analogs of HO[C24][F6C5]Gal [14] and of the fluorinated SerGal derivatives [55], were found to be inactive. These results support the possibility that gp120 preferentially or exclusively binds to glycolipid-rich domains in the liposomal bilayer [51,52], their formation being indeed more favoured in the case of the highly fluorinated derivatives.

## 3. Lipoplexes from double-chain fluorinated lipopolyamines

Gene therapy (i.e. the correction of a genetic disease) relies on the administration to patients of the cloned and functional equivalent gene which encodes for the production of the therapeutic protein. It is now a reality and several protocols are under way in humans. One of the main barriers to gene therapy is the ability to deliver DNA efficiently. Although DNA, which is a high molecular weight polyanionic molecule, can associate with cell membranes, it usually cannot penetrate and transfect cells. DNA must therefore be associated with a carrier. If encouraging results have been obtained with viral vectors, safety concerns about these particles have instigated the development of non-viral gene carriers. A large variety of (poly)cationic lipids, liposomes and (lipo)polymers, eventually associated to molecular conjugates for improving cell targeting, cytoplasmatic delivery and/or nuclear transport, has been used extensively to deliver genes to a large variety of cell lines and tissues ([26–28], and references therein). These (poly)cationic

systems are capable of interacting with polyanionic DNA, condensing or compacting DNA into small-sized complexes (e.g. lipoplexes or polyplexes), neutralising its negative charges and thus favouring its entry into the cell.

The in vivo administration of these DNA complexes is also confronted with a number of problems that are generally associated to colloidal systems but also more specifically to the nature of the drug, i.e. DNA. Efficient transfection, especially in vivo, depends, among others, upon the cationic components, the size of the complexes, their stability in and interactions with biological fluids and the cellular constituents. Little is also known concerning the intracellular traffic of the DNA to the nucleus. Consequently, there is a need to develop new compounds, especially (poly)cationic compounds, with characteristics and properties that are substantially different from those already described.

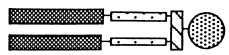
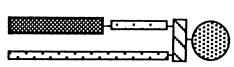
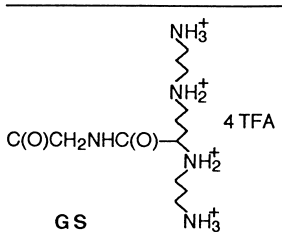
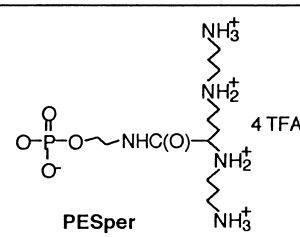
The remarkable and original properties of the fluorinated liposomes as drug carrier and delivery sytems led us to develop several highly fluorinated analogs of DOGS or Transfectam® [29,56] (see structures and code names in Table 6) which is, among the cationic lipids tested so far, one of the most efficient synthetic gene transfer agent [26]. The unique hydrophobic and lipophobic character of the fluorinated lipoplexes they form with DNA was also expected to generate original physico-chemical and biological behaviours.

### 3.1. Lipoplex formation

Table 6 collects the various fluorinated lipospermines which condense DNA and form lipoplexes, whether these lipids are used alone or in conjunction with 1,2-di-oleylphosphatidylethanolamine (DOPE), a helper lipid which was found to enhance transfection with cationic lipids [28].

Their capability to form lipoplexes was extensively studied with the luciferase expressing plasmid pcTG11033 [29], and seldom with the luciferase expressing plasmid pGL3 [30] or chloramphenicol-acetyltransferase (CAT) reporter plasmid [56], all these plasmids (as lipoplexes) have been used for in vitro transfection assays (see next section). The procedure applied for the lipoplex preparation relies on the dilution from organic solutions of the lipids in buffer which contains the plasmid, using various lipid/DNA molar ratios expressed as *N*/*P* ratios (from 10 to 0.8; *N*: number of lipid cation equivalents; *P*: number of DNA phosphate equivalents). The mean particle sizes for the different lipoplexes (which is a most important parameter to control when their in vivo use is intended) were measured

Table 6
Fluorinated double-chain lipospermines forming lipoplexes with DNA

| Schematic Structure | Chemical Structure | Code Name |
|---|---|---|
|  | $F\text{-}(CF_2)_n\text{-}(CH_2)_m$ <br> $F\text{-}(CF_2)_n\text{-}(CH_2)_m$ >N—GS | **DFnCmGS*** <br> n = 4, 6; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m$ <br> $F\text{-}(CF_2)_p\text{-}(CH_2)_q$ >N—GS | **[FnCm][FpCq]GS** <br> n = 4; m = 11 <br> p = 8; q = 2 |
| | $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}$ <br> $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}$ >N—GS | **DFnEmGS** <br> n = 4, 6; m = 11 |
| | $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ <br> $F\text{-}(CF_2)_n\text{-}(CH_2)_m\text{-}O$ >PESper | **DFnCmOPESper** <br> n = 6, 8; m = 11 |
|  | $F\text{-}(CF_2)_n\text{-}(CH_2)_m$ <br> $H\text{-}(CH_2)_q$ >N—GS | **[FnCm][Cq]GS** <br> n = 4; m = 11; q = 14 |
| | $F\text{-}(CF_2)_n\text{-}CH{=}CH\text{-}(CH_2)_{m-2}$ <br> $H\text{-}(CH_2)_q$ >N—GS | **[FnEm][Cq]GS** <br> n = 4, 6; m = 11; q = 14 |
|   **GS** |   **PESper** | * Hydrocarbon analog: <br> **DOGS or Transfectam®:** <br> Fn = H; m = 18 <br> $H\text{-}(CH_2)_m$ <br> $H\text{-}(CH_2)_m$ >N—GS |

