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Physicochemical properties of cationic lipophosphoramidates with an arsonium head group and various lipid chains: A structure–activity approach

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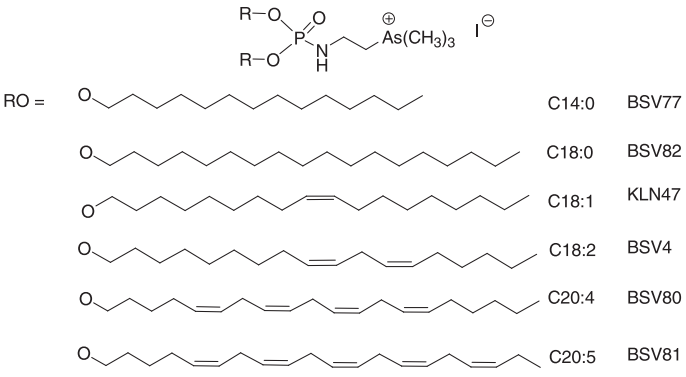
HIGHLIGHTS

- New series of cationic lipophosphoramidates liposomes used as gene vectors
- Influence of chain length and insaturation numbers on physico-chemical properties
- Study of membranes viscosity and fusion, DNA condensation and in vitro transfection
- Comparison of physico-chemical properties and in vitro transfection efficiency
- Best compound for transfection is fluid and efficient for fusion and DNA condensation

GRAPHICAL ABSTRACT

Lipophosphoramidates series for physicochemical properties studies



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ABSTRACT

We studied the physicochemical properties of some cationic lipophosphoramidates used as gene vectors in an attempt to better understand the link between the nature of the hydrophobic chain and both physico-chemical properties and transfection efficiency. These compounds have an arsonium head group and various chain lengths and unsaturation numbers. The synthesis of cationic phospholipids with oleic (Guenin et al., 2000 [1]; Floch et al., 2000 [2]) or linoleic (Fraix et al., 2011 [3]; Le Gall et al., 2010 [4]) chains has already been reported by our group and their efficiency as gene carriers has been demonstrated. Four new compounds were synthesized which incorporated either C14:0, C18:0, C20:4 or C20:5 chains. The membrane fluidity was studied by fluorescence anisotropy measurements. The fusion of liposomes and lipoplexes with membrane models was studied by Förster Resonant Energy Transfer. Finally, DNA condensation was studied and the lipoplexes were tested *in vitro* to quantify their transfection efficiency. From the results obtained on these cationic lipophosphoramidates series, we show that aliphatic chain length and unsaturation number have an important influence on liposomes physicochemical properties and transfection efficiency. However there is no direct link between fluidity and fusion efficiency or between fluidity and DNA condensation. Nevertheless, it seems that for best transfection efficiency the compounds need to combine the properties of fluidity, fusion efficiency and DNA condensation efficiency. This was the case for the C18:1 and C18:2 compounds.

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1. Introduction

Delivery systems based on non-viral vectors such as cationic lipids or cationic polymers are an interesting alternative for gene therapy. Presently, they are less efficient than viral systems but they proved to be generally less toxic, more easily produced and exhibit a greater stability. Both cationic lipids and polymers permit cooperative DNA binding, forming supra-molecular complexes in which the plasmid is highly compacted, allowing efficient introduction into target cells. The field of cationic lipids, pioneered by Felgner [5] and Behr [6] has strongly evolved and many different compounds have been synthesized and screened to improve the transfection efficiency of these molecules [7,8]. Cationic lipids are composed of a cationic polar head, a hydrophobic part and a linker between them. The first approach has been to synthesize cationic amphiphiles with new versions of positively charged polar groups including mono-valent or polyvalent cations such as spermine derivatives [9]. The influence of the hydrophobic domain has also been studied [10] and different types of linkers between the cationic head and the hydrophobic backbone have been used.

Our group has synthesized lipo-phosphonates, which exhibit interesting transfection abilities in both *in vitro* and *in vivo* experiments [11–13]. A first structure variation applied on this vector family has targeted the polar headgroup, through the substitution of the trimethyl-ammonium by trimethyl-phosphonium or trimethyl-arsonium cationic groups [1]. This modification gave rise to a series of vectors which displayed improved transfection efficiencies with decreased toxicities. In subsequent studies, the substitution of the phosphonate functional group by a phosphoramidate lead to a third family of vectors with even better transfection abilities. Specifically, a derivative possessing a phosphoramidate functional group and an arsonium cationic headgroup proved to be efficient for *in vivo* gene delivery [2]. Hypothesis was made that the size difference between a trimethylammonium and trimethylarsonium group [14] generates a modulation of the electrostatic interaction between the cationic lipid and the plasmid. Consequently, a better compromise between stability-required when the lipoplexes are located outside of the cell, and instability needed to favour endosomal escape, is found with the trimetylarsonium-based lipids.

