Articles

Unsaturated Cationic Ortho Esters for Endosome Permeation in Gene Delivery

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Two cleavable cationic lipids were designed to trigger the fusogenicity and membrane permeation of their lipoplexes in endosomes via the formation of inverted hexagonal phases (H_{II}). Both lipids contain a cationic head group and an unsaturated hydrophobic dioleylglycerol moiety joined together by a linear or a cyclic ortho ester linker. At pH 7.4, the lipids formed stable complexes with plasmid DNA together with the conelike helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). The decrease of pH enhanced the hydrolysis of the ortho ester linkers, which removed the cationic head groups and caused the aggregation of the lipoplexes. At pH 5.5, the cationic lipid *N*-[2-methyl-2-(1′,2′-dioleylglyceroxy)dioxolan-4-yl]methyl-*N*,*N*,*N*-trimethylammonium iodide (**2**) with a cyclic ortho ester linker showed exceptional pH-sensitivity and triggered its lipoplex to permeate model biomembranes within the time span of endosome processing prior to lysosomal degradation. Lipid **2** significantly improved gene transfection in cultured cells compared to the pH-*in*sensitive control lipid 1,2-dioleoyl-3-trimethylammoniopropane (DOTAP).

Introduction

The correction of human severe combined immunodeficiency (SCID)[1] by gene therapy in clinical trial has demonstrated its potential to be an important therapeutic modality. Nonetheless, the commonly used viral gene carriers face serious safety issues,[2,3] including immune reactions, inflammation, and carcinogenesis. Intensive research thus continues on nonviral gene delivery systems,[4] in which the exogenous DNA is complexed with synthetic materials, including lipids, polymers, and peptides. Cationic lipoplexes (positively charged nanoparticulate complexes of cationic lipids and DNA) represent the most investigated nonviral gene delivery system. Cationic lipoplexes enter the cells by adsorbing to the cell surface, most probably via the negatively charged proteoglycans,[5] followed by endocytosis.[6,7] Cationic lipoplexes are in general considered safer than viral gene carriers but are hampered in the clinic by substantially lower transfection efficiency.

One important reason for the limited efficiency of cationic lipoplexes is that, after cellular uptake, most of them are processed through the endosomal pathway to the lysosome,[8,9] where extensive degradation takes place. Since many viruses possess designated pH-sensitive proteins to destabilize the endosomes so as to transfer their genomic DNA into the cytosol, such viral proteins and other pH-sensitive synthetic polymers/peptides[10] have been incorporated into cationic lipoplexes to facilitate their escape from the endosomes. This approach achieved encouraging improvements in gene transfection efficiency but caution need to be taken regarding the possible immune or inflammatory reactions to such macromolecules.

The introduction of acid-labile linkers into cationic lipids represents another attractive strategy of endosome destabilization.[11,12] In this approach, the decrease of pH in the endosome catalyzes the hydrolysis of the linker group to yield fragmentation products that in turn destabilize the endosome membranes. There is considerable flexibility in the design of acid-labile cationic lipids. Different hydrophilic headgroups, lipophilic tails, linker groups, and linkage configurations can be implemented to generate lipids of desired physicochemical properties. Different hydrolysis kinetics and hydrolysis products of different membrane-destabilizing capacities can also be devised. The functional groups that have been investigated as acid-labile linkers in cationic lipids include vinyl ethers,[13] hydrazones,[14] and ortho esters.[15]

Ortho esters represent one of the most acid-labile functionalities known in the literature.[16−18] Compared with vinyl ethers and ketals, ortho esters hydrolyze more quickly in response to a lowered pH, owing to a stable dialkoxy carbon cation intermediate (Scheme 1). Polyortho esters developed by Heller and co-workers[19] have shown excellent biocompatibility. The hydrolysis of ortho ester (Scheme 1) comprises of the cleavage of three alkoxy groups. The cleavage of the first and the second alkoxy groups is rapidly catalyzed by elevated proton concentration, whereas the cleavage of the third alkoxy group relies on a slower hydrolysis step of an ester bond. Our previous studies on ortho-ester-based lipids with poly(ethylene glycol) (PEG)[20,21] and phosphocholine headgroups[22] have shown that the hydrolysis of the ortho ester functionality can be exploited to trigger lipid phase changes and liposome content release at endosomal pH within 30−60 min, a time frame consistent with that of endosome trafficking before maturing to lysosomes.[8,23,24] DNA encapsulated in liposomes stabilized by POD, an ortho ester lipid with a PEG headgroup, transfected cells in culture.[25]

