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PAPER

Cationic lipo-thiophosphoramidates for gene delivery: synthesis, physico-chemical characterization and gene transfection activity – comparison with lipo-phosphoramidates†

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The synthesis of cationic lipo-thiophosphoramidates, a new family of cationic lipids designed for gene delivery, is reported herein. This new class of lipids is less polar than its oxygenated equivalent the lipo-phosphoramidates. Fluorescence anisotropy and FRET were used to determine the fluidity and fusogenicity of the lipo-phosphoramidates **3a–b** and lipo-thiophosphoramidates **7a–b**. The determination of both the size and the zeta potential of the nano-objects (liposomes and lipoplexes) and the determination of the DNA binding ability of the liposomes have completed the physico-chemical characterizations of the cationic lipids studied. Finally, the cationic lipids **3a–b** and **7a–c** have been evaluated as synthetic vectors for gene transfection into a variety of mammalian cell lines. The lipo-thiophosphoramidate **7a** proved to be an efficient and low toxicity synthetic vector even when used at low lipid to DNA charge ratios.

1 Introduction

Several strategies have been investigated to carry nucleic acid constructs into cells including the use of either recombinant viruses or synthetic vectors which are two promising approaches for *in vivo* applications.[1] Other methods including electroporation,[2] have been proposed for transfection but so far they are restricted to few *in vivo* applications (*e.g.* vaccination[3]). It is worth noting that transfection with naked DNA is also applicable with reasonable success but this strategy is mainly limited to the transfection of muscle tissues.[4] Due to an easier production control associated with the absence of immune response, the use of synthetic vectors appeared to be a promising and realistic strategy. Schematically, the synthetic vectors can be classified in two categories depending on their chemical structures which can be a polymer or a lipid derivative. In the last category, into which the molecules reported in the present work can be classified, a wide variety of cationic[5] or neutral[6] lipids have been reported. Despite the numerous structures, the common point between these vectors is the presence of two lipid chains (*e.g.* oleyl) or a cholesterol unit, a cationic or polycationic[7] head and a linker between these two parts. More recently, neutral lipids exhibiting capacities to compact and carry DNA into cells have also been reported.[8,9] Whatever the structure of the synthetic vectors, their toxicity must be considered with a great attention. This important issue has oriented chemists to use natural resources for the design of low toxicity synthetic vectors.[10] It is also important to notice that the composition of the formulations used for gene delivery has an important impact on the transfection efficiency. It has been observed that the incorporation of natural (*e.g.* DOPE[11]) or synthetic[12] neutral helper lipids into the formulations can greatly improve the transfection activity. Among all this structural diversity, the lipo-phosphonate[13] or lipo-phosphoramidate[14] vectors developed by our group have been used for *in vitro*[15] or *in vivo*[16] transfection assays where they demonstrated a reasonable efficiency and a low toxicity. In the course of that previous work, it has been observed that the chemical nature of the polar head (ammonium, phosphonium or arsonium) induces crucial consequences on both transfection efficacy and toxicity.[17] In light of the commonly accepted mechanism for transfection, the main steps are: the internalization of the lipoplexes, the plasmid or lipoplexes release from endosome to cytosol and the migration of the plasmid towards the cell nucleus. On the basis of these major steps, it is established that the lipoplexes must have a robust supramolecular assembly to

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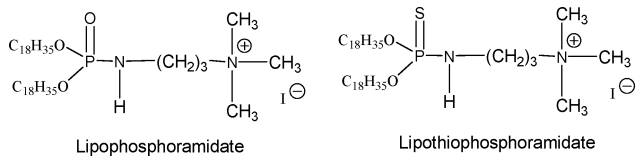
support systemic administration. Nevertheless, their stability must decrease as soon as the cell internalization is achieved. The destabilisation process can arise from the different physiological properties of the internal cell compartments including the pH,[18] redox properties[19] or enzymatic assistance.[20] However, it is highly desired to design lipoplexes possessing a stability that can be fine tuned in order to produce supramolecular assemblies that can circulate in blood vessels for a short period of time without degradation. nevertheless this stability must not be too strong to facilitate plasmid release after cell internalization. Regarding the synthetic vectors designed with a phosphoramidate functional group (Scheme 1), the presence of the highly polar P═O bond confers to the oxygen atom the ability to act as a good hydrogen bond acceptor. Indeed, several studies have shown that the hydrogen bonds involving a P═O moiety are frequently strong in view of the rather short acceptor-donor distances.[21]



Scheme 1 Chemical structure of the lipo-phosphoramidate and lipo-thiophosphoramidate.

The involvement of the phosphoramide functional group in hydrogen bond networks could contribute to the stabilisation of the liposomes or lipoplexes. This hypothesis is supported by the observation at the solid state of strong hydrogen bonds for phosphoramidates involving the oxygen atom of the P═O motif.[22] In particular when the phosphoramidate group is only monosubstituted on the nitrogen atom (presence of a $(RO)_2P(O)$–NH–R motif), strong intermolecular hydrogen bonds are observed in which the oxygen atom of the P═O group acts as acceptor and the nitrogen of phosphoramidate acts as hydrogen bond donor.[22] It can be anticipated that the replacement of the oxygen atom of the P═O group by a sulfur atom (formation of lipo-thiophosphoramidate derivatives), which is a less good hydrogen bond acceptor, could alter the physico-chemical properties of the lipoplexes. Differences have indeed been observed between hydrogen bonds involving either a sp^2 hybridized sulfur atom or their oxygenated analogues despite differences in terms of bond strength.[23] This behaviour is more documented for thioamide or thiourea for which comparisons with their oxygenated analogues (amide or urea) have been reported.[24] The directionality of the hydrogen bonds is also different since the A···H–D angle is smaller when A is a sulfur atom and larger when it is an oxygen atom. In other words, the O_{sp^2}···H–D hydrogen bonds are more linear than their sulfurated analogues.[25] All these different chemical behaviours between an sp^2 hybridized oxygen and sulfur atom transposed to a liposome or lipoplex system involving cationic lipids possessing either lipo-phosphoramide or lipo-thiophosphoramide could modify the robustness of the supramolecular assembly. Furthermore, the difference in polarity of the P═S *versus* P═O bond should produce liposome formulations having a different hydration state around the phosphorus functional group. Hence, the P═S motif can be viewed to some degree as an extension of the hydrophobic domain while the P═O functional group is more clearly an element of the polar head of the amphiphiles. Finally, in view of the global mechanism of transfection, it can be anticipated that the replacement of an oxygen atom by a sulfur atom in the lipo-phosphoramidate vectors could have an important impact on the interaction between the lipoplexes and the biomembranes (plasma and endosomal membranes). It should also be stressed that the *N*-substitution of the phophoramide or thiophosphoramide group by a methyl is also susceptible to modify the liposomal and lipoplex supramolecular robustness since this type of substitution eliminates the possibility of formation of the hydrogen bond network implicated in the auto-assembly of (thio)phosphoramide groups.

We report herein the synthesis of lipo-thiophosphoramidates and lipo-phosphoramidates all characterized by two oleyl chains. These molecules are methylated or not on the nitrogen atom of the (thio)phosphoramidate functional group while all the other structural features are preserved (lipid chain and cationic polar head). The capacity of these cationic lipids to compact DNA and to transfect cell lines are reported and compared. Moreover, the estimation of the fluidity of the liposomes formed by the new lipo-thiophosphoramidates have been evaluated by fluorescent anisotropy measurement.[26] These physicochemical characterizations are compared with those of phosphoramidates.

2 Results and discussion

The five cationic lipids (**3a–b** and **7a–c**) that are reported in the present study are members of two distinct families of compounds: lipo-phosphoramidates (**3a** and **3b**) and lipo-thiophosphoramidates (**7a**, **7b**, and **7c**) (Fig. 1). These five compounds are members of a homogenous series of molecules as the cationic polar head and the spacer are identical for compounds **3a–b** and **7a–b**. Furthermore, the lipid part is always characterized by the presence of two oleyl chains (C18:1-ω9). The choice of this lipid part arises from previous studies that have shown the efficacy for gene delivery of vectors possessing this type of fatty acid chain.[27] Compound **7c** possesses a slightly different chemical structure since an ethylene linker replaces the propylene linker found in the other cationic lipids.

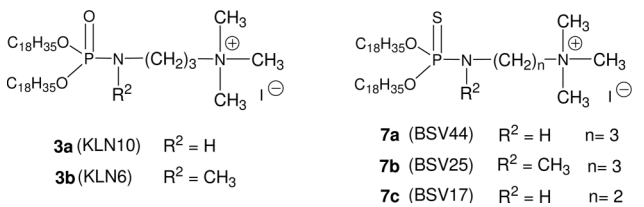


Fig. 1 Chemical structure of the cationic lipo-phosphoramidates (**3a** and **3b**) and lipo-thiophosphoramidates (**7a**, **7b** and **7c**).