To further study this vector family, we thought that the effects of lipid chain variations needed as well to be addressed [3]. In particular, it appeared important to understand the relationships linking together the lipid structure (saturated *versus* unsaturated or poly-unsaturated), the physico-chemical properties of the corresponding liposomes or lipoplexes and their transfection activities [12,15–17]. The nature of the hydrophobic chains, i.e. their length and degree of unsaturation, modifies the fluidity of the cationic lipids as well as the lipids organization [12,18–22]. The way how fluidity influences transfection is not clear. It may influence the lipoplex formation and the interaction of the lipoplex with the cell membrane. Many papers report on mono-unsaturated compounds but only few papers report on poly-unsaturated compounds. Ahmed et al. [23] published a study which describes cationic lipids containing poly-unsaturated lipid chains. They compare stearic, oleic and linoleic chains and suggest that the higher transfection efficiency of the linoleic may be caused by some additional fluidity. Aljaberi et al. [24] also suggest that high *in vitro* transfection activity is mediated by cationic lipids characterized by increased alkyl chain fluidity. The influence of the lipids organization has been pointed out by numerous papers, highlighting the influence of a hexagonal phase H_{II} on transfection efficiency by improving membrane fusion. The phospholipids with two alkyl chains are expected to form bilayers but the number of unsaturations affects the molecular shape and modifies the aggregate structure [25]. It has been reported to increase the H_{II} phase forming ability of phospholipids membranes [26–29]. Such molecular changes may favor the lamellar to non-lamellar transition of cationic lipids and may be a key factor in the mechanism of lipoplex mediated transfection. Tenchov et al. [30] reported that the ethyl-ester of oleyl-decanoyl-ethyl-phosphatidylcholine (C18:1/C10 EPC) was more fusogenic than its fully saturated analog (C18:0/C10 EPC) and the increased fusion rate was correlated with an enhanced transfection efficiency.

From this point of view, the use of mono- or poly-unsaturated alkyl chains as lipidic parts of our cationic lipids might be a valuable strategy to improve the transfection efficiency. In this paper, we study the physico-chemical properties of six lipophosphoramidates to search a relationship between the structure, the physico-chemical properties and the transfection efficiency. Fig. 1 shows that these compounds are characterized by a trimethyl-arsonium polar head and different lipid chains either saturated (i.e. C14:0 and C18:0) or unsaturated (i.e. C18:1 ω9, C18:2 ω6, C20:4 ω6 and C20:5). Here, we report the physico-chemical analysis of the liposomes formed by these compounds including Critical Aggregation Concentration (CAC) determination, size and zeta potential measurements, membrane fluidity measurements by fluorescence anisotropy, as well as an evaluation of the fusogenic properties by Förster Resonant Energy Transfer (FRET). We study also the influence of the co-lipids, cholesterol and DOPE, and the physico-chemical properties of the lipoplexes, i.e. membrane fluidity, and fusogenic properties [3]. The DNA condensation is measured using the ethidium bromide exclusion technique. Finally, the *in vitro* transfection efficiency using A549 and 16HBE cell lines is evaluated.

2. Experimental

2.1. Reagents and chemical

Lipids [L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), Cholesterol (Chol), and 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine (DOPE)] were obtained from Sigma (France). Fluorescent probes used were NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-PE, Abs/Em = 463/536 nm), Rho-PE (Rhodamine-PE, Abs/Em = 560/581 nm), Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, Abs/Em = 552/636 nm) and Laurdan (6-Dodecanoyl-2-dimethylamino-naphthalene, Abs/Em = 364/497 nm), all purchased from Molecular Probes (France). HEPES buffer and chloroform (UV spectroscopy grade) were both from Fluka (France). Lipofectamine is from Invitrogen (France).

Two plasmids DNA were used, pGL3-Ctrl (5.3 kb) and pEGFPLuc (6.4 kb), obtained from Promega (France) and Clontech (UK), respectively. Only the pGL3-Ctrl was used for DNA condensation assays. As those two plasmids are of equivalent sizes, we assumed that similar results are obtained with the pEGFPLuc (this was verified in several tests). For *in vitro* assays, we used the pEGFPLuc which encodes a fusion of an enhanced green fluorescent protein (EGFP; Ex max 488 nm, Em max 507 nm) and the luciferase from the firefly Photinus pyralis.

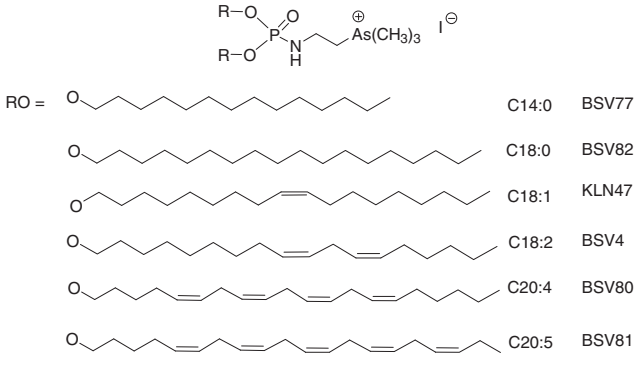

Fig. 1. Chemical structure and label of the lipophosphoramidates used in this study for gene delivery.

2.2. Synthesis

The general procedure for the synthesis of the arsonolipophosphoramidates is the following: to a selected *O,O′*-dialkylphosphoramidate iodoethylamine (1.0×10^{-3} mol) in dry THF (0.7 mL) was added trimethylarsine (2.0×10^{-3} mol). The mixture was stirred at 60 °C for 72 h under nitrogen atmosphere. Solvent and excess of trimethylarsine were evaporated under reduced pressure and the residue was purified by column chromatography on silica gel using methanol in chloroform to give the desired arsonolipophosphoramidate (Fig. 1).

Compound C18:0 (Bret Synthetic Vector 82 "BSV82") was prepared from *O,O*-Dioctadecyl-*N*-(2-iodoethyl)phosphoramidate. Column chromatography $CHCl_3$/MeOH (99/1–95/5). Yields (22%), white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (6H, *t*, ^{3}J = 6.7 Hz), 1.26 (60H, *m*), 1.66 (4H, *m*, ^{3}J = 6.7 Hz), 2.26 (9H, *s*), 3.15 (2H, *m*, ^{3}J = 7.5 Hz), 3.52 (2H, *m*, ^{3}J = 7.5 Hz), 4.02 (4H, *q*, ^{3}J = 6.7 Hz), 4.48 (1H, *bs*). ^{31}P NMR (162 MHz, $CDCl_3$): 8.91. ^{13}C NMR (300 MHz, $CDCl_3$): 10.22, 14.45, 23.02, 25.93, 29.17–30.74, 32.26, 67.51. (MALDI-TOF): *m/z* calc. for $C_{41}H_{88}AsINO_3P$ [M^+-I]: 748.571 Found: 748.631.

