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A Novel Cationic Lipophosphoramide with Diunsaturated Lipid Chains: Synthesis, Physicochemical Properties, and Transfection Activities

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Cationic lipophosphoramidates constitute a class of cationic lipids we have previously reported to be efficient for gene transfection. Here, we synthesized and studied a novel lipophosphoramidate derivative characterized by an arsonium headgroup linked, via a phosphoramidate linker, to an unconventional lipidic moiety consisting of two diunsaturated linoleic chains. Physicochemical studies allowed us to comparatively evaluate the specific fluidity and fusogenicity properties of the liposomes formed. Although corresponding lipoplexes exhibited significant but relatively modest *in vitro* transfection efficiencies, they showed a remarkably efficient and reproducible ability to transfect mouse lung, with *in vivo* transfection levels higher than those observed with a monounsaturated analogue previously described. Thus, these results demonstrate that this diunsaturated cationic lipophosphoramidate constitutes an efficient and versatile nonviral vector for gene transfection. They also invite further evaluations of the transfection activity, especially *in vivo*, of gene delivery systems incorporating the lipid reported herein and/or other lipids bearing polyunsaturated chains.

Introduction

The design of synthetic vectors for gene delivery has been a fast growing field of research since the pioneering work of Felgner and colleagues.[1] To date, numerous studies have reported a wide range of chemical structures able to complex and transfer nucleic acids into different cell types.[2] These compounds are generally subdivided into two categories: cationic lipids[3−6] (often combined with neutral lipids)[7−10] and cationic polymers,[11] the complexes formed with DNA being called lipoplexes and polyplexes, respectively. With regard to the first category of synthetic vectors, their transfection activity depends not only on the structure of the amphiphilic vector (schematically a cationic headgroup linked to a hydrophobic moiety via a spacer) but also on its formulation, e.g., the incorporation of a helper lipid into micelles or liposomes. Indeed, the design of formulations incorporating natural neutral colipids (e.g., DOPE[a]),[12] or especially the design of helper lipids,[8−10] is a widely used strategy which can, in numerous cases, improve gene transfection activity. On the other hand, the improvement of gene transfection efficiency can also be the result of a fine-tuning of the chemical structure of the vector itself. Such an approach is of particular interest when one tries to evaluate the impact of the various structural parts of a given vector family on transfection activity.[13] The identification of structure−activity relationships is indeed an acknowledged approach for the design of new vectors with improved gene transfection activities. Structure−activity investigations have thus been undertaken to elucidate the effect of the different parts of the cationic lipids on their transfection activity. For example, it has been shown that multivalent cationic lipids incorporating a dendritic backbone were able to reach superior gene transfections activities.[14] Similarly, the important role played by gemini lipid systems in mediating efficient gene transfection has also recently been demonstrated.[15−19]

Over the past decade, our laboratory has undertaken to investigate the potential of lipophosphonates as gene delivery vectors. We initially reported that such cationic lipids exhibited significant gene transfection activities both *in vitro* and *in vivo*.[20−24] Next, we made a first structural variation involving the polar headgroup, the trimethylammonium cationic headgroup being substituted by a trimethylphosphonium or -arsonium group. This led to a series of vectors which displayed improved transfection efficiencies and decreased toxicities.[25,26] Such results were actually in agreement with a previous study demonstrating the positive effects, in terms of reduced toxicity, of the replacement of a trimethylammonium by a trimethylarsonium group for the design of antiproliferative drugs.[27] In subsequent studies, we developed a third family of vectors characterized by the substitution of the phosphonate functional group by a phosphoramidate, and we showed that those vectors displayed even better transfection properties.[28−32] In particular, a compound with a phosphoramidate group and an arsonium cationic headgroup proved to be highly efficient for *in vivo* gene delivery.[33]

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[a] Abbreviations: BSV, Brest synthetic vector; CLC, critical liposomal concentratrion; CR, charge ratio; DOPE, dioleoylphosphatidylethanolamine; FRET, Förster resonance energy transfer.

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With a view to further study the properties of that particular vector family, we herein undertook to evaluate the effects of varying the lipidic moiety of our cationic phosphoramidate derivatives. We reasoned that it would be particularly important to better understand the relationships between the lipid structure itself (saturated versus unsaturated or polyunsaturated) and the physicochemical properties of the corresponding liposomes and DNA lipoplexes, as well as their transfection activities (especially *in vivo*). It should be stressed here that numerous studies have clearly pointed out the importance of the lipidic composition of membranes for maintaining cell and organism physiological properties. For example, it is known that the plasma membrane of spermatozoids (a highly efficient vehicle for gene delivery) is enriched in polyunsaturated lipids.[34,35] Other studies have shown that the high abundance of polyunsaturated lipids in the membrane of vegetable cells is crucial for their ability to resist freezing.[36] Finally, several polyunsaturated lipids are also present in very high amounts in various sea organisms (e.g., in microalgae[37] or in scallops[38]). Taken together, these examples strongly suggest that polyunsaturated lipids might confer particular membrane properties (fluidity, fusogenicity, etc.) beneficial under special environmental conditions. This is further supported by other studies reporting that the presence of polyunsaturated lipids decreases the rigidity of cell and model membranes.[39] In particular, it has been shown that *cis* double bonds induce much larger membrane perturbations than *trans* double bonds.[40] Thus, it can be expected that the fusogenic properties of a given vector (or its formulation) may constitute an important physicochemical parameter when considering gene transfer. Accordingly, it has been stated that fusogenicity is a required property for efficient endosomal escape of internalized DNA complexes.[41,42] A commonly used method to improve the fusogenic character of a transfection formulation is the incorporation of the neutral lipid DOPE, as that helper lipid can adopt a hexagonal H_{II} phase or inverted hexagonal H_{II}^{C} phase.[43] An alternative approach may be based on the incorporation, directly into the structure of the cationic lipid, of a chemical group that could increase its fluidity or fusogenicity. Here, the use of unsaturated chains as lipidic parts of a cationic lipid might be a valuable strategy. Interestingly, a recent study by Koynova et al. has reported that the ethyl ester of oleyldecanoylethylphosphatidylcholine (C18:1/C10 EPC) was more fusogenic than its fully saturated analogue (C18:0/C10 EPC), and the increased fusion rate was correlated with an enhanced transfection efficiency.[44]

Yet, the use of polyunsaturated lipid chains in the design of synthetic gene transfection vectors has so far seldom been explored. A first study dealt with the grafting of saturated lipid chains (e.g., palmitic, C16:0, and stearic, C18:0), or unsaturated lipid chains (oleic, C18:1 Δ^9, and linoleic, C18:2 Δ^9 Δ^{12}), on the cationic polymer poly(L-lysine) (PLL);[45] the lipid chain length, rather than the unsaturation degree, was found to be a critical factor for the efficiency of *in vitro* transfection. Another study reported the synthesis of spermine derivatives substituted (via an amide bond) on each of their two central nitrogen atoms by a single lipid chain, which was either a stearic, an oleic, or a linoleic chain.[46] In *in vitro* transfection experiments, the most efficient among these compounds was the derivative bearing two linoleic chains. The authors concluded that the linoleic chains might provide the cationic lipids with some additional fluidity that could favor its phase transition from a L_α to a H_{II} supramolecular organization. Finally, a very recent work[47] has described novel gene carriers consisting of tripod-like cationic amphiphiles with only a single lipid chain. Although vectors with an oleyl, linoleyl, or linolenyl chain were studied, the results showed that the best vectors were characterized by a short saturated (C12:0 and C13:0) or a long saturated (C24:0) lipid chain.

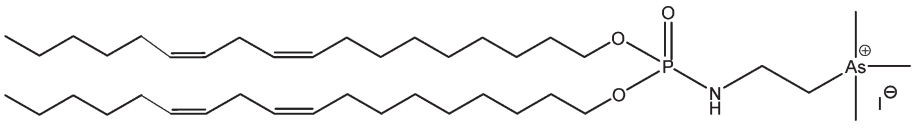
Thus, to further investigate the usefulness of such lipid structures in the design of synthetic vectors for gene transfection (in particular with regard to *in vivo* transfection), we here report the synthesis and properties of a novel lipophosphoramidate derivative (herein termed lipid **4**, but also termed BSV4 in our laboratory) characterized by a lipid moiety, consisting of two linoleic chains (C18:2 Δ^9 Δ^{12}), and by a trimethylarsonium cationic headgroup linked to the lipophosphoramidate moiety by an ethylene spacer. Fluorescence anisotropy[48] and Förster resonance energy transfer (FRET)[49] experiments revealed respectively the high fluidity and good fusogenicity properties of liposomes formed by lipid **4**. Finally, we also report the results of transfection experiments showing that lipid **4**-based liposomes are efficient for transfection, especially *in vivo*, and we discuss the relationships between physicochemical properties and transfection activities. To the best of our knowledge, this is the first report showing that a cationic lipid containing polyunsaturated lipid chains can constitute an efficient vector for *in vivo* gene transfection.

