Lipophilic Pyrylium Salts in the Synthesis of Efficient Pyridinium-Based Cationic Lipids, Gemini Surfactants, and Lipophilic Oligomers for Gene Delivery

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Several new classes of pyridinium cationic lipids were synthesized and tested as gene delivery agents. They were obtained through a procedure that generates simultaneously the heterocyclic ring and the positively charged nitrogen atom, using lipophilic pyrylium salts as key intermediates that react with primary amines, yielding pyridinium salts. The choice of the appropriately substituted primary amine, diamine or polyamine, allows the design of the shape of the final lipids, gemini surfactants, or lipophilic polycations. We report also a comprehensive structure−activity relationship study that identified the most efficient structural variables at the levels of the hydrophobic anchor, linker, and counterion for these classes of pyridinium cationic lipids. This study was also aimed at finding the best liposomal formulation for the new transfection agents.

1. Introduction

Gene therapy is a revolutionary form of therapy[1] that promises to treat diseases at the cellular level using the ultimate drug: the DNA. Its success relies on finding efficient vectors for the transfer and expression (transfection) of the genetic material to the desired location in the living organism.[2] Thus, when the cellular machinery is impaired, a copy of a gene correcting a deficient (abnormal) gene can be transfected into cells, tissues, or organs affected by specific hereditary or acquired diseases. In cancer therapy, on the other hand, one hopes to deliver a gene that selectively kills or inhibits the malignant cells. Permanent incorporation of such a gene into the genome often is not desired. In contrast to conventional therapies, the limiting factor is not the quantity of the DNA but the efficiency of its overall delivery. Side effects are often limiting in conventional therapy. A new paradigm will have to be devised, replacing the classical ADME/Tox[a] paradigm.

Viral vectors are still the most efficient transfection systems available,[3] being structurally evolved to circumvent effectively the various delivery barriers[4] encountered by a foreign plasmid before reaching the nucleus of the target cell. Although current research is attempting to improve viral vectors,[5a,b] their use is still limited by serious side effects such as immunogenicity, mutagenicity, and sometimes fatal toxicity.[5c] Other important disadvantages are the limited size of the plasmid that can be inserted into the virion and the difficulties associated with good manufacturing practice (GMP) production.[5]

An alternative approach to efficient gene delivery vectors is to develop synthetic transfer agents, which fall into two main categories, namely, cationic lipids[6] and cationic polymers.[7] Both classes have much lower immunogenicity and cytotoxicity than viral vectors, allow the use of plasmids of practically unlimited size, and can be manufactured and stored in bulk quantities under GMP-compliant norms.[8]

Cationic lipids are amphiphilic molecules containing a polar (cationic) head linked via a spacer to a hydrophobic tail. When a certain concentration is reached, they self-assemble via cooperative hydrophobic binding forming cationic liposomes. In this form they can interact electrostatically with the negatively charged DNA molecules, forming cationic lipid−DNA complexes (lipoplexes)[9] in which the genetic material is highly compacted and protected from the action of endogenous nucleases. The characteristics of the lipoplexes (lipid nature and composition of the parent liposomes, properties of the plasmid, and the size, shape, and ζ potential of the supramolecular complexes) are essential for achieving high levels of transfection.[10]

With the focus on the nature of the cationic lipid, it must be stressed that after 2 decades of synthetic work that generated several commercial cationic lipid transfection systems such as DOTAP (**1**), DMRIE (**2**), and DOSPA (**3**) (Chart 1), there is still room for improvement of the transfection efficiency while minimizing the cytotoxicity.[4,6,11] In recent years, it has been shown by a few different groups that progress can be achieved via the heterocyclic cationic lipids (**4**−**7**, Chart 2).[12−14] Among these heterocyclic cationic lipids, the pyridinium cationic lipids reported first by Engberts' group[13] and then by ourselves[14] reached or surpassed the transfection efficiency of commercial cationic lipid formulations, both in vitro and in vivo, while maintaining a low cytotoxicity.

Similar to cationic lipids, cationic polymers,[7] when mixed with DNA, form compact polyplexes.[9] Although it is somehow easier to control polyplex properties than those of lipoplexes, owing to the templating features of the polymeric backbone, cationic polymers usually display higher cytotoxic and immunogenic profiles than cationic lipids. Recently, another class emerged to fill the gap between cationic lipids and cationic polymer systems, borrowing advantages from both classes. This new class contains cationic gemini surfactants[15] and oligomeric cationic surfactants.[16]

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[a] Abbreviations: ADME/Tox, absorption, distribution, metabolism, and excretion/toxicity; GMP, good manufacturing practice; DOTAP, *N*-(2,3-dioleoyloxypropyl)-*N*,*N*,*N*-trimethylammonium chloride; DMRIE, *N*-(2,3-dimiristyloxypropyl)-*N*-(hydroxyethyl)-*N*,*N*-dimethylammonium bromide; DOSPA, *N*-(2,3-dioleyloxypropyl)-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethylammonium trifluoroacetate; DOPE, dioleoylphosphatidylethanolamine; Chol or C, cholesterol; DSC, differential scanning calorimetry; PBS, phosphate buffer isotonic saline at pH 7.4; BCA, bicinchoninic acid; bovine BSA, serum albumin; RLU, relative luminescence units; Lipofect, Lipofectamine; Boc, *tert*-butyloxycarbonyl.

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Chart 1

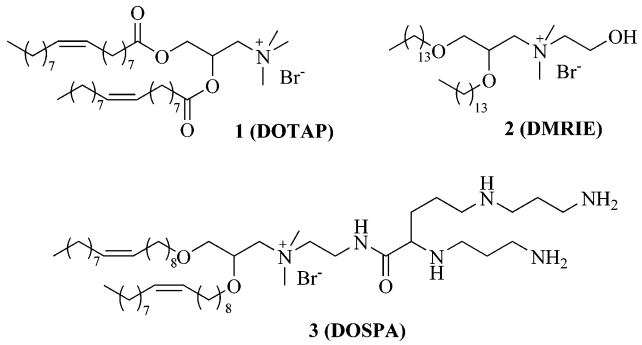


Chart 2

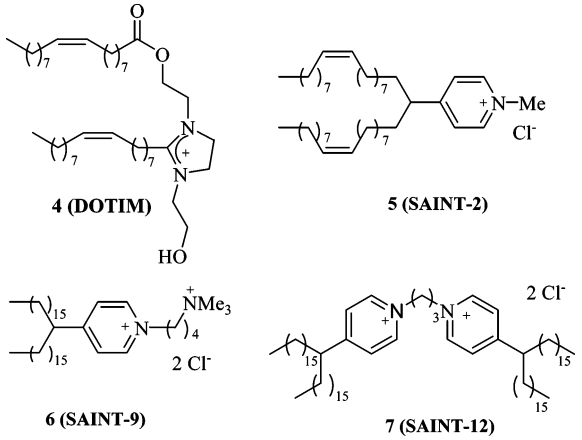
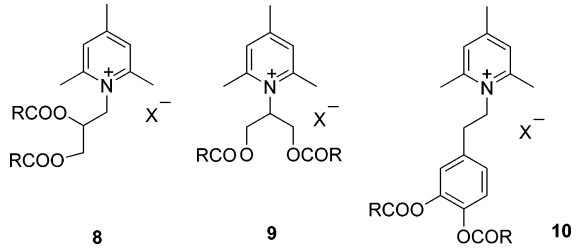


Chart 3



Gemini surfactants are synthetic transfection vectors comprising two simple surfactant moieties linked together at the level of the polar head. Introduced in the 1970s, they experienced a new outburst in the early 1990s because of their unusual properties.[17] They possess a higher charge per mass ratio than cationic lipids and also have superior surfactant properties. They can self-assemble at very low concentrations, forming a variety of supramolecular assemblies (micelles, bilayers, inverted micelles). Because of these special properties, they have been recently used as vectors for gene therapy.[15b] Similar properties were reported for oligomeric surfactants.[18]

Our previous structure−activity relationship study[14b] of three series of pyridinium cationic lipids (**8**−**10**, Chart 3) revealed that the most efficient representative was the *N*-(1,3-dimyristoyloxypropane-2-yl)collidinium derivative **9**. This amphiphilic compound, because of its truncated cone shape, induces a higher radius of curvature of the lipid bilayer, thus generating smaller, more transfection-efficient liposomes. The same features were found to be beneficial by Engbert's group.[13]

These findings prompted us to investigate new pyridinium cationic lipids with the alkyl chains directly attached on the aromatic ring, using the reaction between a lipophilic pyrylium salt[19] and a fatty amine. This original, flexible strategy[14,19] allows various designs while maintaining a truncated cone shape for the cationic amphiphile. It also provides a simple and direct synthetic route to gemini and oligomeric surfactants. We report now a detailed structure−activity relationship study aimed at identifying the most efficient structural variables for these new types of pyridinium cationic vectors. This study also sought the best formulation for these novel transfection agents.

2. Results and Discussion

One of the most important variables in the structure of cationic lipids is the length of the hydrophobic chain.[6,14,20] This parameter influences directly the shape of the individual amphiphilic molecules and acts with the polar head to influence the supramolecular associations that can be generated through the self-assembling process.[21] Therefore, we synthesized a library of lipophilic pyridinium salts in order to evaluate the influence of the chain length on the physicochemical and biological properties of these new transfection vectors. The synthesis (Scheme 1) is based on the original synthetic strategy reported previously,[14a,b] which involves the reaction of pyrylium salts with primary amines to generate the cationic head in a single, high-yield step.[22] Eight lipophilic pyridinium salts with linear alkyl side chains ranging from 10 to 17 carbon atoms and with hexafluorophosphate as counterion were obtained via a modified literature procedure.[19] Briefly, mesityl oxide was monoacylated[22] with fatty acid chlorides **12** in the presence of aluminum chloride to yield the pyrylium chloroaluminates **13**, which were converted into the more stable hexafluorophosphates **14** by hexafluorophosphoric acid. The pyrylium hexafluorophosphates were subsequently reacted with primary linear alkylamines ranging from 10 to 18 carbon atoms, thus generating the library of pyridinium cationic lipids **15**−**28** (Scheme 1 and Table 1).

Physicochemical data of the new compounds show that pyrylium salts **14** display alternating melting points; compounds with an odd number of carbon atoms in the side chain exhibit lower melting points than their congeners with an even number of carbon atoms in the side chain. This behavior, similar to that of fatty acids, is due to the differences in the crystal packing of the amphiphilic molecules. A similar behavior is observed for the critical temperatures of the pyridinium cationic lipids; those compounds with two identical fatty chains (each with an even number of carbon atoms) display slightly higher T_c values than their congeners with an odd and an even number of carbon atoms in the two long side chains (compare **18** with **20** and compare **25** with **26**). Otherwise, the critical temperatures are generally increasing while elongating the alkyl chains.