Compound C20:4 (BSV80) was prepared from *O,O*-Dieicosatetraenoyl-*N*-(2-iodoethyl) phosphoramidate. Column chromatography $CHCl_3$/MeOH (95/5). Yields (96%), brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (6H, *t*, ^{3}J = 6.7 Hz), 1.29 (12H, *m*), 1.56 (4H, *m*, ^{3}J = 6.7 Hz), 1.67 (4H, *m*, ^{3}J = 6.7 Hz), 2.03 (8H, *m*), 2.23 (9 H, *s*), 2.78 (12H, *m*), 3.15 (2H, *t*, ^{3}J = 6.7 Hz), 3.72 (2H, *m*), 4.03 (4H, *q*, ^{3}J = 6.7 Hz), 4.47 (1H, m), 5.41 (16H, *m*). ^{31}P NMR (162 MHz, $CDCl_3$) : 8.73. ^{13}C NMR (300 MHz, $CDCl_3$): 10.02, 14.43, 22.91, 25.98, 27.07, 27.56, 29.11–31.85, 36.41, 67.29, 127.88–130.85. (MALDI-TOF): *m/z* calc. for $C_{45}H_{80}AsINO_3P$ [M^+-I]: 788.509 Found: 788.564.

Compound C20:5 (BSV8) was prepared from *O,O*-Diecosapentaenoyl-*N*-(2-iodoethyl) phosphoramidate. Column chromatography CH_2Cl_2/MeOH (95/5). Yields (64%), brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (6H, *t*, ^{3}J = 6.7 Hz), 1.44 (4H, *m*, ^{3}J = 6.7 Hz), 1.68 (4H, *m*, ^{3}J = 6.7 Hz), 2.07 (8H, *m*), 2.23 (9H, *s*), 2.82 (16H, *m*), 3.15 (2H, *t*, ^{3}J = 6.7 Hz), 3.80 (2H, *m*), 4.03 (4H, *q*, ^{3}J = 6.7 Hz), 4.47 (1H, m), 5.32 (20H, *m*). ^{31}P NMR (162 MHz, $CDCl_3$) : 8.87. ^{13}C NMR (300 MHz, $CDCl_3$): 9.83, 14.30, 20.56, 25.54, 25.63, 26.71, 28.99, 29.99, 30.04, 36.06, 66.99, 127.00–132.04. (MALDI-TOF): *m/z* calc. for $C_{45}H_{76}AsINO_3P$ [M^+-I]: 784.477 Found: 784.543.

Compounds C18:1 (KLN47) and C18:2 (BSV4) synthesis protocol have been already described [1–3].

2.3. Preparation of liposomes

An aliquot of a stock solution of lipids in chloroform at 5 mg/mL was evaporated under reduced pressure during 1 h. HEPES buffer was added and the sample was kept at 4 °C for at least 1 night of hydration. It was then sonicated for at least 5 minutes. In these conditions small unilamellar vesicles are obtained.

2.4. Fluorescence measurements

Fluorescence measurements were conducted on a Cary Eclipse Varian spectrophotometer and fluorescence spectra were recorded (Scan software, Varian). Throughout all this study, a slit width of 5 nm was used for both excitation and emission wavelengths. For anisotropy measurements the manual polariser accessory (Varian) was used. FRET and anisotropy experiments have been described previously [15].

2.5. Zetasizer measurements

Mean particle diameters and zeta potentials (ξ) of liposomes and lipoplexes were measured using a 3000 Zetasizer (Malvern Instruments) at 25 °C after an appropriate dilution of the formulations. For measurements with lipoplexes, each test employed 22 μg of plasmid DNA mixed with the required quantity of liposomes. Every lipoplex preparation is characterized by its Charge Ratio (CR) defined as the ratio of lipids concentration on DNA concentration. For measurements with liposomes, we used the same amount of lipids as for experiments on lipoplexes.

2.6. DNA condensation

Assays were done in 96 well plates either in optiMEM (pH 7.4), in 0.9% NaCl (pH ~5.0), in 5% glucose (pH ~5.0) or in sodium acetate buffers (pH 5.0, 6.0, 7.0 or 7.4). The maximum fluorescence signal was obtained when ethidium bromide (1.5 μg/mL final) was bound to plasmid DNA (1.0 μg/well). DNA was added to the wells containing reagents at different amounts in order to obtain different +/− charge ratios (positive charges of the carrier divided by the negative charges of DNA). The fluorescence signals were measured using a Fluoroskan Ascent FL plate reader (Thermo Electron Instruments, France) at excitation wavelength 530 nm and emission wavelength 590 nm.

2.7. In vitro assays

Epithelial cells (A549 and 16HBE) were grown respectively in DMEM and EMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37 °C, 5% CO2 in a humidified atmosphere. Transfections were performed as previously described by Felgner et al. [5] with the following modification. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 12,500 cells per well in a final volume of 200 μL (i.e. at about 70% confluence). Preliminary experiments were performed as follows. Immediately prior to transfection, the growth medium was removed and replaced with 160 μL of OptiMEM per well. The transfection mixtures (30 μL per well) were then pipetted dropwise to the cell cultures and cells were exposed to transfection reagents for 4 h. Thereafter, cultures were maintained 48 h at 37 °C until reporter gene measurements. Luciferase assays were used as *in vitro* reporter gene measurements. After 48 h, cells were first lysed using the Passive Lysis Buffer (Promega, France), and then centrifuged. The luciferase activitiy in each supernatant was measured using a microtiter plate luminometer (Dynatech Laboratories, Guyancourt, France). Transfection results are expressed in Relative Light Unit per milligrams of protein (RLU mg^{-1}). The late toxicity is expressed as a percentage of the deficit in total proteins in comparison with untransfected cells used as controls.