The cationic lipo-phosphoramidates **3a–b** have been synthesised in a two step sequence according to a protocol previously reported (Fig. 2).[13–16] In the first step, an Atherton–Todd coupling between dioleylphosphite and either *N*,*N*-dimethylpropyl-1,3-diamine or *N*,*N*,*N*′-trimethylpropyl-1,3-diamine gives rise respectively to the lipo-phosphoramidates **2a** and **2b** in good yields (**2a**: 96% **2b**: 63%).

Then, the quaternarization step of **2a** and **2b** is readily obtained by reaction with methyl iodide thus producing the cationic lipo-phosphoramidates **3a–b**. Having in hand the cationic lipo-phosphoramidates **3a–b** or their direct precursors, the

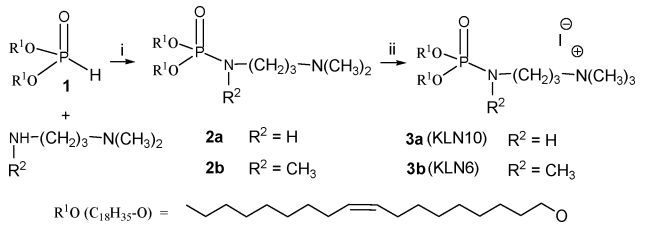


Fig. 2 Synthetic pathway to lipo-phosphoramidates **3a** and **3b**. i) DIPEA, $CBrCl_3$, CH_2Cl_2, 0 °C to R.T., 5 h; ii) CH_3–I, R.T., overnight.

lipo-phosphoramidates **2a** and **2b**, one attractive straightforward synthesis of the thiophosphoramidates might involve the use of a sulfurating agent to transform these phosphoramidates derivatives into their thiophosphoramidate analogues. It was indeed reported in the literature that different types of sulfurated functional groups can be obtained in good yield from their oxygenated equivalents.[28]

In a preliminary study, we attempted this type of direct thionation on compound **4** used as a model (Fig. 3). Among the different methods allowing the sulfuration of oxygenated substrates, we first employed the method of Nivsarka *et al.*[29] that uses hexamethyldisiloxane (HDMO) and P_4S_{10} under microwave activation. The addition of solvent (toluene) or the adaptation of the experimental procedure, according to the protocols published by Curphey *et al.*,[30] were inefficient. A mixture of several phosphorylated compounds was obtained as revealed by ^{31}P NMR and attempts to separate them by column chromatography on silica gel were unsuccessful. Other methods including first the Lawesson reagent used alone or in the presence of HDMO,[31] and secondly hexamethyldisilathiane (HMDT) in the presence of trimethylsilyl triflate (TMSOTf)[32] were also unsuccessful. In conclusion, whatever the method employed for the direct thionation of phosphoramidate **4**, we never managed to isolate more than traces of the expected thiophosphoramidate. Most of the time, several compounds (more than ten) were formed as revealed by ^{31}P NMR, ruling out any chance to isolate more than traces of the expected product.

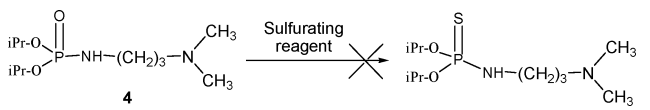


Fig. 3 Attempted thionation of phosphoramide to thiophosphoramide on a model substrate **4**. Sulfurating agent used: Lawesson, HDMO, HMDT with TMSOTf.

Therefore, we considered an alternative synthetic route starting from trichlorothiophosphate (Fig. 4). The first step, which gives rise to *O*,*O*-dioleyl chlorothiophosphate **5** from the reaction of trichlorothiophosphate with oleyl alcohol sodium salt, has required much effort to give reproducible results. Indeed, the formation of oleyl sodium alcoholate was difficult to achieve in a quantitative manner. This difficulty was attributed to the presence of the lipid chains that greatly modified the reactivity and that produced a viscous/heterogeneous solution. Consequently, the stoichiometry of the subsequent reaction that produced **5** was quite difficult to control and the expected dioleyl derivative **5** (^{31}P: 69.7 ppm) was formed jointly with variable amounts of monooleyl ($C_{18}H_{35}$–O–$P(S)Cl_2$; ^{31}P: 58.7 ppm) and trioleyl derivatives ($(C_{18}H_{35}–O)_3P(S)$; ^{31}P: 68.0 ppm). Finally, the best conditions involved the addition

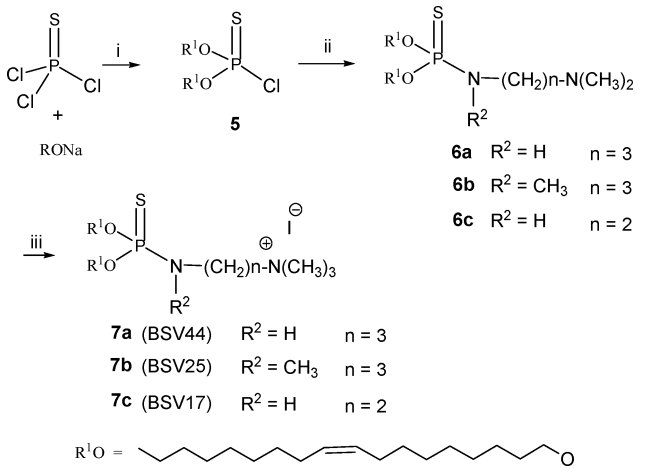


Fig. 4 Synthetic pathway to lipo-thiophosphoramidate **7a**, **7b** and **7c**. i) THF, –80 °C to R.T., 2 h. ii) $H(R^2)N–(CH_2)_n–N(CH_3)_2$, DIPEA, CH_2Cl_2, 0 °C to R.T., overnight. iii) methyl iodide, Et_2O, overnight, R.T.

of the sodium alcoholate solution to trichlorothiophosphate at low temperature (–80 °C) and with a continuous control of the progress of the reaction by ^{31}P NMR. Intermediate **5** was then purified by column chromatography on silica gel to give the pure compound **5** in up to a 76% yield. For the next step, the formation of thiophosphoramidate was easily achieved by the reaction of compound **5** with *N*,*N*-dimethylpropyl-1,3-diamine, *N*,*N*,*N*′-trimethylpropyl-1,3-diamine or *N*,*N*-dimethylethyl-1,2-diamine to produce respectively compounds **6a**, **6b** and **6c** in good yields (94%, 96%, 85% respectively). Finally, the cationic lipo-phosphoramidates **7a–c** were obtained by quaternarisation of compounds **6a–c** with two equivalents of methyl iodide. Compounds **7a–b** were obtained directly with an excellent purity and good yield (98 and 91% respectively) while compound **7c** required a purification on silica gel (80% yield).

The cationic lipo-phosphoramidates **3a–b** and lipo-thiophosphoramidates **7a–c** were formulated as liposomes by hydration of a lipid film and subsequently mixed with plasmid DNA to form lipoplexes. The lipoplexes were formed at different charge ratios (CR)‡ from 1 to 4 and the size and potential zeta were recorded for each CR (full data shown in ESI S3 and S4). A selection of these data (those corresponding to a theoretical CR = 2) is shown in Table 1. For the liposomes, the size of the lipo-phosphoramidate **3a–b** ones was noticeably smaller than for the thiophosphoramidate **7a–c** ones. Interestingly, among the compounds **3a–b** and **7a–c**, the phosphoramidate **3a**, which is the cationic lipid that has probably the highest capacity to generate a strong hydrogen bond network, produces the smallest particles. Compound **3b**, which is the *N*-methylated equivalent of compound **3a**, can not generate a hydrogen bond network by auto-assembly; its size is intermediate between those of **3a** and **7a–c**. These data suggest that the production of smaller liposomes is related to the capacity of the cationic lipid to develop a strong hydrogen bond network (**3a**) or to produce a compact packing that can also be explained by the presence of favourable

‡ The charge ratio *CR* is defined as the number of positive charges (carried by the cationic lipid) by the number of negative charges present on the plasmid DNA. Based on an average molecular weight of 666 g mol^{-1} per base pair (bp), 1 µg of DNA is assumed to carry 3 nmol of negative charges.