by light scattering spectroscopy. The formulations were also analysed by gel electrophoresis which showed the absence of "free" plasmid for an excess of lipid (*N*/*P* ratios from 10 to 2.5), the plasmid being accessible to ethidium bromide in the case of some *N*/*P* 1.25 formulations and for all the *N*/*P* 0.8 formulations.

For example, cationic lipoplexes of a mean size in the 115–180 nm range were formed when a large excess (*N*/*P* ratio of 10–5) of the fluorinated lipospermines listed in Table 6 was used to condense plasmid pcTG11033. The results obtained also for other *N*/*P* ratios suggested further that the fluorinated lipospermines are more effective than DOGS, in terms of compacting this plasmid into small-sized particles [26].

### 3.2. Transfection with fluorinated lipoplexes

The fluorinated lipospermines appear as very promising synthetic vectors for gene delivery. The [F4C11][F8C2]GS lipospermine, which has the highest degree of fluorination among the fluorinated lipospermines investigated, and DOGS demonstrated similar properties for transfecting NIH-3T3 murine fibroblasts, using the CAT reporter plasmid [56]. Lipoplexes formulated with a luciferase reporter plasmid and the fluorocarbon/fluorocarbon lipospermines or the mixed fluorocarbon/hydrocarbon ones do also enable transfection of lung epithelial A549 cells, from human pulmonary carcinoma [29]. Several of these fluorinated lipoplexes showed even a higher transfection efficiency than those based on DOGS (Fig. 9). Their efficiency was further increased when using the helper DOPE lipid. Where the effect on cell viability of the lipoplexes is concerned, no specific cell toxicity was found for the fluorinated ones as compared with that of the DOGS lipoplexes [29].

The palette of structural elements of the fluorinated lipospermines that have been explored allowed us to determine those required for efficient transfection, highlighting the importance of highly fluorinated chains (hence lipophobicity), and the unique properties of unsaturated double-chain lipids. That transfection increases with lipophobicity indicates further that the mixing of the cationic lipids with cellular lipids is not determinant for the gene transfer and expression process. It is rather attributable to some protection effects of DNA resulting from its formulation as lipophobic and hydrophobic lipoplexes with fluorinated lipospermines, which prevent DNA from degradation and interactions with lipophilic and hydrophilic biocompounds.

Although very encouraging, the in vitro transfection efficiencies of the fluorinated lipospermines do not compete with that of viral vectors. This conclusion does not, however, prejudge their in vivo behaviour which remains to be explored.