Recently, By and co-workers[15] have reported cationic lipids based on dioxazocinium ortho ester head groups. The most sensitive of such lipids disassembled at 38 °C within 6 h at pH

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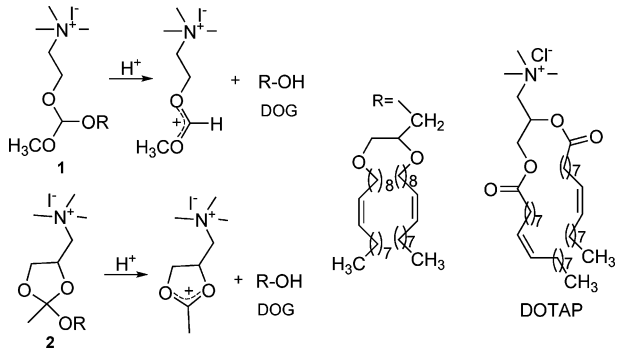
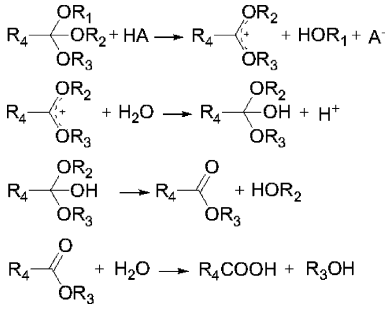


Figure 1. Cationic amphiphiles under study for gene delivery. DOG, dioleylglycerol; CI^-, negatively charged counterion of DOTAP.

Scheme 1



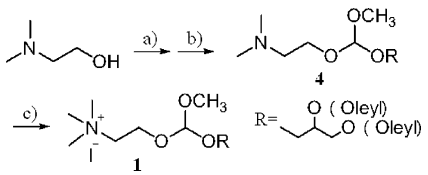
5.0. However, it is still unclear whether the hydrolysis of ortho-ester-based cationic lipids can be translated to improvements in endosome escape and gene transfection of the corresponding lipoplexes.

We report here two novel ortho-ester-based cationic lipids (**1**, **2**, Figure 1) that are designed to trigger their lipoplexes to permeate biomembranes at endosomal pH. The hydrolysis of these lipids at endosomal pH drastically changed the physicochemical properties of their lipoplexes. Compared to the pH-insensitive control lipid 1,2-dioleoyl-*sn*-glycero-3-trimethylammoniopropane (DOTAP),[26,27] compound **2** with an optimized triggering kinetics significantly improved the gene transfection to CV-1 and HTB-129 cells in culture.

Chemistry

Design of Ortho-Ester-Based Cationic Lipids 1 and 2 for Endosome Destabilization in Gene Delivery. Both **1** and **2** contain a cationic quaternary trimethylammonium head group and an unsaturated hydrophobic dioleylglycerol (DOG) moiety joined together by a linear (**1**) or cyclic (**2**) ortho ester linker. The strategy of gene delivery by **1** and **2** involves several steps. First the cationic lipids are assembled into liposomes at neutral pH together with a helper lipid, dioleyolphosphatidylethanolamine (DOPE). The cationic liposomes are then condensed with negatively charged DNA at appropriate molar ratio so that relatively stable cationic lipoplexes, mostly in the sandwich-like lamellar phase (L_α),[28] can be prepared and administered. Such lipoplexes bind to the negatively charged cell surface[5] and enter the cells via endocytosis.[29] Once in the endosomes, the small drop of pH to about 5.5 catalyzes the cleavage of the cationic ammonium group and converts **1** and **2** to DOG, a neutrally charged lipid with a much smaller hydrophilic head group. In cooperation with the conelike helper lipid DOPE,[7] the cleavage imposes a negative curvature to the lipid lamellae and induces the change of the lamellae to the fusogenic inverted hexagonal phase (H_{II}),[28,30] which permeates the endosome membranes. The cleavage would also facilitate the release of DNA from the lipoplexes by reducing the electrostatic interactions between DNA and the lipids.