Results and Discussion

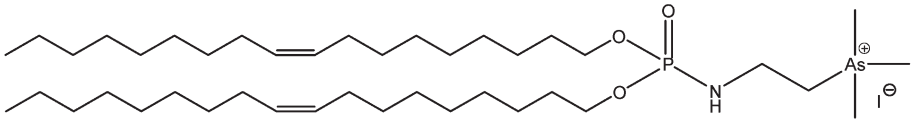
We first describe the synthesis of lipid **4** and report some of its physicochemical characteristics. Next, we report the results of *in vitro* as well as *in vivo* transfection experiments performed to assess the transfection properties of lipid **4**-based lipoplexes: (i) *in vitro*, we studied their efficacy and cytotoxicity using various human epithelial cell lines; (ii) in mice *in vivo*, we not only investigated their efficiency but also performed some biodistribution studies.

1. Synthesis of Lipid 4, a Cationic Diunsaturated Lipophosphoramidate. Lipid **4**, a cationic diunsaturated lipophosphoramidate, whose structure is shown in Figure 1, was readily obtained following a three-step process which is depicted in Figure 2. Schematically, dilinoleylphosphite **1** was synthesized by reacting diphenylphosphite with linoleyl alcohol following a reported procedure.[31] The linoleyl alcohol used for the synthesis of **1** was previously synthesized by reduction of methyllinoleate according to available methodologies.[50,51] Compound **1** was then engaged in a Todd−Atherton reaction[52] involving 2-bromoethylamine bromohydrate to produce the lipophosphoramidate **2**. Compound **2** was reacted with sodium iodide in acetone following a Finkelstein's reaction to yield the iodoalkyl **3**. Finally, in the last step, the trimethylarsonium headgroup was introduced by the reaction of compound **3** with a slight excess of trimethylarsine. This allowed obtaining lipid **4** in good yield, the analytical methods being as indicated in the Experimental Section.

2. Physicochemical Studies. Here, we investigated the physicochemical properties of lipid **4**-based liposomes and also of the lipoplexes obtained when mixing those liposomes with plasmid DNA (pDNA). To calculate the mean theoretical charge ratio (CR, +/−), which is the ratio of the positive charges provided by the arsonium polar headgroup of the vector to the negative DNA phosphate charges, we assumed that 1 μg of pDNA is 3 nmol of negatively charged phosphate and that one permanent positive charge is displayed by lipid **4**.



Lipid 4 (BSV4)



KLN47

Figure 1. Chemical structure of lipid **4** (BSV4), a cationic lipophosphoramidate with diunsaturated linoleic chains, and of its monounsaturated analogue KLN47.[33]

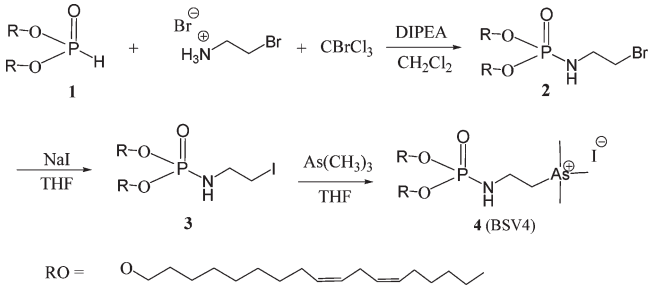


Figure 2. Reaction scheme for the synthesis of the diunsaturated cationic lipophosphoramidate **4** (BSV4).

Liposome Formulation. The protocol for preparation of lipid **4**-based liposomes was first optimized in order to obtain liposomes characterized by an average diameter of about 180 nm. Briefly, lipid **4** was recovered as a thin film in a glass vial, then hydrated for 2 days at +4 °C, and finally vigorously vortexed and sonicated for at least 2 min in a sonicator water bath (see Experimental Section for details).

Critical Liposomal Concentration (CLC). The CLC value of lipid **4** (i.e., the minimal lipid concentration enabling the formation of liposomes) was evaluated in the presence of Nile Red by studying the fluorescence intensity variations of this lipophilic probe at increasing concentrations of lipid **4**. As shown in Figure 3, two series of concentrations were delineated: (i) at the lowest concentrations considered, the fluorescence did not vary, as no lipidic vesicles existed at such concentrations; (ii) on the contrary, above a minimal concentration, the fluorescence increased (a fact indicative of lipid aggregation into liposomes) in a dose-dependent manner (i.e., while the lipid concentration increased). A linear regression applied to those two sets of values enabled the determination of the concentration at which the two lines intersected. This intersection allowed estimating a CLC value for lipid **4** of about $(2.5 \pm 0.5) \times 10^{-6}$ mol/L (CLC uncertainty was estimated using the intersection of the confidence hyperbolas). Such a CLC value is in the same range that the CLC values already reported for natural phospholipids or other cationic lipids.[53] Of note, recent cryotransmission electron microscopy imaging has shown that lipid **4** can indeed form liposomal structures (not shown).

Membrane Viscosity. Anisotropy measurements were conducted using the Laurdan probe, a hydrophobic fluorescent stain, in order to evaluate the viscosity of the membranes formed by lipid **4**. Figure 4 (left panel) shows the anisotropy

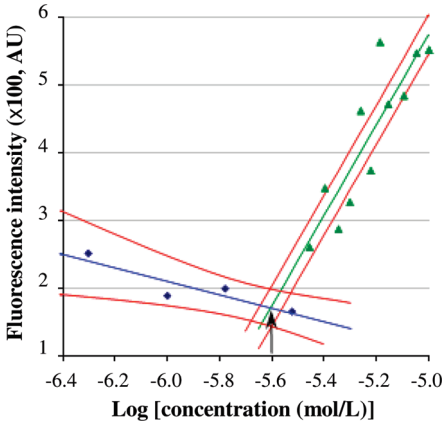


Figure 3. Critical liposomal concentration (CLC) of lipid **4**.

variations at different temperatures for liposomes containing lipid **4** alone or lipid **4** combined with either cholesterol or DOPE (at 1/1 molar ratios). Two sets of values could be distinguished: (i) an upper group which corresponded to mixtures of lipid **4** + cholesterol, characterized by high anisotropy values, and (ii) two lower groups corresponding to lipid **4** alone or lipid **4** + DOPE, characterized by much lower anisotropies, whatever temperature considered. These results indicate that, as previously reported,[54] cholesterol can act as a stiffening lipid reagent as it increases membrane viscosity. On the other hand, addition of DOPE, which is frequently used as a fluidifying colipid,[12,55] did not significantly modify here the membrane viscosity when compared to lipid **4** used alone. Thus, lipid **4**-based membranes probably already display a low viscosity index, which can be increased by its combination with cholesterol but not further decreased by the addition of DOPE. This high fluidity may be related to the two *cis* double bonds borne by each of the two linoleic chains of lipid **4**. Indeed, at 37 °C, the anisotropy value measured with lipid **4** (0.13; Figure 4, left panel) was slightly lower than that observed with its monounsaturated analogue KLN47[33] (0.15; Figure 4, right panel), which differs from lipid **4** only by the presence of two oleic chains (instead of two linoleic chains). Thus, the diunsaturated lipophosphoramidate **4** exhibited a slightly higher fluidity than its monounsaturated analogue. Of note, the melting point of linoleic acid is indeed lower than that of oleic acid (−5 versus +15 °C). Furthermore, the effects observed when combining KLN47 with colipids were similar to those reported above for lipid **4**, i.e., an increased viscosity when

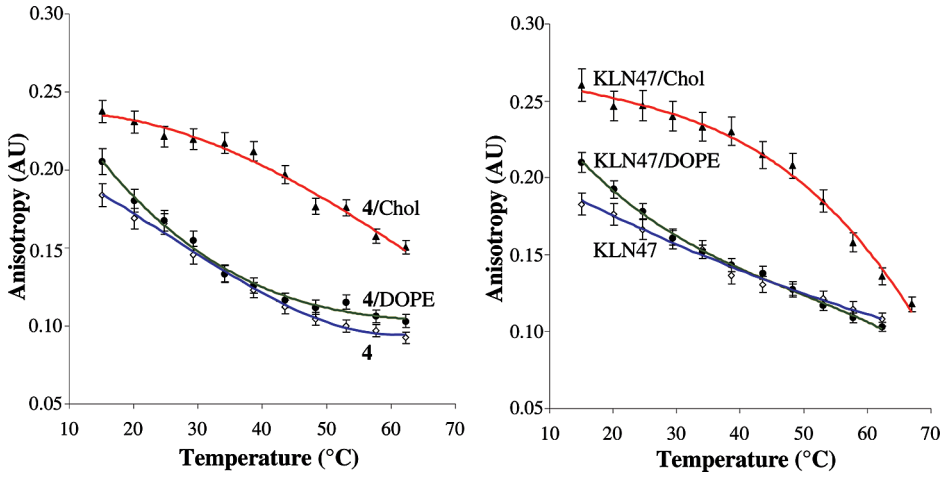


Figure 4. Anisotropy measurements with lipid **4**-based (left panel) or KLN47-based (right panel) liposomes formed of the cationic lipid alone or of the cationic lipid combined with either DOPE or cholesterol (Chol).