To assess how the chain length influences the transfection activity, the lipids were conditioned using a previously optimized protocol for these compounds.[14b,c] Thus, the cationic lipids were mixed with cholesterol at a 1:1 molar ratio, dissolved in chloroform/methanol, and dried under vacuum to generate a lipid film. The dried lipid film was hydrated with sterile phosphate buffer isotonic saline at pH 7.4 (PBS), vortex-mixed, and sonicated to yield cationic liposomes. The resulting liposomal solution was allowed to react with a DNA solution to form the final cationic lipid−DNA complexes (lipoplexes), which were assessed for transfection efficiency. We used a pGL3 plasmid (Promega, Madison, WI), encoding a firefly luciferase gene under the control of the constitutively active SV40 promoter, as the reporter for the transfection efficiency. An optimal[14b,c] electrostatic charge ratio for cationic lipid/DNA of 2:1 was used in all preparations. Cholesterol (Chol) was used as colipid in the liposomal formulations because it had proved to be superior

Scheme 1

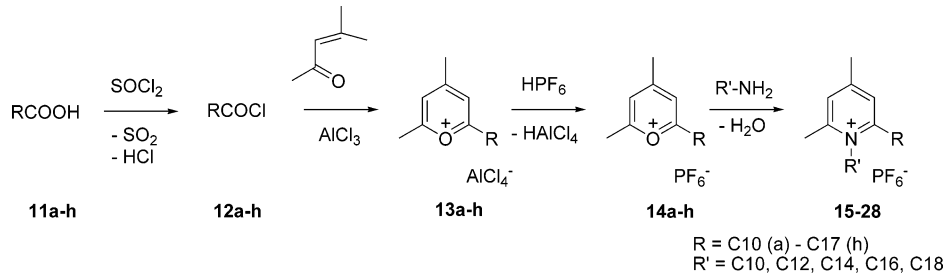


Table 1. Lipophilic Pyrylium Salts **14** and Dialkylpyridinium Cationic Lipids **15**−**28** with Their Critical Temperatures (T_c)

compd	R′	R	mp/T_c (°C)	compd	R′	R	mp/T_c (°C)
14a		C10	67.6	**18**	C12	C11	54.8
14b		C11	62.8	**19**	C10	C12	57.6
14c		C12	78.7	**20**	C12	C12	58.6
14d		C13	73.3	**21**	C10	C13	58.4
14e		C14	85.2	**22**	C12	C13	67.2
14f		C15	79.8	**23**	C14	C14	68.5
14g		C16	88.1	**24**	C14	C15	74.7
14h		C17	81.8	**25**	C16	C15	74.1
15	C10	C10	46.9	**26**	C16	C16	76.2
16	C10	C12	56.7	**27**	C16	C17	82.2
17	C10	C11	52.2	**28**	C18	C17	82.1

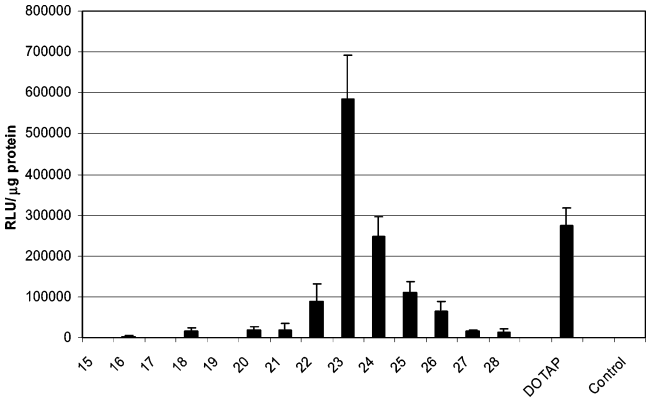


Figure 1. Transfection data for cationic lipids **15**−**28** compared with DOTAP as reference. All lipids were conditioned with cholesterol as a helper lipid, at 1:1 molar ratio. An optimized electrostatic charge ratio of cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

to dioleoylphosphatidylethanolamine (DOPE) for this type of pyridinium cationic lipid.[14b,c] All liposomal preparations were sonicated for 30 min at 65−68 °C (twice for 15 min, with a 15-min pause between) for the new compounds, and at 37 °C for the chosen reference cationic formulation DOTAP/Chol (1:1 molar ratio),[23] similar to our previous studies.[14b,c] Under these conditions liposomes with sizes ranging from 87 to 191 nm were obtained as determined by dynamic light scattering, with a ζ potential of around 30 mV. The resulting lipoplexes had a ζ potential of around −15 mV. We used the lung cancer cell line NCI-H23 as the main test system for in vitro experiments, since our previous transfection studies[14b,c] on several cultured cell lines showed it to be the most sensitive toward pyridinium cationic lipids. The results are summarized in Figure 1.

The maximum transfection efficiency was obtained when two myristyl chains were attached to the pyridinium ring (**23**, Figure 1). The level of transfection was twice that of **24** (which has only one more carbon atom in one of the side chains) or the standard transfection system DOTAP/Chol.

We note than in contrast to our results with these new compounds other authors have reported that in the case of

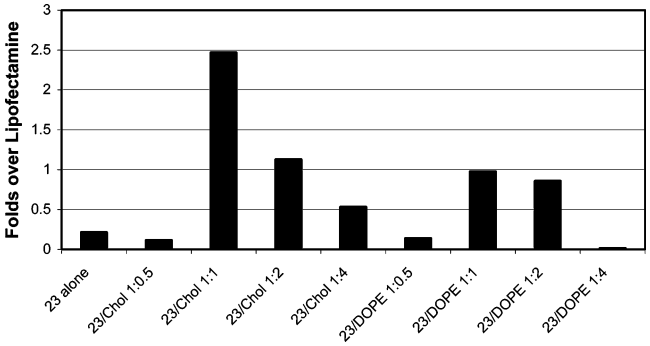


Figure 2. Optimization of helper lipid and its molar ratio relative to the cationic lipid **23** in liposome preparation. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

diacylglycerol cationic lipids, more efficient transfection could be achieved with cationic lipids having hydrophobic tails of unequal lengths than with the symmetrical congeners.[24]

To validate the conditions optimized by the experiment of Figure 1, several formulations based on **23** were tested, using cholesterol or DOPE in various molar ratios as helper lipids. Lipofectamine was used as a reference at 2:1 electrostatic charge ratio to DNA. The results confirmed that the best helper lipid for these compounds is cholesterol, coformulated with the cationic lipid at a 1:1 molar ratio (Figure 2).

Pyridinium Cationic Lipids Coupled with Gemini Surfactants. An important parameter in gene delivery is the amount of compacting cationic species added to the DNA in the delivery complex in order to reduce the dimensions of DNA and mask its negative charge. One wants to keep the amount of cationic entity as low as possible in order to minimize its immunogenicity. As mentioned before, a higher (positive) charge per mass ratio than in cationic lipids can be obtained by using cationic gemini surfactants.[15] Moreover, these amphiphilic compounds have the ability to help the endosomal release of the DNA complexes by destabilizing the endosomal membrane via associations with the gemini anionic lipids.[25] This class of compounds was reported to be efficient in gene delivery;[15b] therefore, we decided to investigate the efficiency of the pyridinium polar head, in a gemini surfactant design, for in vitro gene delivery.

Two libraries of pyridinium gemini surfactants were obtained using the same synthetic strategy (Schemes 2−4). The key intermediate, tetradecyldimethylpyrylium hexafluorophosphate **14e** (whose side chain length was proved to be optimal), was condensed with different primary diamines, generating the linked polar heads in a single, high-yield step.

The linker's structure has an essential role in the size and shape and hence in the supramolecular self-assembling properties of this class of amphiphilic molecules. If hydrophobic, it will tend to pack between the two hydrophobic chains, generating a tapered molecular shape. If hydrophilic, it will be inclined to stay at the hydrophobic/water interface, thus changing the

Scheme 2

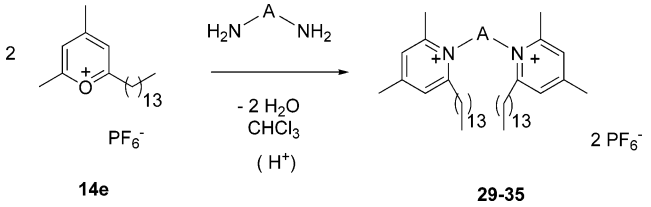


Table 2. Gemini Cationic Lipids **29**−**35**, **41**, and **47** and Their Critical Temperatures (T_c)

compd	spacer	T_c (°C)
29	$(CH_2)_2$	178.2
30	$(CH_2)_3$	186.6
31	$(CH_2)_4$	185.6
32	$(CH_2)_5$	137.8
33	$(CH_2)_6$	189.9
34	$(CH_2)_7$	83.6
35	$(CH_2)_8$	68.8
41a	$(CH_2)_2NH(CH_2)_2$	65.1
41b	$(CH_2)_3NH(CH_2)_3$	45.4
41c	$(CH_2)_3NH(CH_2)_4$	67.2
47	$(CH_2)_3NH(CH_2)_4NH(CH_2)_3$	*a*

[a] Amorphous (glass transition at 9.6 °C).

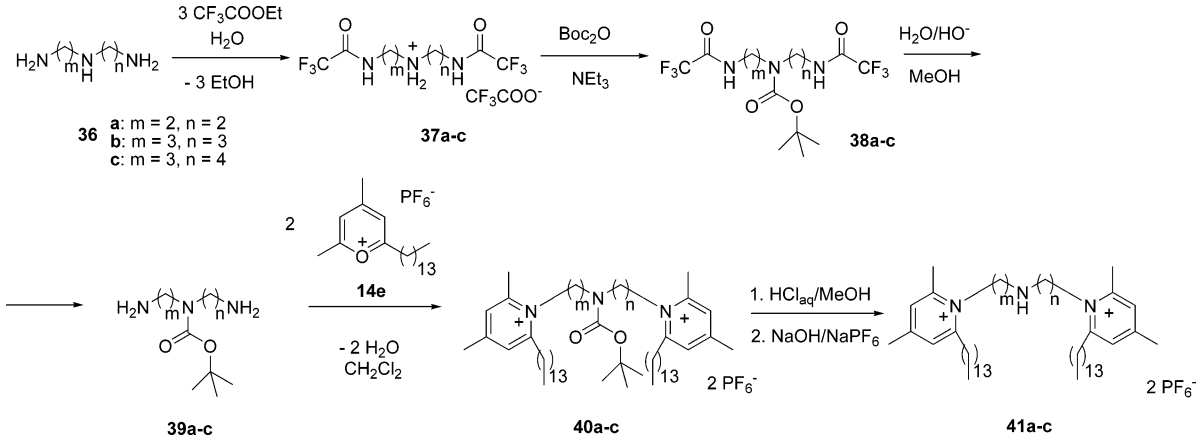
solid angle between the two lipophilic chains and the shape of the amphiphilic molecule.

The first library comprised gemini surfactants **29**−**35** with nonpolar linkers, generated from *n*-alkyldiamines and the pyrylium salt **14e** (Scheme 2). We varied the spacer length from two to eight carbon atoms in order to finely tune the properties of the new species and to assess the influence of the linker (and hence of the shape of the molecule) on the transfection efficiency. The seven representatives thus obtained and their physicochemical properties are presented in Table 2.

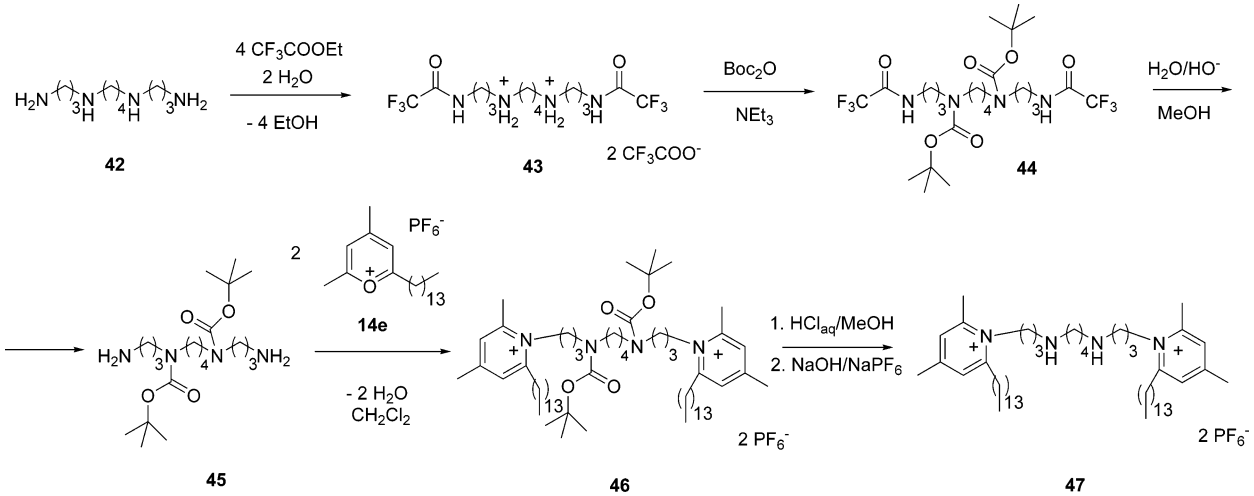
The second library (Schemes 3 and 4) was designed to test the influence of a hydrophilic linker, containing secondary amino groups, on the transfection efficiency of pyridinium gemini surfactants. Since some of these secondary amino moieties are positively charged at the physiologic pH, they will also contribute to the binding and compacting of the DNA and are expected to generate a different transfection profile for compounds **41** and **47**.