Table 1 Zeta potential and size of liposomes and lipoplexes (CR = 2 and 4) formulated in water

Liposome	Size (nm)	Polydispersity index	Zeta potential (mV)
3a	147	0.468	58
3b	186	0.387	51
7a	227	0.232	41
7b	213	0.220	37
7c	226	0.188	25
Lipoplex CR = 2	Size (nm)	Polydispersity index	Zeta potential (mV)
3a	396	0.428	12
3b	244	0.085	–33
7a[a]	>800	—	–2
7b	249	0.128	–31
7c	282	0.103	–25
Lipoplex CR = 4	Size (nm)	Polydispersity index	Zeta potential (mV)
3a	166	0.115	31
3b	195	0.107	22
7a	240	0.369	30
7b[a]	>800	—	10
7c[a]	>800	—	16

[a] At this charge ratio the size was not determined due to the presence of a colloidal instability domain.

dipolar interactions (a P═O bond is much more polarized than a P═S bond). For the lipoplexes, it was observed that at CR = 1, all the nano-objects have a size in the 210–270 nm range and a deeply negative zeta potential (–35 to –45 mV) (see ESI S3 and S4). At CR = 2 (Table 1), the size of the lipoplexes formed by **3a** and **3b** was bigger than that of the corresponding liposomes while this change is much less pronounced for the lipo-thiophosphoramidates **7a–c**. In Table 1, it is shown that the presence of a *N*-methylated (**3b** and **7b**) or *N*-unmethylated phosphoramidate (**3a** and **7a**) has a consequence on the zeta potential. Compounds **3b** and **7b** formed negative nanoobjects (–33 and –31 mV) while the zeta potential of compounds **3a** and **7a** were either positive or close to the neutrality. The negative zeta potential observed could be attributed to the presence of parts of the plasmid DNA on the outer sphere of the lipoplexes. This hypothesis might be explained by the lower capacity of these cationic lipids to compact pDNA. Finally, the stability of lipoplexes at CR = 1 has been determined over 24 h. It was observed, according to the measurement of the size and zeta potential of the lipoplexes (see Figure S5-1 and S5-2 ESI), that these supramolecular assemblies were stable (the maximum of variation observed for the size is 7%).

The physico-chemical behaviour of compounds **3a–b** and **7a–b** formulated as liposomes was next evaluated in order to estimate the consequences (particularly in terms of fluidity) of the presence of either a phosphoramidate or a thiophosphoramidate functional group. First, the CAC (Critical Aggregation Concentration) has been evaluated by using the fluorescence probe Nile Red (9-diethylamino-5*H*-benzo[alpha]phenoxazine-5-one).[33,34] The solubility or critical liposomal concentration of diacyls phospholipids in water is in general quite low and depends on both the chain length and the head group type, the solubility of charged phospholipids being higher.[35] A solubility of 4×10^{-5} mol L^{-1} has been reported for the anionic dimyristoyl-phosphatidylglycerol (14 carbon per chain).[36] CAC found (see ESI-S1) for compounds **3a**, **3b**, **7a** and **7b** are respectively 6.4×10^{-6}, 6.9×10^{-6}, 3.3×10^{-6}, 4.4×10^{-6} mol L^{-1}. Thus a difference in CAC value is observed between the phosphoramidate (**3a–b**) and the thiophosphoramidate (**7a–b**). As expected, the more polar the cationic lipid, the higher is the CAC. On the other hand, the presence of a *N*-methylated or *N*-unmethylated group does not significantly modify the CAC value. Next, the fluidity of lipo-phosphoramidates **3a–b** and lipo-thiophosphoramidates **7a–b** formulated as liposomes was evaluated by anisotropy of fluorescence using Laurdan[37] (6-dodecanoyl-2-dimethylaminonaphthalene) as a probe and following a reported methodology[16] (Fig. 5).

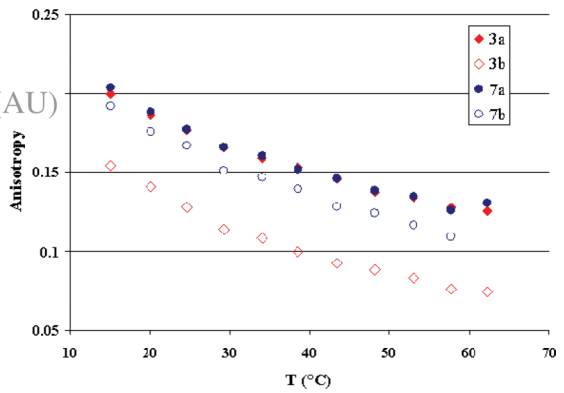


Fig. 5 Anisotropy measurements of **3a–b** and **7a–b** liposomes in function of the temperature (from 15 °C to 65 °C).

To discuss these results we will successively compare: (1) compound **3a** *versus* **3b** and **7a** *versus* **7b** in order to evaluate the influence of a methyl group on the nitrogen atom (2) compound **3b** *versus* **7b** and **3a** *versus* **7a** with the aim to evaluate the consequences of the replacement of an oxygen atom (P═O) by a sulfur atom (P═S). The comparison of the anisotropy for the lipo-phosphoramides **3a** and **3b** indicates that the cationic lipid **3a** produces a more rigid liposomal structure (significantly higher anisotropy in that case – Fig. 5). This observation can be ascribed to the presence of hydrogen bonds involved in the auto-assembly of compound **3a** *via* the phosphoramidate functional groups. For the *N*-methylated phosphoramidate **3b**, such a hydrogen bond network is absent, thus producing more fluid liposomes. For compounds **7a** and **7b**, the comparison of the fluorescence anisotropy values leads to the same conclusion as for compounds **3a–b**. Indeed, the liposomes formed with the *N*-methylated lipo-thiophosphoramidate **7b** are more fluid than those formed by the unmethylated analogue **7a**. The difference in anisotropy between **3a** and **3b** on one side and **7a** and **7b** on the other side nevertheless indicates that the contribution of the hydrogen bond network to the rigidity of the liposomal structure is much more important for the lipo-phosphoramidates compared to the lipo-thiophosphoramidates. This can be explained by the formation of strong hydrogen bonds in the case of phosphoramidates **3a** while weaker hydrogen bonds may exist in the case of the thiophosphoramidate **7a**. For the comparison of the lipids **3b** and **7b**, it is worth noting that the anisotropy is much more important for the *N*-methylated lipo-thiophosphoramidate **7b** than for the

phosphoramidate analogue **3b**. In the case of **7b**, the P═S bond is much less polarized than the P═O bond present in **3b** leading us to consider that the P═S moiety could be included into the hydrophobic domain. Consequently the rigidity of the liposome formed with this compound could be explained by the presence of stronger hydrophobic interactions. Finally, as regards the anisotropy of the *N*-unmethylated derivatives **3a** and **7a**, different factors can be postulated to explain the high values observed in both cases. For compound **3a**, the rigidity of the liposomes may be related to the presence of a strong hydrogen bond network. For compound **7a**, the presence of a weak hydrogen bond network in addition to the extension of the hydrophobic domain are likely to be two parameters contributing to the formation of a rigid liposomal structure.

The results of the FRET efficiency measurements for the lipophosphoramidates (**3a**, **3b**) and lipothiophosphoramidates (**7a**, **7b**) are shown in Fig. 6. First, it must be noticed that the FRET efficiencies were observed in a narrow range of values (52 to 66%), a fact indicating that the fusogenic properties of these compounds are roughly comparable. Nevertheless, some small differences can be noticed. Indeed, the *N*-methylated compound (**3b** and **7b**) based liposomes, were slightly more fusogenic than the corresponding unmethylated compound (**3a** and **7a**) based liposomes. This observation might be explained by the absence of a hydrogen bond network when the liposomes are formed with the *N*-methylated cationic lipids. The comparison of the fluidity (Fig. 5) and fusogenicity (Fig. 6) results indicates that the more fluid derivative is also the more fusogenic (compound **3b**) whereas the less fluid derivatives (**7a** and **3a**) are also the less fusogenic.

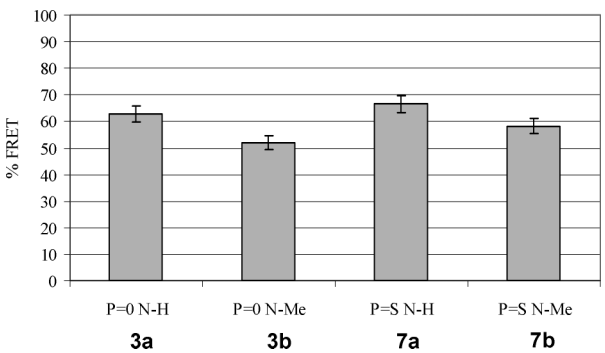


Fig. 6 Evaluation of the fusogenicity of compounds **3a–b** and **7a–b** (formulated as liposomes) by FRET efficiency measurements.