Scheme 2[a]



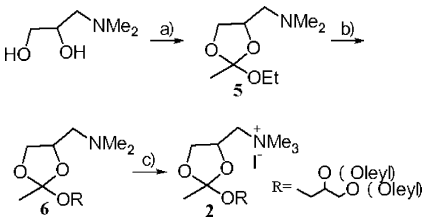
[a] (a) Dioleylglycerol, THF, NaH; (b) CH_3OCHCl_2 (**3**); (c) THF, Na_2CO_3, CH_3I.

The positively charged trimethylammonium head group of **1** and **2** carries out electrostatic interactions with the negatively charged DNA. The unsaturated dioleyl chains are introduced for two reasons. First, they increase the area at the hydrophobic moiety[30] compared to saturated chains and thus impose a more negative curvature on lipoplex lamellae upon hydrolysis; second, they decrease lipid bilayer bending rigidity.[31] Both functions favor the $L_\alpha \rightarrow H_{II}$ phase change. The two ortho ester linkages would have different conformational preferences due to differences in steric hindrance and ring constraints, which could in turn cause differences in the kinetics of hydrolysis.[17]

Synthesis of Ortho-Ester-Based Cationic Lipids. To prepare the linear ortho ester **1**, we discovered the displacement reaction of a commercially available bifunctional molecule, α,α-dichloromethyl methyl ether (**3**), in the literature. Gross and Rieche[32] reported that **3** reacted with deprotonated primary alcohols in THF to form ortho esters as dialkoxymethoxymethanes. However, the displacement of **3** by two different alcohols, as desired in the synthesis of **1**, has not been published. Nonetheless, α,α-dichloromethyl methyl ether is an attractive linker molecule since the displacement reaction should, in theory, be compatible with tertiary amines. Thus, the bifunctional displacement of **3** was attempted in order to conjugate dimethylethanolamine with the lipidic alcohol DOG (Scheme 2). Since DOG needs to be prepared by multistep synthesis as described by Sullivan,[33] the conditions of the displacement reaction were optimized to maximize the use of this intermediate. DOG (1 equiv) and dimethylethanolamine (7 equiv) were mixed and deprotonated with 8.8 equiv of NaH. **3** (4 equiv) was then added to yield the dimethylated tertiary amine **4** (21% purified yield after silica gel chromatography). Quantitative methylation of **4** with iodomethane readily yielded **1** as an iodide salt with minimum workup.

The synthesis of **2** was derived from the work of Heller and associates,[34] who synthesized polyortho esters with repeating dioxolane units by the condensation of 1,2,6-hexanetriol and triethyl orthoacetate. Mechanistically, the 1,2-ethanediol moiety of 1,2,6-hexanetriol first cyclized with triethyl orthoacetate to form the 2-ethoxy-4-(1-hydroxybutane-4-yl)dioxolane intermediate. Intermolecular displacement of the 2-ethoxy group by the remaining hydroxy group from the triol completed the polymerization reaction. Inspired by this reaction, we synthesized **2** (Scheme 3) by first cyclizing 3-dimethylaminopropane-1,2-diol with triethyl orthoacetate to form the analogous dioxolane intermediate **5**. Acid-catalyzed displacement of the ethoxy group of **5** by DOG gave the dimethylated tertiary amine precursor (**6**) of **2**. Methylation of **6** with iodomethane readily yielded **2** as an iodide salt. This amphiphile with an unsaturated dioleyl lipophilic tail is not readily crystallized due to its low melting point. Degradation of **2** at mildly acidic pHs also prohibits its purification by silica gel chromatography. We opted to first purify the tertiary amine precursor **6** by fractionating its reaction mixture with an Amberlite XAD-7 plug. Fractions containing

Scheme 3[a]



[a] (a) Triethyl orthoacetate, pTsOH, cyclohexane; (b) dioleylglycerol, cyclohexane, pTsOH; (c) THF, Na_2CO_3, CH_3I.

6 were pooled and further purified with a Sephadex LH-20 column in a basified mobile phase to yield **6** in high purified yield (98%). The acid-labile lipid **2** was then prepared by quantitative methylation of the purified **6** with minimum workup.