The synthetic approach used in this case involved polyamines **36a**−**c** and **42**, with various numbers of methylene units between the amino moieties, as starting materials, which were selectively protected with *tert*-butyloxycarbonyl (Boc) groups on the secondary amino moieties using a well-established literature procedure.[26] Thus, compounds **36a**−**c** and **42** were condensed with ethyl trifluoroacetate when the primary amino moieties were selectively protected with trifluoroacetyl groups. Treatment of trifluoroacetamides **37a**−**c** and **43** with di-*tert*-butyl dicarbonate in the presence of triethylamine afforded compounds **38a**−**c** and **44**, respectively, in which the secondary amino groups were Boc-protected. A selective hydrolysis in basic medium generated the diamines **39a**−**c** and **45**, which were subsequently condensed with the key intermediate **14e** to generate the pyridinium gemini compounds **40a**−**c** and **46**. The

Scheme 3



Scheme 4



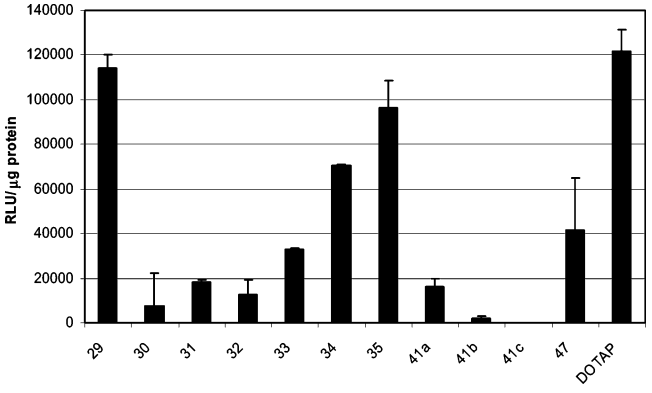


Figure 3. Transfection data for gemini cationic lipids **29**−**35**, **41**, and **47** compared with DOTAP as reference. All lipids were conditioned with cholesterol as a helper lipid, at 1:1 molar ratio. An electrostatic charge ratio cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

removal of Boc protecting groups from these precursors via HCl/MeOH yielded the final polycationic amphiphiles **41a**−**c** and **47**. Their physicochemical characteristics are shown in Table 2.

The use of this synthetic strategy was made necessary because of the interference of the secondary amino groups[22] in a direct reaction between polyamines **36** or **42** and pyrylium salt **14e**, which translates into a complex mixture of products, which are very difficult to separate.

The data in Table 2 show that the critical temperatures of the cationic amphiphiles depend markedly on the structure of the linker. As a general trend, gemini cationic lipids with alkyl linkers display higher transition temperatures than their congeners bearing polar secondary amino moieties in the spacer. Within the gemini series **29**−**35** it can be observed that when the spacer is elongated from two to eight carbon atoms, two opposite effects can be noticed. Thus, when the spacing is increased from two to three carbon atoms in the linker, the critical temperature increases because of a better separation of the positive charges on the pyridinium rings. It remains elevated with further elongation of the spacer up to six methylene units, after which it decreases abruptly, possibly because of a change in the packing of hydrophobic elements.

In the case of hydrophilic linkers, substitution of a methylene unit by an amino group decreased the critical temperature (compare **32** with **41a** and compare **34** with **41b**). Interestingly, when the linker is sufficiently long, the differences between its characteristics are blurred (compare **35** with **41c**), the thermal stability of the compounds being dictated predominantly by other structural elements. Compound **47**, with two secondary amino moieties in the linker, is a viscous liquid at room temperature. Therefore, it would be expected that the gemini surfactants with hydrophilic spacers **41** and **47** would display higher transfection efficiencies than their hydrophobic congeners **29**−**35**, since the fluidity of the complexes with DNA would be much higher under physiological conditions.[10] However, after the lipids were tested as a 1:1 molar ratio mixture with cholesterol, the reverse was observed (Figure 3). Polycationic compounds **41** and **47** displayed much lower transfection efficiencies than their hydrophobic congeners **29**−**35**. This fact may be explained by considering that a transfection event implies both an effective compaction of the DNA via electrostatic association of the lipid with the DNA (favored in the case of polycationic surfactants) and an efficient release of the plasmid from the complex after passing the cell's membrane, which is slowed by the presence of multiple positive charges on the lipid's polar head. Another

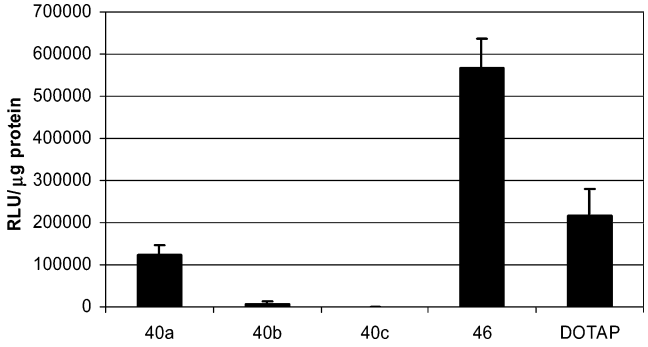


Figure 4. Transfection data for Boc-gemini cationic lipids **40** and **46**, compared with DOTAP as reference. All lipids were conditioned with cholesterol as helper lipid, at 1:1 molar ratio. An electrostatic charge ratio cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

possibility is the inactivation of the lipoplexes in contact with serum, similar to the case of Lipofectamine.[27] Other groups reported similar findings.[28]

In the case of gemini lipids **29**−**35** another interesting behavior was observed (Figure 3). On elongation of the linker from two carbon atoms (compound **29**, which is somehow similar in shape to compound **23**) to three carbon atoms (compound **30**), there was a steep decrease of transfection. Further elongation by four or five carbon atoms gave little improvement, but thereafter the transfection efficiency increased monotonically with the linker length. Even so, the transfection efficiency of gemini amphiphile **35** was lower than of its congener **29**, which transfected similarly to the standard transfection system DOTAP/Chol 1:1 (Figure 3, "DOTAP"). Changing the helper lipid and its molar ratio in the formulation, as well as the lipid/DNA charge ratio, did not improve the transfection efficiency (data not shown). The transfection results (with the exception of **29**) are similar to the ones reported by MacDonald and co-workers[28b] for quaternary ammonium-based gemini surfactants.

Since the polycationic lipids **41** and **47** displayed much lower transfection efficiency than the related gemini congeners **29**−**35**, we tested the transfection efficiency of their precursors **40** and **46**, hypothesizing that they would give higher transfection efficiency. These compounds have the secondary amino moieties Boc-protected, behaving therefore as normal gemini cationic surfactants. We found that their critical temperatures are higher than their congeners having *n*-alkyl linkers with the same dimensions (Table 3).

Table 3. Gemini Cationic Lipids **40** and **46** and Their Critical Temperatures (T_c)

compd	spacer	T_c (°C)
40a	$(CH_2)_2NBoc\ (CH_2)_2$	139.9
40b	$(CH_2)_3NBoc\ (CH_2)_3$	134.4
40c	$(CH_2)_3NBoc\ (CH_2)_4$	143.5
46	$(CH_2)_3NBoc(CH_2)_4NBoc(CH_2)_3$	103.3

Compounds **40** and **46** were conditioned (1:1 molar) with cholesterol, assayed under the same conditions as those used for the gemini series **29**−**35**. The results of transfections (Figure 4) confirmed the working hypothesis. The transfection efficiency was found to depend on the structure of the linker; the best results were obtained with **40a** and **46** (Figure 4).

Interestingly, **40a** was at least 5 times more active than its alkyl congener **32**. The Boc derivative **46** was the most active, being about 3 times more efficient than the commercial formulation DOTAP/Chol 1:1. These facts indicated that ef-

Scheme 5

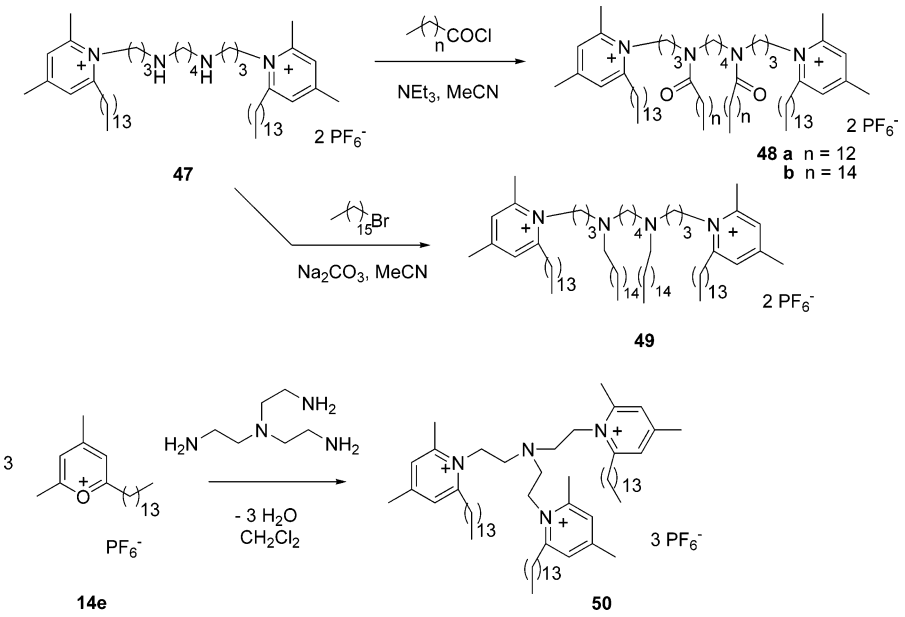


Table 4. Multitail Cationic Lipids **48**−**50** and Their Critical Temperatures (T_c)

compd	*n*	T_c (°C)
48a	12	125.8
48b	14	124.9
49		69.4
50		121.2

ficient pyridinium gemini cationic lipids can be obtained using hydrophilic linkers decorated with lipophilic moieties.

These findings prompted us to test the effect of substituting the Boc groups in **46** with more lipophilic moieties, introduced either by acylation of **47** (with myristoyl or palmitoyl chloride) or by alkylation of the same intermediate with palmityl bromide. Compounds **48a**, **48b**, and **49** were thus obtained in good yields (Scheme 5). All these compounds were expected to form tapered dendritic units, either flat or conical. Another conical lipophilic dendritic amphiphile, the tris(2,4-dimethyl-6-myristylpyridinium-1-ethylenyl)amine **50**, was synthesized by condensation of the myristylpyrylium salt **14e** with tris(aminoethyl)amine (Scheme 5).

The physicochemical properties of these dendritic molecules are presented in Table 4. As expected, the two bispyridinium-bisamides **48a** and **48b** have critical temperatures slightly higher than that of their Boc congener **46**, a fact that can be related to the association of the four hydrophobic tails. In contrast, the tetraalkyl derivative **49**, lacking the two amido groups, displays a much lower transition temperature. The trispyridinium **50** has a relatively high critical temperature probably because of the presence of the third heterocyclic ring, which confers a better packing.

The transfection efficiency of these dendritic molecules was tested on the same NCI-H23 cell line, using Lipofectamine as reference and a constant lipid/DNA electrostatic charge ratio of 2:1. The lipids were either tested alone or coformulated with cholesterol or DOPE as colipids at molar ratios of 1:1 or 1:2 (Figure 5).

Data from Figure 5 reveal that **48a**, **48b**, and **49** have the same transfection profile, being most active when coformulated with DOPE in 1:1 molar ratio. In this form, the lipid **49** was more efficient than the commercial transfection system Lipofectamine. At the 1:1 molar ratio, DOPE was found to be

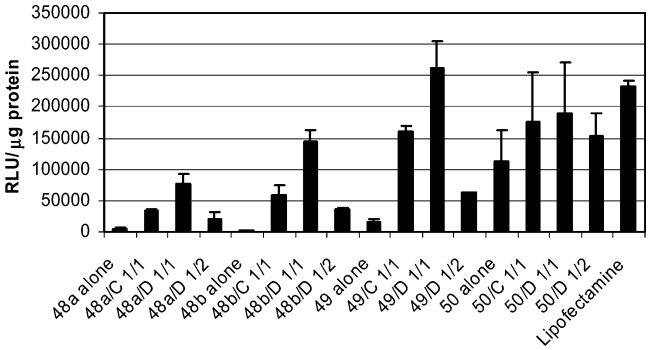


Figure 5. Transfection data for multitail cationic lipids **48**−**50** compared with Lipofectamine as reference. Cholesterol (C) was coformulated at 1:1 molar ratio with the test lipid. DOPE (D) was coformulated at 1:1 or 1:2 molar ratio. An electrostatic charge ratio cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

superior to cholesterol as a colipid for all these multitail compounds. When the amount of DOPE was doubled, the transfection efficiency decreased markedly to about one-third of the initial value. The transfection efficiencies of the lipids alone are very small. By contrast, the tris(myristylpyridinium) **50** displayed a more homogeneous transfection profile, being significantly active even without helper lipids. Taking into consideration all these facts and knowing that the Lipofectamine formulation consists of the polycationic lipid DOSPA (Chart 1), coformulated with DOPE, we hypothesize that the mechanism of action of pyridinium polycationic lipids is similar to the one of DOSPA and therefore Lipofectamine.