Next, DNA binding abilities of the cationic lipids were estimated by using electrophoretic mobility of plasmid DNA at a variable charge ratio from 0.5 to 8 and compared with free pDNA (Fig. 7). First, it is observed that all the cationic lipids tested have the ability to compact pDNA. Nevertheless, differences are observed between them. First, the presence of a methyl group on the nitrogen atom of the phosphoramidates **3b** (**3a** *versus* **3b**) induces a lower binding capacity at a same charge ratio. Indeed, pDNA is fully compacted at CR = 2 for **3a** while the same result is only obtained at CR = 4 for compound **3b**. This observation may be rationalised by the assistance of the unsubstituted phosphoramidate functional group present in **3a** in the pDNA compaction through hydrogen bonds involving the -PO–NH- motif. The comparison of phosphoramidate **3a** and thiophosphoramidate **7a** also indicates

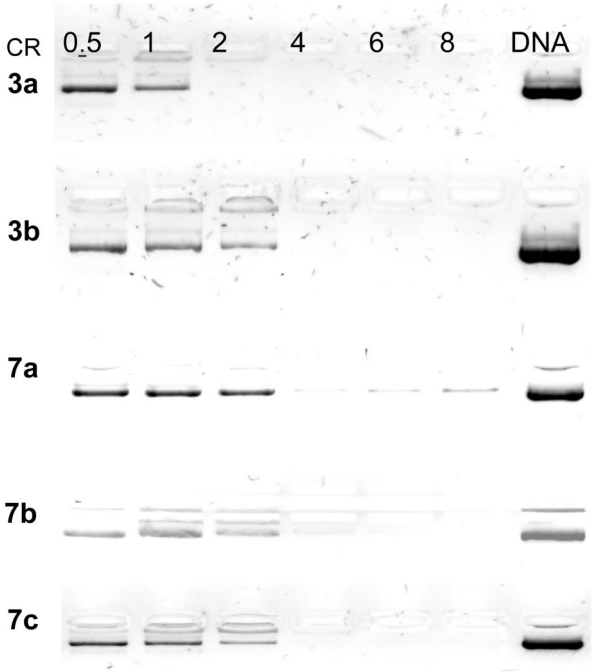


Fig. 7 DNA binding ability of lipo-phosphoramidate **3a–b** and lipo-thiophosphoramidate **7a–c** at charge ratio (CR) = 0.5, 1, 2, 4, 6, 8 and comparison with free DNA.

that **3a** exhibits a higher capacity to compact DNA since a lower charge ratio is required to observe a full retardation. The implication of electrostatic interactions and more robust hydrogen bonds in the case of **3a** might be postulated to explain this difference. Finally, the presence of a methyl group on the nitrogen atom in the thiophosphoramidate family of compounds does not modify significantly the compaction properties (**7a** *versus* **7b**). Not surprisingly, when taking into account the chemical structure similarities, compound **7c** has almost the same action on pDNA retardation as observed for compound **7a**.

Finally, the formulations prepared have been tested for the transfection of three different cell lines at different Charge Ratios (CR). Globally, all the cationic lipids tested have the capacity to transfect efficiently. Interestingly, it is worth noting that even at low CR (1 and 2) the transfection efficacies are generally good. The transfection results shown in Fig. 8–10 are summarized in Table 2 in which the vectors are compared two by two: (entry 1): *N*-unmethylated phosphoramidate **3a** is compared with *N*-unmethylated thiophosphoramidate **7a**; (entries 2 and 3): the consequences of the presence of a methyl group on the nitrogen atom are summarized for the two families of cationic lipids;

Table 2 Summary of the comparison of the transfection efficiencies of lipo-phosphonates **3a–b** and lipo-thiophosphoramidates **7a–b**

Entry	Comparison	HeLa	A549	16HBE14o(−)
1	**3a** *versus* **7a**	**3a** ~ **7a**	**3a** < **7a**	**3a** < **7a**
2	**3a** *versus* **3b**	**3a** ~ **3b**	**3a** ~ **3b**	**3a** < **3b**
3	**7a** *versus* **7b**	**7a** > **7b**	**7a** ~ **7b**	**7a** > **7b**
4	**3b** *versus* **7b**	**3b** > **7b**	**3b** < **7b**	**3b** > **7b**
5	best vector at CR = 2	**7a**	**7a** ~ **7b**	**7a** ~ **3b**

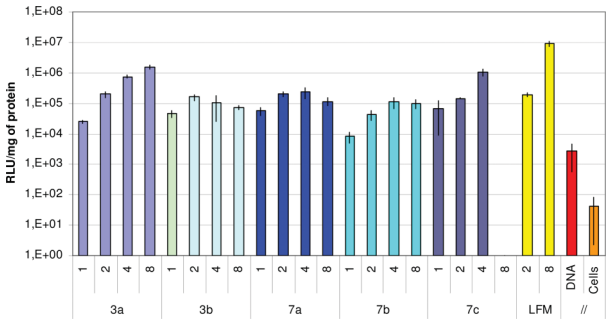


Fig. 8 *In vitro* transfection efficiency of **3a–b** and **7a–c** liposomes with the HeLa cell line. Lipofectamine (LFM) was used as reference.

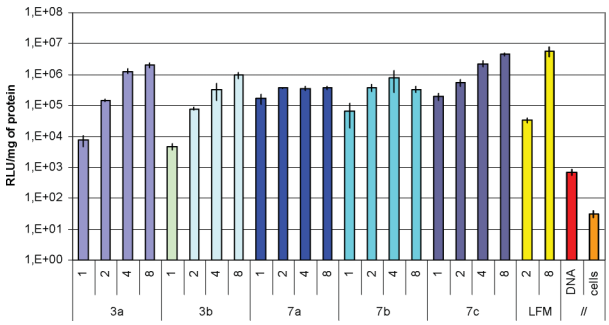


Fig. 9 *In vitro* transfection efficiency of **3a–b** and **7a–c** liposomes with the A549 cell line. Lipofectamine (LFM) was used as reference.

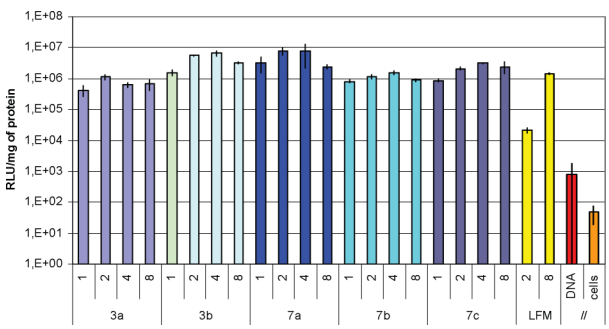


Fig. 10 *In vitro* transfection efficiency of **3a–b** and **7a–c** liposomes with the 16HBE14o(–) cell line. Lipofectamine (LFM) was used as reference.

(entry 4): the phosphoramidate and thiophosphoramidate are compared when the nitrogen atom is methylated.

From these comparisons, it can be observed that the cationic lipid **7a** was always among the best vectors for the transfection of the three cell lines tested at the theoretical CR of 2. It is also noteworthy that compound **7a** was also efficient at CR = 1 or 4, a fact indicating that the efficiency observed at CR 2 is not due to the existence of a colloidal instability at this CR (Table 1) since at CR = 1 or 4 a colloidal stability was observed (See ESI Figure S3-1). Additionally, compound **7c**, in which the propylene linker present in compound **7a** has been replaced by an ethylene motif, has also been evaluated as pDNA carrier. The results obtained with this vector lead to the conclusion that the length of the linker has a small impact on the transfection efficiency at low charge ratio. Indeed, only at the highest CR, the HeLa cells are more efficiently transfected with **7c**.

The evaluation of the cytotoxicity (using a chemiluminescent assay) of the cationic lipids **3a–b** and **7a–c** is summarized in Fig. 11.

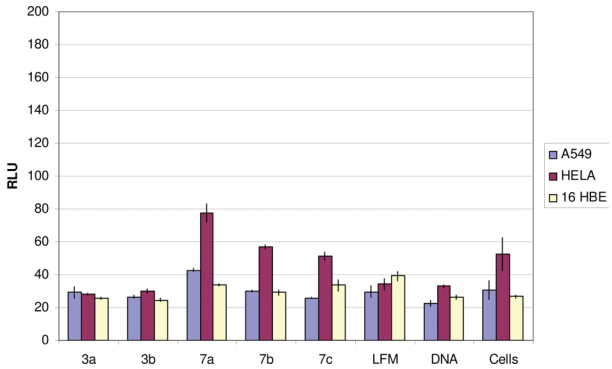


Fig. 11 Comparison of the cytotoxicity produced by lipoplexes formed at CR = 2 with either lipo-phosphoramidates **3a–b**, thiophosphoramidates **7a–c**, or lipofectamine (LFM) and considering three cell lines.

From these results, it can be concluded that, whatever the cell line considered, there is no obvious difference between the various formulations even if a very slight increase of the toxicity is observed for the cationic lipids **7a–c** for HeLa cells. Globally, the cytotoxic impact of these molecules was particularly low and was in the same range as that observed for both lipofectamine (LFM) treated and untreated cells (data not shown).