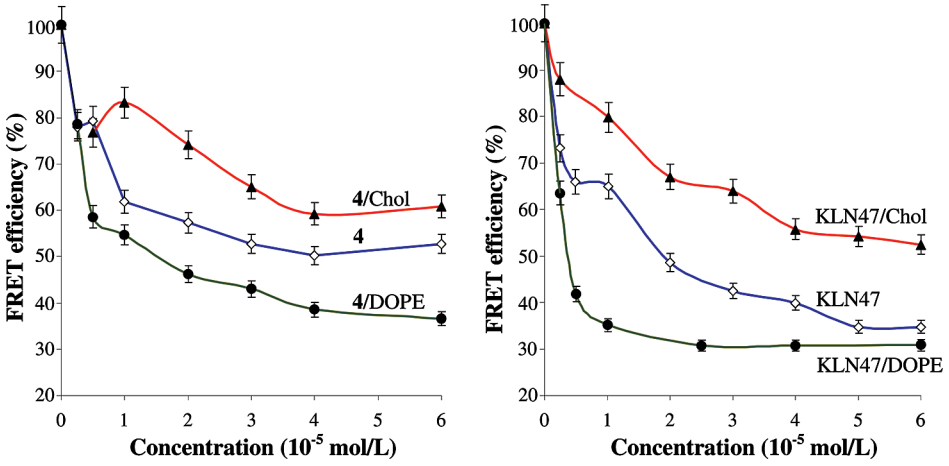


Figure 5. FRET efficiency with lipid **4**-based (left panel) or KLN47-based (right panel) liposomes formed of the cationic lipid alone or of the cationic lipid combined with either DOPE or cholesterol (Chol).

combined with cholesterol but no significant changes when combined with DOPE. These observations are also in good agreement with previous studies showing that the addition of DOPE most changes liposome membrane fluidity when there is, without DOPE, a significant level of viscosity.[56]

Liposome Fusion. To estimate the fusogenicity of lipid **4**-based liposomes, we conducted Förster resonance energy transfer (FRET) experiments.[57] Figure 5 (left panel) shows the FRET efficiency variations obtained at different lipid **4** concentrations. It indicates that a FRET efficiency of 53 ± 3% can be reached with lipid **4**-based liposomes, a fact suggesting that a high membrane fusion rate does exist between the model liposomes (incorporating the donor and acceptor fluorescent probes) and the test lipid **4**-based liposomes. Combination of lipid **4** with DOPE (at a 1/1 molar ratio) was found to decrease the FRET efficiency at 37 ± 3%, indicating that the high fusion rate of lipid **4**-based liposomes can even be further increased by the colipid DOPE. Conversely, addition of cholesterol (at a 1/1 molar ratio) increased the FRET efficiency at 61 ± 3%, thus indicating a lower ability of lipid **4**/cholesterol liposomes to fuse with model membranes. In comparison, KLN47-based liposomes (Figure 5, right panel) were significantly more fusogenic than lipid **4**-based liposomes (FRET efficiency of 35 ± 3% versus 53 ± 3%); it is also noteworthy here that the fusogenicity of KLN47-based liposomes, although it could be somewhat increased by the addition of DOPE, was already approximately similar to that of lipid **4**/DOPE-based liposomes. The FRET results also showed that, similarly to lipid **4**-based liposomes (see above), the addition of cholesterol decreased the KLN47-based liposome fusion rates, both observations being consistent with the stiffening effect of cholesterol. Finally, it should be stressed here that the anisotropy and FRET data reported above have basically shown that lipid **4**-based liposomes were slightly more fluid but significantly less fusogenic than KLN47-based liposomes. These lipid **4** characteristics may be linked to their enhanced transfection activity *in vivo* following systemic administration (see below).

DNA Condensation. First, ethidium bromide exclusion assays were performed to evaluate the ability of the lipid **4** to condense plasmid DNA. Indeed, upon condensation, ethidium bromide is expelled from DNA, and thus, the fluorescence signal is decreased. The results obtained (see Figure 6, left panel) showed that, when prepared in Opti-MEM (medium used for *in vitro* transfection), the lipid **4**-based liposomes were able to condense DNA with a high efficiency. Indeed, the fluorescence signal rapidly decreased as the CR increased, the minimum fluorescence value (~20%) being already reached at a relatively low lipid/

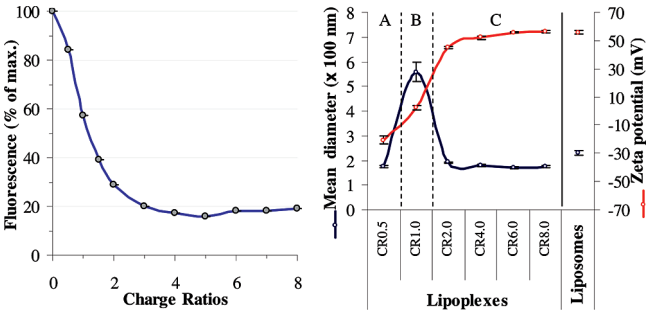


Figure 6. DNA condensation by lipid **4**-based liposomes (left panel). Colloidal stability and zeta potential of lipid **4**-based lipoplexes as a function of their charge ratio (right panel).

DNA ratio (i.e., as soon as CR2). DNA retardation assays on agarose gel electrophoresis were next performed to confirm the DNA condensation ability of lipid **4**-based liposomes. The aforementioned condensation measurements were actually clearly correlated with the observed DNA retardation, which was partial at CR0.5 and CR1.0, and became complete at higher CRs. Thus, lipid **4**-based liposomes can efficiently complex (neutralization) as well as condense (compaction) plasmid DNA. It should be stressed here that DNA condensation by the monounsaturated analogue KLN47 was actually quite similar.

In addition, we also compared the DNA condensation ability of lipid **4** with that of the commercially available cationic lipid lipofectamine (LFM), not only in OptiMEM medium but also in 0.9% NaCl (isotonic saline solution), a medium more suitable for *in vivo* administration. Schematically, lipid **4** and LFM-based liposomes had similar condensation abilities in OptiMEM. However, in 0.9% NaCl, lipid **4**-based liposomes exhibited a decreased DNA condensation ability, whereas that of LFM remained unchanged (Supporting Information Figure S1). Thus, in isotonic saline solution, DNA compaction by lipid **4**-based liposomes appeared weaker than by LFM-based liposomes. Along these lines, we also studied the dismantling of lipid **4** and LFM-based lipoplexes when incubated with dextran sulfate, i.e., when adding a counteranion capable of competing with the DNA for electrostatic interaction with the cationic lipids. Here again, clear differences were observed between lipid **4** and LFM-based lipoplexes formed in isotonic saline solution (Supporting Information Figure S1). When increasing the concentration of dextran sulfate, it was indeed observed that the DNA was released "more slowly" from lipid **4**-based lipoplexes than from LFM complexes, although, as indicated above, it was less condensed in the former ones. This may be explained by important differences between lipid **4** and LFM-based lipoplexes formed in isotonic saline solution. It is noteworthy here that, with regard to DNA condensation by LFM in isotonic saline solution (with and without dextran sulfate), there was a good correlation between ethidium bromide exclusion and agarose gel retardation, both methods showing that dextran sulfate can effectively hinder DNA condensation (Supporting Information Figure S2). On the other hand, as shown in Supporting Information Figure S3, DNA condensation by lipid **4** appeared to be more progressive and less unstable over time than condensation by LFM. Taken together, these results strongly suggest that there are important differences between our lipid **4** and the commercially available LFM with regard to the formation, the structure, and the dismantling of the complexes formed with plasmid DNA, differences which may strongly impact on their respective transfection activities (see below).