A comparison between the transfection results for polycationic amphiphiles **49** and **50** and their congeners **41** and **47** reveals the important contribution of the hydrophobic elements in the design of polycationic surfactants. Moreover, an analysis of the most efficient transfection systems obtained in the gemini surfactant series (compounds **29** and **48b**) shows that they actually share common structural elements (Figure 6). Thus, **48b** (as well as **48a** and **49**) can be considered to be dimers of a cationic lipid having a charged pyridinium group (red circle in Figure 6) and a second polar group (blue circle) at the water/lipid interface. The two polar elements are spaced by a short

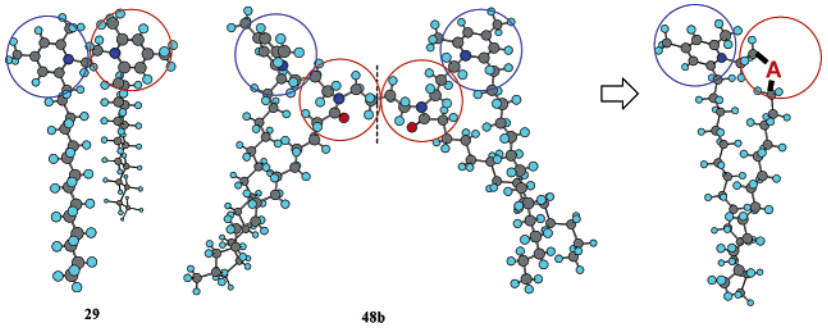
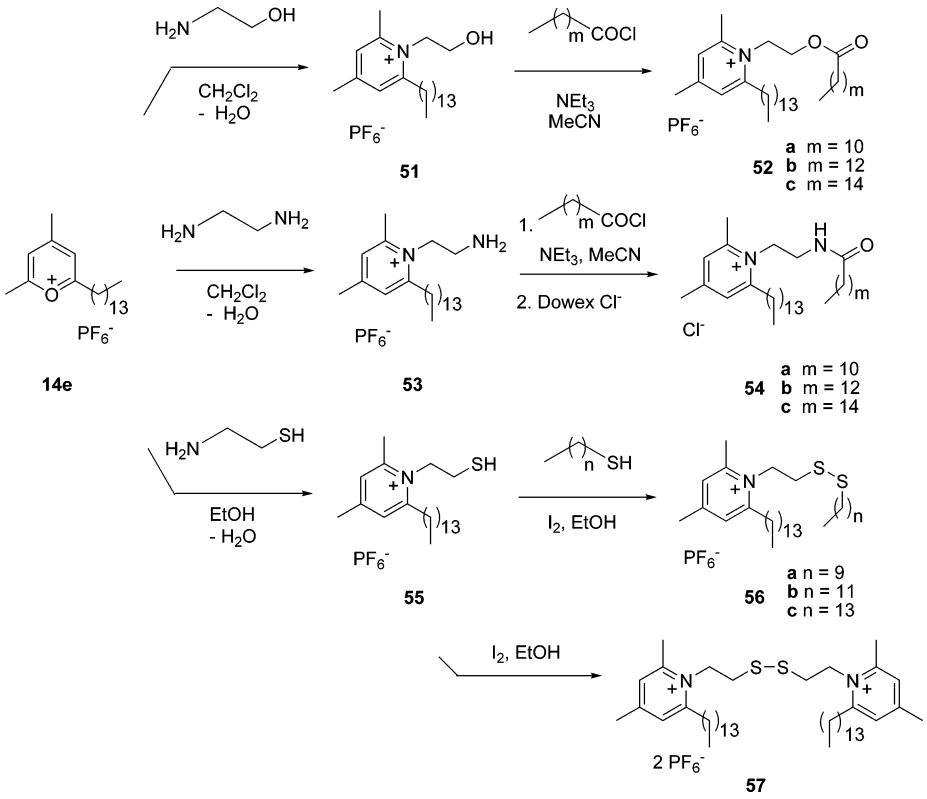


Figure 6. Energy-minimized structures of gemini lipids **29** and **48b.** It can be considered that both structures share a common structural motif (shown at right), consisting of a pyridinium polar head (indicated by a large blue circle) and a second polar moiety (indicated by a red circle) placed at the water/hydrophobic interface, spaced by a short linker (two or three carbon atoms). For segment A in the right-hand structural motif, we chose ester and amide polar groups (see text).

Scheme 6



linker (with two or three carbon atoms). In **29**, the second pyridinium ring can be considered to act as the secondary polar group.

Therefore, we decided to investigate different constructs that match the basic design depicted in Figure 6. We used ester and amide polar groups (segment A in Figure 6) because they are biodegradable and can reduce the cytotoxicity of the new cationic lipids. The synthesis is outlined in Scheme 6.

Thus, the starting myristylpyrylium salt **14e** was condensed either with ethanolamine or with ethylenediamine to yield the hydroxy- or aminoethylpyridinium salts **51** and **53**, which were subsequently acylated with fatty acid chlorides to generate the final cationic lipids **52** and **54**. We varied the second chain length from 12 to 16 carbon atoms in order to find the optimum size for the best transfection efficiency in these new classes of cationic lipids.

Also, to test the validity of the new design, a lipophilic disulfide group was introduced instead of the polar ester and amidic groups, via condensation of **14e** with 2-aminoethanethiol followed by the oxidative cross-coupling with I_2/EtOH of the *N*-ethylmercaptopyridinium salt **55** with *n*-alkylthiols. In contrast with compounds **52** and **54**, which possess a hydrophilic element in the second chain that induces a tapered shape in aqueous media, disulfides **56** were expected to self-assemble in a way similar to lipophilic pyridinium salts **15**−**28**. We used *n*-alkylthiols with chain lengths ranging from 10 to 14 carbon atoms, thus generating lipophilic pyridinium salts structurally similar to the most efficient congeners **15**−**28**. Additionally, the same key intermediate **55** was oxidized alone with iodine in ethanol to yield the "dimeric" disulfidic gemini pyridinium salt **57**.

Pyridinium amides **54** were obtained as mixtures of counterions; thus, they had to be subsequently transformed into chlorides by anion exchange over a Dowex resin in chloride form. We had demonstrated in our previous study[14b] that there are essentially no differences in the transfection efficiency and cytotoxicity between hexafluorophosphate and chloride anions, the chloride anion being more biocompatible. The characteristics of these new series of cationic lipids are outlined in Table 5.

We tested the three new series of cationic lipids **52**, **54**, and

Table 5. Cationic Lipids of Type **52**, **54**, **56**, and **57** with Their Critical Temperatures (T_c)

compd	T_c (°C)	counterion	compd	T_c (°C)	counterion
52a	61.3	PF_6^-	**56a**	77.4	Cl^-
52b	68.5	PF_6^-	**56b**	81.0	PF_6^-
52c	77.8	PF_6^-	**56b**	75.1	Cl^-
54a	41.9	Cl^-	**56c**	81.8	PF_6^-
54b	49.9	Cl^-	**56c**	86.9	Cl^-
54c	46.8	Cl^-	**57**	107.0	PF_6^-
56a	76.0	PF_6^-	**57**	139.3	Cl^-

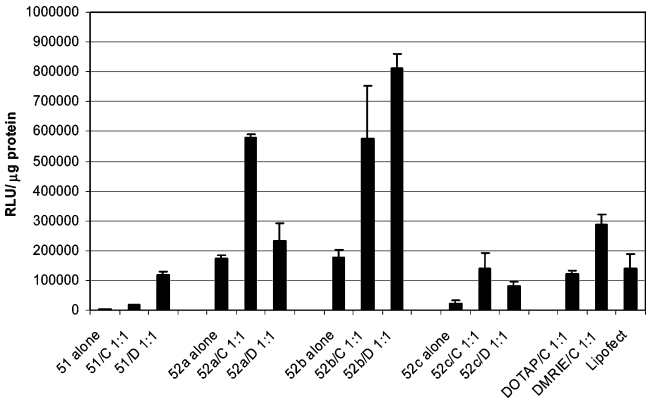


Figure 7. Transfection efficiency for compounds **51** and **52** compared with standard transfection reagents. In all preparations an electrostatic charge ratio of cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

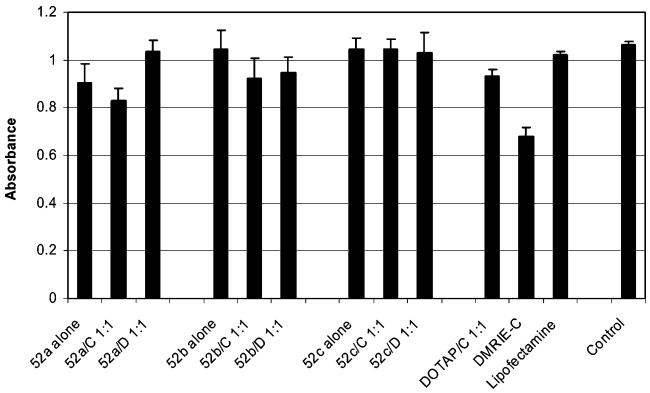


Figure 8. Cytotoxicity for **52** compared with standard transfection reagents. In all preparations an electrostatic charge ratio of cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

56 against several commercial formulations (DOTAP/Chol 1/1, DMRIE/Chol 1/1, Lipofectamine) on the NCI-H23 cell line at a constant electrostatic charge ratio of cationic lipid/DNA of 2:1. The new lipids were tested alone or coformulated with cholesterol or DOPE in a 1:1 molar ratio, since this ratio was the most efficient in previous cases. A WST-1 cytotoxicity assay[29] was performed in parallel (Figures 7−10). Lower absorbance in this latter assay corresponds to higher toxicity.

The disulfides **56** and **57**, together with the precursor **55** were also tested in the same conditions. However, they were found to be devoid of any significant transfection efficiency (efficiency less than 3000 RLU/μg of protein), showing moreover significant cytotoxic effects (data not shown). The transfection efficiency did not improve when the counterion was changed from PF_6^- to Cl^-, using the DOWEX anion exchanger resin. These findings are similar to the conclusions of a previous study[30a] but at variance with the results of other groups that used a strategically placed disulfide group to enhance the transfection efficiency in various redox-sensitive designs that

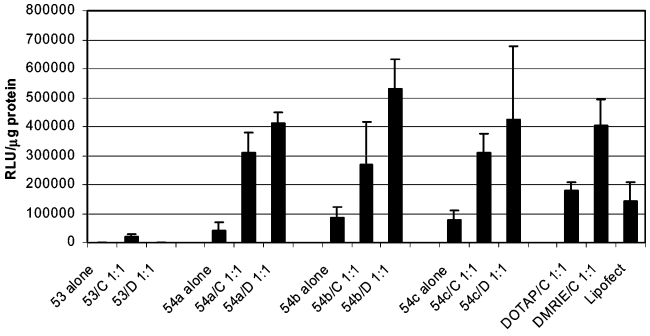


Figure 9. Transfection efficiency for compounds **53** and **54** compared with standard transfection reagents. In all preparations an electrostatic charge ratio of cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

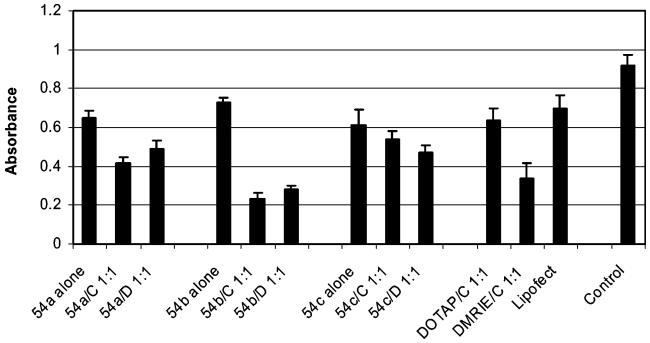


Figure 10. Cytotoxicity data for **54** compared with standard transfection reagents. In all preparations an electrostatic charge ratio of cationic lipid/DNA of 2:1 was used. NCI-H23 cell extracts were assayed 24 h later for luminescence (RLU/μg of protein).

exploit the high intracellular levels of glutathione,[30b−e] revealing once again the importance of structure−activity correlation studies in the design of novel cationic lipid-based transfection systems.