3. Results and discussion

3.1. Critical aggregation concentration

Cationic lipids are amphiphilic compounds and any change in the structure of the compounds will modify the balance between the lipophilic and hydrophilic part influencing their tendency to form self-assembled nanoparticles. Gruneich et al. [31] have correlated the transfection efficiency of a series of cholesterol-based cationic lipids with the hydrophobicity of their lipid moiety by calculating their partition coefficient in octanol saturated water (log P). A similar reasoning was used by Le Gall et al. for the study of a series of lipidic neamine derivatives which incorporated aliphatic chains [32]. MacDonald et al. [33] also published results suggesting a new approach for enhancing gene delivery, based on identifying the optimal hydrophobicity of cationic lipid derivatives. So, we decided to calculate the critical aggregation concentration (CAC) for all six compounds in order to evaluate the influence of the hydrocarbon chain structure on the aggregation of cationic lipids. The CAC corresponds to the concentration from which aggregates are formed and it can be calculated by measuring the fluorescence of the hydrophobic probe Nile red [34]. This hydrophobic probe has a fluorescence emission that is enhanced and blue-shifted

in hydrophobic matrices. That is why its fluorescence intensity suddenly increases upon the formation of phospholipids aggregates, where Nile Red locates because of its hydrophobic character. By plotting the fluorescence intensity of Nile Red against the logarithm concentration of liposomes the CAC value can be found as the onset of the fluorescence intensity increase. The CAC is given by the intersection of two regression lines: a horizontal line obtained when the probe is free in aqueous solution and a positive slope line proportional to the concentration of liposomes. Its uncertainty can be calculated from the intersection of the confidence intervals of both regression lines [4].

Table 1 shows that the CAC of the six compounds is in the range of 2 μmol L^{-1} to 10 μmol L^{-1} (Table 1). These values are higher than those measured for neutral phospholipids of the same chain length. A value of 4.6×10^{-10} mol L^{-1} has been reported for dipalmitoylphosphatidylcholine (C16:0) [35] and 5 μmol L^{-1} for didecanoylphosphatidylcholine (C10:0) [36]. Consequently, the solubility is strongly enhanced by the positive charge of the arsonium headgroup as reported for other charged phospholipids [37]. As expected from prediction models [38], a higher solubility is obtained for the myristic compound (C14:0) whose hydrophobic domain is shorter. In spite of their longer hydrophobic chains, the C20 compounds have CAC values in the same range as those of unsaturated C18, probably because of their numerous unsaturations [39].

3.2. Membrane fluidity

Spectral and anisotropy measurements using fluorescent probes have been used commonly to assess membrane fluidity and changes in membrane phases [40–42]. Laurdan is a probe that has proven useful for these studies [40,43]. We measured the fluorescence anisotropy in order to evaluate the membrane fluidity and the main phase transition temperature T_m. To measure T_m the evolution of the anisotropy versus the temperature is fitted by an arc-tangent type model. The derivation of this model allows to obtain the inflexion point of the anisotropy gap from high to low value. This inflection point corresponds to the T_m value. Fig. 2 shows that a high anisotropy (about 0.3) is measured for the C18:0 compound which strongly decreases at 55 °C. At room temperature, this compound forms a gel phase and the decrease of anisotropy at 55 °C is due to the transition to the liquid crystalline state as expected for stearic chains [44]. The shorter aliphatic chain compounds (C14:0) has a lower anisotropy (about 0.17) at room temperature, and T_m is equal to 22 °C. Fig. 2 shows that the anisotropy at room temperature is in the same range for the C18:1 and C18:2 compounds. However, we could not observe the corresponding decrease of anisotropy for these compounds because their T_m is lower than 10 °C. C20:4 and C20:5 compounds have the same evolution of anisotropy versus temperature as the C18:1 and C18:2 compounds (data not shown to keep Fig. 2 simple). The T_m values were confirmed by differential scanning calorimetry measurements (data not shown).

For compounds with high anisotropy at room temperature (C18:0) the addition of increasing amounts of DOPE (25% to 75%) decreases progressively the anisotropy, whereas the addition of cholesterol smoothed the anisotropy variation versus the temperature at the phase transition. Fig. 3 shows the results obtained for an addition of a 50% ratio of DOPE and cholesterol. On the opposite, cationic phospholipids with low anisotropy (C18:2) showed that the addition of DOPE did not affect anisotropy whereas the addition of cholesterol increased significantly the anisotropy at 37 °C from $r=0.12$ to $r=0.20$.

We have conducted the same experiments on lipoplexes (data not shown). For C18:0 lipoplexes we observed the same evolution of the anisotropy versus the temperature as for liposomes and similar T_m values. We did not observe any significant modification for C18:1 and C18:2 compounds and the T_m were also lower than 10 °C. The same observations were made for C20:4 and C20:5 compounds. For C14:0 lipoplexes, the curve of the anisotropy was shifted 0.05 units and increased to 27 °C instead of 22 °C for liposomes.

Table 1
Comparison of the main results obtained for liposomes (critical aggregation concentration: CAC) and for lipoplexes (size and Zeta potential at CR = 4).