Preparation of Liposomes and Lipoplexes with Ortho-Ester-Based Cationic Lipids.[35] The cationic lipids (**1** or **2**) were hydrated together with an equal mole of the conelike helper lipid DOPE by agitation in a pH 7.4 buffer. Chloride ion (20 mM) was included in the hydration buffer to facilitate the suspension of the lipids, presumably by replacing the less hydratable iodide counterion. Subsequent extrusions through polycarbonate membranes with pores 200 nm in diameter readily formed homogeneous cationic liposomes (Table 1). The liposomes were then mixed with plasmid DNA (N/P = 5/1, where N/P is the molar ratio of the quaternary ammonium of cationic lipids and the phosphate of DNA) to form cationic lipoplexes. A lipoplex preparation comprising DOTAP, a commercially available analogue of **1** and **2**, was prepared in the same manner as a pH-*in*sensitive control. All the prepared lipoplexes (Table **1**) carried excess positive surface charges, as indicated by their highly positive ζ-potential values. The ζ-potential of the lipoplexes is similar to that of the corresponding liposomes, probably due to the large excess of the cationic lipids (N/P = 5/1) in the lipoplexes. The size of the lipoplexes ranges between 230 and 270 nm. All the lipoplex preparations can be stored at room temperature for several hours with no obvious changes in these colloidal properties.

pH-Dependent Hydrolysis of Ortho-Ester-Based Cationic Lipids in Liposomes and Lipoplexes. The lipids as in their corresponding cationic liposomes and lipoplexes were analyzed by HPLC after the liposomes, and the lipoplexes had been incubated at neutral or acidic pHs for different time periods. DOG was confirmed (Figure **1**) as the dominant hydrolysis product of both **1** (∼85%) and **2** (∼98%) on the basis of the integration of area under the curve for all the lipid peaks detected by UV absorbance. The percentage of hydrolysis (H%) of **1** and **2** as a function of incubation time is shown in Figure 2A,B. All the curves exhibited tight linearity ($r > 0.9$), indicating apparent zero-order hydrolysis kinetics at constant pH in aqueous buffers. For both **1** and **2**, the hydrolysis kinetics in the cationic liposome is similar to that in the lipoplex with virtually identical rates.

The acidic pH accelerated the hydrolysis of both **1** and **2**. For example, it took about 90 h to hydrolyze the first 50% of **2** at pH 7.4; at pH 5.5, the same extent of hydrolysis needed only 5 h. This is consistent with the acid sensitivity of the ortho ester functionalities. Interestingly, the hydrolysis rate of **2** was faster than that of **1** at both pH 5.5 and 7.4. For example, at pH 5.5, it took about 5 h to hydrolyze the first 50% of **2** but about 70 h to hydrolyze the first 50% of **1**.

pH-Triggered Aggregation of Lipolexes Comprising Ortho-Ester-Based Cationic Lipids. To assess whether the acid-catalyzed hydrolysis of the ortho ester lipids can trigger the changes in the colloidal properties of their lipoplexes, the hydrodynamic diameter of the lipoplexes was monitored at neutral and acidic pHs. At pH 7.4, 37 °C, both the ortho-ester-based lipoplexes (**1**/DOPE/DNA and **2**/DOPE/DNA, 50 mol % DOPE in total lipids, N/P = 5/1) and the pH-*in*sensitive control lipoplex (DOTAP/DOPE/DNA, 50 mol % DOPE in total lipids, N/P = 5/1) maintained their original size for 35 h. At acidic pHs, the hydrolysis of the ortho ester lipids induced the aggregation of the lipoplexes (particle diameter increased to more than 1000 nm, Figure 3) after a certain lag time. The control lipoplex DOTAP/DOPE/DNA did not aggregate at pH 5.5 during the entire period of monitoring (40 h, data not shown).

The lipoplex **2**/DOPE/DNA comprising **2** with a cyclic ortho ester linker showed drastically higher sensitivity to acidic pHs compared to the lipoplex **1**/DOPE/DNA comprising **1** with a linear ortho ester linker, which correlates with the faster hydrolysis of **2** at acidic pH 5.5 compared to **1** (Figure **2**). At pHs 5.0, 5.5, and 6.0, the aggregation of **2**/DOPE/DNA took place within 16, 32, and 44 min, respectively, whereas the aggregation of **1**/DOPE/DNA took place within 570 and 1050 min at pHs 5.0 and 5.5, respectively. No obvious aggregation of **1**/DOPE/DNA was observed at pH 6.0 within 2000 min.

