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PAPER

Lipothiophosphoramidates for gene delivery: critical role of the cationic polar headgroup†

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When considering a family of cationic lipids designed for gene delivery, the nature of the cationic polar head probably has a great influence on both the transfection efficacy and toxicity. Starting from a cationic lipothiophosphoramidate bearing a trimethylammonium headgroup, we report herein the impact on gene transfection activity of the replacement of the trimethylammonium moiety by a trimethylphosphonium or a trimethylarsonium group. A series of three different human epithelial cell lines were used for the experimental transfection studies (HeLa, A549 and 16HBE14o(−)). The results basically showed that such structural modifications of the cationic headgroup can lead to a high transfection efficacy at low lipid/DNA charge ratios together with a low cytotoxicity. It thus appears that the use of a trimethylarsonium cationic headgroup for the design of efficient gene carriers, which was initially proposed in the lipophosphoramidate series, can be extended to other series of cationic lipids and might therefore have great potential for the development of novel non-viral vectors in general.

1. Introduction

The use of DNA and RNA as therapeutics offers encouraging prospects[1] but efficient vectors for nucleic acid delivery are still required. Various strategies, including physical methods[2] (*e.g.* electroporation), the use of recombinant viruses[3] or synthetic vectors,[4] have been tested and promising results have already been reported for both *in vitro* and *in vivo* transfection experiments. Nevertheless, development of vectors which can be readily produced and easily administered still requires further study. Accordingly, synthetic vectors constitute an attractive class of gene carriers since their synthesis requires the same technical procedures as those used to produce drugs. These synthetic vectors for nucleic acid delivery include a wide variety of molecular structures, such as cationic lipids,[5] neutral lipids,[6] cationic polymers,[7] dendrimers[8] or neutral polymers.[9] The binary combination of these vectors can, in some cases, increase transfection efficacy (*e.g.* cationic lipid + cationic polymer[10]). Moreover, the association of a helper lipid (*e.g.* DOPE,[11] imidazole derivatives[12]) with a cationic lipid is another strategy used to increase transfection efficacy *via* a modification of the physico-chemical properties of the nanoparticles formed by the vector with the nucleic acid to be transferred. Beside the optimization of the formulation and the production of new families of helper lipids, a slight change in the chemical structure of a cationic lipid can greatly impact its transfection capacity.[13] This has been shown in particular by results obtained by our group with cationic lipophosphonate-based vectors.[14] Indeed, the replacement of a trimethylammonium polar head by a trimethylphosphonium or trimethylarsonium was doubly beneficial as it increased the transfection efficiency and reduced the toxicity of the vector[15] (this replacement was initially attempted in view of the results of Stekar *et al.* showing its benefit for decreasing the toxicity of edelfosine[16]). It is important to note that transfection improvements were also observed by the replacement of a trimethylammonium group by a pyridinium polar head.[17] Secondly, the replacement of the phosphonate functional group by a phosphoramidate moiety[18] also induced a gain of efficiency and this without increasing toxicity. As others,[19] we also observed that a slight modification of the lipid part in the structure of a given cationic lipid can improve the transfection efficacy *in vivo*.[20] More recently, we have designed thiophosphoramidate-based cationic lipids[21] with the aim to design synthetic vectors capable of satisfying the compromise required between stability of the lipoplexes (needed in blood circulation and more generally when the

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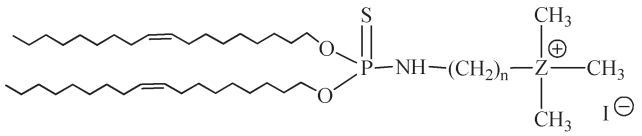


2 (BSV17) n = 2 ; Z = N
3 (BSV44) n = 3 ; Z = N
6a (BSV14) n = 2 ; Z = P
6b (BSV28) n = 3 ; Z = P
7 (BSV21) n = 2 ; Z = As

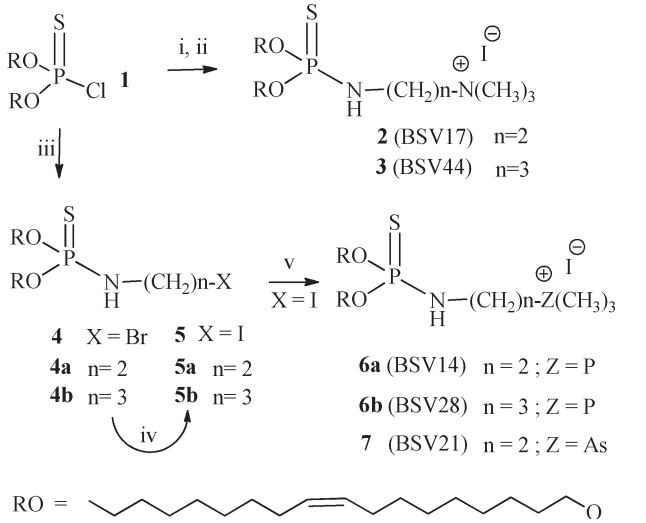
Scheme 1 Chemical structure of the lipothiophosphoramidates.

nano-object is located outside the cells) and their intracellular instability (required in particular for endosomal escape of pDNA after cellular uptake). Fluorescence anisotropy measurements clearly showed that this minor structural change (thiophosphoramidate *versus* phosphoramidate) modified the physico-chemical behaviour of the vectors, in particular their fluidity (the modification being also function of the substitution of the nitrogen atom of the phosphoramide functional group).[21] Most importantly, such lipothiophosphoramidates with two oleyl alcohol chains and one trimethylammonium polar head exhibited interesting transfection activities, most notable at low charge ratios.