Chain length	Number of insaturations	CAC (μmol $L^{-1}\pm1$)	Size (nm ± 20) at CR = 4	Zeta potential at CR = 4 (mV ± 2)
14	0	8	220	42
18	0		180	41
18	1	3	150	49
18	2	3	140	51
20	4	2	240	37
20	5	4	280	39

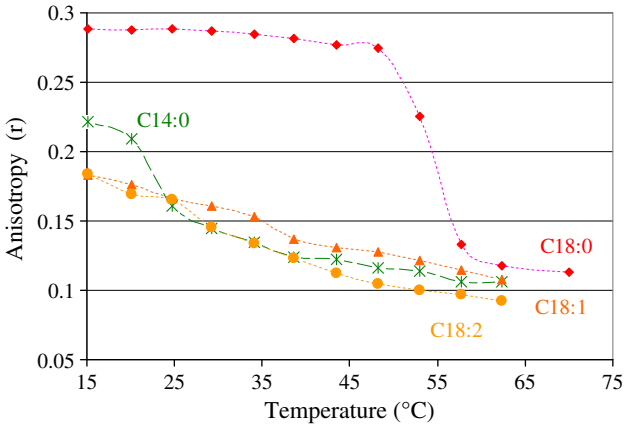

Fig. 2. Laurdan fluorescence anisotropy of liposomes as a function of temperature. The inflection point of the curves corresponds to the phase transition temperature, T_m. No inflection points were observed for C18:1 and C18:2. Therefore, both compounds have T_m lower than 15 °C. For C18:0 the T_m is of 55 °C whereas for C14:0 is of 22 °C.

3.3. Membrane fusion

Once internalized during the transfection process, the cationic lipids–DNA complexes have to escape the endosomal compartment for effective delivery [45]. The mechanism of endosomal release is thought to involve lipid mixing between the endosomal and cationic lipid membranes which leads to membrane disruption and release of DNA into the cytoplasm. This fusion between membranes proved

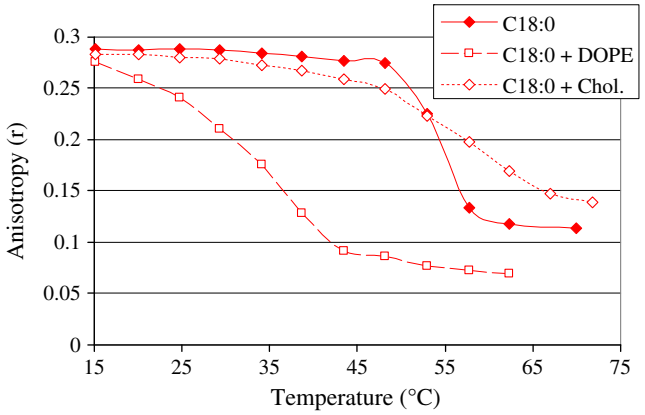

Fig. 3. Effect of the addition of the co-lipids DOPE and cholesterol on membrane anisotropy of the C18:0 liposomes. The laurdan fluorescence anisotropy is obtained at a molar ratio 1/1 of DOPE and Cholesterol. The addition of DOPE decreases anisotropy and T_m, whereas the addition of cholesterol decreased the variation of anisotropy versus temperature at the phase transition.

influenced by the structure of the lipid probably because of its influence on the phase organization. It was also found to be significantly influenced by the target membrane composition [46]. The phosphatidylserine content of the membrane is important because it possesses a negative charge and the fusion of cationic lipids with negatively charged liposomes depends on the fraction of anionic lipids in the bilayer. The fusion of cationic liposomes, or lipoplexes, with a model membrane labelled with two fluorescent probes was studied by FRET assays [47–49]. Since the FRET efficiency between the probes depends on their spatial separation, any fusion event between such double-labelled liposome with a cationic liposome devoid of any fluorophore decreases the efficiency of energy transfer. The FRET efficiency was calculated from the fluorescence emission intensity of NBD-PE at 530 nm using the following equation:

$$E = 1 - F/F_0$$

Fluorescence intensities were recorded in the presence (F) and absence (F_0) of Rhod-PE. The relative fluorescence intensity (RFI, in percent) is calculated using the following equation:

$$\mathrm{RFI} = (\mathrm{Emix}/\mathrm{Eab}) \cdot 100$$

Emix and Eab are the FRET efficiencies calculated in the presence (Emix) or absence (Eab) of cationics lipids. Consequently, after addition of the maximal cationic lipid concentration (60 μmol L^{-1}) a RFI of about 80% can be interpreted as a low fusion rate and inversely a RFI of about 50% corresponds to an important fusion rate.

As labelled membrane [3,15], we used liposomes containing phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, cholesterol and NBD-PE and Rhod-PE as respectively donor and acceptor probes (PC/PE/PS/Chol/NBD-PE/Rhod-PE) in a relative mass proportion of (44/25/10/20/0.8/0.2). Fig. 4 shows that the compounds with long aliphatic chains (C20:4 and C20:5) have a high RFI hence a low fusion rate; whereas the other compounds (C14:0; C18:0; C18:1; C18:2) have an important fusion rate, C18:1 being the highest. Similar results were obtained with lipoplexes without any significant difference between the RFI of liposomes compared to lipoplexes.

Fig. 5 shows that the addition of co-lipids (50% ratio, total lipid concentration = 60 μmol L^{-1}) has a significant effect on fusion rate of liposomes. For C18:0, C18:1 and C18:2 compounds one can see that DOPE addition increases the fusion rate, whereas cholesterol addition decreases it.