Permeation of a Model Biomembrane by Lipoplexes Comprising Ortho-Ester-Based Cationic Lipids. To evaluate their ability to destabilize endosomes, the lipoplexes (**1**/DOPE/DNA, **2**/DOPE/DNA, and DOTAP/DOPE/DNA) were incubated with a model liposome[36] encapsulating the fluorescent dyes ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and DPX (*p*-xylene-bis-pyridinium bromide). The lipid composition (50 mol % POPC, 20 mol % POPE, 5 mol % POPS, 10 mol % L-α-PI, 15 mol % cholesterol) of the model liposome imitates that of biomembranes[37] (Figure 4). The permeation of the model liposome releases the encapsulated ANTS/DPX and dequenches their fluorescence.[38] In the time span of endosome processing[8,23,24] (30−60 min) before merging with lysosomes (where extensive degradation takes place), the cyclic ortho-ester-based lipoplex **2**/DOPE/DNA induced a rapid leakage (∼70% in 30 min) of the model liposomes at endosomal pH 5.5. At pH 7.4, the lipoplex induced <20% leakage in 30 min and <30% in 60 min. The linear ortho ester lipoplex **1**/DOPE/DNA induced only 12.5% leakage at pH 5.5 and 6.8% leakage at pH 7.4 in 60 min. Thus, the kinetics of membrane permeation by the ortho ester lipoplexes correlates with that of pH-triggered lipid hydrolysis and lipoplex aggregation. The DOTAP-based lipoplex induced ≤15% leakage at acidic or neutral pHs in 60 min, consistent with the previous finding that, at 50 mol % DOPE, the DOTAP/DOPE/DNA lipoplexes with excess positive surface charges mostly stay in the less fusogenic L_α phase.[28] The slower but notable leakages by the ortho ester lipoplexes even at pH 7.4 and by the pH-insensitive DOTAP/DOPE/DNA lipoplexes probably resulted from partial charge neutralization between the cationic lipoplexes and the negatively charged model liposomes[39] and/or the partitioning of the bilayer-destabilizing lipid DOPE from the lipoplexes to the model liposomes.[36]

Gene Transfection by Lipoplexes Comprising Ortho-Ester-Based Cationic Lipids. CV-1 (monkey kidney fibroblast) and HTB-129 (human breast cancer) cells in culture were treated with ortho-ester-based lipoplexes (**1**/DOPE/DNA and **2**/DOPE/DNA, N/P = 5/1) to test whether their pH-sensitivity can improve the gene transfection efficiency. The DOTAP/DOPE/DNA (N/P = 5/1) lipoplex was also administered as a pH-insensitive control. CV-1 is a mammalian tissue cell line

Table 1. Colloidal Properties of Ortho-Ester-Based Liposomes and Lipoplexes[a]

	liposomes (X/DOPE)			lipoplexes (X/DOPE/DNA)		
X	diameter (nm)	polydispersity index	ζ-potential (mV)	diameter (nm)	polydispersity index	ζ-potential (mV)
1	188 ± 2.6	0.14	58.7 ± 1.4	248 ± 1.8	0.14	58.3 ± 0.6
2	147 ± 1.9	0.24	61.0 ± 1.1	266 ± 2.8	0.13	52.4 ± 0.4
DOTAP	166 ± 2.3	0.23	50.0 ± 2.8	232 ± 0.7	0.11	56.8 ± 1.4

[a] Samples were measured in 10 mM HEPES buffer, pH 7.4, and three measurements were performed for each sample. Colloidal size and ζ-potential are reported as mean ± standard deviation of three measurements.

