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ORIGINAL PAPER

Fluorescence Study of Lipid-based DNA Carriers Properties: Influence of Cationic Lipid Chemical Structure

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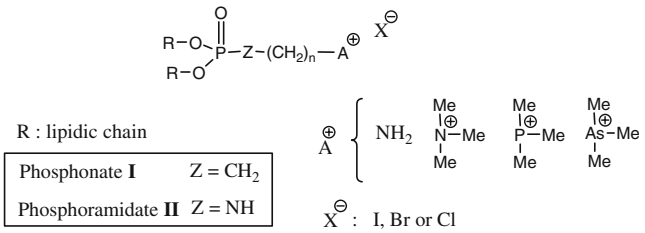
Abstract We report here a study on the physicochemical properties of cationic phospholipids liposomes used for lipoplex formulation and DNA transfer. The original cationic phospholipids synthesized in our laboratory are first presented with the liposome formulation process. The second part deals with the liposomes fusogenic properties studied by fluorescence resonant energy transfer (FRET). The nature of the cationic polar head and the formulation with or without a neutral colipid have a great influence on the FRET signal. The third part reports the study of the viscosity of the liposome by fluorescence anisotropy measurements. It has been observed that the vectors having a saturated lipid chain exhibit a more pronounced anisotropy than those having unsaturated lipid chains. Finally, liposomes formed by a mixture of phospholipids and DC-Chol (a rigid lipid) leads to increase the anisotropy denoting a more rigid liposome.

Keywords Fluorescence · FRET · Anisotropy · Cationic phospholipids · Liposomes

Introduction

Gene therapy will probably change medicine by treating the diseases at their core levels. The development of this technology relies in designing optimal systems for DNA transfer and expression. The recent progress reporting the use of cationic lipids as efficient DNA carriers constitute a promising alternative [1–3] to the virus based vectors [4]. In our laboratory, original cationic lipids have been designed. These vectors are characterised by the presence of two lipidic chains placed on a phosphorus group (phosphonate I or phosphoramidate II) which is also bonded to a cationic head via a linker. The cationic head can be formed by a permanent cationic charge (ammonium, phosphonium, arsonium [5]) or by an amine which will be protonated at physiological pH [6]. More recently, lipophosphoramidates with two permanent cationic sites have been reported [7].



The evaluation of these cationic lipids as DNA carriers reveals their efficiencies, in the course of both in vitro [8, 9] and in vivo [10] transfection essays, associated, in several cases, with a low toxicity. With the aim to rationalise and to correlate the transfection efficiency of our home-made cationic lipids with their chemical structure, the description of these vectors with some physicochemical parameters is needed.

Fluorescence techniques are of interest to study the liposomes and lipoplexes (ADN-lipids complexes) formed from these cationic lipids. Förster Resonant Energy Transfer (FRET) allows studying the fusion of cationic

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liposomes with membrane models [11]. Fluorescence anisotropy is a second convenient tool available to study the physicochemical properties of liposomes. This technique allows the estimation of the « viscosity » of phospholipid bilayers [12]. It is worth mentioning that fusogenic properties and viscosity are two parameters which do have a great influence on the transfection process. We report in this paper some results presented at Salzburg (10th International Conference on Methods and Applications of Fluorescence, Salzburg, 9–12 September 2007), completed with new physicochemical studies on new phosphoramidates vectors, and new neutral vectors which have been very recently synthesized.

Materials and methods

Products

Fluorescent probes used are nitrobenzoxadiazole-phosphatidylethanolamine (NBD-PE), Rhodamine-PE and diphenylhexatriene (DPH) both from Molecular Probes. All lipids were from Sigma. Stock solutions of each lipid (5 $g.L^{-1}$) were prepared in chloroform and stored in dark, at 4 °C. Hepes buffer and Chloroform (UV spectroscopy grade) are both from Fluka.