Colloidal Stability of the Complexes Formed with Plasmid DNA. Zetametry measurements were conducted in order to further characterize the DNA complexes formed by lipid **4**-based liposomes. First, we found that lipid **4**-based liposomes alone (i.e., without DNA) were characterized by a mean diameter of about 200 nm and a positive charge (zeta potential, ξ) of approximately +55 mV (Figure 6, right panel). Second, we measured the size and zeta potential of a series of lipid **4**-based lipoplexes prepared at CRs ranging from 0.5 to 8.0. The results obtained (Figure 6, right panel) were in agreement with the three-zone model of colloidal stability previously described for various cationic lipid/DNA complexes, including lipopolyamine/DNA[58−61] or BGTC/DNA lipoplexes.[62−64] The three different zones (named A, B, and C) were determined by the cationic lipid/nucleic acid CR. According to this model, in zone A at low CR, negatively charged and colloidally stable complexes with partially condensed nucleic acids are formed. Zone B contains neutral, large, and colloidally unstable aggregates. In zone C, the particles are positively charged, small, and again colloidally stable. Interestingly, we observed here that zone B was relatively narrow and characterized by complexes with a mean theoretical CR of about 1. In zone C, which ranged from CR2 up to CR8, complexes of similar sizes were obtained. They were characterized by a small mean diameter of about 175 nm, thus slightly smaller than liposomes. With regard to the zeta potential ξ, Figure 6 (right panel) indicates that ξ rapidly increased with the lipoplex CR, with (i) negatively charged species obtained at CR0.5 (DNA in excess), (ii) neutral species effectively obtained at CR1, and (iii) positively charged species formed at CR2, with a positive charge of approximately +55 mV, similar to that of lipid **4**-based liposomes. Thus, when mixed with DNA at a CR of 2 or higher, lipid **4**-based liposomes are able to rapidly complex plasmid DNA, forming stable aggregates characterized by a clearly positive surface charge and a relatively small mean diameter.

3. Transfection Experiments. The transfection activity of lipid **4** lipoplexes was evaluated in a series of experiments *in vitro*, using different cell lines, as well as *in vivo*, with Swiss mice.

***In Vitro* Transfection.** As lung-directed gene therapy for cystic fibrosis is at the forefront of gene therapy research, we chose to mainly use in the present study the A549 and 16HBE 14o-cell lines which are both derived from human lung epithelial cells. As gene transfection by cationic lipids is presumed to involve electrostatic interactions between positively charged lipoplexes and negatively charged cell surface residues, we first examined the influence of the lipoplex CR on the transfection activity. Thus, a series of lipoplexes characterized by various CRs (ranging from 0.5 to 8.0) were prepared by mixing the required amounts of lipid with a constant amount of plasmid DNA expressing the reporter gene luciferase. Schematically, as shown in Figure 7, significant levels of luciferase expression were measured when using positively charged lipid **4**-based lipoplexes. However, when compared with LFM-based lipoplexes, lipid **4** yielded luciferase levels which were either lower or of the same order of magnitude, depending on the cell line and the CR used. It is nevertheless noteworthy that, at low CR, lipid **4**-based lipoplexes were as efficient as LFM-based lipoplexes (or even more) for 16HBE 14o-cells, which are less transformed cells

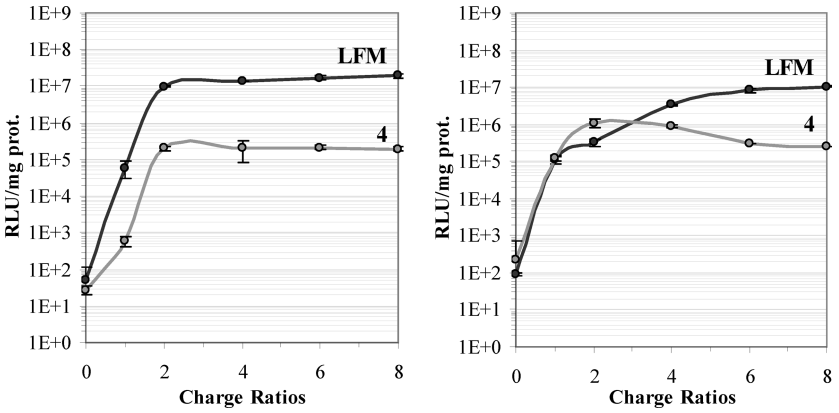


Figure 7. *In vitro* transfection efficiency of lipid **4**-based and of lipofectamine- (LFM-) based lipoplexes with A549 (left panel) or 16HBE 14o-(right panel) cells.

presumed to better mimic the normal bronchial epithelial cells than A549 cells. Moreover, we also evaluated the lipid **4** transfection activity with some other cell lines, in particular SKMEL28 and A375 cells which are both derived from human skin melanomas. The results obtained showed that lipid **4**-based lipoplexes could also mediate efficient delivery of reporter plasmid into those cell types, leading to luciferase levels similar to those of LFM. Thus, lipid **4** is a versatile cationic lipid able to transfect various mammalian cell lines. Finally, as the plasmid used in these *in vitro* transfection experiments expressed not only the luciferase gene but also the green fluorescent protein (GFP) reporter gene (see Experimental Section), we could also determine the percentage of transfected cells by flow cytometry. Basically, lipid **4**-based lipoplexes yielded up to 10% GFP-positive cells, the percentage observed depending on the cell type and the CR used.

As it is agreed that gene transfection by cationic lipids may be associated with some level of cytotoxicity, we also performed *in vitro* cytotoxicity measurements using the same cell lines (see above). The cytotoxic effects induced by lipid **4**-based liposomes were quantified by measuring (i) the release from the damaged cells of an enzyme normally located in the cytoplasm and (ii) the total amount of proteins in the cell lysate, which is an index of the cell number (see Experimental Section). We observed that the cytotoxicity of the lipid **4**-based lipoplexes was clearly dose-dependent. It was actually similar to that of LFM at low CRs but became significantly more pronounced at high CRs, as it increased with the dose of lipid **4**, as shown in Figure 8 for the release into the culture medium of the cytosolic enzyme adenylate kinase. This cytotoxicity increase at high CRs may explain, at least in part, why the lipid **4** transfection activity reached its maximum at CR about 2, then plateaued, or decreased (Figure 7). Indeed, the gene transfection efficiency depends upon the toxic effects induced by the reagents employed, as these effects may alter the normal cellular metabolism and, in turn, hamper transgene expression. Of note, LFM is a 3/1 (w/w) liposomal formulation of the polycationic lipid DOSPA with the neutral lipid DOPE, whose incorporation is known to be in general beneficial for gene transfection. In particular, the incorporation of DOPE enables the reduction of the surface charge density of the DNA complexes, and this was proposed as a potential solution for reducing their cytotoxicity.[2−4] Accordingly, it will be interesting to evaluate in the future the transfection activity of formulations of lipid

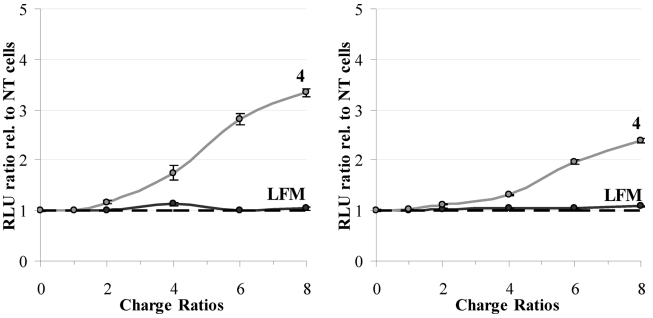


Figure 8. Cytotoxicity of lipid **4**-based lipoplexes with A549 (left panel) or 16HBE 14o-(right panel) cells.

4 with a colipid, in particular DOPE, as well as that of multivalent analogues of lipid **4** (see below).

***In Vivo* Transfection.** Here, we first administrated lipid **4**-based lipoplexes via the systemic route (mouse tail vein), and we used *in vivo* bioluminescence imaging to detect, and quantify, transgene (luciferase) expression. As the lipoplex CR is known to be a critical parameter not only for *in vitro* (see above) but also for *in vivo* transfection, we prepared different positively charged lipid **4**-based lipoplexes by mixing, in 0.9% NaCl, a constant amount (50 μg) of pTG11033 plasmid DNA with the required amounts of lipid **4**. Of note, pTG11033 contains not only the firefly luciferase cDNA under control of the strong viral CMV promoter but also a HMG-1 intron for enhanced transgenic protein synthesis via increased mRNA export from the nucleus. These complexes were subsequently injected into the tail vein of a series of mice. *In vivo* bioluminescence imaging (quantified as photons per second) was performed using a cooled charge-coupled device (CCD) camera customized to achieve a high sensitivity enabling the detection of photon emissions from lungs of living animals (NightOwl II; Berthold). In addition, lung homogenates were also prepared from some treated animals and assayed for luciferase expression (quantified as relative light units (RLU) per milligram of total protein).