In contrast to the disulfide series, the esters **52** and the amides **54** showed a good transfection profile, the esters being generally more active and less cytotoxic than amides, despite having a higher T_c (mostly due to the PF_6^- anion) and a less biocompatible counterion (Table 5, Figures 7 and 9). In both cases the transfection efficiency peaked at the myristoyl derivatives, similar to **23** and **29**, a fact that proves that the structural assumptions were correct. Thus, **52b** and **54b**, when conditioned with DOPE as colipid at a 1:1 molar ratio, were 1.5−6 times more efficient than commercial transfection systems, including the very efficient DMRIE-C formulation.[20] In particular, the transfection efficiency of **52b** was associated with a very low (practically negligible) cytotoxicity, which is particularly important for further in vivo applications. The amide series **54**, although also very transfection efficient, showed a higher cytotoxic side effect, probably due in part to the possible in vivo degradation product **53**, which may be much more toxic than its hydroxyl congener **51** (Figures 8 and 10).

Good results were also obtained when cholesterol was used as colipid (at 1:1 molar ratio to cationic lipid), especially in the ester series **52**, where its effect seems to be correlated with the second chain's length. The members of the amide series **54** showed a homogeneous transfection profile when conditioned with cholesterol, and were rather insensitive to the modifications of the secondary chain length. These differences between the two hydrophilic groups might be explained considering that the ester group is a hydrogen-bond acceptor and therefore sensitive to the hydrogen-bond-donating properties of the cholesteric OH group, whereas the amide group is a hydrogen-bond donor and

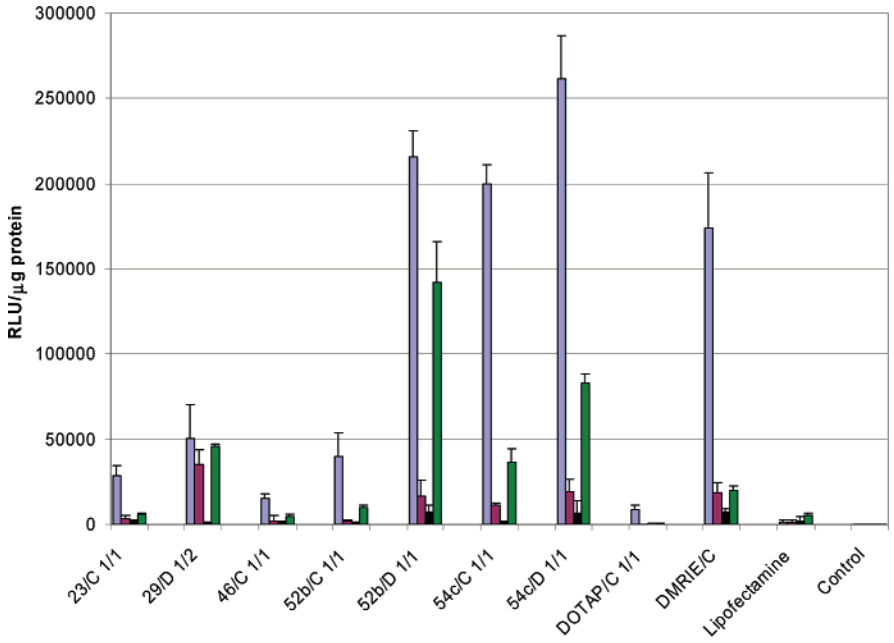


Figure 11. Transfection data for the most efficient cationic lipids in MCF-7 (blue bars), MDA-231β (red bars), DU-145 (black bars), and SWB-95 (green bars) tumor cell lines against different commercial formulations. In all preparations an electrostatic charge ratio of cationic lipid/DNA of 2:1 was used.

thus rather inert toward these interactions. The liposomes generated from lipids **52** and **54** had sizes between 74 and 157 nm, with a ζ potential ranging from + 23 to + 44 mV; their corresponding lipoplexes had a negative ζ potential, around −15 mV, for the lipids alone or conditioned with cholesterol, and around −50 mV, when conditioned with DOPE. Mention must be made that these new lipids are able to transfect NCI-H23 cells with low cytotoxicity even without a helper lipid.

Since it is known that the transfection efficiency of synthetic vectors varies with the cell line, we tested the most efficient cationic amphiphilic formulations on four additional tumor cell lines. We chose two breast cancer cell lines, MCF-7 and MDA-231β, a prostate tumor line (DU-145), and a glioma cell line (SWB-95), all representative of important targets in cancer therapy. The transfection conditions were kept constant for all preparations.

The results (Figure 11) showed that optimized formulations based on the new cationic lipids synthesized in this study reached or surpassed the efficiency of commercial transfection systems DOTAP/Chol, DMRIE-C, and Lipofectamine. For the MCF-7 cell line, the best results were obtained with the cationic lipids **52b** (conditioned with DOPE at 1:1 molar ratio) and **54c** (conditioned with DOPE or cholesterol, at the same 1:1 molar ratio), which surpassed the most efficient commercial system DMRIE-C. Interestingly, **52b**/Chol 1/1, which was very efficient on the NCI-H23 cell line, showed a lower activity on MCF-7 cells.

The gemini surfactant **29** was the most efficient system on the MDA-231β cell line, being 2 times more efficient than DMRIE-C and largely surpassing DOTAP and Lipofectamine. Ester **52b** and amide **54c** also gave good transfection results. The latter compound also proved to be efficient on the DU-145 cell line, with transfection levels similar to those of DMRIE-C.

The most spectacular results were obtained on the brain carcinoma SWB-95 cell line, known to be very difficult to transfect. Formulations based on **29**, **52b**, and **54c** were 2−6 times more efficient than the most effective commercial formulation, DMRIE-C. The best results were obtained in this case with the ester **52b**, conditioned with DOPE at 1:1 molar ratio, which was 6 times more efficient than DMRIE-C, largely surpassing the other two commercial formulations tested.

3. Conclusions

An extensive structure−activity relationship study was carried out to identify the structural parameters required for the best transfection efficiency in different classes of gene transfer agents. The optimization of liposomal formulations and the transfection efficiency of the corresponding lipoplexes on several tumor cell lines were discussed. Incorporation of groups that may be removed enzymatically (such as ester and amide) in the structure of the lead compounds **52b** and **54c** reduced the cytotoxicity and enhanced the transfection efficiency by overcoming the intracellular delivery barriers.

By making use of the high reactivity of pyrylium salts toward primary amines, we present an alternative to traditional approaches for constructing cationic lipids that are efficient as gene delivery agents. The approach is versatile, allowing access to cationic lipids, gemini surfactants, and oligomeric surfactants. The simplicity of the procedure makes it useful in supramolecular chemistry, when hydrophobicity-mediated self-assembling elements are required in the design of novel materials with various applications.

4. Experimental Protocols

Chemistry. General. The phase transition temperatures (T_c) for cationic lipids were determined by differential scanning calorimetry, using a TA-Instruments Q100 DSC and a heating rate of 5 °C/min. The same instrument was used for recording the melting points for the pyrylium salts and other intermediates. A Mel-Temp II (Laboratory Devices) melting point device was also used for this purpose. NMR spectra were recorded at ∼300 K with a Varian Unity Plus spectrometer equipped with a 5 mm indirect detection probe, operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. Chemical shifts are reported as δ values, using tetramethylsilane (TMS) as internal standard for proton spectra and the solvent resonance for carbon spectra. Assignments were made on the basis of signal intensity, selective decoupling, COSY (^{1}H−^{1}H), TOCSY, APT, HMQC, and HMBC sequences. Elemental analyses

were performed by combustion, using a Perkin-Elmer 2400 series II CHNS analyzer.

Fatty acids, triethylamine, acetic anhydride, acetic acid, acyl chlorides, and other solvents were from Acros (Fisher Scientific, Pittsburgh, PA). Thin-layer chromatography (TLC) was performed on silicagel 60-F_{254} plates (Merck, Whitehouse Station, NJ), eluted with MeOH/$CHCl_3$ 20:80 (v/v).

General Procedure for the Synthesis of 4,6-Dimethyl-2-alkylpyrylium Hexafluorophosphates 14 (Adapted from ref 19a). The fatty acid (0.1 mol) was refluxed with 45 mL of thionyl chloride for 90 min. Excess thionyl chloride was removed by rotevaporation under reduced pressure; 20 mL of hexane was added and then evaporated again under reduced pressure to remove the traces of any remaining thionyl chloride. Anhydrous aluminum chloride (13.5 g, 0.1 mol) was added to the crude fatty acid chloride under stirring and external cooling (ice bath). The mixture became homogeneous in about 10 min. Then 12 mL (0.15 mmol) of mesityl oxide was added dropwise, and the reaction mixture was stirred at room temperature for 12 h (fuming occurs because of HCl generation). The resulting dark viscous mass was hydrolyzed by carefully adding 50 mL of ice-cold 5% hydrochloric acid and 50 mL of diethyl ether, with external cooling (ice bath). The ethereal layer was separated and extracted a second time with 20 mL of diluted hydrochloric acid, then discarded. The combined aqueous solution of pyrylium tetrachloroaluminate was extracted with 20 mL of diethyl ether to remove ketonic byproducts, separated, and treated with 15 mL of hexafluorophosphoric acid when the crude pyrylium hexafluorophosphate separated as a brown oil. After extraction with chloroform, separation, drying on anhydrous sodium sulfate, and evaporation of the solvent, the crude compound was recrystallized twice from ethanol to afford the pure pyrylium hexafluorophosphate.

4,6-Dimethyl-2-decylpyrylium hexafluorophosphate 14a: mp 67.6 °C; yield 27%. Anal. ($C_{17}H_{29}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-undecylpyrylium hexafluorophosphate 14b: mp 62.8 °C; yield 21%. Anal. ($C_{18}H_{31}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-dodecylpyrylium hexafluorophosphate 14c: mp 78.7 °C; yield 31%. Anal. ($C_{19}H_{33}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-tridecylpyrylium hexafluorophosphate 14d: mp 73.3 °C; yield 19%. Anal. ($C_{20}H_{35}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-tetradecylpyrylium hexafluorophosphate 14e: mp 85.2 °C; yield 32%. Anal. ($C_{21}H_{37}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-pentadecylpyrylium hexafluorophosphate 14f: mp 79.8 °C; yield 25%. Anal. ($C_{22}H_{39}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-hexadecylpyrylium hexafluorophosphate 14g: mp 88.1 °C; yield 26%. Anal. ($C_{23}H_{41}O^+PF_6^-$) C, H.

4,6-Dimethyl-2-heptadecylpyrylium hexafluorophosphate 14h: mp 81.8 °C; yield 20%. Anal. ($C_{24}H_{43}O^+PF_6^-$) C, H.

General Procedure for the Synthesis of 4,6-Dimethyl-1,2-dialkylpyridinium Hexafluorophosphates 15−28. 4,6-Dimethyl-2-alkylpyrylium hexafluorophosphate **14** (3.5 mmol) was dissolved in 15 mL of chloroform and treated with a solution of fatty alkylamine (3.5 mmol) in 3 mL of chloroform. Triethylamine (0.5 mL) was then added to the brown-yellow solution, which was subsequently refluxed for 5 min, treated with 1 mL of acetic acid, and refluxed for another hour. The homogeneous mixture was treated with 0.2 mL of concentrated aqueous ammonia to convert any unreacted pyrylium salt into the corresponding highly soluble pyridine, refluxed for 5 min, cooled, and extracted two times with 10 mL of water to dissolve the precipitated inorganic salts. The combined aqueous extracts were back-extracted once with 10 mL of chloroform and discarded. The chloroform extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60, using a 20/80 MeOH/$CHCl_3$ (v/v) mobile phase, afforded the pure compound, which was crystallized from ethanol to yield the final cationic lipid.