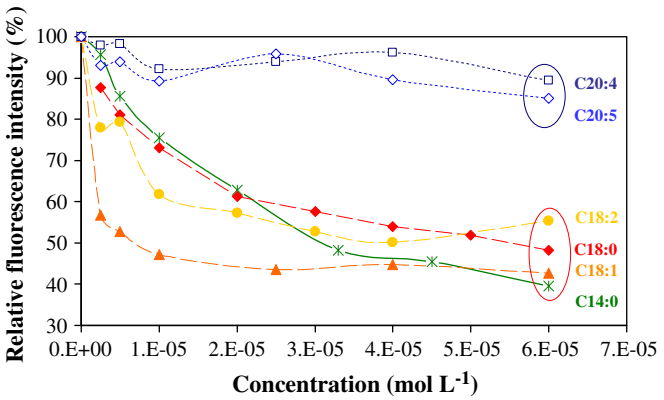

Fig. 4. Relative Fluorescence Intensity (RFI) as a function of the added concentration of the liposomes obtained with the six cationic phospholipids. A total lipid concentration of 6×10^{-5} mol L^{-1} corresponds to a 50% ratio. The compounds with long aliphatic chains (C20:4 and C20:5) have a high RFI and consequently a low fusion rate. Inversely, all other have an important fusion rate.

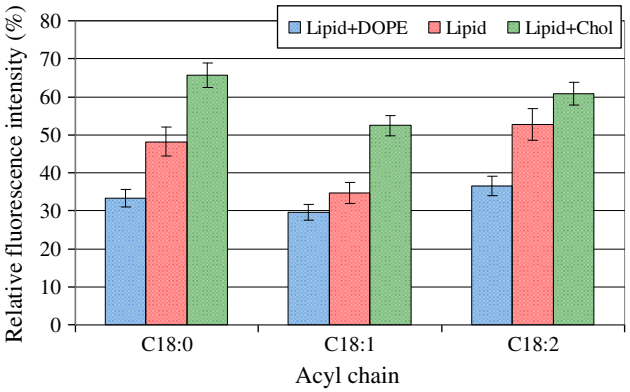

Fig. 5. Relative Fluorescence Intensity (RFI) of C18:0, C18:1 and C18:2 in presence and absence of the co-lipids DOPE and cholesterol at 50% ratio (i.e. total lipid concentration = 6×10^{-5} mol L^{-1}). The addition of co-lipids has a significant effect on the fusion rate of liposomes. DOPE increases the fusion rate, whereas cholesterol decreases it.

3.4. DNA condensation

The condensation of plasmid DNA by the cationic agents was investigated using as fluorescent probe the ethidium bromide (EtBr). The intercalation of DNA with EtBr increases its fluorescence quantum yield. When the DNA condenses on lipoplexes, the EtBr is displaced from the EtBr–DNA complexes and its fluorescence quantum yield decreases. The maximum fluorescence is obtained just before lipoplex addition and is normalized to 100% [50]. The condensation efficiency was estimated for different lipid/DNA charge ratios versus time. Fig. 6 shows for C18:2 that higher charge ratios give better DNA condensation and that the maximum condensation efficiency is reached faster. An optimum appears for CR = 4 after 6 h. For a higher CR = 8 the condensation becomes less efficient. Fig. 7 shows that very different profiles of condensation efficiency are obtained for the cationic phospholipids when CR = 4. The best condensing compound is the C18:1, which allows a complete condensation after only 2 h and whose complexes remain stable during the next 12 h. Its efficiency is even higher than the commercial lipofectamine and its complexes seem more stable. The C18:2 is also an efficient condensing compound but the condensation is not complete and is not as fast as for the C18:1. A 90% efficiency is observed after 5 h and remains stable. However, C20:4 and C20:5 compounds are much less efficient than C18:1 and C18:2 as well as the C18:0 compound which seems a very poor DNA condensing compound.

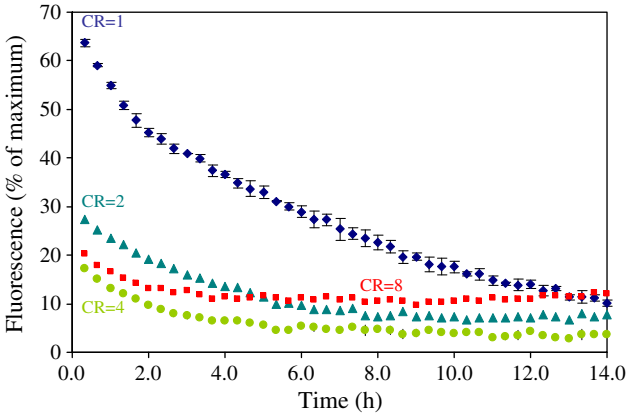

Fig. 6. Condensation of C18:2 liposomes with DNA versus time obtained for different charge ratios (CR). DNA condensation was determined by ethidium bromide exclusion assay. The more fluorescence decreases, the higher is the condensation rate. An optimum condensation efficiency appears for CR = 4 after 6 h. DNA condensation becomes less efficient for CR ≥ 8.

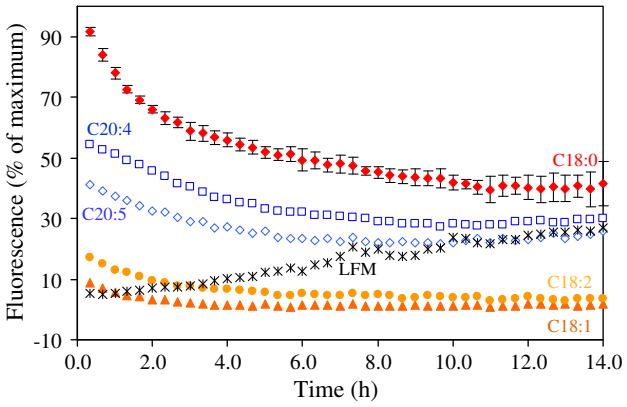

Fig. 7. Condensation of liposomes and lipofectamine (LFM) with DNA at CR = 4. The best condensing compound is the C18:1 which allows a complete condensation after 2 h and whose complexes remain stable during 12 h. The C18:2 is also an efficient condensing compound but the condensation is not complete and is not as fast as for the C18:1. C20:4 and C20:5 compounds are less efficient than C18:1 and C18:2. C18:0 is the worst condensing compound.