As with our previous cationic lipids,[23,26] preliminary experiments showed that the highest luciferase activity in the lung area was obtained with a CR between 4 and 6. Thus, we used lipoplexes formed at CR6 to study the transgene expression kinetics by *in vivo* bioluminescence. For comparative purposes, KLN47[33] (Figure 1), the monounsaturated analogue of lipid **4**, was used under identical experimental conditions. A series of mice were intravenously injected with

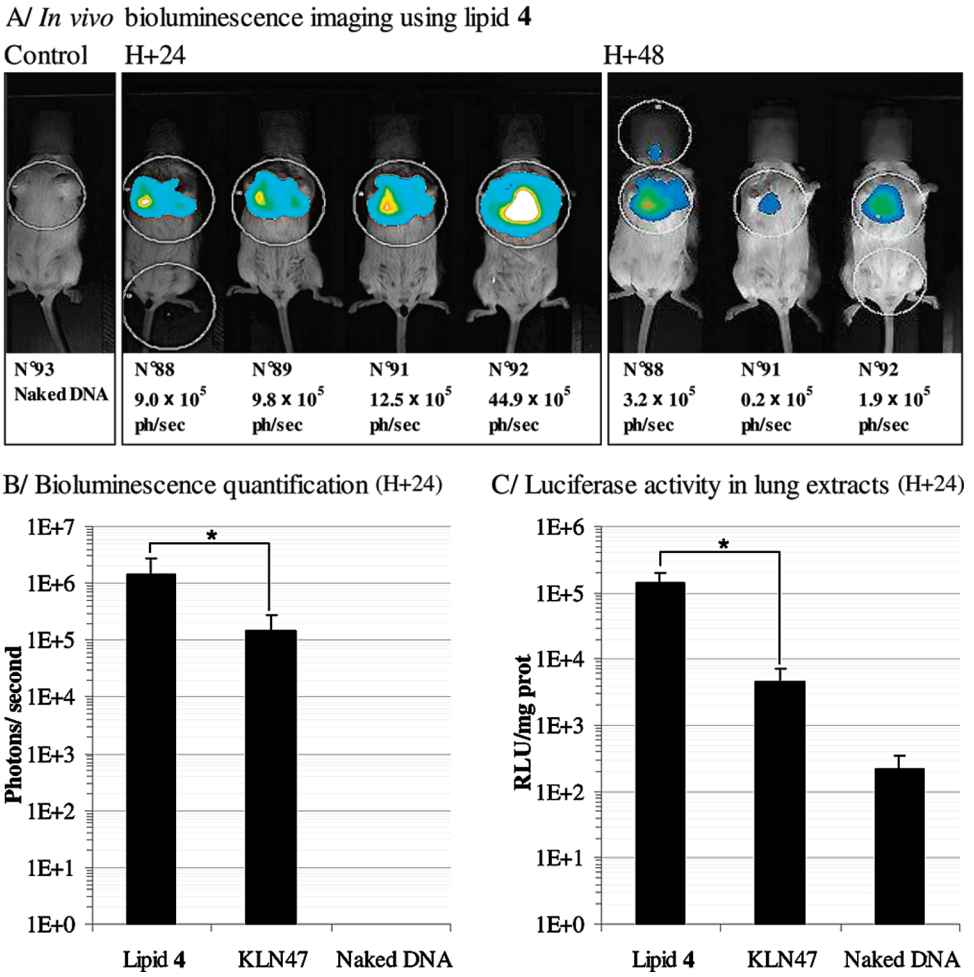


Figure 9. *In vivo* transfection efficiency of lipid **4**-based and KLN47-based lipoplexes.

such complexes (lipid **4** group, $n = 11$; KLN47 group, $n = 4$) and repeated imaging was performed, from 24 h up to 3 days. As a control, other mice ($n = 4$) received an equivalent amount of naked uncomplexed DNA. As shown in Figure 9A, an *in vivo* bioluminescence signal was detected only in the lung area of the animals which received lipid **4**-based lipoplexes. With regard to kinetics, the highest photon emission was measured 24 h after administration ($(1.46 \pm 1.29) \times 10^6$ photons/s, $n = 11$; Figure 9A,B). At 48 h, the bioluminescence signal had decreased (Figure 9A) by one magnitude ($(1.70 \pm 1.29) \times 10^5$ photons/s, $n = 6$), and no signal was detected at 72 h ($n = 6$). Compared with lipid **4**-based lipoplexes, 24 h postinjection, positive ($(1.48 \pm 1.26) \times 10^5$ photons/s, $n = 4$) but significantly lower (Student's t test, p value $= 3.6 \times 10^{-3}$) bioluminescence signals were measured in the mice treated with KLN47 lipoplexes (Figure 9B). Thus, bioluminescence imaging showed that lipid **4** is more efficient than KLN47 for *in vivo* lung transfection, and this was confirmed by measuring luciferase expression from harvested lung tissue (see below). Finally, in the naked DNA control group, we did not detect any *in vivo* bioluminescence signal (Figure 9B), a finding highlighting the role of DNA condensation by the cationic vector for *in vivo* transfection via the systemic route.

Also, as shown in Figure 9A, we observed a stronger bioluminescence signal in the right lung area, a fact that may be explained by signal absorption by the heart and the smaller size of the left lung. Of note, a post-mortem examination of two animals revealed some macroscopic patches of liver necrosis, as already observed by others after systemic administration of lipoplexes.[65] Considering the biodistribution of lipid **4** (see below), it may be hypothesized here that its *in vivo* toxicity in mice may mainly result from hepatotoxic effects caused by the cationic lipid itself and/or some of its metabolites.

After imaging at 24 h, several mice from each group ($n \geq 3$) were sacrificed in order to evaluate the transgene expression from the harvested lungs. As shown in Figure 9C, luciferase expression reached $(1.43 \pm 0.59) \times 10^5$ and only $(4.60 \pm 2.58) \times 10^3$ RLU/mg of total protein in the lipid **4** and KLN47-treated animals, respectively, whereas it was found to be equal to $(2.22 \pm 1.31) \times 10^2$ RLU/mg of total protein in the animals which had received naked DNA. These results confirm the *in vivo* bioluminescence data reported above, both showing that lipid **4** is more efficient than KLN47 for *in vivo* lung transfection following systemic administration. These results also highlight the significant improvement of the present type of cationic lipophosphoramidates (lipid **4**) in transfection efficiency compared to our first generation of cationic phosphonolipids (such as lipid GLB43)[24]. Indeed, we have previously reported that, under similar experimental conditions, lipid GLB43 led to $\sim 2.0 \times 10^3$ RLU/mg of total protein. With the second generation phosphonolipids characterized by an arsonium group substituting the quaternary ammonium headgroup (e.g., lipid EG372), the luciferase expression in lung homogenates was $\sim 4.0 \times 10^3$ RLU/mg of total protein.[26] Next, when comparing the *in vivo*

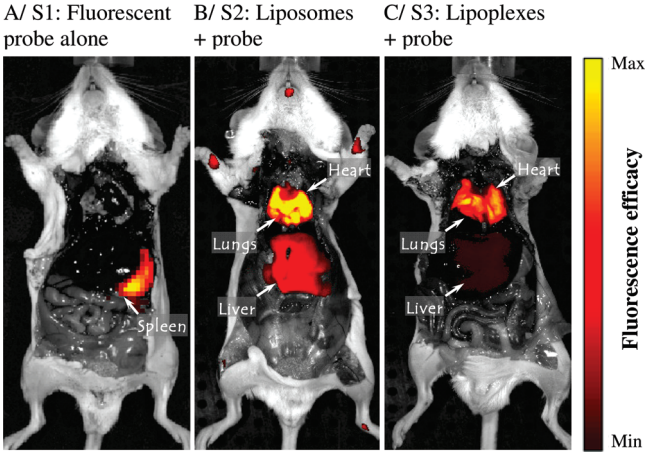


Figure 10. Biodistribution in mice of lipid **4**-based liposomes and lipid **4**-based lipoplexes after intravenous injection.

transfection efficiencies of lipid **4** and KLN47, the present results demonstrate the benefit of diunsaturated linoleic chains as the lipidic part of lipophosphoramidates for obtaining even higher *in vivo* transgene expressions. Here, when taking into account the respective viscosity and fusogenicity properties of lipid **4** and KLN47-based liposomes reported above, it may be hypothesized that the high fluidity combined with a good (but not too high) fusogenicity are favorable characteristics of lipid **4** for efficient *in vivo* gene transfection into the lung via the bloodstream, where numerous physical and chemical interactions can occur. Finally, our results also strongly invite further studies on the usefulness of incorporating polyunsaturated aliphatic chains into cationic lipid-based gene delivery systems.

With regard to the transient character of *in vivo* luciferase expression, it is noteworthy that experiments in animals were performed with pTG11033, a plasmid (i) which is not CpG free and (ii) in which the luciferase reporter gene is under control of the immediate-early cytomegalovirus (CMV) enhancer/promoter element. According to the literature,[66] these plasmid characteristics are important features which can explain, at least in part, the brief duration of transgene expression observed here. However, it cannot be excluded that toxic effects of the cationic vector itself also play a role. The respective roles of those different parameters will be further investigated in the future.