Materials

Fluorescence measurements

Classical fluorescence measurements were conducted on a Cary Eclipse Varian spectrophotometer and fluorescence spectra were recorded on a computer (Scan software, Varian). The emission and excitation wavelengths could vary from 190 to 1100 nm; both the scanning rate (from 30 to 24,000 nm min^{-1}) and the detector gain (up to 1,000 V) can be changed to optimize the fluorescence intensity, which was thus expressed in arbitrary units (a.u.). A 3D option provided access to emission–excitation matrices. Throughout all this study, a slit width of 5 nm was used for both the excitation and the emission wavelengths. Anisotropy measurements were conducted with the same fluorimeter equipped with the manual polariser accessory (Varian).

Statistical analysis

The experimental results' uncertainty are equal to $t.\sigma/\sqrt{n}$, where t is Student's t (α=5%, $n-1$ is the number of degrees of freedom), and σ is the standard deviation.

The Student t test relating to the difference between two means is used. $\overline{x}_i$, σ_i, and n_i are respectively the mean of the measured values, their standard deviation and the number of experiments. The weighted standard deviation (σ_w) and the experimental t of the difference (t_D) are computed with the following formulae. If t_D is higher than $t_{Student}$ tabulated with $n_1 + n_2 - 2$ degrees of freedom (d.o.f.) and a chosen risk α, one can conclude that the means are significantly different.

$$\sigma_w = \sqrt{\frac{(n_1 - 1) \times \sigma_1^2 + (n_2 - 1) \times \sigma_2^2}{n_1 + n_2 - 2}}$$

$$t_D = \frac{|\overline{x}_1 - \overline{x}_2|}{\sigma_w} \times \sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$$

Experimental part and results

The following paragraphs will present the experimental methodology and the results obtained for the estimation of the liposome fusion ability and of the membrane viscosity depending both on different specific liposome formulations.

Liposomes formulation

For these experiments the specific cationic phospholipids synthesised in our laboratory have been used. The different lipids structures have been selected in order to evaluate their influence on liposome fusion and membrane viscosity. These cationic lipids, presented in Table 1, differ by their aliphatic chain length (14 or 18 carbons), their insaturation number (0 or 1) and the nature of their cationic head which can be ammonium (N^+), guanidinium ($NH{-}C(NH_2) = NH_2^+$), methylimidazolium ($C_4H_6N_2^+$), phosphonium (P^+) or arsonium (As^+). These cationics heads have been selected for their properties which are discussed below. The size of these cations can be classified from the smaller to the bigger as follow: ammonium < phosphonium < arsonium. Therefore the charge density is higher for an ammonium head when compared with an arsonium cationic head. Consequently ammonium head will have higher dipolar interaction with the solvent (water) than phosphonium and arsonium. This different charge density can also induce different behaviour when mixed with pDNA (not the focus of this study); moreover they can also modify the membrane properties which have not been studied yet. With the goal to compare the physicochemical properties of the phosphonates and phosphoramidates with commercial cationic or helper lipids, DOTAP, DC-Chol and DOPE lipids are also included in this study. To prepare the liposomes, aliquots of phospholipids solutions (1 $g.L^{-1}$) were mixed and dried under reduced pressure. The dried lipid film formed was hydrated with a 5 mM Hepes buffer containing NaCl (150 mM). Suspensions were vigorously