To further investigate the potential of lipothiophosphoramidates as a new class of cationic lipids, we report herein for the first time the use of lipothiophosphoramidates, characterized by either a trimethylphosphonium (**6a–b**) or a trimethylarsonium **7** polar head, for gene delivery (Scheme 1). For comparative purposes, compound **2** (characterized by a trimethylammonium headgroup but with the same spacer as compounds **6a** and **7**) was also evaluated, as well as compound **3** (where the spacer is slightly longer than in compound **2**).[21] The replacement of the trimethylammonium group by a trimethylphosphonium or trimethylarsonium group was based on our recent work showing that this modification was beneficial as it led both to an increased transfection efficiency and to a reduced cytotoxicity in a series of lipophosphoramidate vectors[15,22] Thus, with a view to extend our previous observations, we aimed in the present work to evaluate in particular the effects of such a modification in the case of lipothiophosphoramidates, another class of cationic lipids for gene transfection.

2. Results and discussion

The synthesis of the lipothiophosphoramidates **6a–b** and **7** which are characterized, respectively, by the presence of a trimethylphosphonium or trimethylarsonium polar head is shown in Fig. 1. *O*,*O*-Dioleylchlorothiophosphate **1** and the cationic lipid **2** and **3** were obtained following a previously reported procedure.[21] Briefly, the reaction of **1** with *N*,*N*-dimethyl-1,2-ethanediamine, followed by the quaternarization of the tertiary amine, produced the cationic lipothiophosphoramide **2**. The modification of the cationic polar head was achieved *via* a three step sequence from compound **1** (Fig. 1). First, the reaction of **1** with either 2-bromoethylamine or 3-bromopropylamine produced respectively compounds **4a** or **4b**. Next, a halogen exchange (Finkelstein reaction) produced the iodide



4 X = Br **5** X = I
4a n= 2 **5a** n= 2
4b n= 3 **5b** n= 3
2 (BSV17) n=2
3 (BSV44) n=3
6a (BSV14) n = 2 ; Z = P
6b (BSV28) n = 3 ; Z = P
7 (BSV21) n = 2 ; Z = As

Fig. 1 Synthetic pathway of trimethylamonium-lipothiophosphoramidates **2** and **3** trimethylphosphonium-lipothiophosphoramidates **6a–b** and trimethylarsonium-lipothiophosphoramidate **7**. (i) $H_2N(CH_2)_nN(CH_3)_2$, DIPEA, CH_2Cl_2, 0 °C→RT, overnight, 85%; (ii) CH_3I, Et_2O, RT, 2 days, 80%. (iii) $HBr.H_2N(CH_2)_nBr$, DIPEA, CH_2Cl_2, 0 °C→RT overnight, 83% for n = 2, quantitative for n = 3; (iv) NaI, acetone, RT, 3 days, 80% for n = 2, overnight, 90% for n = 3; (v) Z = As, $AsMe_3$, 40 °C 12 days, 27%; Z = P, PMe_3, toluene, RT, 3 days, 56% for n = 2, 5 days, 53% for n = 3.

intermediates **5a** and **5b**. Finally, in a last step, **5a** or **5b** reacted with trimethylphosphine or trimethylarsine, thereby giving the trimethylphosphonium derivatives **6a–b** and the trimethylarsonium **7**. It is worth noting that the conditions, for this last step, have been optimized and changed when compared to the synthesis of phosphoramidates analogues. Indeed, heating at reflux (50 °C), especially for the synthesis of **7**, produced unexpected compounds that could be due to the thiophilicity of trimethylarsine. This problem was solved by carrying out the reaction at 40 °C. Consequently, the amount of trimethylarsine used was increased to get a full conversion.

Liposomal solutions were prepared by hydration of a lipid film prepared from one of the cationic lipids reported in Scheme 1. After a hydration period, the solutions were vortexed and sonicated. The time of hydration was optimized since 24 h (at 4 °C), which is the usual time with many vectors, did not produce homogenous liposomal solutions. We have previously observed such difficulties in hydration with the lipothiophosphoramidates possessing a trimethylammonium polar head. Here also, the use of a longer hydration time (> 5days) solved these difficulties for hydration of compounds **2**, **3**, **6a** and **6b**. Note, the presence of the P═S bond, which is much less polar than the P═O bond present in the phosphoramidate series of compounds previously studied by our research team,[18,20] is likely to be the reason underlying the longer hydration times required for the lipothiophosphoramidate family of cationic lipids (see ESI-1†); the low polarity of the P═S bond probably does not favor the formation of electrostatic interactions and hydrogen bonds with water. For compound **7**, the preparation of liposomal solutions by the hydration of a lipid film was unsuccessful however long the hydration time (up to 45 days). In that

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

Liposomes	Size (nm)	Polydispersity index	Zeta potential (mV)
2	189	0.188	25
6a	253	0.416	34
6b	132	0.502	37
7	241	0.152	60
Lipoplexes CR = 1	Size (nm)	Polydispersity index	Zeta potential (mV)
2	256	0.187	−39
6a	219	0.259	−35
6b	201	0.156	−24
7	>800	—	19
Lipoplexes CR = 2	Size (nm)	Polydispersity index	Zeta potential (mV)
2	282	0.103	−25
6a	229	0.169	−38
6b	264	0.192	14
7	320	0.200	32
Lipoplexes CR = 4	Size (nm)	Polydispersity index	Zeta potential (mV)
2	>800	—	16
6a	>800	—	−5
6b	122	0.287	39
7	206	0.160	40