4,6-Dimethyl-1,2-didecylpyridinium hexafluorophosphate 15: T_c = 46.9 °C; yield 65%. Anal. ($C_{27}H_{50}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-dodecyl-2-decylpyridinium hexafluorophosphate 16: T_c = 56.7 °C; yield 71%. Anal. ($C_{29}H_{54}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-decyl-2-undecylpyridinium hexafluorophosphate 17: T_c = 52.2 °C; yield 69%. Anal. ($C_{28}H_{52}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-dodecyl-2-undecylpyridinium hexafluorophosphate 18: T_c = 54.8 °C; yield 60%. Anal. ($C_{30}H_{56}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-decyl-2-dodecylpyridinium hexafluorophosphate 19: T_c = 57.6 °C; yield 72%. Anal. ($C_{29}H_{54}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1,2-didodecylpyridinium hexafluorophosphate 20: T_c = 58.6 °C; yield 63%. Anal. ($C_{31}H_{58}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-decyl-2-tridecylpyridinium hexafluorophosphate 21: T_c = 58.4 °C; yield 59%. Anal. ($C_{30}H_{56}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-dodecyl-2-tridecylpyridinium hexafluorophosphate 22: T_c = 67.2 °C; yield 62%. Anal. ($C_{32}H_{60}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1,2-ditetradecylpyridinium hexafluorophosphate 23: T_c = 68.5 °C; yield 70%. Anal. ($C_{35}H_{66}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-tetradecyl-2-pentadecylpyridinium hexafluorophosphate 24: T_c = 74.7 °C; yield 61%. Anal. ($C_{36}H_{68}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-hexadecyl-2-pentadecylpyridinium hexafluorophosphate 25: T_c = 74.1 °C; yield 63%. Anal. ($C_{38}H_{72}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1,2-dihexadecylpyridinium hexafluorophosphate 26: T_c = 76.2 °C; yield 59%. Anal. ($C_{39}H_{74}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-hexadecyl-2-heptadecylpyridinium hexafluorophosphate 27: T_c = 82.2 °C; yield 71%. Anal. ($C_{40}H_{76}N^+PF_6^-$) C, H, N.

4,6-Dimethyl-1-octadecyl-2-heptadecylpyridinium hexafluorophosphate 28: T_c = 82.1 °C; yield 65%. Anal. ($C_{42}H_{80}N^+PF_6^-$) C, H, N.

General Procedure for the Synthesis of Pyridinium Gemini Surfactants 29−35. 4,6-Dimethyl-2-tetradecylpyrylium hexafluorophosphate (**14e**, 1 g, 2.22 mmol) was dissolved in 10 mL of chloroform and treated with 0.75 mmol of diamine. Triethylamine (0.2 mL) was then added to the brown-orange solution, which was subsequently refluxed for 5 min, treated with 0.5 mL of acetic acid, and refluxed for another hour. The homogeneous mixture was treated with 0.2 mL of concentrated aqueous ammonia, refluxed for 5 min, cooled, and extracted with 10 mL of water to dissolve the precipitated inorganic salts. The combined aqueous extracts were back-extracted once with 10 mL of chloroform and discarded. The chloroform extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Recrystallization from ethanol, followed by flash chromatography on silica gel 60 (MeOH/$CHCl_3$ gradients 5/95 to 20/80 v/v as mobile phase) afforded the pure compound, which was crystallized from methanol or ethanol to yield the final gemini surfactant.

1,2-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)ethane dihexafluorophosphate 29: T_c = 178.2 °C; yield 57%. Anal. ($C_{44}H_{78}N_2^{+}2PF_6^-$) C, H, N.

1,3-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propane dihexafluorophosphate 30: T_c = 186.6 °C; yield 53%. Anal. ($C_{45}H_{80}N_2^{+}2PF_6^-$) C, H, N.

1,4-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)butane dihexafluorophosphate 31: T_c = 185.6 °C; yield 57%. Anal. ($C_{46}H_{82}N_2^{+}2PF_6^-$) C, H, N.

1,5-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)pentane dihexafluorophosphate 32: T_c = 137.8 °C; yield 62%. Anal. ($C_{47}H_{84}N_2^{+}2PF_6^-$) C, H, N.

1,6-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)hexane dihexafluorophosphate 33: T_c = 189.9 °C; yield 52%. Anal. ($C_{48}H_{86}N_2^{+}2PF_6^-$) C, H, N.

1,7-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)heptane dihexafluorophosphate 34: T_c = 83.6 °C; yield 61%. Anal. ($C_{49}H_{88}N_2{}^{+}2PF_6{}^{-}$) C, H, N.

1,8-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)octane dihexafluorophosphate 35: T_c = 68.8 °C; yield 64%. Anal. ($C_{50}H_{90}N_2{}^{+}2PF_6{}^{-}$) C, H, N.

General Procedure for the Synthesis of Pyridinium Gemini Surfactants 41 and 47. A. General Procedure for the Synthesis of Trifluoroacetyl-Protected Polyamines 37 and 43 (after Ref 26). In a round-bottom flask 15 mmol of polyamine **36** or **42** were dissolved in 10−20 mL of MeCN and treated dropwise, under stirring, with ethyl trifluoroacetate (35 + 15*k* mmol, where *k* is the number of secondary amino moieties) and water (18*k* mmol). The reaction mixture was subsequently refluxed for 3−4 h (TLC control, MeOH/$CHCl_3$ 20/80 v/v) and then cooled, and most of the solvent was removed under vacuum. Crystallization of the desired product occurred upon cooling and was completed by gradually adding CH_2Cl_2 (30−40 mL) to the mother liquor, under stirring. The solid was filtered, washed with cold CH_2Cl_2, and dried under vacuum to afford the pure trifluoroacetate salts.

***N*¹,*N*⁷-Bis(trifluoroacetyl)diethylenetriamine trifluoroacetate 37a:** yield 93%; mp 89−90 °C; lit.[26a] mp 89−90 °C.

***N*¹,*N*⁹-Bis(trifluoroacetyl)dipropylenetriamine trifluoroacetate 37b:** yield 97%; mp 120−121 °C; see ref 26i for the free base.

***N*¹,*N*⁸-Bis(trifluoroacetyl)spermidine trifluoroacetate 37c:** yield 92%; mp 145−146 °C; lit.[26f] mp 144−146 °C; lit.[26a] mp 146−147 °C.

***N*¹,*N*¹⁴-Bis(trifluoroacetyl)spermine ditrifluoroacetate 43:** yield 94%; mp 198−199 °C; lit.[26a] mp 195−198 °C; lit.[26g] mp 199−200 °C.

B. General Procedure for the Boc Protection of the Secondary Amino Moiety of Trifluoroacetamides 37 and 43 (after Ref 26). The trifluoroacetate salt **37** or **43** from the previous step A (12 mmol) was dissolved/suspended in triethylamine (25 + 12*k* mmol, where *k* is the number of secondary amino moieties) and treated dropwise under argon, at 0 °C (ice bath), with a solution of $(Boc)_2O$ (12.6*k* mmol) in THF (3−8 mL). The cooling bath was removed, and the homogeneous yellowish solution was stirred at room temperature for 3−6 h (TLC control, MeOH/$CHCl_3$ 20/80 v/v). Then the reaction was quenched with water (150−250 mL) and the desired product was extracted with ethyl acetate (3 × 150 mL). The combined organic extracts were washed with water (70 mL) and dried on Na_2SO_4. Evaporation of the solvent yielded the crude compound as a yellow oil, which was crystallized from ether/hexane mixtures to afford pure white crystals of Boc-protected trifluoroacetamides.

***tert*-Butyl-*N*,*N*-bis(2-trifluoroacetamidoethyl)carbamate 38a:** yield 97%; mp 122−123 °C; lit.[26h] mp 113−115 °C;

***tert*-Butyl-*N*,*N*-bis(3-trifluoroacetamidopropyl)carbamate 38b:** [26d,26i] yield 90%; mp 67−68 °C.

***tert*-Butyl-*N*-(3-trifluoroacetamidopropyl)-*N*-(4-trifluoroacetamidobutyl)carbamate 38c:** yield 98%; mp 74−75 °C; lit.[26f] mp 74 °C; lit.[26a] mp 74−75 °C.

***N*,*N*′-Di-*tert*-butyloxycarbonyl-*N*,*N*′-bis(3-trifluoroacetamidopropyl)butanediamine 44:** yield 94%; mp 107−108 °C; lit.[26c] mp 93.5−95 °C; lit.[26g] mp 104−105 °C.

C. General Procedure for the Selective Deprotection of the Primary Amino Moiety of Trifluoroacetamidoalkyl Carbamates 39 and 45 (after Ref 26). The carbamate **38** or **44** from step B (10 mmol) was dissolved in methanol (120 mL), cooled to 5−10 °C (ice bath), and treated dropwise with a methanolic 0.2 M NaOH solution (130 mL). When addition was complete, the cooling bath was removed and the mixture was stirred at 22 °C overnight. The methanol was removed in a vacuum, and the oily solution was extracted with a mixture of MeOH/$CHCl_3$ (1/9 v/v, 3 × 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated to dryness to afford the pure bis-aminoalkylcarbamate **39** or **45** (TLC control) as a pale-yellow oil (yields 93−97%), which was used directly into the next step.

D. General Procedure for the Synthesis of Lipophilic Pyridinium Gemini Carbamates 40 and 46. A solution of bis-aminoalkylcarbamate **39** or **45** (1 mmol) in $CHCl_3$ (5 mL) was treated with 1.25 g of 4,6-dimethyl-2-tetradecylpyrylium hexafluorophosphate **14e** (2.5 mmol) dissolved in 5 mL of $CHCl_3$, under stirring, when a yellow-brown solution was formed. The homogeneous reaction mixture was refluxed for 1 h, cooled, and treated with 0.3 mL of concentrated aqueous ammonia. After refluxing for another 5 min, the mixture was allowed to reach room temperature and was diluted with chloroform to a final volume of 25 mL. The solution was washed with water (10 mL), dried on Na_2SO_4, and evaporated to dryness. Recrystallization from ethanol, followed by flash chromatography on silica gel 60 (MeOH/$CHCl_3$ gradients 5/95 to 25/75 v/v as mobile phase) afforded the pure compound, which was crystallized from ethanol to yield the final gemini pyridinium carbamate.

***tert*-Butyl-*N*,*N*-bis(2-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)ethylenyl) carbamate dihexafluorophosphate 40a:** T_c = 139.9 °C; yield 84%. Anal. ($C_{51}H_{91}N_3O_2{}^{+}2PF_6{}^{-}$) C, H, N.

***tert*-Butyl-*N*,*N*-bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propylenyl) carbamate dihexafluorophosphate 40b:** T_c = 134.4 °C; yield 86%. Anal. ($C_{53}H_{95}N_3O_2{}^{+}2PF_6{}^{-}$) C, H, N.

***tert*-Butyl-*N*-(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)-propylenyl)-*N*-(4-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)butylenyl) carbamate dihexafluorophosphate 40c:** T_c = 143.5 °C; yield 79%. Anal. ($C_{54}H_{97}N_3O_2{}^{+}2PF_6{}^{-}$) C, H, N.

***N*,*N*′-Di-*tert*-butyloxycarbonyl-*N*,*N*′-bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propyl)butanediamine dihexafluorophosphate 46:** T_c = 103.3 °C; yield 81%. Anal. ($C_{62}H_{112}N_4O_4{}^{+}2PF_6{}^{-}$) C, H, N.

E. General Procedure for the Synthesis of Pyridinium Polycationic Amphiphiles 41 and 47. The pyridinium gemini carbamate **40** or **46** (0.5 mmol) was dissolved in methanol (20 mL) and was treated dropwise, under stirring, with a solution obtained by diluting 1 mL of 37% aqueous HCl with 4 mL of MeOH. The mixture was stirred at room temperature until TLC showed complete deprotection of the amino group (0.5−4 h). The solvent was evaporated in a vacuum, and the residue was taken into 25 mL of CH_2Cl_2 and extracted with 10% aqueous NaOH saturated with $NaPF_6$. The aqueous layer was separated and reextracted with 20 mL of CH_2Cl_2, then discarded. The combined CH_2Cl_2 extracts were dried on Na_2SO_4 and evaporated to dryness. Flash chromatography on silica gel 60 (NH_4OH/MeOH/CH_2Cl_2 gradients 5/15/80 to 5/35/60 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH mixtures to yield the final gemini pyridinium amphiphile.