Table 1 Lipids used for the liposome's formulation

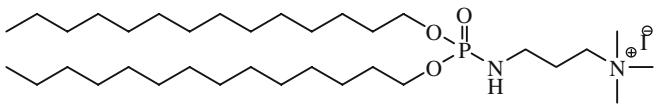
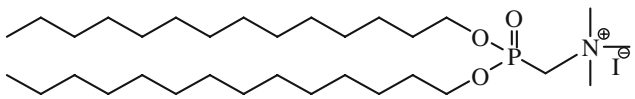
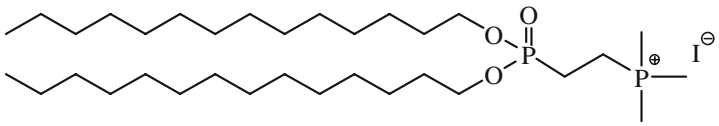
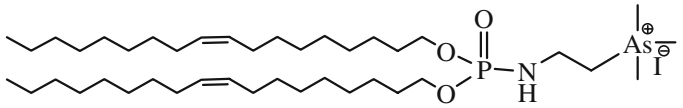
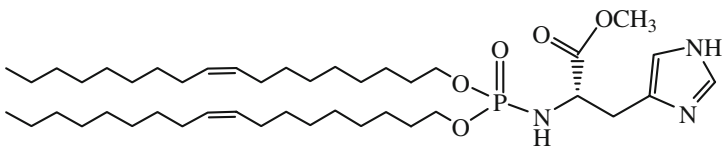
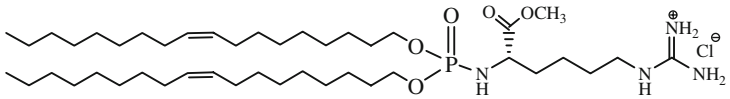
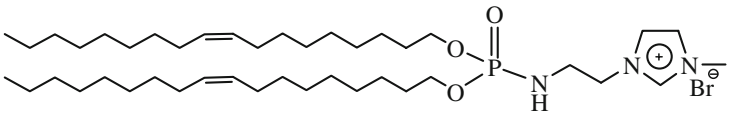
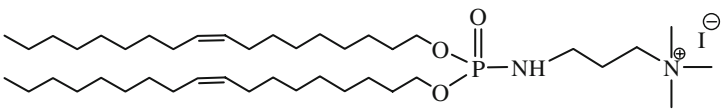
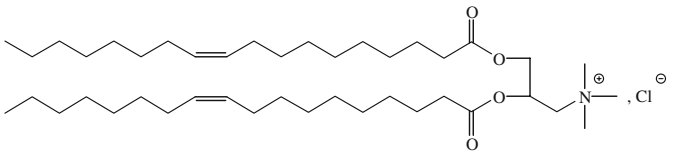
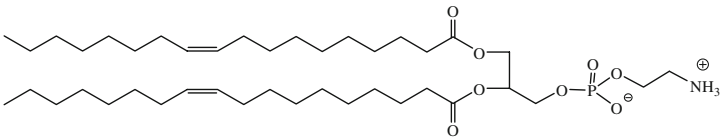
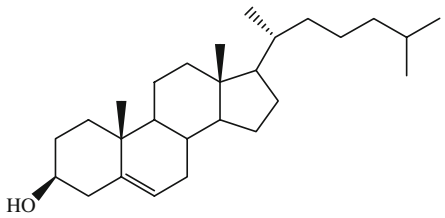
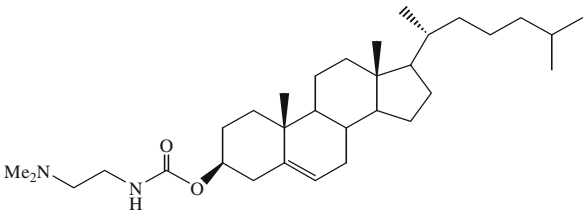
Number	Chain length and insaturations	Polar head	Formula
1	14:0	N^+ amonium	
2	14:0	N^+ amonium	
3	14:0	P+ phosphonium	
4	18:1	As^+ arsonium	
5	18:1	Neutral imidazole	
6	18:1	NH_2^+ guanidinium	
7	18:1	N^+ imidazolium	
8	18:1	N^+ ammonium	
DOTAP	18:1	N^+ amonium	

Table 1 (Continued)

DOPE	18:1	N^+ amonium	
Choles-terol		alcohol	
DC-Chol		amine	

vortexed for 5 min and sonicated at 40 °C for 10 min to form liposomes.