When using the commercial cationic polymer ExGen 500 (a linear 22 kDa polyethylenimine (PEI); Fermentas Life Sciences, France), whose transfection efficiency (but also toxicity) is well-known, we observed *in vivo* bioluminescence signals of the same order of magnitude as those obtained with lipid **4** $(1.0-2.0) \times 10^6$ photons/s, 24 h postinjection). In addition, we also found that LFM-based lipoplexes administered under the same experimental conditions were inefficient to transfect mice lungs *in vivo* (not shown). Thus, whereas LFM appeared more suitable for *in vitro* transfection, lipid **4** was more versatile as it was efficient *in vitro* as well as *in vivo*. This may be linked to differences in the properties of LFM and lipid **4**-based lipoplexes, such as a faster dismantling of the LFM lipoplexes in complex biological media (see above). Taken together, these data confirm that, as it is widely recognized, *in vitro* results do not necessarily allow the prediction of the *in vivo* behavior of a given gene delivery system.[3,4] They also invite working out lipid **4**-based formulations for improved *in vivo* transfection. Accordingly, we are attempting to design lipid **4**-based formulations optimized for different routes of *in vivo* administration in an ongoing study.

Biodistribution Experiments. Finally, we performed *in vivo* biodistribution experiments to study the relationships between biodistribution of the lipid **4**-based lipoplexes and the observed *in vivo* transgene expression pattern. Indeed, bioluminescence imaging allowed us to detect a significant signal only in the lung area (see above). We have previously shown that, after intravenous delivery of DNA complexes, *in vivo* luciferase expression was only detectable in lung homogenates and it was related to the biodistribution of lipoplexes.[67] In the present work, we attempted to better define the correlation between the *in vivo* bioluminescence pattern and biodistribution, by using lipid **4**-based liposomes and lipid **4**-based lipoplexes, tagged with the lipophilic fluorescent probe DiR. Thus, tail vein injections were performed in Swiss mice with different formulations which consisted of either (i) the DiR probe alone (Figure 10, panel A), (ii) lipid **4**-based liposomes + DiR probe (panel B), or (iii) lipid **4**-based lipoplexes + DiR probe (panel C). Approximately 1 h later, the mice were sacrificed, and their organs were exposed in order to be visualized using an IVIS Spectrum apparatus (Caliper; 700 nm excitation filter, 780 nm emission filter). It should be noticed here that fluorescence signals shown in Figure 10 are not at the same scale between the three panels. In panel A, a weak signal was only detected in the spleen area. Panel B shows strong fluorescence signals in the lung, heart, and liver areas. Panel C depicts a main fluorescence signal localized in the lung and heart areas, and a less intense signal in the liver area. These results suggest that, despite being administered *in vivo* via the systemic delivery route, lipid **4**-based liposomes displayed a preferential tropism for the lungs (panel B). This was even more obvious with lipid **4**-based lipoplexes, the signal in the spleen area observed with DiR alone having disappeared and the liver signal observed with the liposomes being here strongly decreased compared to the signal in the lungs (panel C). Therefore, the bioluminescence signals observed in the lung area of the mice probably resulted from a predominant distribution of lipid **4**-based lipoplexes to the lung. Accordingly, transfection of secondary areas (e.g., the liver, as sometimes reported for other cationic lipids)[68] should be weak (or null) with lipid **4**. In conclusion, lipid **4**-based liposomes may constitute an attractive vector for gene transfection into the lung *in vivo*. In ongoing experiments, we are at present investigating the airway cell type(s) actually transfected with the lipid **4**-based lipoplexes.

Conclusion and Perspectives

We have herein further evaluated the transfection properties of lipophosphoramidate derivatives, a class of cationic lipids we have previously reported to be efficient for gene transfection.[20−26,28−32] Indeed, we have synthesized and studied a novel monocationic lipophosphoramidate characterized by an unconventional lipidic moiety consisting of two *cis* diunsaturated linoleic chains (C18:2 $\Delta^9\,\Delta^{12}$). Physicochemical studies revealed the specific fluidity and fusogenicity characteristics of lipid **4**-based liposomes, in comparison with liposomes made of KLN47, the monounsaturated analogue of lipid **4**. Most importantly, although lipid **4**-based lipoplexes exhibited significant but relatively modest *in vitro* transfection

efficiency with various cell lines, they were however consistently and reproducibly efficient for gene transfection in the mouse lung *in vivo* (after tail vein injection).

Taken together, these results call for further studies on the impact on transfection efficiency of the incorporation of polyunsaturated fatty acid chains into the structure of lipophosphoramidate vectors. They also invite studying vectors combining a polyunsaturated lipidic moiety with a multivalent headgroup (e.g., spermine) instead of a monovalent arsonium group as in lipid **4**. Indeed, the introduction of a multivalent headgroup should allow to form lipoplexes of a given charge ratio by using a lower amount of cationic lipid, thereby possibly decreasing the transfection-induced cytotoxicity. On the other hand, it will also be interesting to study the effects of the incorporation of a colipid (such as DOPE) into lipid **4**-based formulations. Indeed, when compared with lipid **4**-based liposomes, lipid **4**/DOPE-based liposomes showed a greater fusogenicity (see above), and preliminary results also strongly suggest that they may be more efficient for *in vitro* gene transfection. Along these lines, in an approach aiming to design multimodular gene delivery systems, polyunsaturated cationic lipids such as lipid **4** might be advantageously combined with other cationic vectors (lipids or polymers) in order to fine-tune the overall physicochemical properties of the resulting DNA complexes. This is supported by the fact that positive transfection results have already been obtained with lipid **4**-based lipopolyplexes in preliminary experiments. Finally, future studies should allow assessing more extensively the usefulness of gene delivery systems incorporating the highly versatile lipid **4** for efficient *in vivo* gene transfection, in particular with regard to the various routes of administration, the different cellular targets, and the general *in vivo* toxicity.

Experimental Section

Chemistry: Preparation and Characterization of Cationic Lipid 4. The cationic lipophosphoramidate **4**, whose structure is shown in Figure 1, was readily obtained following a three-step process which is depicted in Figure 2. It was purified by chromatography on silica gel. It was finally characterized by NMR (^{1}H, ^{31}P, ^{13}C), including HMBC and HMQC experiments, and also by high-resolution mass spectrometry (HRMS). These NMR and HRMS data were in agreement with a purity level ≥95%. Pertinent spectroscopic and analytical data are given below.

Synthesis of *O*,*O*-Dilinoleyl-*N*-(2-bromoethyl)phosphoramidate (2). Diisopropylethylamine (DIPEA; 2.88 mL, 16.5 mmol) was slowly added to a solution cooled at 0−5 °C of dilinoleylphosphite **1** (4.35 g, 7.5 mmol), bromotrichloromethane (2 mL, large excess), and 2-bromoethylamine bromohydrate (1.69 g, 8.2 mmol) in dichloromethane. At the end of the addition, the solution was further stirred for 1 h at 0−5 °C and 1 h at 20 °C. The volatiles were removed *in vacuo*, and hexane was added (20 mL). The precipitate was removed by filtration on Celite. The filtrate was concentrated to produce compound **2** as a viscous oil (4.98 g, 95% yield) which was engaged in the next step without further purification. ^{1}H NMR ($CDCl_3$) 0.89 (t, $^{3}J_{HH}$ = 6.9 Hz, 6H, CH_3-CH_2), 1.22−1.44 (m, 32H), 1.63−1.70 (m, 4H), 2.01−2.08 (m, 8H), 2.77 (t, $^{3}J_{HH}$ = 5.9 Hz, 4H, =CH−CH_2−CH=), 3.04 (m, 1H, NH), 3.25−3.36 (m, 2H), 3.45 (t, $^{3}J_{HH}$ = 5.8 Hz, 2H, CH_2−Br), 3.98 (dd, $^{3}J_{HH} \sim {}^{3}J_{HP}$ = 6.5 Hz, 4H, $-CH_2-O-$), 5.28−5.42 (m, 8H). ^{13}C{^{1}H} NMR ($CDCl_3$) 14.0 (s, 2C), 22.4 (s, 2C), 26.3−31.4 (22 C), 34.4 (s 1C), 44.5 (s, 1C), 65.3 (d, $^{2}J_{CP}$ = 6.4 Hz, 2C), 127.7 and 128.3 (2s, 4C), 130.1 and 130.6 (2s, 4C). ^{31}P{^{1}H} NMR ($CDCl_3$) 8.7.

Synthesis of *O*,*O*-Dilinoleyl-*N*-(2-iodoethyl)phosphoramidate (3). Compound **2** (4.98 g, 7.1 mmol) and sodium iodide (1.39 g, 9.3 mmol) were mixed in tetrahydrofuran (15 mL). The solution was heated at reflux for 2 h. After cooling, the volatiles were removed *in vacuo*. After addition of hexane (20 mL), the solution was filtered on Celite to remove sodium bromide. The filtrate was concentrated to produce compound **3** as a viscous oil (5.31 g, 100% yield) which was engaged in the next step without further purification. ^{1}H NMR ($CDCl_3$) 0.86 (t, $^{3}J_{HH}$ = 6.8 Hz, 6H, CH_3-CH_2), 1.22−1.43 (m, 32H), 1.60−1.68 (m, 4H), 1.99−2.08 (m, 8H), 2.74 (t, $^{3}J_{HH}$ = 6.5 Hz, 4H, =CH−CH_2−CH=), 2.99 (m, 1H, NH), 3.19−3.27 (m, 4H), 3.98 (dd, $^{3}J_{HH} \sim {}^{3}J_{HP}$ = 6.4 Hz, 4H, $-CH_2-O-$), 5.27−5.42 (m, 8H). ^{13}C{^{1}H} NMR ($CDCl_3$) 2.9 (s, 1C), 14.1 (s, 2C), 22.3 (2C), 26.1−31.9 (22C), 45.2 (s, 1C), 65.9 (d, $^{2}J_{CP}$ = 6.4 Hz, 2C), 127.6 and 128.5 (2s, 4C), 130.3 and 130.7 (2s, 4C). ^{31}P{^{1}H} NMR ($CDCl_3$) 8.5.