***N*,*N*-Bis(2-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)ethylenyl)-amine dihexafluorophosphate 41a:** T_c = 65.1 °C; yield 49%. Anal. ($C_{46}H_{83}N_3{}^{+}2PF_6{}^{-}$) C, H, N.

***N*,*N*-Bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propylenyl)amine dihexafluorophosphate 41b:** T_c = 45.4 °C; yield 52%. Anal. ($C_{48}H_{87}N_3{}^{+}2PF_6{}^{-}$) C, H, N.

***N*-(3-(4,6-Dimethyl-2-tetradecylpyridinium-1-yl)propylenyl)-*N*-(4-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)butylenyl)amine dihexafluorophosphate 41c:** T_c = 67.2 °C; yield 47%. Anal. ($C_{49}H_{89}N_3{}^{+}2PF_6{}^{-}$) C, H, N.

***N*,*N*′-Bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propyl)-butanediamine dihexafluorophosphate 47:** T_{glass} = 9.6 °C; yield 45%. Anal. ($C_{52}H_{96}N_4{}^{+}2PF_6{}^{-}$) C, H, N.

General Procedure for the Synthesis of Dendritic Pyridinium Amphiphiles 48. The pyridinium gemini amphiphile *N*,*N*′-bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propyl)butanediamine dihexafluorophosphate **47** (0.32 g, 0.3 mmol) was dissolved in MeCN (10 mL) together with 0.1 g (1 mmol) of NEt_3. The solution was cooled to 0 °C (ice bath) and was treated dropwise, under stirring, with a solution obtained by diluting 0.9 mmol of myristoyl or palmitoyl chloride with 2 mL of MeCN. The reaction mixture was allowed to reach room temperature, and the stirring was continued until TLC showed complete acylation (2 h). The solvent was rotevaporated. The residue was taken into 25 mL of CH_2Cl_2 and extracted with 10 mL of 10% aqueous NaOH saturated with $NaPF_6$, and the organic layer was dried on Na_2SO_4. Evaporation of the solvent afforded the crude product, which was crystallized from

EtOH (5 mL). Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradient 5/95 to 20/80 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH mixtures to yield the final dendritic pyridinium amphiphile.

***N,N′*-Bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propylenyl)butane-*N,N′*-bismyristoylamide dihexafluorophosphate 48a (*n* = 12):** T_c = 125.8 °C; yield 55%. Anal. ($C_{80}H_{148}N_4{}^+2PF_6{}^-$) C, H, N.

***N,N′*-Bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propylenyl)butane-*N,N′*-bispamitoylamide dihexafluorophosphate 48b (*n* = 14):** T_c = 124.9 °C; yield 62%. Anal. ($C_{84}H_{156}N_4{}^+2PF_6{}^-$) C, H, N.

General Procedure for the Synthesis of Dendritic Pyridinium Amphiphile 49. The pyridinium gemini amphiphile *N,N′*-bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propyl)butanediamine dihexafluorophosphate **47** (0.32 g, 0.3 mmol) was dissolved in MeCN (10 mL) with 0.3 g (1 mmol) of 1-bromohexadecane. The mixture was refluxed under inert atmosphere until TLC showed complete alkylation (10 h). The solvent was removed by rotevaporation. The residue was taken into 25 mL of CH_2Cl_2, extracted with 10 mL of 10% aqueous NaOH saturated with $NaPF_6$, and dried on anhydrous Na_2CO_3. Evaporation of the solvent afforded the crude product, which was crystallized from EtOH (5 mL). Flash chromatography on silica gel 60 ($NH_4OH/MeOH/CH_2Cl_2$ gradients 5/15/80 to 5/35/60 v/v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH mixtures to yield the final dendritic pyridinium amphiphile.

***N,N′*-Bis(3-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)propylenyl)butane-*N,N′*-bispalmitylamine dihexafluorophosphate 49:** T_c = 69.4 °C; yield 65%. Anal. ($C_{84}H_{160}N_4{}^+2PF_6{}^-$) C, H, N.

General Procedure for the Synthesis of Dendritic Tris-Pyridinium Amphiphile 50. 4,6-Dimethyl-2-tetradecylpyrylium hexafluorophosphate (**14e**, 0.9 g, 2 mmol) was dissolved in 10 mL of CH_2Cl_2 and treated with a solution obtained by dissolving 73 mg (0.5 mmol) of tris(2-aminoethyl)amine in 2 mL of CH_2Cl_2. The resulting homogeneous brown-orange solution was subsequently refluxed for 5 min, treated with 0.5 mL of acetic acid, and refluxed for another hour. The reaction mixture was cooled to 22 °C, treated with 0.2 mL of concentrated aqueous ammonia, and refluxed for another 5 min. After cooling, it was diluted with 20 mL of CH_2Cl_2 and extracted with 10 mL of water to dissolve the precipitated inorganic salts. The combined aqueous extracts were back-extracted once with 10 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product, which was crystallized from ethanol. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 5/95 to 30/70 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH (1/2 v/v mixture) to yield the final crystalline amphiphilic trispyridinium salt.

Tris(2-(4,6-dimethyl-2-tetradecylpyridinium-1-yl)ethyl)amine trishexafluorophosphate 50: T_c = 121.2 °C; yield 57%. Anal. ($C_{69}H_{123}N_4{}^+3PF_6{}^-$) C, H, N.

General Procedure for the Synthesis of Pyridinium-Ester Cationic Lipids 52. A. Synthesis of Hydroxyethylpyridinium Hexafluorophosphate 51. 4,6-Dimethyl-2-tetradecylpyrylium hexafluorophosphate (**14e**, 1.98 g, 4.4 mmol) was dissolved in 20 mL of CH_2Cl_2 and treated, while stirring, with a solution obtained by dissolving 0.24 g (4 mmol) of 2-aminoethanol in 15 mL of CH_2Cl_2. Triethylamine (0.1 mL) was added to the reaction mixture, and the resultant homogeneous brown-orange solution was subsequently refluxed for 5 min, treated with 1 mL of acetic acid, and refluxed for another hour. The reaction mixture was cooled to room temperature, treated with 1 mL of concentrated aqueous ammonia, and refluxed for another 5 min. After cooling, it was diluted with 65 mL of CH_2Cl_2 and extracted with water (2 × 25 mL) in order to dissolve the precipitated inorganic salts. The combined aqueous extracts were back-extracted once with 20 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 10/90 to 30/70 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH to yield the final hydroxyethylpyridinium salt in 86% yield.

4,6-Dimethyl-2-tetradecyl-1-(2-hydroxyethyl)pyridiniumhexafluorophosphate 51: mp 55.6 °C. Anal. ($C_{23}H_{42}NO^+PF_6{}^-$) C, H, N.

B. Acylation of Hydroxyethylpyridinium Hexafluorophosphate 51 with Fatty Acid Chlorides. The hydroxyethylpyridinium hexafluorophosphate **51** (0.4 g, 0.8 mmol) was dissolved in 10 mL of MeCN with 0.84 mmol of *n*-alkyl acid chloride. Triethylamine (91 mg, 0.9 mmol) was added to the reaction mixture as a solution in 3 mL of acetonitrile, while stirring under external cooling (ice bath). The cooling bath was then removed, and the resultant suspension was subsequently refluxed until TLC showed complete acylation (6−10 h). The solvent was removed in a vacuum, and the residue was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaPF_6$ (25 mL). The aqueous layer was back-extracted with 25 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 5/95 to 25/75 v/v as mobile phase) afforded the pure compound, which was crystallized from EtOH to yield the final pyridinium cationic lipid.

4,6-Dimethyl-2-tetradecyl-1-(2-dodecanoyloxyethyl)pyridinium hexafluorophosphate 52a: T_c = 61.3 °C; yield 87%. Anal. ($C_{35}H_{64}NO_2{}^+PF_6{}^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(2-tetradecanoyloxyethyl)pyridinium hexafluorophosphate 52b: T_c = 68.5 °C; yield 90%. Anal. ($C_{37}H_{68}NO_2{}^+PF_6{}^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(2-hexadecanoyloxyethyl)pyridinium hexafluorophosphate 52c: T_c = 77.8 °C; yield 91%. Anal. ($C_{39}H_{72}NO_2{}^+PF_6{}^-$) C, H, N.

General Procedure for the Synthesis of Pyridinium Amide Cationic Lipids 54. A. Synthesis of Aminoethylpyridinium Hexafluorophosphate 53. 4,6-Dimethyl-2-tetradecylpyrylium hexafluorophosphate (**14e**, 2.25 g, 5 mmol) was dissolved in 50 mL of CH_2Cl_2 and treated under stirring with a solution obtained by dissolving 0.3 g (5 mmol) of ethylenediamine in 20 mL of CH_2Cl_2. Triethylamine (0.1 mL) was added to the reaction mixture, and the resultant homogeneous orange-brownish solution was subsequently refluxed for 5 min, treated with 1 mL of acetic acid, and refluxed for another hour. The reaction mixture was cooled to room temperature, treated with 1 mL of concentrated aqueous ammonia, and heated to reflux for another 5 min. After cooling, it was extracted with water (2 × 25 mL) in order to dissolve the precipitated inorganic salts. The combined aqueous extracts were back-extracted once with 20 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($NH_4OH/MeOH/CH_2Cl_2$ gradients 5/20/75 to 5/40/55 v/v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH to yield the final aminoethylpyridinium salt in 53% yield.

4,6-Dimethyl-2-tetradecyl-1-(2-aminoethyl)pyridinium hexafluorophosphate 53: mp 61.4 °C. Anal. ($C_{23}H_{43}N_2{}^+PF_6{}^-$) C, H, N.

B. Acylation of Aminoethylpyridinium Hexafluorophosphate 53 with Fatty Acid Chlorides. The aminoethylpyridinium hexafluorophosphate **53** (0.2 g, 0.4 mmol) was dissolved in 10 mL of MeCN with 50 mg (0.5 mmol) of triethylamine and treated dropwise, while stirring under external cooling (ice bath), with 0.44 mmol of *n*-alkyl acid chloride dissolved in 3 mL of MeCN. The cooling bath was removed, and the resulting suspension was refluxed until TLC showed complete acylation (3−6 h). The solvent was removed in vacuo, and the residue was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaPF_6$ (25 mL). The aqueous layer was back-extracted with 25 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 20/80 to 30/70 v/v as mobile phase) afforded the pure compound, which was crystallized from ethanol to yield the final

pyridinium cationic lipid in 75−85% yield. Although the NMR analysis of the resultant lipids showed pure compounds, elemental analysis showed a mixture of counterions (Cl^- and PF_6^-), which was confirmed by DSC. Therefore, the compounds were dissolved in a minimum amount of 96% EtOH and passed through a DOWEX 1X8-400 anion exchange column (Cl^- form), packed in the same solvent. Elution with 96% EtOH afforded the pure compounds as chlorides, which were concentrated and crystallized from EtOH.

4,6-Dimethyl-2-tetradecyl-1-(2-dodecanoylamidoethyl)pyridinium chloride 54a: T_c = 41.9 °C; yield 51%. Anal. ($C_{35}H_{65}N_2O^+Cl^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(2-tetradecanoylamidoethyl)pyridinium chloride 54b: T_c = 49.9 °C; yield 55%. Anal. ($C_{37}H_{69}N_2O^+Cl^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(2-hexadecanoylamidoethyl)pyridinium chloride 54c: T_c = 46.8 °C; yield 48%. Anal. ($C_{39}H_{73}N_2O^+Cl^-$) C, H, N.