Liposomes fusion study

To estimate the liposome fusion we used the FRET technique [3]. FRET depends upon the Förster interactions that occur between two fluorophores when the emission band of one (the energy donor) overlaps with the excitation band of the second (the energy acceptor) when the probes are in close proximity. FRET efficiency then depends on the distance between the two probes. If a couple of two lipidic fluorophores, one energy donor (NBD-PE for instance) and one energy acceptor (Rhod-PE for instance) is introduced into a liposome, any fusion event of such a doubly labelled liposome with a second liposome (devoid of any fluorophore) will decrease the efficiency of resonance energy transfer. Thus, any decrease in FRET efficiency provides evidence for membrane fusion [4]. The efficiency of energy transfer was calculated from the fluorescence emission intensity of NBD-PE at 530 nm by using Equation 1:

$$E = 1 - F/F_0 \tag{1}$$

Fluorescence intensities were recorded in the presence (F) and absence (F_0) of Rhod-PE. The relative fluorescence intensity, ER (in percent) is calculated using relation 2; where Emix and Eab are the FRET efficiency calculated in the presence (Emix) or absence (Eab) of cationics lipids.

$$ER = (E\text{mix}/E\text{ab}) \times 100 \tag{2}$$

As labelled membrane we used liposomes made with L-α-phosphatidylcholine (PC) and fluorescent probes, or liposomes made with PC, L-α-PE, L-α-phosphatidyl-L-serine (PS), cholesterol (Chol) and fluorescent probes; (PC/PE/PS/Chol/NBD-PE/Rhod-PE approximately 44/25/10/20/0.8/0.2 m/m), a lipid composition close to that of the plasma membrane. They are then mixed with unlabeled cationic lipids liposomes at increasing concentrations from 10^{-6} to 10^{-4} mol/L, with DOPE added (1:1 m/m) or not, in each case. The final Rhod-PE and NBD-PE concentrations are 6.10^{-8} and 3.10^{-7} mol.L^{-1}. The labelled membrane final concentration is 15 mg.L^{-1}, corresponding approximately to 2.10^{-5} M for PC.

The Rhod-PE/lipid ratio was chosen after determination of the FRET efficiency versus Rhod-PE/PC molar ratio. A ratio closed from 0.003 was chosen in order that lipids fusion undergoes a significant decrease of FRET efficiency. NBD-PE concentrations did not affect the FRET efficiency as described before [12].

Figure 1 describes the evolution of the FRET efficiency in percentage versus the concentration of cationic phospholipids. It shows first that *6*, which has a guanidinium

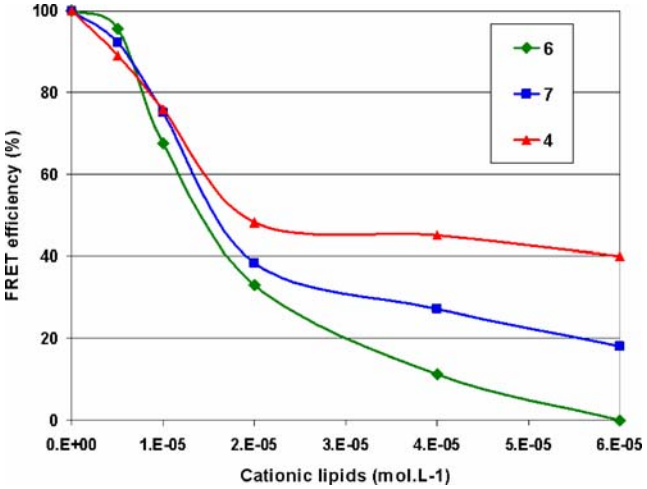


Fig. 1 Evolution of the FRET efficiency in percentage versus the concentration of cationic phospholipids

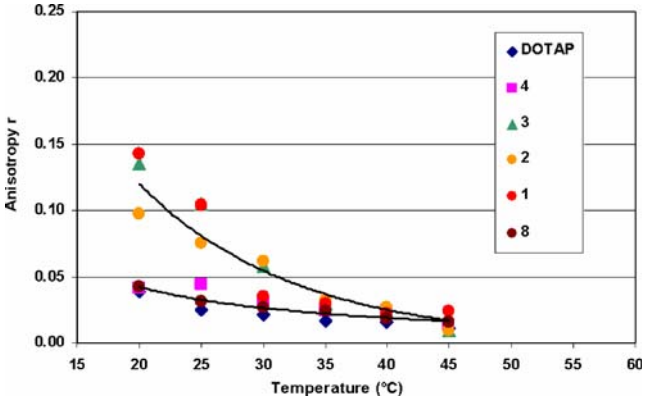


Fig. 3 Evolution of the DPH emission anisotropy versus the temperature for different liposome formulations

head, can completely eliminate FRET. This indicates an important membrane fusion rate between the model liposome and the liposomes formed by the cationic lipid *6*. Secondly, an important FRET efficiency decrease reaching 20% for a cationic lipids concentration of 6.10^{-5} M/L is observed for compound *7* formulated as liposome. The fusion rate is still important but cationic lipid *7* is less efficient than *6*. At least the phospholipid *4* reaches a minimum FRET efficiency of 40% indicating a lower membrane fusion rate.