3.5. Colloidal stability of the complexes formed with plasmid DNA

Zetametry measurements were also conducted in order to characterize some additional physicochemical properties of the DNA complexes formed. First, we found that liposomes alone were characterized by a mean diameter size comprised between 135 and 280 nm and a positive potential around 40 mV (Fig. 8 and Table 1). Second, zeta potential (ξ) and size (particle mean diameter) variations of lipoplexes were determined in parallel for a series of increasing charge ratios (CR) of lipids to DNA (i.e. from CR = 0.5 up to CR = 6.0 as upper CR are too far from the optimum condensation conditions). The results were coherent with a three-zone model of colloidal stability which had already been described for lipopolyamine/DNA or BGTC/DNA lipoplexes. The three different zones (named A, B, and C) were determined by the cationic lipid/nucleic acid CR [51,52]. According to this model, in zone A at low CR, negatively charged and colloidally stable complexes with partially condensed nucleic acid are formed. Zone B contains neutral, large, and colloidally unstable aggregates. In zone C, the particles are positively charged, small, and again colloidally stable. Interestingly, we observed here that zone B was closely restricted to complexes formed at CR around 1 or 1.5 (i.e. when virtually neutral species are formed, see below). In zone C, which ranged here from CR = 2 up to CR = 6, complexes of similar sizes were obtained, characterized with a mean diameter value of about 200 nm. Zeta potentials were also determined on the same samples. ξ rapidly increased with the lipoplexes CR, with negatively charged species obtained at CR = 0.5 (DNA in excess), neutral species at charge ratios around 1 and finally positively charged species at CR = 2 with a positive charge close to the maximum measured, between 45 and 50 mV, thus recovering the values of liposomes alone. In all, when mixed with DNA at CR = 2 or above, liposomes formed with the different lipids are able to rapidly complex DNA, producing stable species characterized by a full positive surface charge and a relatively small mean diameter. These properties are indeed similar to those of previously published cationic lipids (e.g. BGTC [53]).

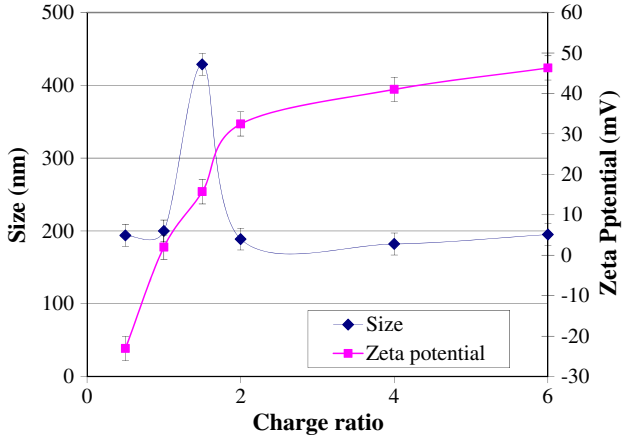

Fig. 8. Evolution of the size (nm) and the Zeta potential (mV) for C18:0 lipoplexes as a function of the charge ratio. The particle mean diameters were of about 200 nm, except for CR = 1.5. Zeta potentials were also determined on the same samples. ξ rapidly increased with the CR of the lipoplexes.

3.6. In vitro transfection experiments

As lung-directed gene therapy for Cystic Fibrosis is at the forefront of gene therapy research, we chose to mainly use, in the present study, the A549 and 16HBE cell lines which are both derived from human lung epithelial cells. Thus, a series of lipoplexes characterized by various CRs (ranging from 0.5 to 8.0) were prepared by mixing the required amounts of lipid with a constant amount of plasmid DNA expressing the reporter gene luciferase.

First, the impact of lipoplex formulations on cell viability was investigated. We found that the toxicity index (expressed as a percentage of deficit in protein content) increases with the CR. For CR = 4, Fig. 9 (upper panel) shows three groups in terms of toxicity. The first group (C14:0 and C18:0) is completely safe, the second one (C18:1 and C20:5) has a medium toxicity and the third group (C18:2, C20:4 and Lipofectamine) has the highest toxicity.

We have also investigated the transfection efficiency and the best results were obtained for CR = 4 (which corresponds to the most efficient DNA condensation). Fig. 9 (lower panel) shows the results obtained for all compounds when CR = 4. They take also into account

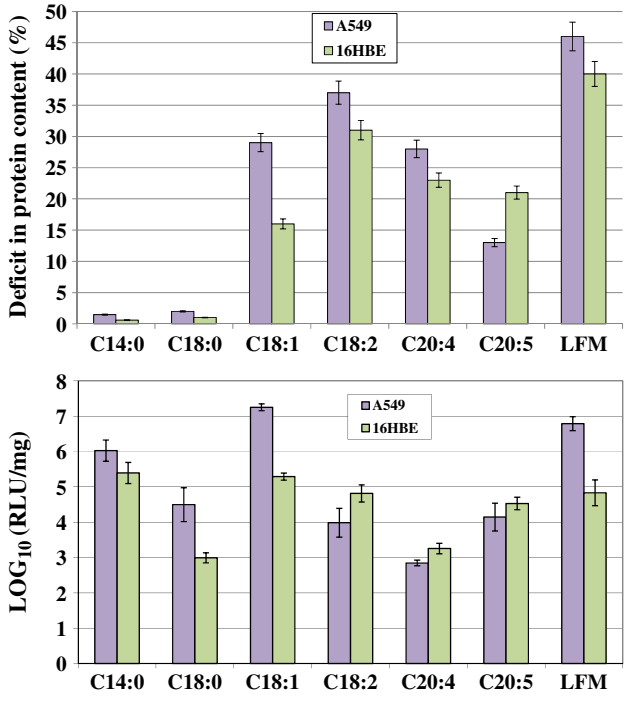

Fig. 9. Toxicity index (deficit in protein content in %, upper panel) and transfection efficiency (lower panel) expressed as Relative Light Units (RLU) per milligrams of proteins. Concerning toxicity, C14:0 and C18:0 appears to be completely safe whereas C18:2, C20:4 and Lipofectamine have the highest toxicity. Concerning the transfection efficiency, C18:1 shows the best efficiency on both cell lines and is even better than for lipofectamine (LFM).