3 Conclusion

We report herein the synthesis of cationic lipo-phosphoramidates and lipo-thiophosphoramidates possessing the capacity to compact and transfect plasmid DNA. The selected molecular structures (with similar polar head groups and lipid parts) of the lipo-phosphoramidates and lipo-thiophosphoramidates allow comparative analyses aiming to evaluate the influence of the phosphoramidate and thiophosphoramidate functional groups on both the physico-chemical properties of these vectors and their gene transfection efficiency and toxicity. To the best of our knowledge, this work reports for the first time the use of lipo-thiophosphoramidates as synthetic vectors for nucleic acid delivery. Their synthesis has been achieved by using dioleylchlorothiophosphate **5** as a key intermediate. The physico-chemical analysis of these synthetic vectors formulated as liposomes has been performed by using both fluorescence anisotropy and FRET efficiency measurements. It was observed that the fluidity and fusogenicity of the liposomes were governed by different factors including the presence (or the absence) and the robustness of a hydrogen bond network, the size of the hydrophobic domain as well as electrostatic interactions. From the physico-chemical studies, it is clearly evidenced that the fluidity and fusogenicity can be fine-tuned *via* the use of either a phosphoramidate or a thiophosphoramidate functional group. The transfection activity studies point out that the cationic lipo-thiophosphoramidate **7a** is an efficient pDNA carrier. Its transfection activity behavior appears different from that of the phosphoramides **3a** and **3b** since, even at a low theoretical charge ratio (CR = 1 or 2), a high transfection efficacy was observed. It may thus be concluded that a minor modification of the chemical structure of the cationic lipids (phosphoramidate *versus* thiophosphoramidate) may have a direct impact on both their physico-chemical properties and their gene transfection ability. In ongoing work, we are at present evaluating the efficiency of those novel vectors for *in vivo* gene transfection in the mouse.

4 Experimental

4.1 General

Solvents were dried with a solvent purification system MBraun-SPS (THF, CH_2Cl_2) or freshly distilled on appropriate driers. All compounds were fully characterized by ^1H (500.13, 400.133 or 300.135 MHz), ^{13}C (125.773 or 75.480 MHz) and ^{31}P (161.970 or 121.498 MHz) NMR spectroscopy (Bruker AC 300, Avance DRX 400 and Avance DRX 500 spectrometers). Coupling constants J are given in Hertz. The following abbreviations were used: s for singlet, d doublet, t triplet, q quadruplet, qt quintuplet, m for multiplet and dt for doublet of triplets. When needed, ^{13}C heteronuclear HMQC and HMBC were used to unambiguously establish molecular structures. Mass spectroscopy analyses were performed by CRMPO (Université de Rennes 1, Rennes-France) by using a MS/MS high resolution Micromass ZABSpecTOF or at Brest (service commun de spectrometrie de masse) on a Bruker Autoflex MALDI TOF-TOF III LRF200 CID. Commercial compounds [oleyl alcohol 85%, thiophosphoryl chloride, *N*,*N*-dimethyl-1,3-propandiamine, *N*,*N*,*N*′-trimethyl-1,3-propandiamine, *N*,*N*-dimethylethylendiamine, methyl iodide, bromotrichloromethane] were used as received except DIPEA which was distilled over KOH. Dioleylphosphite **1** has been synthesized following the reported methods.[38] The synthesis of compound **2a** has been achieved following reported methods.[7] The mean particle diameter and zeta potential (ξ) of the liposomes and lipoplexes were measured using a 3000 Zetasizer (Malvern Instruments) at 25 °C (see ESI†).

4.2 Synthesis of *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-*N*-methylphosphoramidate 2b

To a cooled (0 °C) mixture of *N*,*N*,*N*′-trimethyl-1,3-propandiamine (128 mg; 1.1 mmol; 1.1 eq.) and DIPEA (190 µL; 1.1 mmol; 1.1 eq.) in 15 mL of dry CH_2Cl_2 was added, under N_2 atmosphere, dioleylphosphite (583 mg; 1.0 mmol; 1 eq.) and bromotrichloromethane (110 µL; 1.1 mmol; 1.1 eq.). The solution was stirred for 4 h while the temperature was slowly increased up to R.T. CH_2Cl_2 was removed and the residue was dissolved in diethyl ether (50 mL). The solution was filtered to remove insoluble particles and washed with brine (3 × 100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. Purification by column chromatography on silica gel (chloroform and methanol gradient 95/5 to 90/10) afforded the expected compound **2b** as a colourless wax in 63% yield. ^1H NMR (500.133 MHz; $CDCl_3$): 0.87 (6H, t, $^3J_{H-H}$: 6.7 Hz, C*H*$_3$–CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.64 (4H, m, C*H*$_2$–CH$_2$–O), 1.71 (2H, qt, $^3J_{H-H}$: 7.5 Hz, N(CH$_3$)–CH$_2$–C*H*$_2$), 1.99 (8H, m, C*H*$_2$–CH═CH–C*H*$_2$), 2.24 (6H, s, N(C*H*$_3$)$_2$), 2.30 (2H, t, $^3J_{H-H}$: 7.5 Hz, C*H*$_2$–N(CH$_3$)$_2$), 2.64 (3H, d, $^3J_{H-P}$: 9.9 Hz, N(C*H*$_3$)), 3.03 (2H, m, N(CH$_3$)–C*H*$_2$), 3.90 (4H, m, C*H*$_2$–O), 5.33 (4H, m, C*H*═C*H*). ^{13}C NMR (125.773 MHz; $CDCl_3$): 14.1 (s, *C*H$_3$–CH$_2$), 22.7 (s, CH$_3$–*C*H$_2$), 26.2 (s, N(CH$_3$)–CH$_2$–*C*H$_2$), 27.2 (s, *C*H$_2$–CH═CH–CH$_2$ and/or CH$_2$–CH═CH–*C*H$_2$), 30.4 (d, $^3J_{P-C}$: 6.7 Hz, C*H*$_2$–CH$_2$–O), 31.9 (s, CH$_3$–CH$_2$–*C*H$_2$), 25.7 to 32.5 (*C*H$_2$ fatty chain and), 33.5 (s, N(*C*H$_3$), 45.4 (s, N(*C*H$_3$)$_2$), 47.3 (d, $^2J_{P-C}$: 4.1 Hz, N(CH$_3$)–*C*H$_2$), 57.0 (s, *C*H$_2$–N(CH$_3$)$_2$), 66.2 (d, $^2J_{P-C}$: 5.8 Hz, *C*H$_2$–O), 129.8 and 130.0 (s, *C*H═CH and CH═*C*H). ^{31}P NMR (161.970 MHz; $CDCl_3$): 11.1. MS (MALDI-TOF): *m*/*z* calcd for $C_{42}H_{86}N_2O_3P$ (M + H) 697.6376; found 697.512.

4.3 Synthesis of 3-(*O*,*O*-dioleylphosphoramidoyl)-propyltrimetyl-ammonium iodide 3a (KLN10)

O,*O*-Dioleyl-*N*-(3-dimethylaminopropyl)phosphoramidate **2a** (2.26 g; 3.3 mmol; 1 eq.) and an excess of methyl iodide (2 mL; 32 mmol) was stirred at R.T. for one night. The excess of methyl iodide was removed under vacuum. Purification by column chromatography on silica gel (chloroform and methanol gradient 95/5 to 90/10) afforded the expected compound as a yellow wax in 55% yield. ^1H NMR (400.133 MHz, $CDCl_3$): 0.87 (6H, t, $^3J_{H-H}$: 6.0 Hz, C*H*$_3$–CH$_2$), 1.15–1.40 (44H, m, C*H*$_2$ fatty chain), 1.62 (4H, qt, $^3J_{H-H}$: 6.8 Hz, C*H*$_2$–CH$_2$–O), 1.99 (8H, m, C*H*$_2$–CH═CH–C*H*$_2$), 2.09 (2H, m, NH–CH$_2$–C*H*$_2$), 3.10 (2H, m, NH–C*H*$_2$), 3.40 (9H, s, N$^+$(C*H*$_3$)$_3$), 3.78 (2H, m, C*H*$_2$–N$^+$(CH$_3$)$_3$), 3.96 (4H, dt, $^3H_{H-H}$ = $^3J_{P-H}$: 6.8 Hz, C*H*$_2$–O), 5.35 (4H, m, C*H*═C*H*). ^{13}C NMR (75.480 MHz, $CDCl_3$): 14.4 (s, *C*H$_3$–CH$_2$), 22.9 (s, CH$_3$–*C*H$_2$), 25.9 (s, NH–CH$_2$–*C*H$_2$), 27.5 (s, *C*H$_2$–CH═CH–CH$_2$ and/or CH$_2$–CH═CH–*C*H$_2$), 30.7 (d, $^3J_{P-C}$: 6.9 Hz, *C*H$_2$–CH$_2$–O), 32.2 (s, CH$_3$–CH$_2$–*C*H$_2$), 29.5 to 32.5 (*C*H$_2$ fatty chain), 38.2 (s, NH–*C*H$_2$), 54.4 (s, N$^+$(*C*H$_3$)$_3$), 65.3 (s, *C*H$_2$–N$^+$(CH$_3$)$_3$), 67.2 (d, $^2J_{P-C}$: 5.5 Hz, *C*H$_2$–O), 130.0 and 130.2 (s, *C*H═CH and CH═*C*H). ^{31}P NMR (161.970 MHz, $CDCl_3$): 9.3. MS (MALDI-TOF): *m*/*z* calcd for $C_{42}H_{86}N_2O_3P$ (M^+) 697.6371; found 697.586.