As the three cationic vectors present the same aliphatic chain structure (C18:1) one can conclude that the differences observed are due to the polar head. Interestingly, this behaviour is correlated with the relative toxicity of these three vectors which has been recently classified [13] as follow (Colorimetric MTT assay establishing the percentage of viable cells after transfection): *6*>*7*>*4*. Therefore, from these early results, it appeared that a great FRET decrease is correlated with a toxicity increase.

Figure 2 compares the FRET efficiency using *7* formulated with different neutral co-lipids (DOPE, *5*, Chol), at molar ratio 1/1. *7* has been selected because recent studies show good transfection efficiencies with this lipophosphoramidate [13]. Of note, compounds *5*, which is a phosphor-histidine derivative, proved to be an efficient neutral colipid [13].

The addition of DOPE does not modify the FRET efficiency of compound *7*-liposome which stay at 20% at the highest concentration of *7*. On the other hand, with the addition of cholesterol the FRET is much less reduced (25% at 6.10^{-5} Mol.L^{-1}; σ_w=0.0070 t_D=5.87 $t_{Student}$=2.23 with 4 d.o.f.), meanings a less fusion efficiency. Finally, the use of *5* as colipid decreases significantly the FRET efficiency to 10% (σ_w=0.0070 t_D=5.87 $t_{Student}$=2.23 with 4 d.o.f.), which can be interpreted as an improvement of the membrane fusion rate.

The FRET studies clearly show that the cationic lipids tested have different fusogenic behaviour. Moreover, the formulation of one cationic lipid with different neutral or cationic helper lipid can either decrease or increase the fusogenic properties of the formulation demonstrating that the choice of the helper lipid can have a great importance towards a transfection process. Further study will be done to optimise the helper lipid nature and the liposomes formulation.

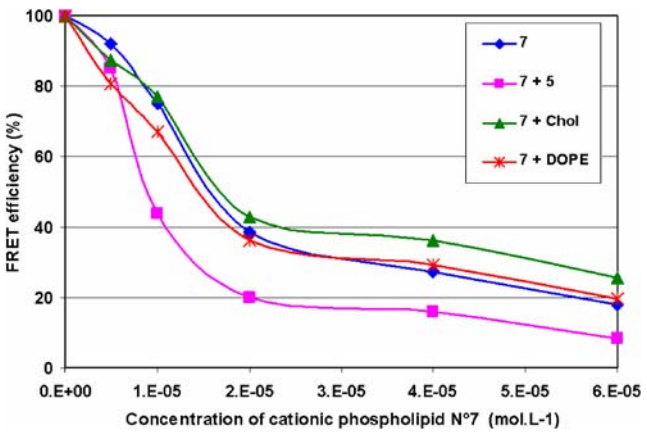


Fig. 2 Comparison of the FRET efficiency using *7* formulated with different neutral co-lipids (*DOPE*, *5*, *Chol*), at molar ratio 1/1

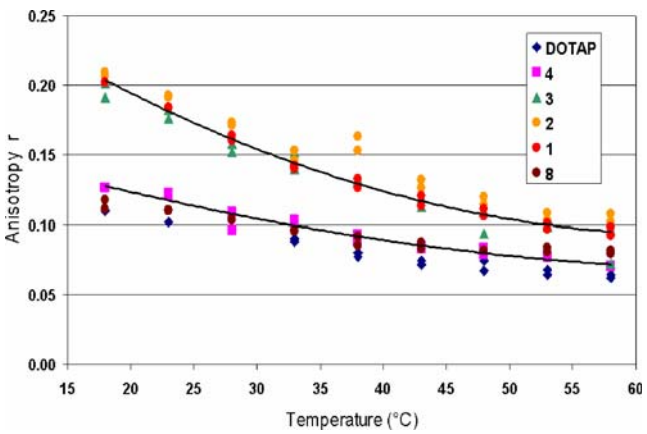


Fig 4 The same anisotropic measurements conducted in presence of DC-Chol acting as a second cationic lipid