the toxicity results as they are expressed in Relative Light Unit per milligrams of proteins (RLU mg^{-1}). We measured significant levels of luciferase expression. The best efficiency is observed for the C18:1 compound on both cell lines. An efficiency of 5×10^7 RLU mg^{-1} is obtained on the A549 cell line and C18:1 transfection results are always better than lipofectamine reference. C18:2 transfection results are in the same efficiency range of 5×10^4 RLU mg^{-1} for both cell lines and comparable to lipofectamine results for 16HBE cell line. On the opposite, C18:0 gives better transfection results on A549 cell line (5×10^4 RLU mg^{-1}) than on 16HBE (8×10^2 RLU mg^{-1}). Finally, the C20 compounds have similar transfection efficiencies on both cell lines and C20:4 is the less efficient. Transfection experiments were also carried out on both cell lines with 50% ratio of DOPE and cholesterol. However, both co-lipids showed no significant effect on transfection efficiency.

4. Discussion and conclusion

An homogeneous family of original phosphoramidates has been synthesized with different hydrophobic chains including saturated and poly-unsaturated chains of different lengths (from 14 carbons to 20). It allows to study the physico-chemical properties and the transfection activity following a rational approach [54] that can help to understand some of the key factors that contribute to the transfection efficiency of a given cationic vector.

4.1. Physico-chemical properties

The solubilities of the different compounds are not very different, the CAC values ranging from 2 μmol L^{-1} to 8 μmol L^{-1}. The liposomes formed after hydration and sonication have a diameter in the range of 130 to 280 nm with a positive zeta potential around 40 mV. Our studies by fluorescence anisotropy have shown that all the compounds except the stearic compound (C18:0) give a fluid bilayer at 37 °C, the temperature used for transfection experiments. The main transition temperature was determined for the saturated compounds, at 23 °C for the myristic compound (C14:0) and 55 °C for the stearic one. The addition of co-lipids allowed modulating the fluidity. An increase of the fluidity was observed when DOPE is added to the stearic compound whereas it is decreased by cholesterol.

Our lipid mixing studies using a FRET assay mainly showed that the C20 compounds do not fuse with the model membranes whereas efficient fusion is observed for the other compounds (FRET efficiency around 40% and 55%), in particular for C18:1. From these results we can see that there is no clear correlation between the fluidity of the membrane and the fusion efficiency. Then we can suppose that the main parameter governing fusion efficiency may be the shape of the lipid. Previous works have shown that the shape of the lipid influence the fusion through the ability to promote an hexagonal phase when interacting with the model membrane [26–29]. The shape of polyunsaturated compounds is expected to be very different from the other compounds and it may be the reason why low FRET efficiencies are observed for these compounds.

As expected, DOPE addition increased the fusion rate for all the lipids tested. This is coherent with its ability to adopt an inverted hexagonal phase which is considered to promote membrane fusion [55]. On the opposite, the addition of cholesterol decreased the fusion rate for the different phospholipids. That result is different from what has been observed by Carraciolo et al. and Zudovska et al. [56,57]. These authors suggest that the molar fraction of cholesterol in the membrane is responsible for the decrease of the hydration layer then enhancing the fusion of lipoplexes with the membrane. Our family of compounds, characterized by a tri-methylarsonium head group, does not exhibit such a behavior.

The ability of liposomes to condense DNA showed that the C18:1 and C18:2 compounds are the most efficient compounds to condense DNA. Weaker results were obtained with the C20 compounds and the stearic one. A minimum charge ratio of 2 was required for efficient condensation to obtain stable lipoplexes with a positive zeta potential and a diameter around 200 nm. Moreover an optimum charge ratio of 4 allowed to obtain the better DNA condensation rate. C18:1 and C18:2 compounds have the best physicochemical results which are supposed to promote transfection efficiency.

4.2. Transfection efficiency

In vitro transfection experiments using 16 HBE and A549 cell lines showed that better results are obtained for higher charge ratios (from CR = 2) and that the oleic compound gives the best results, even better than those of the commercial lipofectamine. The linoleic one is the second best compound on 16HBE cells whereas the saturated stearic compound gives better results on A549 cells. Consistently with the aforementioned DNA condensation results, transfection efficiencies using both C20 compounds were rather low.

No correlation has been established between the *in vitro* transfection efficiency and the hydrophobicity of the compound. Furthermore, although all compounds form liposomes with similar diameters, they do not condense DNA with the same ability and consequently do not transfect cells in the same manner. The compounds which efficiently condense DNA give better results in transfection at medium charge ratio (CR = 4).

As concluded by Zhi et al. in their review [10], finding the "best hydrophobic domain", whenever it exists, requires consider the balances between opposing factors such as short-long chains or saturated-unsaturated chains. As observed in that study, these factors strongly influence the physico-chemical parameters. The newly synthesized C20 compounds present low fusogenicity and a low DNA condensation rate; they are consequently of low interest for transfection. On the opposite, the C18:1 and C18:2 compounds are both fluid and efficient for DNA condensation. C18:1 was found to be the most efficient for the fusion assay and for in vitro transfection experiments. The shape of these two compounds may promote an hexagonal phase during the membrane fusion thus increasing the transfection efficiency. Their transfection efficiencies have been confirmed *in vivo* [3].

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