Synthesis of Dilinoleylphosphatidyl-2-aminoethyltrimethylarsonium Iodide (4) (BSV4). Trimethylarsine (1.2 mL, 11.2 mmol) was added to a solution of compound **3** (5.32 g, 7.1 mmol) in anhydrous tetrahydrofuran (2 mL) placed in a Schlenk tube equipped with an efficient reflux condenser (−20 °C). The solution was placed in an oil bath heated at 60 °C for 48 h. The volatiles were removed *in vacuo*. The residue was dissolved in chloroform (2 mL) and purified by chromatography on silica gel ($CHCl_3$/MeOH, 98/2) to produce compound **4** as a viscous oil (3.87 g, 63% yield). ^{1}H NMR ($CDCl_3$) 0.89 (t, $^{3}J_{HH}$ = 6.9 Hz, 6H, CH_3-CH_2), 1.22−1.43 (m, 32H), 1.59−1.73 (m, 4H), 2.01−2.11 (m, 8H), 2.22 (s, 9H), 2.77 (t, $^{3}J_{HH}$ = 6.1 Hz, 4H, =CH−CH_2−CH=), 3.15−3.21 (m, 2H), 3.49−3.56 (m, 2H), 3.98 (dd, $^{3}J_{HH} \sim {}^{3}J_{HP}$ = 6.6 Hz, 4H, $-CH_2-O-$), 4.29−4.36 (m, 1H), 5.28−5.42 (m, 8H). ^{13}C{^{1}H} NMR ($CDCl_3$) 9.8 (s, 3C), 14.0 (s, 2C), 22.5 (2 C), 25.6−31.4 (23C), 36.0 (s, 1C), 67.0 (s, 2C), 127.8 and 127.9 (2s, 4C), 129.9 and 130.1 (2s, 4C). ^{31}P{^{1}H}($CDCl_3$) 8.7. HRMS (ES-TOF) *m*/*z* calcd for $C_{41}H_{80}NO_3PAs$ (M^+) 740.50918; found 740.5088.

Plasmid DNA. For physicochemical and transfection studies, three different plasmids were used: pGL3-Ctrl (5.6 kb), pEGFP-Luc (6.4 kb), and pTG11033 (9.6 kb) obtained from Promega (France), Clontech (U.K.), and Transgene (France), respectively. The pGL3-Ctrl was used for DNA condensation assays. For *in vitro* assays, we used the pEGFP-Luc, which encodes a fusion of two reporter genes, the enhanced green fluorescent protein (EGFP; Abs/Em = 488/507 nm) and the firefly *Photinus pyralis* luciferase; this plasmid allowed us to evaluate the transfection activity by both flow cytometry and measurement of luciferase activity. Of note, the pEGFP-Luc, whose size is similar to that of the pGL3-Ctrl, yielded similar condensation results. For *in vivo* transfection experiments, we used the pTG11033 which contains not only the firefly luciferase cDNA under control of the strong viral CMV promoter but also a HMG-1 intron for enhanced transgene protein synthesis via increased mRNA export from the nucleus. All of these plasmids were amplified in *Escherichia coli* (DH5α) and purified using the Qiagen Giga Prep Plasmid Purification protocol (Qiagen, Germany). Plasmid purities were checked by electrophoresis on 1.0% agarose gel. DNA concentrations were estimated spectroscopically by measuring the absorption at 260 nm and confirmed by gel electrophoresis. Plasmid preparations showing a value of $OD_{260}/OD_{280} > 1.8$ were used.

Formulations of Lipid 4-Based Liposomes and Preparation of Lipoplexes. The compound synthesized in this study was formulated alone or in combination with neutral colipids, either DOPE or cholesterol, at a 1/1 molar ratio. To prepare liposomes, the lipid **4** was first introduced into glass vials; next DOPE or cholesterol was added as required and then dissolved in chloroform. The solvent was subsequently evaporated under vacuum in order to obtain dry lipid films. A required volume of either 5 mM HEPES buffer (for physicochemical studies), water (for *in vitro* transfections), or 0.9% NaCl (for *in vivo*

transfections) was added to the lipid films; the vials were then sealed and stored at 4 °C. Before use, solutions were subjected to several cycles of sonication in a bath sonicator (Prolabo, France) and vigorous vortex mixing, in order to form small vesicles. To prepare the cationic lipid/DNA complexes, plasmid DNA was first diluted in either water (for Zetasizer measurements), OptiMEM (for *in vitro* transfections), or 0.9% NaCl (for *in vivo* transfections) before being added to the lipid solutions. These mixtures were kept at room temperature for at least 30 min before use, in order to allow the formation of DNA complexes. Lipoplexes characterized by different charge ratios (CR) were prepared, the CR being defined as the ratio of the vector positive charge, carried by the arsonium headgroup, to the negative DNA phosphate charges.

Critical Liposomal Concentration. The critical liposomal concentration (CLC) was determined using the Nile Red probe (9-diethylamino-5*H*-benzo[*a*]phenoxazin-5-one; Molecular Probes, France), a hydrophobic stain that, when intercalated into lipid membranes and excited at 485 nm, emits a strong fluorescence at 525 nm. Thus, studying the variation of probe fluorescence quantum yield enabled the estimation of the minimum lipid concentration allowing the formation of lipid **4**-based liposomes, i.e., the CLC value for that lipid. Increasing quantities of lipid **4** (final concentrations from 5×10^{-7} to 3×10^{-5} mol/L) were mixed with Nile Red (at 4×10^{-7} mol/L), and corresponding fluorescences were quantified.

Anisotropy Measurements. Fluorescence anisotropy (r) of the Laurdan probe (6-dodecanoyl-2-dimethylaminonaphthalene, Abs/Em = 364/497 nm; Molecular Probes, France) depends on the diffusion correlation time θ, according to the relation (eq 1):

$$r(t) = r(0)\mathrm{e}^{-(t/\theta)} \tag{1}$$

For steady-state anisotropy measurements, the relation becomes

$$r = \frac{r(0)}{1+(\tau/\theta)} \tag{2}$$

θ depends on the volume V of the molecule, the fluorescence lifetime τ, the absolute temperature T, and the medium viscosity η (eq 3):

$$\theta = \frac{\eta V}{kT} \tag{3}$$

Thus, the fluorescence anisotropy of the Laurdan probe is correlated with the medium viscosity η by the relation (eq 4) for steady-state anisotropy measurements (with k, Boltzmann constant). This relation indicates that a fast anisotropy decrease correlates with a low membrane viscosity.

$$\frac{1}{r} = \frac{1}{r(0)} + \frac{(\tau kT)}{(r(0)\eta V)} \tag{4}$$

Förster Resonance Energy Transfer (FRET) Measurements. For these tests, we used the fluorescent probes NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-PE, Abs/Em = 463/536 nm) and Rhod-PE (rhodamine-PE, Abs/Em = 560/581 nm), obtained from Molecular Probes (France). Förster interactions occur between two fluorophores in close proximity when the emission band of one (the energy donor) overlaps with the excitation band of the second (the energy acceptor). FRET efficiency thus depends on the distance between the two probes. If a couple of two lipidic fluorophores (e.g., the energy donor NBD-PE and the energy acceptor Rhod-PE) is introduced into a liposome, any fusion event of such a doubly labeled liposome with a second liposome (the "test" liposome devoid of any fluorophore) will decrease the efficiency of resonance energy transfer. Thus, any decrease in FRET efficiency can provide evidence for membrane fusion.[69] The efficiency (E) of the FRET was calculated from the fluorescence emission intensity of NBD-PE at 530 nm using eq 5:

$$E = 1 - \frac{F}{F_0} \tag{5}$$

Fluorescence intensities were recorded in the presence (F) and absence (F_0) of Rhod-PE. The relative fluorescence intensity (ER, in percent) was calculated using eq 6, where E_{mix} and E_{ab} are the FRET efficiencies calculated in the presence or absence of cationic lipids, respectively.