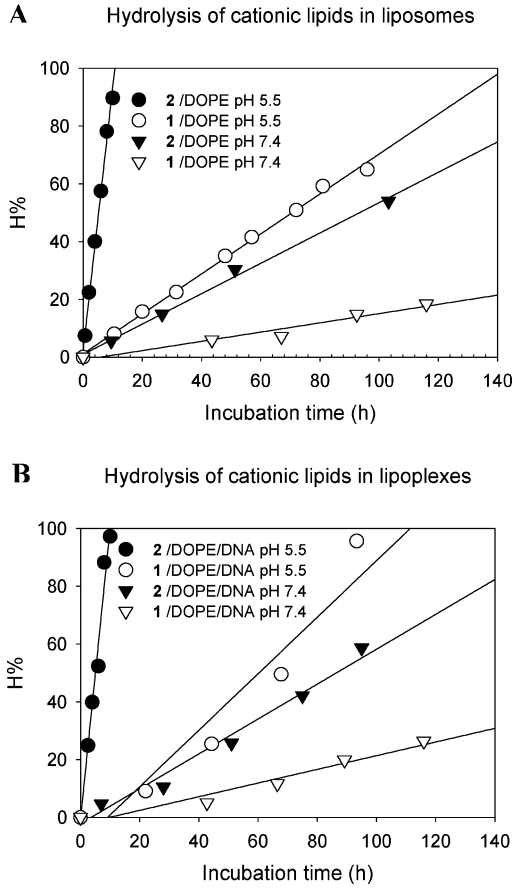


Figure 2. Hydrolysis of **1** and **2** in liposomes (A) containing 50 mol % DOPE and in the corresponding lipoplexes (N/P = 5/1, B) at pHs 5.5 and 7.4.

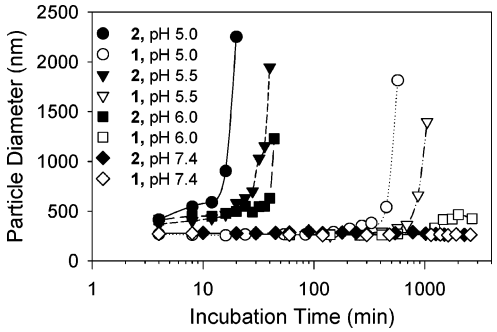


Figure 3. Acidic pH-triggered aggregation of ortho-ester-based lipoplexes. Each lipoplex contains 50 mol % DOPE; N/P = 5/1.

that has been used to assess the efficiency of numerous nonviral gene delivery systems including DOTAP-derived lipoplexes.[40−43] HTB-129 cells are derived from an epidermal cancer (human breast ductal carcinoma) whose suppression by cancer gene therapy would offer considerable therapeutic opportunities.[44] The lipoplex **2**/DOPE/DNA with an optimized triggering kinetics in both the aggregation and the membrane-permeation studies

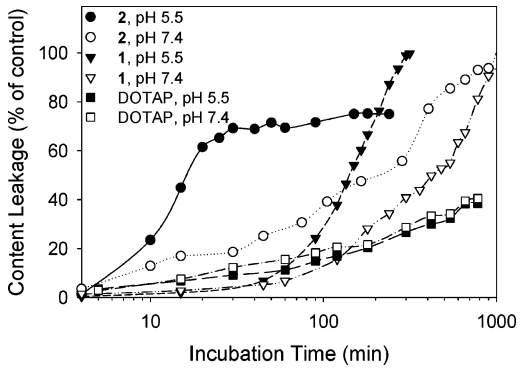


Figure 4. Acidic pH-triggered permeation of model lipid bilayer by ortho-ester-based lipoplexes. Each lipoplex contains 50 mol % DOPE; N/P = 5/1. DOTAP lipoplex is a pH-insensitive control.

significantly improved the gene transfection compared to the pH-insensitive DOTAP/DOPE/DNA control lipoplex. In both cell lines, the highest expression of the luciferase gene is achieved by the **2**/DOPE/DNA lipoplex at optimal dosage of plasmid DNA per well. In CV-1 cells, the highest gene expression was achieved by **2**/DOTAP/DOPE at 0.3 μg DNA/well, approximately 50-fold ($p < 0.01$, Student's *t*-test) as much as that by the same dose of DOTAP/DOPE/DNA lipoplex, and 5-fold ($p < 0.02$, Student's *t*-test) as much as that by the optimal dose (3.0 μg DNA/well) of DOTAP/DOPE/DNA. In HTB-129 cells, the highest gene expression was achieved by **2**/DOTAP/DOPE at 3.0 μg DNA/well, approximately 20-fold ($p < 0.005$, Student's *t*-test) as much as that by the same dose of DOTAP/DOPE/DNA lipoplex, and 3-fold ($p < 0.01$, Student's *t*-test) as much as that by the optimal dose (1.0 μg DNA/well) of DOTAP/DOPE/DNA. The **1**/DOPE/DNA lipoplex also offered higher luciferase gene expression than DOTAP/DOPE/DNA in CV-1 cells, especially at lower doses (0.1−1.0 μg DNA/well). However, there is no convincing improvement of the gene transfection by **1**/DOPE/DNA in HTB-129 cells.