case, the cumulative effects resulting from the presence of both a low polar P═S bond and a cationic charge "diluted" in a larger volume (because of the larger size of the arsenic atom[22] when compared to a phosphorus or a nitrogen atom), may explain why hydration of cationic lipid **7** was not achieved. Hence, we formulated compound **7** with the neutral helper lipid DOPE (dioleylphosphatidylethanolamine) in a 1 : 1 molar ratio as the co-lipid should help with the hydration process. Accordingly, homogeneous liposomal solutions were obtained. Nevertheless, formulation of compound **7** alone was successfully achieved by using ethanolic injection instead of using the hydration of a lipid film. Consequently, the aqueous liposomal solutions contained only small amounts of ethanol that was not removed before the *in vitro* transfection assays. All the liposomal and lipoplexe solutions prepared were characterized by dynamic light scattering (DLS – size analyser) and zeta potential determination (see ESI-2†); a selection of data is reported in Table 1 (see also ESI†). As indicated, the size and zeta potential of the liposomes and lipoplexes (prepared at the different theoretical charge ratios (CR)[24] of 1, 2 and 4) ranged from 130 to 300 nm. These data are consistent with the size of liposomes and lipoplexes prepared from other lipothiophosphoramidates[21] or lipophosphoramidates.[18] Notably, in many cases, the zeta potential is negative up to a theoretical charge ratio of 2, a fact which might be explained by a partial compaction of the pDNA. Similar observations have been made with other classes of vectors.[23] The capability of lipothiophosphoramidates to bind pDNA was evaluated by agarose gel electrophoresis shift assay at different theoretical charge ratios (Fig. 2). For compound **7** (formulated alone), a full compaction of pDNA is obtained at CR = 8 while in the other cases, even at such a high CR, pDNA was not fully compacted. Nevertheless, a great difference of compaction was usually observed between CR = 2 and 4, with most of the plasmid DNA being condensed by the cationic lipid at CR = 4. The gene transfection efficacy of these solutions of lipoplexes was then evaluated on three human cell lines (Fig. 3). In this series of transfection experiments, compound **2** (lipophosphoramidate with a trimethylammonium polar head) and the commercial standard cationic lipid Lipofectamine™ (LFM) were used as controls. Each compound was evaluated at different charge ratios. Compound **7** was tested when formulated alone or with DOPE (The results with DOPE are shown in the ESI†). With **7** formulated alone, the best transfection efficacy was observed at CR = 1 or 2. At these CRs mixing with DOPE at a 1 : 1 molar ratio produced the same transfection efficacy or improved it for one cell line (HeLa) (See ESI-3†). Interestingly, the transfection level was high and, at comparable charge ratio, better than when using the reference compound **2** or Lipofectamine. In some cases (A549 and 16HBE14o(−)), its efficacy was even 100 times higher than Lipofectamine (*e.g.* at CR = 2). For the lipothiophosphoramidates bearing a trimethylphosphonium polar head

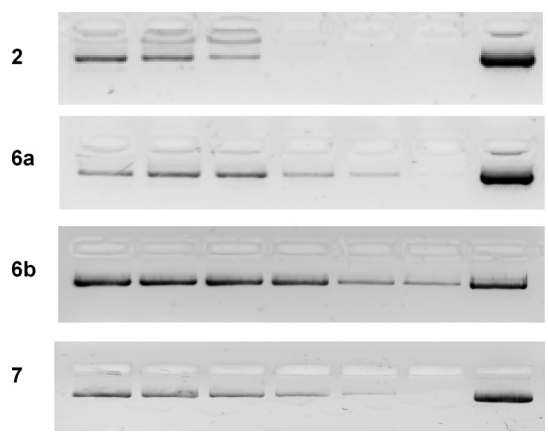


Fig. 2 Agarose gel electrophoresis shift assay of lipoplexes prepared by mixing lipothiophosphoramide and pDNA (9.6kb, pCMV-Luc encoding firefly luciferase protein) at different theoretical charge ratios.

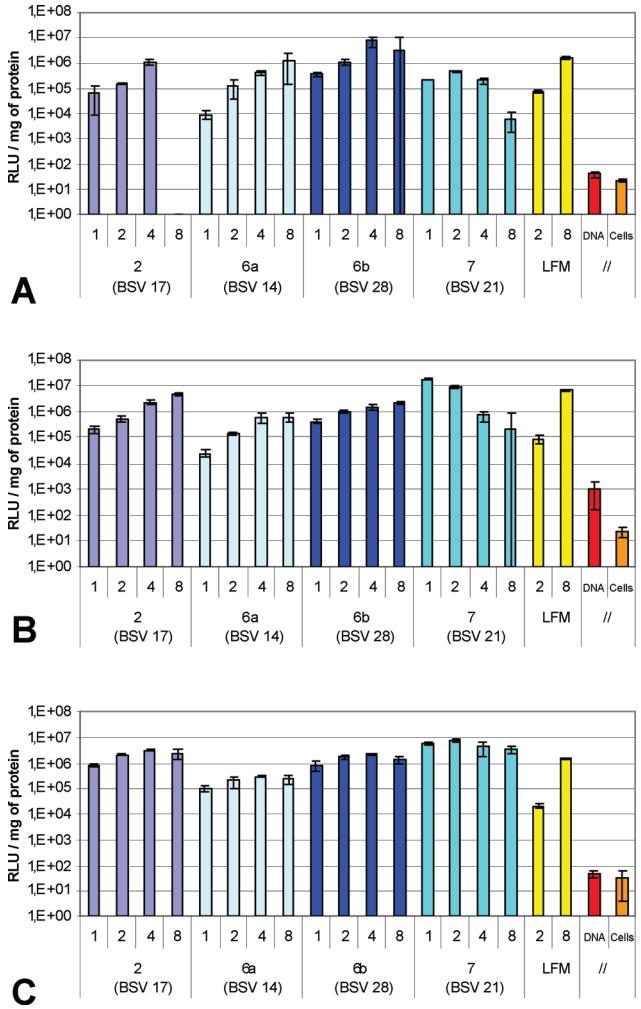


Fig. 3 *In vitro* transfection efficiency of **2** (BSV17), **6a** (BSV14), **6b** (BSV28), **7** (BSV21), Lipofectamine (used as reference), DNA alone and untransfected cells are used as negative controls. The results are expressed as a function of the charge ratio (1, 2, 4 or 8). **A**-HeLa cell line. **B**- A549 cell line; **C**- 16HBE14o(−) cell line.