Membrane viscosity study

To evaluate the membrane viscosity, DPH is used as hydrophobic fluorescent probe located inside the lipid bilayer. Fluorescence anisotropy (r) of the DPH probe depends on the diffusion correlation time θ following the relation *3*:

$$r(t) = r_0 \times e^{-t/\theta} \quad (3)$$

For steady state anisotropy measurements:

$$r = \frac{r_0}{1 + (\tau/\theta)} \quad (4)$$

Theta (θ) depends on the volume of the molecule V, the fluorescence lifetime τ, the temperature and the viscosity η of the medium (Equation 5):

$$\theta = \eta V/kT \quad (5)$$

Then, the fluorescence anisotropy of DPH is correlated with the medium viscosity η by the relation *6* for steady state anisotropy measurements (with *k Boltzmann* constant, *T* absolute temperature). This relation indicates that a fast anisotropy decrease is correlated with a low membrane viscosity.

$$\frac{1}{r} = \frac{1}{r_0} + \frac{(\tau \times k \times T)}{(r_0 \times \eta \times V)} \quad (6)$$

On Fig. 3 showing the evolution of the DPH emission anisotropy versus the temperature for different liposomes formulations two groups appear. For the first one the anisotropy varies from 0.12 to 0.02 between 20 and 45 °C. In this group we find compounds *1*, *2* and *3* which have all a C14:0 aliphatic chains and a permanent cationic formed by an ammonium or a phosphonium. For the second group the anisotropy is lower (from 0.04 to 0.02) indicating a higher membrane fluidity. The second group is composed by the following cationic lipids: *4*, *8* and DOTAP. Of note, these lipids are characterized by the presence of a C18:1 lipid chains and different polar head (As+, N+, and N+ respectively). The first conclusion of this comparison study is that the nature of the polar head does not have a crucial influence on the membrane viscosity. Inversely, the viscosity is strongly related to the nature of the lipidic chain. Indeed, the presence of one insaturations (C18:1) decrease, in agreement with previous studies [14], the viscosity of the membranes.

Figure 4 reports the same anisotropic measurements conducted in presence of DC-Chol acting as a second cationic lipid. We can observe in this case the formation of the same two groups of vectors with the same evolution and the same hierarchy. In the upper group *1*, *2* and *3* follow the same evolution and reach a mean anisotropy of 0.095 at 58 °C; while the lower group (*4*, *8* and DOTAP) reach a mean of 0.071 at the same temperature. The two groups are significantly different at α risk of 5% (σ_w=0.0070 t_D=5.87 $t_{Student}$=2.23 with 10 d.o.f.). However the anisotropy is globally higher moving from 0.2 to 0.09 for the first group and from 0.17 to 0.05 for the second group when compared with the same experiments carried out without DC-Chol. This shows that the addition of DC-Chol increase the membrane viscosity in all cases without particular interactions with the phospholipid tested. Similar results have been obtained using cholesterol as helper lipid (data not shown), in agreement with previous studies [15].

Conclusion

The FRET experiments have shown that the three cationic lipids studied are able to fuse with membrane models and that addition of co-lipids only slightly affect the results. The polar head nature appears to have a significant effect on fusion efficiency. The guanidinium head shows the highest fusion rate while the imidazolium head and the arsonium head are less efficient. However as important fusogenic properties induce also membrane destabilisation and toxicity a compromise will have to be searched to optimise DNA delivery.

The anisotropy experiments have shown that all cationic lipids with C14:0 chains give more rigid membrane than C18:1 lipids. The same difference is observed in presence of cholesterol which still increases the membrane rigidity. The cis-insaturations present in the lipid chains is probably the main responsible of such a difference.

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