4.4 Synthesis of 3-(*O*,*O*-dioleylphosphoramidoyl)-*N*-methylpropyltrimetylammonium iodide 3b (KLN6)

An excess of methyl iodide (0.5 mL) was added to *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-*N*-methyl-phosphoramidate **2b** (293 mg; 0.4 mmol; 1 eq.) in 2 mL of EtO_2. The mixture was stirred at R.T. overnight. Diethyl ether and methyl iodide were removed and the expected compound was obtained as a yellow wax in 95% yield. ^1H NMR (400.113 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H-H}$: 8.0 Hz, C*H*$_3$–CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.66 (4H, m, C*H*$_2$–CH$_2$–O), 2.00 (8H, m, C*H*$_2$–CH═CH–C*H*$_2$), 2.11 (2H, m, N(CH$_3$)–CH$_2$–C*H*$_2$), 2.67 (3H, d, $^3J_{P-H}$: 12.0 Hz, N(C*H*$_3$), 3.22 (2H, m, N(CH$_3$)–C*H*$_2$), 3.43 (9H, s, N$^+$(C*H*$_3$)$_3$), 3.67 (2H, m, C*H*$_2$–N$^+$(CH$_3$)$_3$), 3.94 (4H, m, C*H*$_2$–O), 5.34 (4H, m, C*H*═C*H*). ^{13}C NMR (75.480 MHz, $CDCl_3$): 14.4 (s, *C*H$_3$–CH$_2$), 23.0 (s, CH$_3$–*C*H$_2$), 25.9 (s, N(CH$_3$)–CH$_2$–*C*H$_2$), 27.5 (s, *C*H$_2$–CH═CH–CH$_2$ and/or CH$_2$–CH═CH–*C*H$_2$), 30.7 (d, $^3J_{P-C}$: 6.8 Hz *C*H$_2$–CH$_2$–O), 32.2 (s, CH$_3$–CH$_2$–*C*H$_2$), 25.6 to 33.0 (*C*H$_2$ fatty chain), 34.6 (s, N(*C*H$_3$)), 45.4 (d, $^2J_{P-C}$: 7.5 Hz N(CH$_3$)–*C*H$_2$), 54.4 (s, N$^+$(*C*H$_3$)$_3$), 65.2 (s, *C*H$_2$–N$^+$(CH$_3$)$_3$), 67.0 (d, $^2J_{P-C}$: 6.2 Hz, *C*H$_2$–O), 130.1 and 130.3 (s, *C*H═CH and CH═*C*H). ^{31}P NMR (161.970 MHz; $CDCl_3$): 10.4. MS (MALDI-TOF): *m*/*z* calcd for $C_{43}H_{88}N_2O_3P$ (M^+) 711.6527; found 711.514.

4.5 Synthesis of *O*,*O*-diisopropyl-*N*-(3-dimethylaminopropyl)-phosphoramidate 4

To a cooled (0 °C) mixture of *N*,*N*-dimethylpropyl-1,3-diamine (500 µL; 4.0 mmol; 1.3 eq.) and DIPEA (700 µL; 4.0 mmol; 1.3 eq.) in 20 mL of dry CH_2Cl_2 were added, under N_2 atmosphere, diisopropylphosphite (500 µL; 3.0 mmol; 1 eq.) and

bromotrichloromethane (400 μL; 4.0 mmol; 1.3 eq.). The solution was stirred for 4 h while the temperature was slowly increased up to R.T. CH_2Cl_2 was removed and the residue was dissolved in diethyl ether (50 mL). The solution was filtered to remove insoluble particles. The expected compound was obtained as a yellow liquid in 67% yield. This compound has been used subsequently without additional purification. ^{1}H NMR (300.135 MHz; $CDCl_3$): 1.29 and 1.31 (12H, 2 d, $^3J_{H-H}$: 6.2 Hz, (CH_3)$_2$–CH–O), 1.66 (2H, qt, $^3J_{H-H}$: 6.7 Hz, NH–CH_2–CH_2), 2.24 (6H, s, N(CH_3)$_2$), 2.39 (2H, t, $^3J_{H-H}$: 6.7 Hz, CH_2–N(CH_3)$_2$), 2.97 (2H, m, NH–CH_2), 4.58 (2H, m, (CH_3)$_2$–CH–O). ^{31}P NMR (121.498 MHz; $CDCl_3$): 7.7.

4.6 Synthesis of *O*,*O*-dioleylchlorothiophosphate 5

Excess of sodium (2.6 g; 113.0 mmol) was added under N_2 atmosphere to a solution of oleyl alcohol (6.18g; 23.2 mmol; 4 eq.) in 50 mL of dry THF. This solution was stirred for 2 h at R.T. and heated under reflux for 2 days in presence of a controlled flow of nitrogen leading, at the end, to 30 mL of a very viscous alcoholate solution. After cooling to R.T., an aliquot (between 3 to 6 mL) of oleyl alcoholate solution was added to a solution of thiophosphoryl chloride (600 μL; 5.8 mmol; 1eq.) in 5 mL of dry THF cooled at −80 °C. The solution was stirred for 15 min and the reaction was monitored by ^{31}P NMR. This operation was repeated until the NMR control displays only the expected signal (27 mL of the 30 mL of the initial alcoholate solution were needed). After warming up the solution at R.T., THF was removed under vacuum and the residue was purified by column chromatography on silica gel (hexane and ethyl acetate gradient 100/0 to 90/10). The expected compound **5** was obtained as a colourless oil in 76% yield (2.78 g). ^{1}H NMR (300.133 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H-H}$: 6.7 Hz, CH_3–CH_2), 1.10–1.30 (44H, m, CH_2 fatty chain), 1.74 (4H, qt, $^3J_{H-H}$: 7.0 Hz, CH_2–CH_2–O), 2.00 (8H, m, CH_2–CH=CH–CH_2), 4.21 (4H, m, CH_2–O), 5.35 (4H, m, CH=CH). ^{13}C NMR (75.480 MHz; $CDCl_3$): 14.1 (s, CH_3–CH_2), 22.7 (s, CH_3–CH_2), 27.2 (s, CH_2–CH=CH–CH_2 and/or CH_2–CH=CH–CH_2), 29.6 (s, CH_2–CH_2–O), 25.4 to 32.6 (s, CH_2 fatty chain), 70.0 (s, CH_2–O), 129.8 and 130.0 (s, CH=CH and CH=CH). ^{31}P NMR (121.498 MHz; $CDCl_3$): 69.7. HRMS (ESI-TOF): *m*/*z* calcd for $C_{36}H_{71}{}^{35}ClO_2PS$ (M + H) 633.4610; found 633.4597.

4.7 Synthesis of *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-thiophosphoramidate 6a

O,*O*-Dioleylchlorothiophosphate **5** (1.28 g; 2.0 mmol; 1 eq.) was added under N_2 atmosphere to a cooled (0 °C) mixture of DIPEA (360 μL; 2.2 mmol; 1.1 eq.) and *N*,*N*-dimethylpropyl-1,3-diamine (280 μL; 2.2 mmol; 1.1 eq.) in 20 mL of dry CH_2Cl_2. The solution was stirred overnight while the temperature was slowly increased up to R.T. CH_2Cl_2 was removed and the residue was dissolved in diethyl ether (50 mL). The solution was filtered to remove insoluble particles and washed with brine (3 × 40 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The expected compound was isolated as a pale yellow oil in 94% yield. This compound has been used subsequently without additional purification. ^{1}H NMR (500.134 MHz; $CDCl_3$): 0.87 (6H, t, $^3J_{H-H}$: 6.8 Hz, CH_3–CH_2), 1.10–1.30 (44H, m, CH_2 fatty chain), 1.65 (6H, m, CH_2–CH_2–O and NH–CH_2–CH_2), 1.99 (8H, m, CH_2–CH=CH–CH_2), 2.24 (6H, s, N(CH_3)$_2$), 2.38 (2H, t, $^3J_{H-H}$: 6.7 Hz, CH_2–N(CH_3)$_2$), 3.02 (2H, m, NH–CH_2), 3.96 (4H, m, CH_2–O), 5.33 (4H, m, CH=CH). ^{13}C NMR (125.816 MHz; $CDCl_3$): 14.1 (s, CH_3–CH_2), 22.7 (s, CH_3–CH_2), 27.2 (s, CH_2–CH=CH–CH_2 and/or CH_2–CH=CH–CH_2), 30.2 (d, $^3J_{P-C}$: 8.8 Hz, CH_2–CH_2–O), 31.8 (s, CH_3–CH_2–CH_2), 25.7 to 32.5 (CH_2 fatty chain and NH–CH_2–CH_2), 41.0 (s, NH–CH_2), 45.3 (s, N(CH_3)$_2$), 58.0 (s, CH_2–N(CH_3)$_2$), 66.8 (d, $^2J_{P-C}$: 5.1 Hz, CH_2–O), 129.8 and 130.0 (s, CH=CH and CH=CH). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.4. MS (MALDI-TOF): *m*/*z* calcd for $C_{41}H_{84}N_2O_2PS$ (M + H) 699.5991; found 699.581.