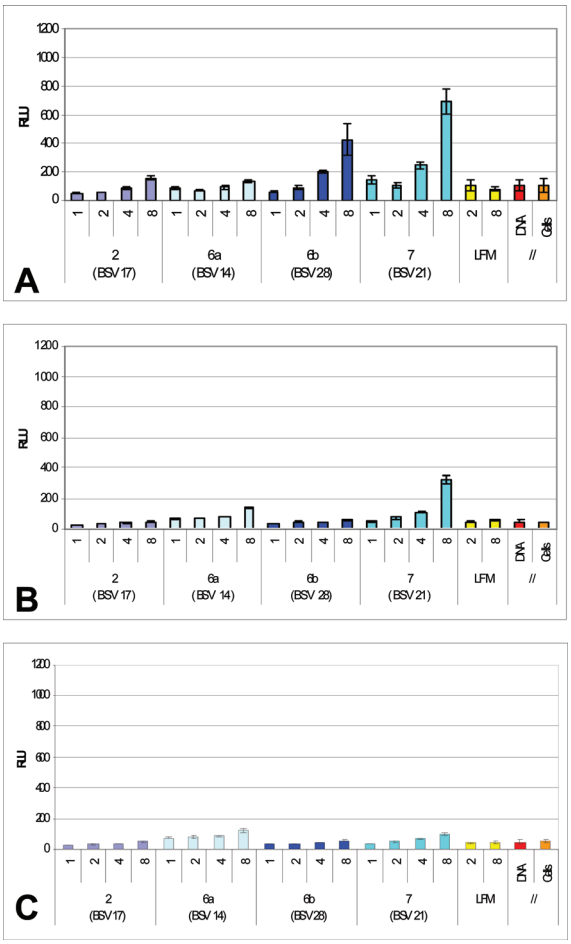


Fig. 4 *In vitro* early toxicity of **2** (BSV17), **6a** (BSV14), **6b** (BSV28), **7** (BSV21), Lipofectamine (used as reference), DNA alone and untransfected cells are used as negative controls. The results are expressed as a function of the charge ratio (1, 2, 4 or 8). **A**-HeLa cell line. **B**- A549 cell line; **C**- 16HBE14o(−) cell line.

(compounds **6a** and **6b**), high transfection efficacies were also observed. In both cases, the transfection efficacies were, at low theoretical charge ratios, better than those obtained with Lipofectamine. The comparison of the transfection efficacy of vectors **6a** and **6b** revealed that the number of carbon atoms between the thiophosphoramidate functional group and the cationic polar head had only a weak influence. Most importantly, with these two vectors, high transfection efficacies were observed for the A549 and 16HBE14o(−) cell lines even at very low theoretical CR. The influence of the nature of the cationic polar head on the transfection efficacy does not lead to any general conclusion since the results are cell line-dependant.

Nevertheless, it should be stressed that the cationic lipid **7**, with a trimethylarsonium was globally very efficient, when it was formulated alone or with DOPE at a CR = 2, and was more efficient than the trimethylammonium analogue **2**.

The evaluation of the cytotoxicity (through the measurement of the leakage from damaged cells of a normally cytoplasmic enzyme) induced by the cationic lipids **2** and **6a–b** and **7** is presented in Fig. 4. These results show that only minor differences exist when comparing this series of lipothiophosphoramidate compounds. Schematically, below a charge ratio of 4, a limited to very limited toxicity was evidenced (in the same range as that observed for Lipofectamine) while at a higher CR, significant toxicity appeared. Furthermore, it was observed that HeLa cells were more sensitive than the two other cell lines tested. This observation contrasts with the toxicity induced by the lipophosphoramidates series of vectors since no extra toxicity on HeLa cells was previously observed.[21] Nevertheless at the lowest efficient theoretical charge ratio (*e.g.* 1 and 2) the toxicity is as low as it is for LFM or lipophosphoramidate compounds. The observation that, when increasing the CR, a progressive reduction of the transfection efficiency was accompanied with an increase of the cytotoxicity (which is clearly evidenced from a CR4 on) clearly indicates that compound **7** is a very interesting transfection reagent for which the best compromise (high TE with no or low cytotoxicity) is already reached at a quite low CR (actually close to the theoretical neutrality) above which side-effects may override the efficiency. This interesting finding suggests that efficient gene transfer activities may be obtained

with reduced amounts of vectors, with the obvious important consequence being a potential reduction of undesirable toxic effects in more complex biological contexts, such as those encountered when performing *in vivo* gene transfection.