General Procedure for the Synthesis of Pyridinium Disulfide Cationic Lipids 56. A. Synthesis of Mercaptoethylpyridinium Hexafluorophosphate 55. Aminothioethanol hydrochloride (0.34 g, 3 mmol) was added, while stirring, to a solution of 0.2 g of EtONa in 10 mL of absolute ethanol, followed by 1.42 g of 4,6-dimethyl-2-tetradecylpyrylium hexafluorophosphate (**14e**, 3.15 mmol) dissolved in ethanol (10 mL). The resulting homogeneous orange-brownish solution was refluxed for 10 min, treated with 0.4 mL of acetic acid, and refluxed for another hour. The reaction mixture was cooled to room temperature, and 0.25 mL of concentrated aqueous ammonia was added. After the mixture was refluxed for another 5 min, the solvent was removed by rotevaporation, and the residue was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaPF_6$ (25 mL). The aqueous layer was back-extracted with 25 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined, dried on sodium sulfate, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 3/97 to 20/80 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH to yield the final mercaptoethylpyridinium salt in 66% yield.

4,6-Dimethyl-2-tetradecyl-1-(2-mercaptoethyl)pyridinium hexafluorophosphate 55: mp 71.9 °C. Anal. ($C_{23}H_{42}NS^+PF_6^-$) C, H, N.

B. Oxidative Coupling of Mercaptoethylpyridinium Hexafluorophosphate 55 with *n*-Alkyl Thiols. The mercaptoethylpyridinium hexafluorophosphate **55** (0.25 g, 0.5 mmol) was suspended in 20 mL of EtOH with 3 mmol of *n*-alkylthiol, and CH_2Cl_2 was added until all the solids were dissolved (4−8 mL). The solution was cooled to 0 °C (ice bath), and a solution of iodine (0.46 g, 1.8 mmol) in 10 mL of EtOH was added dropwise, under nitrogen atmosphere and with vigorous stirring, until the yellow-brown color of free iodine was persistent. The solvent was removed in vacuo, and the residue was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaHCO_3$ (25 mL). The aqueous layer was back-extracted with 25 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined and treated with 20% aqueous $NaHSO_3$ (25 mL), dried on Na_2SO_4, and evaporated to dryness to yield the crude product. Trituration with hexane followed by flash chromatography on silica gel 60 (petroleum ether/acetone gradients 30/70 to 0/100 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH to yield the final disulfidic pyridinium cationic lipid. The PF_6^- counterion can be exchanged to Cl^- using the same procedure as for the preparation of amides **54**, the pure chlorides being crystallized from Et_2O/EtOH.

4,6-Dimethyl-2-tetradecyl-1-(3,4-dithiatetradecyl)pyridinium hexafluorophosphate 56a: T_c = 76.0 °C; yield 58%. Anal. ($C_{33}H_{62}NS_2^+PF_6^-$) C, H, N. Chloride: T_c = 77.4 °C; yield 82% (from hexafluorophosphate). Anal. ($C_{33}H_{62}NS_2^+Cl^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(3,4-dithiahexadecyl)pyridinium hexafluorophosphate 56b: T_c = 81.0 °C; yield 63%. Anal. ($C_{35}H_{66}NS_2^+PF_6^-$) C, H, N. Chloride: T_c = 75.1 °C; yield 76% (from hexafluorophosphate). Anal. ($C_{35}H_{66}NS_2^+Cl^-$) C, H, N.

4,6-Dimethyl-2-tetradecyl-1-(3,4-dithiaoctadecyl)pyridinium hexafluorophosphate 56c: T_c = 81.8 °C; yield 65%. Anal. ($C_{37}H_{70}NS_2^+PF_6^-$) C, H, N. Chloride: T_c = 86.9 °C; yield 78% (from hexafluorophosphate). Anal. ($C_{37}H_{70}NS_2^+Cl^-$) C, H, N.

C. Oxidative Coupling of Mercaptoethylpyridinium Hexafluorophosphate 55. A solution of mercaptoethylpyridinium hexafluorophosphate **55** (0.25 g, 0.5 mmol) in 15 mL of EtOH/CH_2Cl_2, 2/1, was cooled to 0 °C (ice bath) and treated dropwise, under nitrogen atmosphere and with vigorous stirring, with a solution of iodine (0.076 g, 0.3 mmol) in 3 mL of EtOH until the yellow-brown color of free iodine was persistent. The solvent was removed in a vacuum, and the residue was partitioned between CH_2Cl_2 (25 mL) and saturated aqueous $NaHCO_3$ (25 mL). The aqueous layer was back-extracted with 25 mL of CH_2Cl_2 and discarded. The methylene chloride extracts were combined and treated with 20% aqueous $NaHSO_3$ (25 mL), dried on Na_2SO_4, and evaporated to dryness to yield the crude product. Flash chromatography on silica gel 60 ($MeOH/CHCl_3$ gradients 3/97 to 25/75 v/v as mobile phase) afforded the pure compound, which was crystallized from Et_2O/EtOH to yield the final disulfidic pyridinium cationic lipid. The PF_6^- counterion can be exchanged to Cl^- using the same procedure as for the preparation of amides **54**, the pure chloride being crystallized from Et_2O/EtOH.

1,6-Bis(4,6-dimethyl-2-tetradecylpyridinium-1-yl)-3,4-dithiahexane dihexafluorophosphate hexafluorophosphate 57: T_c = 107.0 °C; yield 71%. Anal. ($C_{46}H_{82}N_2S_2^+PF_6^-$) C, H, N. Chloride: T_c = 139.3 °C; yield 79% (from hexafluorophosphate). Anal. ($C_{46}H_{82}N_2S_2^+Cl^-$) C, H, N.

Preparation and Evaluation of Liposomes. Dioleoylphosphatidylethanolamine (DOPE), cholesterol (Chol), and *N*-(2,3-dioleoyloxypropyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP) were purchased from Sigma (St. Louis, MO). The purity of the lipids was checked by TLC and also by differential scanning calorimetry using a TA Instruments Q100 DSC instrument (New Castle, DE).

For the preparation of liposomes a protocol optimized for this type of compound[14b,c] was used. The cationic amphiphile (with or without helper lipid) was dissolved in chloroform/methanol (2/1, v/v) to reach a final concentration of 1 mM for the charged species. From this stock solution, a volume of 50 μL was transferred to a 1 mL microcentrifuge tube (USA Scientific, catalog no. 1415-2500), and the solvent was evaporated in a SpeedVac evaporator under vacuum for 1 h. Traces of organic solvent were removed by drying the lipid film in a vacuum desiccator for 3 h over Drierite. The dried film was hydrated overnight after adding 500 μL of sterile phosphate buffer isotonic saline at pH 7.4 (PBS) (GibcoBRL/Invitrogen, Carlsbad, CA), purging the tube with sterile nitrogen, and vortex-mixing it for 1 min. Liposomes were generated the next day by sonicating the tube in a water bath sonicator (Branson, model 1210) for 30 min (two reprises of 15 min sonication, with 15 min pause between) at 65−68°C for formulations based on lipids **15**−**35**, **40**, **41**, **46**−**50**, **52**, **54**, **56**, and **57** in a mixture with cholesterol or DOPE and at 37 °C for DOTAP/cholesterol. After sonication the preparations were allowed to reach 22 °C and assessed on the specified cells within 30 min from the preparation. Lipofectamine and DMRIE liposomes were prepared according to the manufacturer's specifications.

The size of the lipoplexes was determined in parallel by dynamic light scattering, using a DynaPro particle sizer (Protein Solutions, Piscataway, NJ) and employing the Dynamics data collection and analysis software. In each experiment the liposomal preparation (20 μL) was diluted with PBS to a final volume of 200 μL. From this solution an aliquot (15 μL) was introduced in a quartz cuvette precleaned with distilled water and methanol and dried to eliminate any contaminants. The cuvette was sealed with Parafilm, and the mean average diameter of the vesicles was determined at 25 °C as the result of at least three reliable readings.

The ζ potentials of the liposomes and lipoplexes were determined using a Zetasizer 2000 instrument (Malvern Instruments, U.K.) using the Zeta2000 software. The liposomal/lipoplex formulation (200 μL) was diluted with PBS to a final volume of 2 mL and injected into the measuring cuvette of the instrument. Calibration

was performed using the DTS5050 carboxylated polystyrene latex. The final value for the ζ potential of the particles was the average of at least three separate readings.

Preparation of Lipoplexes. The plasmid used in this study was pGL3 (Promega, Madison, WI), encoding a firefly luciferase gene under control of the constitutively active SV40 promoter. The plasmid was amplified in *Escherichia coli* and purified using a Genopure Plasmid MaxiKit (Roche, Indianapolis, IN).

For generation of the the lipoplexes, 2.8 μL of plasmid DNA solution (0.5 μg/μL) was diluted with 100 μL of Optimem (Gibco/Invitrogen) to make plasmid DNA stock solutions. A liposome solution was prepared separately by diluting 40 μL of the initial liposomal stock solution (0.1 mM in cationic lipid) with Optimem in order to reach a final volume of 50 μL. To this liposome solution, 50 μL of the DNA stock solution was added, and (after mixing) the tube was incubated for 30 min at 22 °C. The content was then diluted with 500 μL of Optimem (final volume of the lipoplex stock solution was 600 μL).

Cell Transfection. The lipoplexes were tested for their ability to transfect five cancer cell lines: a lung carcinoma (NCI-H23, found to be the most sensitive to pyridinium-based transfection formulations), two breast carcinomas (MCF-7 and MDA-231β), a prostate carcinoma (DU-145), and a brain glioma (SWB-95). The cells were maintained in 10% fetal bovine serum (FBS) enriched medium at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The following media were used: RPMI 1640 (CellGro, Houston, TX) for NCI-H23, Iscove's modified Dulbecco's medium (Gibco/Invitrogen) for MCF-7, Dulbecco's modified Eagle medium (Gibco/Invitrogen) for MDA-231β, minimum essential medium (Gibco/Invitrogen) for DU-145, and minimum essential medium Eagle α modification (Sigma) for the SWB-95 cell line. Twenty-four hours prior to transfection, the cells were transferred to 96-well microtiter plates (Costar, catalog no. 3596) at a density of 20 000 cells/well. Each well received 100 μL of appropriate medium, and the plate was incubated in the same conditions as above. All experiments were done in triplicate or quadruplicate. The error bars in figures represent one standard deviation from the average value.

Immediately before transfection the medium was removed, and the cells from each well were briefly washed with 200 μL of sterile PBS. After removal of the PBS solution, each well received 100 μL of lipoplex stock solution, and the plate was returned to the incubator for 1 h. An additional 100 μL of medium (20% FBS) was added to each well, and the plate was incubated for a further 24 h, after which the luciferase content and protein content were assessed.

Luciferase and Protein Content Assay. Twenty-four hours after transfection, the medium was aspirated and the wells were washed briefly with 200 μL of PBS. After removal of PBS, the cells were lysed by adding 100 μL of 1X reporter lysis buffer (Promega) to each well and incubating the plate at 37 °C for 10 min. The cell lysate was collected and used for luciferase and protein assays.

For the luciferase assay, 20 μL of cell lysate was transferred to a test tube and assessed directly by means of a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) using a luciferase assay kit from Promega.

The protein content was quantified using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The BCA assay was prepared as specified in its manufacturer's instructions. An amount of 40 μL of cell lysate was treated with 1 mL of BCA reagent in an acryl cuvette, and the solution was incubated for 1 h at 37 °C. The light absorption of the solution was then read at 562 nm by means of a Beckman DU-600 UV−vis spectrometer (Palo Alto, CA), and the protein content was estimated by comparison to bovine serum albumin standards. The luciferase activity was normalized by the protein content and expressed as relative luminescence units/μg of protein (RLU/μg protein).

Viability Assay. To quantify the relative cytotoxicity of the nonviral cationic vectors, a WST-1 standard viability method[29] was performed along with the luciferase and BCA assays. Five wells of cells for each liposomal preparation were set up in parallel and transfected identically as in the pGL3 assay. Twenty-four hours after transfection, 20 μL of WST-1 tetrazolium dye solution (Roche, Mannheim, Germany) was added to each well (still containing the serum and the liposomal preparation). A blank was prepared by mixing 100 μL of Optimem, 100 μL of serum, and 20 μL of tetrazolium dye solution, and the plate was incubated at 37 °C in the CO_2 incubator. After 3 h the colorimetric measurement was performed at 450 nm (with a reference wavelength of 595 nm that was subtracted) by means of a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The value corresponding to the blank was deducted from the value corresponding to each well.

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Supporting Information Available: Analytical data and results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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