4.8 Synthesis of *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-*N*-methylthiophosphoramidate 6b

The same protocol described for **6a** is used with the following quantities: *O*,*O*-dioleylchlorothiophosphate **5** (632 mg; 1.0 mmol; 1 eq.), *N*,*N*,*N*′-Trimethylpropyl-1,3-diamine (160 μL; 1.1 mmol; 1.1 eq.) and DIPEA (180 μL; 1.1 mmol; 1.1 eq.). The expected compound was obtained as a turbid yellowish liquid in 96% yield. This compound has been used subsequently without additional purification. ^{1}H NMR (400.103 MHz; $CDCl_3$): 0.87 (6H, t, $^3J_{H-H}$: 8.0 Hz, CH_3–CH_2), 1.10–1.30 (44H, m, CH_2 fatty chain), 1.67 (6H, m, CH_2–CH_2–O and N(CH_3)–CH_2–CH_2), 2.00 (8H, m, CH_2–CH=CH–CH_2), 2.23 (6H, s, N(CH_3)$_2$), 2.28 (2H, t, $^3J_{H-H}$: 8.0 Hz, CH_2–N(CH_3)$_2$), 2.72 (3H, d, $^3J_{P-H}$: 12.0 Hz, N(CH_3)), 3.18 (2H, m, N(CH_3)–CH_2), 3.93 (4H, m, CH_2–O), 5.34 (4H, m, CH=CH). ^{13}C NMR (125.816 MHz; $CDCl_3$): 14.1 (s, CH_3–CH_2), 22.7 (s, CH_3–CH_2), 26.4 (s, N(CH_3)–CH_2–CH_2), 27.2 (s, CH_2–CH=CH–CH_2 and/or CH_2–CH=CH–CH_2), 30.1 (d, $^3J_{P-C}$: 7.5 Hz, CH_2–CH_2–O), 31.9 (s, CH_3–CH_2–CH_2), 25.7 to 32.5 (CH_2 fatty chain and), 33.5 (d, $^2J_{P-C}$: 3.8 Hz, N(CH_3), 45.5 (s, N(CH_3)$_2$), 48.2 (d, $^2J_{P-C}$: 3.8 Hz, N(CH_3)–CH_2), 57.1 (s, CH_2–N(CH_3)$_2$), 66.3 (d, $^2J_{P-C}$: 6.3 Hz, CH_2–O), 129.8 and 130.0 (s, CH=CH and CH=CH). ^{31}P NMR (161.970 MHz; $CDCl_3$): 76.9. MS (MALDI-TOF): *m*/*z* calcd for $C_{42}H_{86}N_2O_2PS$ (M + H) 713.6148; found 713.598.

4.9 Synthesis of *O*,*O*-dioleyl-*N*-(2-dimethylaminoethyl)-thiophosphoramidate 6c

The same protocol described for **6a** is used with the following quantities: *O*,*O*-dioleylchlorothiophosphate **5** (1.02 g; 1.6 mmol; 1 eq.), *N*,*N*-dimethylethyl-1,2-diamine (300 μL; 1.8 mmol; 1.1 eq.) and DIPEA (195 μL; 1.8 mmol; 1.1 eq.). The expected compound was obtained as a turbid white liquid in 85% yield. This compound has been used subsequently without additional purification. ^{1}H NMR (300.135 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H-H}$: 6.2 Hz, CH_3–CH_2), 1.10–1.30 (44H, m, CH_2 fatty chain), 1.63 (4H, m, CH_2–CH_2–O), 1.98 (8H, m, CH_2–CH=CH–CH_2), 2.21 (6H, s, N(CH_3)$_2$), 2.37 (2H, t, $^3J_{H-H}$: 5.8 Hz, CH_2–N(CH_3)$_2$), 3.00 (2H, m, NH–CH_2), 3.97 (4H, m, CH_2–O), 5.33 (4H, m, CH=CH). ^{13}C NMR (75.478 MHz; $CDCl_3$): 14.9 (s, CH_3–CH_2), 22.7 (s, CH_3–CH_2), 27.2 (s, CH_2–CH=CH–CH_2 and/or CH_2–CH=CH–CH_2), 30.2 (d, $^3J_{P-C}$: 7.8 Hz, CH_2–CH_2–O), 31.9 (s, CH_3–CH_2–CH_2), 25.7 to 32.5 (CH_2 fatty chain and NH–CH_2–CH_2), 39.2 (s, NH–CH_2), 45.3 (s, N(CH_3)$_2$), 59.6 (d, $^3J_{P-C}$: 7.2 Hz, CH_2–N(CH_3)$_2$), 66.9 (d, $^2J_{P-C}$: 4.3 Hz, CH_2–O), 129.8 and 130.0 (s, CH=CH and CH=CH). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.9. MS (MALDI-TOF): *m*/*z* calcd for $C_4H_{82}N_2O_2PS$ (M + H) 685.5835; found 685.567.

4.10 Synthesis of 3-(*O*,*O*-dioleylthiophosphoramidoyl)-propyltrimetylammonium iodide 7a (BSV44)

Methyl iodide (160 µL; 2.6 mmol; 3 eq.) was added to a solution of *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-thiophosphoramidate **6b** (600 mg; 0.9 mmol; 1 eq.) in 10 mL of diethyl ether. The mixture was stirred at R.T. for one night. The excess of methyl iodide and the diethyl ether were removed under vacuum. The expected compound was obtained as a white wax in 98% yield. ^{1}H NMR (500.133 MHz, $CDCl_3$): 0.87 (6H, t, $^{3}J_{H-H}$: 6.5 Hz, C*H*$_3$–CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.64 (4H, m, C*H*$_2$–CH$_2$–O), 1.99 (8H, m, C*H*$_2$–CH=CH–C*H*$_2$), 2.10 (2H, m, NH–CH$_2$–C*H*$_2$), 3.19 (2H, m, NH–C*H*$_2$), 3.41 (9H, s, N^+(C*H*$_3$)$_3$), 3.76 (2H, m, C*H*$_2$–N^+(CH$_3$)$_3$), 3.95 (4H, m, C*H*$_2$–O), 5. 35 (4H, m, C*H*=C*H*). ^{13}C NMR (125.773 MHz, $CDCl_3$): 14.1 (s, *C*H$_3$–CH$_2$), 22.7 (s, CH$_3$–*C*H$_2$), 25.2 (s, NH–CH$_2$–*C*H$_2$), 27.2 (s, *C*H$_2$–CH=CH–CH$_2$ and/or CH$_2$–CH=CH–*C*H$_2$), 30.2 (d, $^{3}J_{P-C}$: 7.8 Hz, *C*H$_2$–CH$_2$–O), 31.8 (s, CH$_3$–CH$_2$–*C*H$_2$), 29.3 to 32.5 (*C*H$_2$ fatty chain), 38.4 (s, NH–*C*H$_2$), 54.2 (s, N^+(*C*H$_3$)$_3$), 65.2 (s, *C*H$_2$–N^+(CH$_3$)$_3$), 67.5 (d, $^{2}J_{P-C}$: 5.8 Hz, *C*H$_2$–O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz, $CDCl_3$): 73.2. HRMS (ESI-TOF): *m*/*z* calcd for $C_{42}H_{86}N_2O_2PS$ (M^+) 713.6148; found 713.6137.