3. Conclusion

We report here the synthesis of cationic lipothiophosphoramidates bearing either a trimethylphosphonium or a trimethylarsonium cationic moiety. These compounds can easily be obtained from the dioleylchlorothiophosphate **1** in three steps. Their formulation as liposomes, which required a long hydration duration or the use of ethanolic injection, leads to nano-objects characterized by an average size ranging from 130 to 320 nm. Such a long hydration time may be related to the presence of the thiophosphoramidate functional group. As regards to the complexation of our phosphonium or arsonium-based lipids with pDNA, the results of a series of assays (agarose gel electrophoresis, size and zeta potential measurements) showed that a supramolecular complex was formed but that full DNA compaction was only reached at high charge ratios (CRs). However, despite the partial compaction of pDNA at CR = 1, 2 or 4, efficient gene transfection into three different human epithelial cell lines was obtained with the phosphonium **6a–b** and arsonium **7** cationic lipids and could thus be compared with the transfection activity of compound **2**, a vector characterized by a trimethylammonium polar head. The phosphonium derivatives had similar (or even higher) (derivative **6b**) or slightly lower (derivative **6a**) transfection efficiencies at comparable CR to their ammonium counterpart **2**. On the other hand, the trimethylarsonium-based cationic lipid **7** was more efficient, especially at low CRs, than compound **2**. Our study showed in particular that the arsonium derivative **7** (formulated alone or in association with DOPE) was the best vector at CR = 2. Note that the transfection efficacy observed with lipid **7** was better than that of the commercial reagent Lipofectamine used here as a positive control. Finally, the cytotoxicity of this series of vectors appeared to be dependent upon the cell line considered but only low toxicities were observed at the optimal charge ratio of 2. To further assess the potential of our novel vectors, we are at present performing *in vivo* mouse experiments with the view to evaluate their *in vivo* gene transfection efficacy. In a broader perspective, our study suggests that trimethylphosphonium and trimethytrimethylarsonium may constitute two types of cationic polar heads that are worth consideration for the development of efficient nonviral vectors for gene delivery.

4 Experimental

4.1 General

Solvents were dried with a solvent purification system MBraun-SPS (THF, CH_2Cl_2). All compounds were fully characterized by ^1H (500.13 or 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). The lipids were purified by flash chromatography (GRACE REVELERIS Flash Chromatography System) equipped with UV and DLS detectors allowing us to attest the high purity (> 95%) of the purified compounds. 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 and ^{31}P 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, 2-bromoethylamine hydrobromide, 3-bromopropylamine hydrobromide, sodium iodide trimethylphosphine, trimethylarsine) were used as received except DIPEA which was distilled over KOH. *O*,*O*-dioleylchlorothiophosphate **1**, 2-(*O*,*O*-dioleylthiophosphatidyl)-ethyltrimetylammonium iodide **2** and 3-(*O*,*O*-dioleylthiophosphatidyl)-propyltrimetylammonium iodid **3** have been synthesized according to a reported procedure.[21] 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*-(2-bromoethyl) thiophosphoramidate 4a

DIPEA (410 μL; 2.5 mmol; 2.5 eq.) was added under N_2 atmosphere to a solution of 2-bromoethylamine hydrobromide (307 mg; 1.5 mmol; 1.5 eq.) in 20 mL of dry CH_2Cl_2. The mixture was cooled to 0 °C and a solution of chlorothiophosphate **1** (632 mg; 1.0 mmol; 1 eq.) in 5 mL of dry CH_2Cl_2 was added. The solution was stirred for one night while the temperature was slowly increased up to RT. The mixture was washed with brine (3 × 30 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The expected compound was afforded as a colorless oil in 83% yield. ^1H NMR (500.303 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H\text{-}H}$ = 7.0 Hz, C*H*$_3$-CH$_2$), 1.20–1.40 (44H, m, C*H*$_2$ fatty chain), 1.66 (4H, m, *CH*$_2$-CH$_2$-O), 2.00 (8H, m, C*H*$_2$-CH=CH-C*H*$_2$), 3.38 (2H, m, NH-C*H*$_2$), 3.43 (2H, m, C*H*$_2$-Br), 3.98 (4H, m, C*H*$_2$-O), 5.35 (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$_2$), 27.3 (s, *C*H$_2$-CH=CH-CH$_2$ and/or CH$_2$-CH=CH-*C*H$_2$), 30.1 (d, $^3J_{P\text{-}C}$ = 7.5 Hz, *C*H$_2$-CH$_2$-O), 32.0 (s, CH$_3$-CH$_2$-*C*H$_2$), 25.5 to 32.7 (*C*H$_2$ fatty chain), 34.1 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, *C*H$_2$-Br), 43.5 (s, NH-*C*H$_2$), 67.3 (d, $^2J_{P\text{-}C}$ = 6.3 Hz, *C*H$_2$-O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.3; MS (ESI): *m*/*z* calcd for $C_{38}H_{76}INO_2PS$ (M-Br + OH + H^+) 658; found 658.

4.3 Synthesis of *O*,*O*-dioleyl-*N*-(3-bromopropyl) thiophosphoramidate 4b

DIPEA (410 μL; 2.5 mmol; 2.5 eq.) was added under N_2 atmosphere to a solution of 3-bromopropylamine hydrobromide (328 mg; 1.5 mmol; 1.5 eq.) in 20 mL of dry CH_2Cl_2. The mixture was cooled to 0 °C and a solution of chlorothiophosphate **1** (632 mg; 1.0 mmol; 1 eq.) in 5 mL of dry CH_2Cl_2 was added. The solution was stirred for one night while the