$$\mathrm{ER} = \frac{E_{mix}}{E_{ab}} \times 100 \tag{6}$$

As a doubly labeled membrane, we used liposomes incorporating L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), and cholesterol (Chol), all purchased from Sigma (France), mixed with the fluorescent probes NBD-PE and 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). These liposomes were then mixed with unlabeled cationic liposomes at increasing concentrations, from 10^{-6} to 10^{-4} mol/L of lipid **4**. The Rhod-PE and NBD-PE final concentrations were 6×10^{-8} mol/L and 3×10^{-7} mol/L, respectively. The final concentration of labeled membrane was 15 mg/L, corresponding approximately to 2×10^{-5} mol/L for PC. The Rhod-PE/lipid ratio was chosen after determination of the FRET efficiency versus Rhod-PE/PC molar ratio. A ratio closed to 0.003 was chosen so that any lipid fusion resulted in a significant decrease of FRET efficiency. NBD-PE concentrations did not affect the FRET efficiency, as previously described.[48]

DNA Condensation and Relaxation. The condensation of plasmid DNA by the cationic reagents studied and thereafter the efficiency of dextran sulfate (as a counteranion) to disorganize the lipoplexes formed were investigated using ethidium bromide intercalation into DNA. Upon condensation, ethidium bromide is expelled from DNA, and thus the fluorescence signal decreases. Conversely, DNA relaxation from the complexes results in recovery of fluorescence.[70] These assays were performed in 96-well plates either in OptiMEM (pH 7.4) or in 0.9% NaCl (pH ~5.0). The maximum fluorescence signal was obtained when ethidium bromide (1.5 μg/mL final) was bound to free plasmid DNA (1.0 μg/well). DNA was added to the wells containing different amounts of the reagents in order to form lipoplexes characterized by different CRs. The fluorescence signals were measured using a Fluoroskan Ascent FL plate reader (ThermoElectron Instruments, France) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. After formation of the lipoplexes, increasing amounts of dextran sulfate were added to the complexes, and the subsequent DNA relaxation was followed by measuring the fluorescence increase.

Zetasizer Measurements. The mean particle diameter and zeta potential (ξ) of the liposomes and lipoplexes were measured using a 3000 Zetasizer (Malvern Instruments) at 25 °C after an appropriate dilution of the formulations. Briefly, for measurements with lipoplexes, each assay used 40 μg of plasmid DNA mixed in water with the required quantity of lipid **4**-based liposomes in order to form lipoplexes with CRs ranging from 0.5 to 8.0. For measurements with liposomes, we used a lipid quantity equivalent to a CR1.0 mixture in water.

Cell and Culture Conditions. Two different cell lines were mainly used: (i) the A549 cell line, bronchial alveolar type II epithelial cells derived from a human pulmonary carcinoma, obtained from the American Type Culture Collection (ATCC No. CCL-185); (ii) 16HBE 14o-cells, human bronchial epithelial cells, kindly provided by D. Gruenert (USA).[71] They were grown in Dulbecco's modified Eagle's medium (DMEM, for A549 cells) or Eagle's minimum essential medium (EMEM, for 16HBE 14o-cells), supplemented with 100 units/mL penicillin,

100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. DMEM, EMEM, FBS, penicillin, and streptomycin were purchased from Invitrogen (U.K.).

***In Vitro* Transfections.** The *in vitro* transfection activities were evaluated in transient transfection experiments, as previously described by Felgner et al.,[1] with the following modifications. Twenty-four hours before transfection, cells were seeded in 96-well plates at a density of 12500 cells per well in a final volume of 200 μL (i.e., at about 70% confluence). Immediately prior to transfection, the growth medium was removed and replaced with 160 μL of OptiMEM (Invitrogen, U.K.) per well. The transfection mixtures (40 μL per well) were then added dropwise to the cell cultures, and the cells were exposed to the transfection reagents for 4 h. Thereafter, the medium containing the transfection mixtures was replaced with complete growth medium, and the cultures were maintained for 48 h at 37 °C until reporter gene measurements. For the evaluation of the effect of serum on transfection efficiency, the transfections were performed in the presence of serum, i.e., in complete growth medium with no medium change before the addition of the complexes onto the cell cultures. The commercially available lipofectamine (LFM; Invitrogen, U.K.), which corresponds to a liposome formulation of DOSPA/DOPE, 3/1 (w/w), was used as a reference reagent in these assays.

***In Vitro* Cytotoxicity.** Cytotoxicity was evaluated by two different methods. (1) The Toxilight assay (Cambrex, Belgium) is a chemiluminescent test allowing to quantitatively measure the release into the growth medium, from damaged cells, of an enzyme (adenylate kinase) normally located in the cytoplasm. This toxicity assay was carried out as specified by the manufacturer a few hours after addition of the complexes onto the cells (in order to estimate early cytotoxicity). The relative light units measured here were proportional to the number of viable cells. Untreated cells were used as a reference. (2) The total amount of extractable cell proteins, at 48 h after addition of the complexes onto the cells, was estimated using the BC assay kit (Interchim, France) and used as an index of the cell number present in each well. The cell density is indeed the result of (i) the number of cells initially plated, (ii) the normal, or induced, mortality depending on the treatment(s) applied, and (iii) the cell growth, normal or delayed. Here, cytotoxicity data were expressed as the percentage of missing proteins compared to the total protein content of untreated cells. The deficit in total protein content 48 h later was considered as an estimation of the final, cumulated, toxicities.

***In Vitro* Transfections: Luciferase Assays.** Forty-eight hours after transfection, the cells were first lysed using 1X passive lysis buffer (PLB; Promega, France) and then centrifuged, and the luciferase activity in each supernatant was measured using the luciferase assay system (Promega, France) with a microtiter plate luminometer (Dynatech Laboratories, France). The total protein content of each supernatant was quantified using the BC assay kit (Interchim, France). Results were expressed as RLU (relative light units) per milligram of total protein.

***In Vivo* Transfections.** Six- to nine-week-old female Swiss mice (Elevage Janvier, France) were housed and maintained at the University animal facility; they were processed in accordance with the Laboratory Animal Care Guidelines (NIH Publication 85-23, revised 1985) and with the agreement of the regional veterinary services (authorization FR; 29-024). Lipid **4** or KLN47-based lipoplexes were prepared at room temperature in 0.9% NaCl. The mice were placed in a restrainer, and 200 μL of complexes incorporating 50 μg of pDNA per mouse was intravenously injected, via their tail vein, within 5−10 s, using a $^1/_2$ in. 26-gauge needle and a 1 mL syringe. A total of 11 mice were treated with lipid **4**-based lipoplexes, 4 mice were injected with KLN47-based lipoplexes, and 4 mice received naked uncomplexed DNA.

***In Vivo* Bioluminescence: Noninvasive Imaging of Luciferase Activity.** Mice to be imaged first received an intraperitoneal injection of highly purified synthetic D-luciferin (4 mg in 200 μL of water; Interchim, France). Five minutes later, the animals were anesthetized with isofluorane administered through a nose cone. Ten minutes after luciferin injection, luminescence images were acquired using an *in vivo* imaging system (NightOWL NC320; Berthold) and associated software (WinLight 32; Berthold) with a binning of 8×8 and exposure times ranging from 4 to 8 min, according to the degree of luminescence. Luminescence images were then superimposed onto still images of each mouse. Signals were quantitated within the regions of interest as units of photons per second.

Luciferase Activity in Lung Homogenates. Twenty-four or forty-eight hours after transfection, some mice were killed by cervical dislocation, and their lungs were removed for analysis. Luciferase expression was evaluated as previously described.[72] Briefly, tissue pieces were washed in 1× PBS and rapidly frozen in liquid nitrogen, then disrupted, and finally collected in 1× PLB. Complete lysis was achieved by vigorous shaking at 4 °C for 45 min, and the supernatant was obtained by centrifugation. Luciferase activity and total protein content were then evaluated as indicated before. Results were expressed as RLU per mg of total protein.

***In Vivo* Biodistribution Study.** *In vivo* biodistribution studies of lipid **4**-based liposomes or lipid **4**-based lipoplexes were performed in mice using the lipophilic fluorescent probe DiR [DiIC18(7), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide; Invitrogen, U.K.]. This probe is a hydrophobic stain that, when intercalated into lipid membranes and excited at 750 nm, emits a strong fluorescence in the near-infrared, at 780 nm. Practically, mice were injected with 0.9% NaCl solutions containing either (1) the lipophilic fluorescent probe DiR alone, (2) lipid **4**-based liposomes mixed with DiR, or (3) lipid **4**-based lipoplexes mixed with DiR. Mice S2 and S3 (Figure 10) received equivalent quantities of lipid **4**, sufficient to reach a CR of 4.0 in the case of S3. About 1 h later, mice were sacrificed by cervical dislocation, and their organs were exposed in order to be visualized via an IVIS Spectrum device (Xenogen, France) with a 700 nm excitation filter and a 780 nm emission filter.

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Supporting Information Available: Additional information about DNA condensation into, and its release from, lipoplexes formed by lipid **4**-based liposomes or by the commercially available lipofectamine- (LFM-) based liposomes, under various experimental conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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