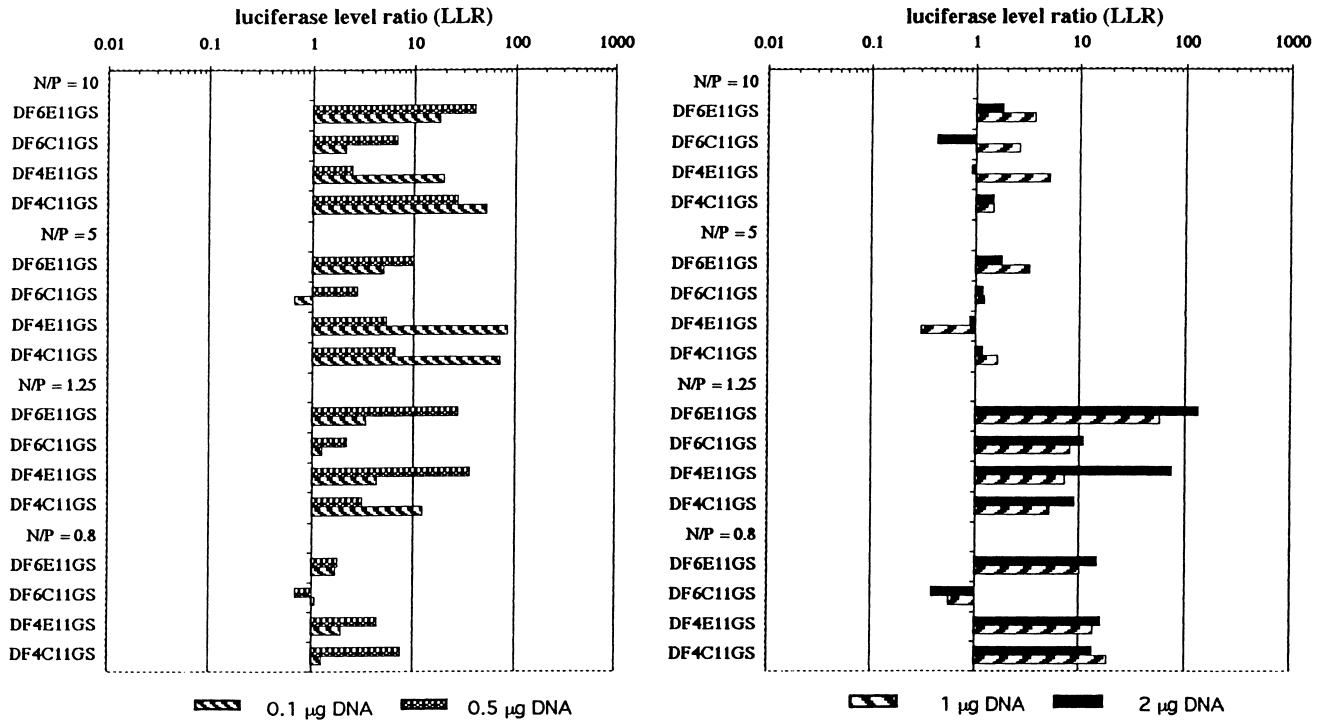



Fig. 9. Transfection efficiency of the fluorinated lipoplexes vs. that of their corresponding DOGS-lipoplexes. The luciferase level ratio LLR is the ratio of luciferase amount measured for the formulation with the fluorinated lipoplexes vs. that measured for the corresponding DOGS-lipoplexes. The efficiency is significantly higher if LLR is $\geq 5$, significantly lower if LLR $\leq 0.2$, or comparable if $0.2 < \text{LLR} < 5$ ($P \leq 0.05$). The fluorinated lipoplexes are made from the double-chain fluorinated DFnE11GS or DFnC11GS lipospermines. Data from [29].

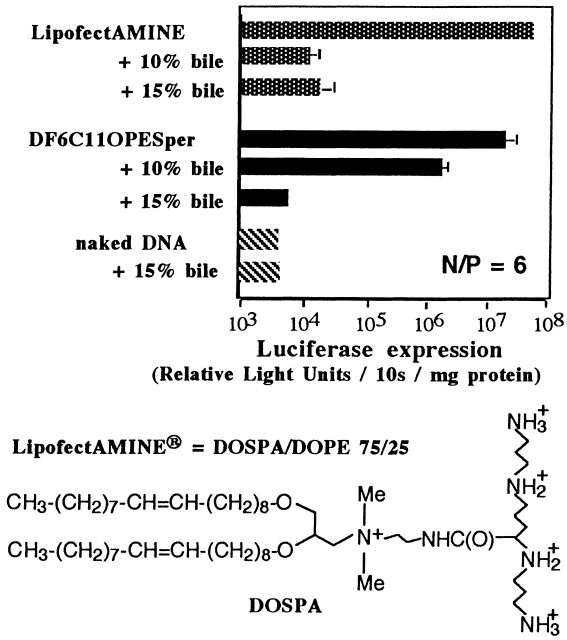



Fig. 10. Transfection of 3T3 murine fibroblasts with fluorinated DF6C11OPESper/pGL3(=DNA)-lipoplexes vs. that of LipofectAMINE®/pGL3-lipoplexes in the presence of bile (data from [30]).

### *3.3. Transfection with fluorinated lipoplexes in the presence of bile salts*

Gene transfer into biliary cells is a challenging issue for gene therapy of the hepato-biliar disease associated with cystic fibrosis. Among other features, the gene vector must resist the lytic action of enzymes and the detergent effects of the biliary salts. In view of the higher resistance found for the fluorinated liposomes towards the lytic action of bile salts, fluorinated lipoplexes appeared as appealing candidates for such an issue. As illustrated in Fig. 10, the fluorinated DF6C11OPESper lipoplexes enabled transfection of fibroblast cells in the presence of bile to a larger extent than conventional LipofectAMINE® lipoplexes (the composition of LipofectAMINE® is given in Fig. 10). If both lipids proved as efficient in transfecting cells in the absence of bile, only the fluorinated lipoplexes allowed cell transfection in the presence of 10% of bile [30].

## 4. Conclusions/prospects

Fluorinated liposomes made from highly fluorinated double-chain phospho- or glyco-lipids as well as fluorinated lipoplexes made from highly fluorinated polycationic lipospermines and reporter genes have displayed a number of interesting physico-chemical and biological properties. These systems are very attractive alternatives as drug or gene carrying, targeting and delivering devices. Future developments should explore their in vivo potential.

The major handicap to the utilisation of highly fluorinated amphiphiles in pharmaceuticals is their novelty. Hence, the need for carrying out the full range of indispensable subacute and chronic toxicity, adsorption, distribution, metabolism and excretion studies. Preliminary tolerance tests have so far given encouraging results. If the results of the pharmacology/toxicology studies are satisfactory, fluorinated amphiphiles could play a significant role in future drug delivery and targeting devices.

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