temperature was slowly increased up to RT. The mixture was washed with brine (3 × 30 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated. The expected compound is afforded as a colorless turbid oil in a quantitative yield. ^{1}H NMR (300.135 MHz; $CDCl_3$): 0.87 (6H, t, $^3J_{H\text{-}H}$ = 6.6 Hz, C*H*$_3$-CH$_2$), 1.20–1.40 (44H, m, C*H*$_2$ fatty chain), 1.66 (4H, qt,$^3J_{H\text{-}H}$ = 6.8 Hz C*H*$_2$-CH$_2$-O), 2.01 (10 H, m, C*H*$_2$-CH=CH-C*H*$_2$ and NH-CH$_2$-C*H*$_2$), 3.10 (2H, dt,^{3}J$_{H\text{-}H}$ = ^{3}J$_{P\text{-}H}$ = 6.6 Hz, NH-C*H*$_2$), 3.46 (2H, t,$^3J_{H\text{-}H}$ = 6.4 Hz, C*H*$_2$-Br), 3.98 (4H, m, C*H*$_2$-O), 5.35 (4H, m, C*H*=C*H*). ^{13}C NMR (100.625 MHz; $CDCl_3$): 14.1 (s, *C*H$_3$-CH$_2$), 22.7 (s, CH$_3$-*C*H$_2$), 27.2 (s, *C*H$_2$-CH=CH-CH$_2$ and/or CH$_2$-CH=CH-*C*H$_2$), 30.1 (d, ^{3}J$_{P\text{-}C}$ = 8.0 Hz, *C*H$_2$-CH$_2$-O), 30.4 (s, NH-CH$_2$-*C*H$_2$), 31.9 (s, CH$_3$-CH$_2$-*C*H$_2$), 25.5 to 32.6 (*C*H$_2$ fatty chain), 33.9 (s, *C*H$_2$-Br), 39.9 (s, NH-*C*H$_2$), 67.0 (d, ^{2}J$_{P\text{-}C}$ = 6.3 Hz, *C*H$_2$-O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.5.

4.4 Synthesis of *O*,*O*-dioleyl-*N*-(iodoethyl)thiophosphoramidate 5a

Sodium iodide (200 mg; 1.33 mmol; 2 eq.) was added to a solution of *O*,*O*-dioleyl-*N*-(2-bromoethyl)thiophosphoramidate **4a** (482 mg; 0.67 mmol; 1 eq.) in 5 mL of acetone. The mixture was stirred at RT for 3 days. Acetone was removed. The residue was dissolved in petroleum ether (75 mL) and filtered on celite. The filtrate was concentrated and the expected compound was afforded as a colorless oil in 80% yield. ^{1}H NMR (400.130 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H\text{-}H}$ = 6.7 Hz, C*H*$_3$-CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.65 (4H, m, C*H*$_2$-CH$_2$-O), 2.00 (8H, m, C*H*$_2$-CH=CH-C*H*$_2$), 3.22 (2H, m, C*H*$_2$-I), 3.32 (2H, m, NH-C*H*$_2$), 3.98 (4H, m, C*H*$_2$-O), 5.36 (4H, m, C*H* = C*H*). ^{13}C NMR (75.480 MHz; $CDCl_3$): 7.4 (s, *C*H$_2$-I), 14.1 (s, *C*H$_3$-CH$_2$), 22.7 (s, CH$_3$-*C*H$_2$), 27.2 (s, *C*H$_2$-CH=CH-CH$_2$ and/or CH$_2$-CH=CH-*C*H$_2$), 30.0 (s, *C*H$_2$-CH$_2$-O), 31.9 (s, CH$_3$-CH$_2$-*C*H$_2$), 18.6 to 31.9 (*C*H$_2$ fatty chain), 44.2 (s, NH-*C*H$_2$), 67.3 (s, *C*H$_2$-O), 129.8 and 130.0 (s, *C*H=CH and CH = *C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.0. HRMS (ESI-TOF): *m*/*z* calcd for $C_{38}H_{76}INO_2PS$ (M+H) 768.4379; found 768.4394.

4.5 Synthesis of *O*,*O*-dioleyl-*N*-(iodopropyl) thiophosphoramidate 5b

Sodium iodide (300 mg; 2.0 mmol; 2 eq.) was added to a solution of *O*,*O*-dioleyl-*N*-(3-bromopropyl)thiophosphoramidate **4b** (735 mg; 1.0 mmol; 1 eq.) in 15 mL of acetone. The mixture was stirred at RT over night. Acetone was removed. The residue was dissolved in ether petroleum (60 mL) and filtered on celite. The filtrate was concentrated and the expected compound was afforded as a colorless turbid oil in a 90% yield. ^{1}H NMR (300.135 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H\text{-}H}$ = 6.6 Hz, C*H*$_3$-CH$_2$), 1.20-1.40 (44H, m, C*H*$_2$ fatty chain), 1.66 (4H, m, *C*H$_2$-CH$_2$-O), 2.01 (10 H, m, C*H*$_2$-CH=CH-C*H*$_2$ and NH-CH$_2$-C*H*$_2$), 3.06 (2H, m, NH-C*H*$_2$), 3.22 (2H, t,$^3J_{H\text{-}H}$ = 6.7 Hz C*H*$_2$-I), 3.98 (4H, m, C*H*$_2$-O), 5.35 (4H, m, C*H*=C*H*). ^{13}C NMR (100.625 MHz; $CDCl_3$): 2.9 (s, CH2-I), 14.1 (s, *C*H$_3$-CH$_2$), 22.7 (s, CH$_3$-*C*H$_2$), 27.2 (s, *C*H$_2$-CH=CH-CH$_2$ and/or CH$_2$-CH=CH-*C*H$_2$), 30.1 (d, ^{3}J$_{P\text{-}C}$ = 8.0 Hz, *C*H$_2$-CH$_2$-O), 31.9 (s, CH$_3$-CH$_2$-*C*H$_2$), 25.5 to 32.6 (*C*H$_2$ fatty chain), 34.6 (s, NH-CH$_2$-*C*H$_2$), 41.9 (s, NH-*C*H$_2$), 67.1 (d, ^{2}J$_{P\text{-}C}$ = 6.3 Hz, *C*H$_2$-O), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$): 72.5.