4.11 Synthesis of 3-(*O*,*O*-dioleylthiophosphoramidoyl)-*N*-methylpropyltrimetylammonium iodide 7b (BSV25)

Methyl iodide (130 µL; 2.0 mmol; 2.1 eq.) was added to a solution of *O*,*O*-dioleyl-*N*-(3-dimethylaminopropyl)-*N*-methylthiophosphoramidate **6b** (708 mg; 1.0 mmol; 1 eq.) in 15 mL of diethyl ether. The mixture was stirred at R.T. for one night. The excess of methyl iodide and the diethyl ether were removed under vacuum. The expected compound was obtained as a white wax in 91% yield. ^{1}H NMR (500.303 MHz, $CDCl_3$): 0.88 (6H, t, $^{3}J_{H-H}$: 6.5 Hz, C*H*$_3$–CH$_2$), 1.20–1.40 (44H, m, C*H*$_2$ fatty chain), 1.64 (4H, m, C*H*$_2$–CH$_2$–O), 2.00 (8H, m, C*H*$_2$–CH=CH–C*H*$_2$), 2.10 (2H, m, N(CH$_3$)–CH$_2$–C*H*$_2$), 2.74 (3H, d, $^{3}J_{H-P}$: 10.0 Hz, N(C*H*$_3$)), 3.39 (2H, m, N(CH$_3$)–C*H*$_2$), 3.46 (9H, s, N^+(C*H*$_3$)$_3$), 3.63 (2H, m, C*H*$_2$– N^+(CH$_3$)$_3$), 3.91 (4H, m, C*H*$_2$–O), 5.34 (4H, m, C*H*=C*H*). ^{13}C NMR (125.816 MHz, $CDCl_3$): 14.2 (s, *C*H$_3$–CH$_2$), 22.7 (s, CH$_3$–*C*H$_2$), 22.4 (s, N(CH$_3$)–CH$_2$–*C*H$_2$), 27.2 (s, *C*H$_2$–CH=CH–CH$_2$ and/or CH$_2$–CH=CH–*C*H$_2$), 30.1 (d, $^{3}J_{P-C}$: 7.5 Hz *C*H$_2$–CH$_2$–O), 31.9 (s, CH$_3$–CH$_2$–*C*H$_2$), 25.6 to 32.7 (*C*H$_2$ fatty chain), 33.9 (s, N(*C*H$_3$)), 47.3 (d, $^{2}J_{P-C}$: 7.5 Hz, N(CH$_3$)–*C*H$_2$), 54.0 (s, N^+(*C*H$_3$)$_3$), 66.2 (s, *C*H$_2$–N^+(CH$_3$)$_3$), 67.2 (d, $^{2}J_{P-C}$: 6.3 Hz, C*H*$_2$–O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.496 MHz; $CDCl_3$): 76.3. HRMS (ESI-TOF): *m*/*z* calcd for $C_{43}H_{88}N_2O_2PS$ (M^+) 727.63042; found 727.6302.

4.12 Synthesis of 2-(*O*,*O*-dioleylthiophosphoramidoyl)-ethyltrimetylammonium iodide 7c (BSV17)

Methyl iodide (900 µL; 14.5 mmol; 10.6 eq.) was added under N_2 atmosphere to a solution of **6c** (933 mg; 1.4 mmol; 1 eq.) in 15 mL of diethyl ether. The mixture was stirred at R.T. for 2 days. The excess of methyl iodide and the diethyl ether were removed under vacuum. A purification by column chromatography on silica gel (dichloromethane and methanol 90/10) afforded the expected compound as a white wax in 80% yield. ^{1}H NMR (500.133 MHz; $CDCl_3$): 0.87 (6H, t, $^{3}J_{H-H}$: 6.7 Hz, C*H*$_3$–CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.64 (4H, m, C*H*$_2$–CH$_2$–O), 2.00 (8H, m, C*H*$_2$–CH=CH–C*H*$_2$), 3.47 (9H, s, N^+(C*H*$_3$)$_3$), 3.65 (2H, m, NH–C*H*$_2$), 3.91 (2H, t, $^{3}J_{H-H}$: 5.8 Hz, C*H*$_2$–N^+(CH$_3$)$_3$), 3.97 (4H, m, C*H*$_2$–O), 4.51 (1H, m, N*H*), 5.34 (4H, m, C*H*=C*H*). ^{13}C NMR (125.773 MHz; $CDCl_3$): 14.2 (s, *C*H$_3$–CH$_2$), 22.6 (s, CH$_3$–*C*H$_2$), 27.2 (s, *C*H$_2$–CH=CH–CH$_2$ and/or CH$_2$–CH=CH–*C*H$_2$), 30.2 (s, *C*H$_2$–CH$_2$–O), 31.9 (s, CH$_3$–CH$_2$–*C*H$_2$), 25.6 to 32.7 (*C*H$_2$ fatty chain), 36.8 (s, NH–*C*H$_2$), 54.9 (s, N^+(*C*H$_3$)$_3$), 66.5 (s, *C*H$_2$–N^+(CH$_3$)$_3$), 67.7 (s, C*H*$_2$–O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (202.471 MHz; $CDCl_3$): 73.1. HRMS (ESI-TOF): *m*/*z* calcd for $C_{41}H_{84}N_2O_2PS$ (M^+) 699.5991; found 699.6009.

4.13 Liposome formulation

The cationic lipids **3a–b** and **7a–c** were formulated as liposomes by using the hydration of the lipid film. First a concentrated solution of a desired lipid in chloroform (*e.g.* **7a**, 6.31 mM, 475.2 µL) was placed in a glass tube and the solvent was evaporated to produce a thin lipid film. Then water was added on this film. After an optimized hydration period at 4 °C (See ESI-S6) the solution was vortexed (1 min) and sonicated (6 × 10 min) at 45 kHz using a VWR ultrasonic bath. The size and the zeta potential of these formulation were determined for each liposomal solution. (see Table 1 and ESI S3 and S4).

4.14 CAC determination

The concentration from which phospholipids aggregates are formed is determined by measuring the fluorescence of the Nile Red hydrophobic probe[33] (9-diethylamino-5*H*-benzo[alpha]phenoxazine-5-one; Molecular Probe, France). Nile red is highly solvatochromic exhibiting an increase in fluorescence quantum yield with decreasing solvent polarity accompanied by a blue shift in the peak emission [35] Nile Red fluorescence intensity rises upon the formation of phospholipids aggregates as illustrated in Figure S1 (see ESI-S1).

4.15 Fluorescence anisotropy measurements

The membrane viscosity was evaluated by fluorescence anisotropy which was determined by using Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) as a probe following a protocol that has been reported elsewhere[16] and that is summarized in ESI (S2).

4-16 Förster resonance energy transfer (FRET) measurements

The efficiency (E) of the FRET.[39] was calculated from the fluorescence emission intensity of NBD-PE at 530 nm using the following equation where fluorescence intensities were recorded in the presence (F) and absence (F_0) of Rhod-PE:

$$E = 1 - F/F_0$$

The relative fluorescence intensity (ER, in %) was calculated using the next relation, where E_{mix} and E_{ab} are the FRET efficiencies calculated in the presence or absence of cationic lipids, respectively:

$$ER = (E_{mix}/E_{ab}) \times 100$$

As labelled membrane, we used liposomes made with PC, PE, PS, Chol and fluorescent probes (PC/PE/PS/Chol/NBD-PE/Rhod-PE in a relative mass proportion of approximately 44/25/10/20/0.8/0.2), *i.e.* a lipid composition close to that of the plasma membrane. They were then mixed with unlabeled cationic liposomes at increasing concentrations, from 10^{-6} to 10^{-4} mol L^{-1}. The Rhod-PE and NBD-PE final concentrations were 6×10^{-8} mol L^{-1} and 3×10^{-7} mol L^{-1}, respectively. The labeled membrane final concentration was 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 close to 0.003 was chosen so that lipid fusion underwent a significant decrease of FRET efficiency. NBD-PE concentrations did not affect the FRET efficiency, as it was described before.

4.17 DNA binding ability

To 1 μg of plasmid DNA in Optimem (Gibco) were added cationic lipids at concentrations corresponding to an N/P charge ratio ranging from 1 to 8. The mixture was incubated for 30 min at room temperature. The complexes were subjected to electrophoresis in 1% agarose gel at 100 V, 90 mA. The gel was stained with ethidium bromide (10 mg mL^{-1}) and visualized on a UV illuminator (Fischer Bioblock).

4.18 *In vitro* reporter gene measurements: luciferase assays

Cells were seeded 24 h before transfection onto a 24-well plate at a density of 100000 cells per well and incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. Transfection was performed as described by Felgner *et al.*[40] (*1*), with the following modifications. Appropriate amounts of the cationic lipids and the plasmid vector were complexed in OptiMem, and about 200 μL was added to each well. Lipofectamine (LFM) was used as a positive control. After 2 h 30 min incubation at 37 °C, 20 μL of the surrounding is picked up to evaluate the early toxicity of the formulations. The rest of the medium was removed, and fresh medium was added. Following a further 48 h at 37 °C, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega). Assays were carried out as described by the manufacturer. The total protein content of the cell culture was determined using the BC assay kit (Uptima). Data are expressed as relative light units (RLU) per milligram of total proteins (mean ± SD with $n = 3$).

4.19 Evaluation of the early toxicity

The early toxicity of the different lipid/DNA complexes was determined by using a chemiluminescent assay (TOXILIGHT - Cambrex, Liège, Belgium). This was based on the release of adenylate kinase (AK) from damaged cells into the surrounding medium. The reaction involves two steps. The first one involves the addition of ADP as a substrate for AK. In the presence of AK, the ADP is converted to ATP for assay by bioluminescence. Then, the bioluminescence method utilizes an enzyme Luciferase, which catalyzes the emission of photons from ATP and luciferin. By combining both reactions, the emitted light intensity is directly related to the AK concentration. The relative light units (RLU) were inversely proportional to the number of living cells. Untransfected cells were used as a reference.

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