4.6 Synthesis of 2-*N*-(*O*,*O*-dioleylthiophosphatidyl)-ethyltrimethylphosphonium iodide 6a

A solution of trimethylphosphine in toluene (1 M) (4 mL; 4 mmol; 2 eq.) was added under N_2 atmosphere on *O*,*O*-dioleyl-*N*-(iodoethyl)thiophosphoramidate **5a** (1.536 g; 2 mmol; 1 eq.). The mixture was stirred at RT for 3 days. Toluene and the excess of trimethylphosphine were removed under vacuum. The trimethylphosphine oxide was removed by Kugelrohr distillation (120 °C, 3 × 10^{-2} torr). Purification by column chromatography on silica gel (chloroform and methanol gradient 98 : 2 to 90 : 10) afforded the expected compound as a yellowish wax in 56% yield. ^{1}H NMR (300.135 MHz; $CDCl_3$): 0.88 (6H, t, $^3J_{H\text{-}H}$ = 6.7 Hz, C*H*$_3$-CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.63 (4H, m, C*H*$_2$-CH$_2$-O), 2.00 (8H, m, C*H*$_2$-CH=CH-C*H*$_2$), 2.21 (9H, d, $^2J_{H\text{-}P}$ = 14.1 Hz, $^+$P(C*H*$_3$)$_3$), 3.00 (2H, m, C*H*$_2$-$^+$P(CH$_3$)$_3$), 3.50-3.65 (2H, m, NH-C*H*$_2$), 3.98 (4H, dt, $^3J_{H\text{-}H}$ = 6.8 Hz and ^{3}J$_{H\text{-}P}$: 8.3 Hz, C*H*$_2$-O), 4.51 (1H, m, N*H*), 5.36 (4H, m, C*H*=C*H*). ^{13}C NMR (125.773 MHz; $CDCl_3$): 10.5 (d, $^1J_{C\text{-}P}$ = 54.2 Hz, $^+$P(*C*H$_3$)$_3$), 14.2 (s, *C*H$_3$-CH$_2$), 22.7 (s, CH$_3$-*C*H$_2$), 26.0 (s, *C*H$_2$-$^+$P(CH$_3$)$_3$), 27.3 (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$_2$), 29.0 to 32.7 (*C*H$_2$ fatty chain), 35.6 (s, NH-*C*H$_2$), 67.7 (s, O-*C*H$_2$), 129.8 and 130.0 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$): 25.9 ($^+$*P*(Me)$_3$), 73.1 ((RO)$_2$*P*(S)NH). HRMS (ESI-TOF): *m*/*z* calcd for $C_{41}H_{84}NO_2P_2S$ (M^+) 716.5698; found 716.5700.

4.7 Synthesis of 3-*N*-(*O*,*O*-dioleylthiophosphatidyl)-propyltrimethylphosphonium iodide 6b

A solution of trimethylphosphine in toluene (1 M) (2 mL; 2 mmol; 2.2 eq.) was added under N_2 atmosphere to *O*,*O*-dioleyl-*N*-(iodopropyl)thiophosphoramidate **5b** (705 mg; 0.9 mmol; 1 eq.). The mixture was stirred at RT for 5 days. Toluene and the excess of trimethylphosphine were removed under vacuum. The trimethylphosphine oxide was removed by Kugelrohr distillation (120 °C, 3 × 10^{-2} torr). Purification by column chromatography on silica gel (chloroform and methanol gradient 98 : 2 to 90 : 10) afforded the expected compound as a yellowish wax in 53% yield. ^{1}H NMR (400.089 MHz; $CDCl_3$): 0.85 (6H, t, $^3J_{H\text{-}H}$ = 6.8 Hz, C*H*$_3$-CH$_2$), 1.10–1.30 (44H, m, C*H*$_2$ fatty chain), 1.63 (4H, m, C*H*$_2$-CH$_2$-O), 2.00 (10H, m, C*H*$_2$-CH=CH-C*H*$_2$ and NH-CH$_2$-C*H*$_2$), 2.19 (9H, d, $^2J_{H\text{-}P}$ = 14.0 Hz, $^+$P(C*H*$_3$)$_3$), 2.57 (2H, m, C*H*$_2$-$^+$P(CH$_3$)$_3$), 3.13 (2H, m, NH-C*H*$_2$), 3.84 (1H, m, N*H*), 3.93 (4H, dt, $^3J_{H\text{-}H}$ = $^3J_{H\text{-}P}$ = 6.8 Hz, C*H*$_2$-O), 5.35 (4H, m, C*H*=C*H*).^{13}C NMR (75.478 MHz; $CDCl_3$): 9.8 (d, $^1J_{C\text{-}P}$ = 54.6 Hz, $^+$P(*C*H$_3$)$_3$), 14.3 (s, *C*H$_3$-CH$_2$), 21.8 (s, *C*H$_2$-$^+$P(CH$_3$)$_3$), 22.8 (s, CH$_3$-*C*H$_2$), 23.6 (s, NH-CH$_2$-*C*H$_2$), 27.4 (s, *C*H$_2$-CH=CH-CH$_2$ and/or CH$_2$-CH=CH-*C*H$_2$), 30.3 (d, $^3J_{P\text{-}C}$ = 7.6 Hz, *C*H$_2$-CH$_2$-O), 32.1 (s, CH$_3$-CH$_2$-*C*H$_2$), 21.1 to 32.8 (*C*H$_2$ fatty chain), 41.6 (d,$^2J_{C\text{-}P}$ = 15.8 Hz, NH-*C*H$_2$), 67.5 (d, $^2J_{P\text{-}C}$ = 4.7 Hz, *C*H$_2$-O), 130.0 and 130.2 (s, *C*H=CH and CH=*C*H). ^{31}P NMR (121.498 MHz; $CDCl_3$):

27.8 ($^{+}P(Me)_3$), 72.9 ((RO)$_2P$(S)NH). HRMS (ESI-TOF): *m/z* calcd for $C_{42}H_{86}NO_2P_2S$ (M^+) 730.5855; found 730.5864.

4.8 Synthesis of 2-*N*-(*O*,*O*-dioleylthiophosphatidyl)-ethyltrimethylarsonium iodide 7

In a reactor equipped with an efficient reflux condenser (-20 °C), trimethylarsine (550 µL; 5.2 mmol; 4 eq.) was added under N_2 atmosphere to *O*,*O*-dioleyl-*N*-(iodoethyl)thiophosphoramidate **5a** (1.00g; 1.3 mmol; 1 eq.). The mixture was stirred at 30-40 °C for 12 days. After cooling to RT, the excess of trimethylarsine was removed under vacuum. The residue was dissolved in n-hexane and the supernatant liquid was collected (three times). N-hexane was removed and a purification by column chromatography on silica gel (chloroform and methanol gradient 99 : 1 to 90 : 10) afforded the expected compound as a white wax in 27% yield. ^{1}H NMR (400.133 MHz; $CDCl_3$): 0.87 (6H, t, $^3J_{H\text{-}H}$ = 8.0 Hz, CH_3-CH_2), 1.20–1.40 (44H, m, CH_2 fatty chain), 1.64 (4H, m, CH_2-CH_2-O), 1.99 (8H, m, CH_2-CH=CH-CH_2), 2.24 (9H, s, $^{+}As(CH_3)_3$), 3.19 (2H, m, CH_2-$^{+}As(CH_3)_3$), 3.58 (2H, m, NH-CH_2), 3.98 (4H, dt, $^3J_{H\text{-}H}$ = 8.0 Hz and $^3J_{H\text{-}P}$ = 8.0 Hz, CH_2-O), 4.54 (1H, m, N*H*), 5.34 (4H, m, C*H* = C*H*). ^{13}C NMR (100.625 MHz; $CDCl_3$): 10.0 (s, $^{+}As(CH_3)_3$), 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), 29.2 (s, CH_2-$^{+}As(CH_3)_3$), 30.2 (d, $^3J_{P\text{-}C}$ = 7.0 Hz, CH_2-CH_2-O), 31.9 (s, CH_3-CH_2-CH_2), 20.6, to 32.7 (CH_2 fatty chain), 36.7 (s, NH-CH_2), 67.7 (d,$^2J_{P\text{-}C}$ = 5.0 Hz, CH_2-O), 129.8 and 130.0 (s, CH=CH and CH=CH). ^{31}P NMR (161.985 MHz; $CDCl_3$): 73.4. HRMS (ESI-TOF): *m/z* calcd for $C_{41}H_{84}AsNO_2PS$ (M^+) 760.5176; found 760.5178

4.9 Liposome formulation and DNA binding

The cationic lipids **2**, **6a–b** and **7** + DOPE were formulated as liposomes by using the film hydration technique. First a concentrated solution of the desired lipid in chloroform (*e.g.* **6b**, 12.5 mM, 119.6 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 (sSee ESI-1†) the solution was vortexed (1 min) and sonicated (*e.g.* **6b**, 5 × 10 min) at 45 kHz using an ultrasonic bath (VWR).

For cationic lipid **7**, liposomes were formulated by using the ethanolic injection method to obtain a liposomal solution with a concentration of 1.5 mmol L^{-1}. Next, 25 µL of a concentrated solution of desired the lipid in ethanol (*e.g.* 59.3 mM, 1.5 mL) were injected in 975 µL of water with smooth stirring. The solution was vortexed (1 min) and sonicated (2 × 10 min) at 45 kHz. The size and the zeta potential of these formulations were determined for each liposomal solution. (see Table 1).

These liposomal solutions were mixed with pDNA as follows: to 1 µg of pDNA in Opti-MEM® (Gibco®) were added cationic lipids at concentrations corresponding to ± charge ratios ranging from 1 to 8. These mixtures were incubated for 30 min at RT. 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}) in order to be thereafter visualized using an UV transilluminator (Fisher Bioblock).

4.10 *In vitro* reporter gene measurements: luciferase assays

The protocol of the experiments was similar to those previously reported.[21] Briefly, cells were seeded 24 h before transfection onto a 24-well plate at a density of 100,000 cells per well and incubated overnight in a humidified 5% CO2 atmosphere at 37 °C. About 200 µL of a solution of lipoplex was added to each well. Lipofectamine (LFM) was used as a positive control. After 2 h 30 min of incubation at 37 °C, 20 µL of the surrounding was picked up to evaluate the toxicity of each formulation (see below) The culture medium was removed to be replaced with fresh. Following a further 48 h incubation at 37 °C, the cells were lysed in order to be assayed for luciferase expression using a chemiluminescent assay (Luciferase Assay System, Promega). The total protein content of the cell lysate 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).

4.11 Evaluation of the toxicity

The toxicity of the different lipid/DNA complexes was determined by using a chemiluminescent assay (ToxiLight™ - Cambrex, Liège, Belgium). Briefly, this test consists of the measurements of the release of a normally cytoplasmic enzyme (adenylate kinase, AK) from damaged cells into the surrounding medium. The reaction involves two steps. The first one requires 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) are conversely proportional to the intensity of damage induced to the cells consequent of their exposure with the complexes studied. Untransfected cells were used to evaluate the AK background activity indicative of an absence of toxicity.

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