Discussion

Investigations over the past 2 decades have established the paradigm that gene delivery is a complicated process in which multiple biophysical and biochemical barriers must be overcome.[9,45] Endosomal processing coupled with degradation in the lysosome represents one of the major barriers,[8] and a number of mechanisms of endosome destabilization have been exploited to improve the efficiency of synthetic gene delivery systems. For cationic liposomes and their lipoplexes, the most studied mechanism of endosome destabilization is the formation of inverted hexagonal columnar phases (H_{II})[7,28,29,46,47] featuring hexagonal stacking of lipid tubules, where the hydrophobic tails of the lipid molecules face the surface of the tubules. The H_{II} phases tend to aggregate with one another and destabilize lipid bilayers, such as those of biomembranes.[30] X-ray scattering studies by Koltover et al.[28] have shown that the formation of the H_{II} phases is favored by high mole percentage of the conelike

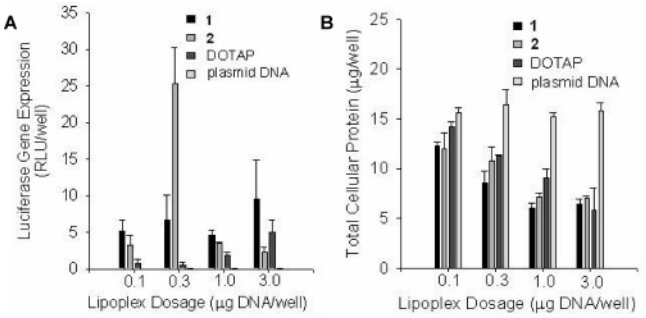


Figure 5. Luciferase gene expression (A) and cytotoxicity (shown by the decrease of cellular proteins, B) of ortho-ester-based lipoplexes in CV-1 cells. Each lipoplex contains 50 mol % DOPE; N/P = 5/1. DOTAP lipoplex is a pH-insensitive control.

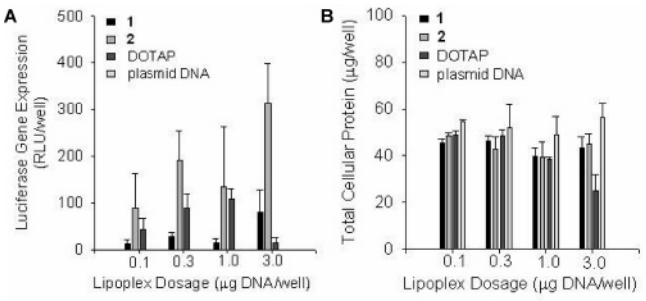


Figure 6. Luciferase gene expression (A) and cytotoxicity (shown by the decrease of cellular proteins, B) of ortho-ester-based lipoplexes in HTB-129 cells. Each lipoplex contains 50 mol % DOPE; N/P = 5/1. DOTAP lipoplex is a pH-insensitive control.

lipid DOPE in the lipoplexes, thus explaining the empirical observation that conelike lipids (eg. DOPE, cholesterol) with a small hydrophilic head group and a relatively large hydrophobic moiety often enhance the gene transfection of cationic lipids. More recently, X-ray scattering and cryoelectron microscopy studies on a highly efficient "gemini" cationic lipid[47] showed that lipoplexes comprising this lipid converted from the lamellar phase to the H_{II} phases when the pH is lowered from neutrality to the endosomal pH around 5.5.

In this paper, the characterization of the ortho ester amphiphiles has been focused on their sensitivity to endosomal pH and the kinetics of the lipid phase changes induced by the hydrolysis of such amphiphiles. The decrease of pH enhanced the hydrolytic cleavage of the cationic head group from the ortho-ester-based lipids **1** and **2** to generate the conelike lipid DOG, which favors the formation of the H_{II} phase. A faster generation of DOG, either caused by lowering the pH or by the structural differences between **1** and **2**, is correlated with earlier onsets of lipoplex aggregation and more efficient model bilayer permeation. In addition, we carried out ^{31}P NMR studies on **1**/DOPE liposomes (1:1 molar ratio) incubated at pH 5.5, which showed a delayed and cooperative shift of the DOPE headgroup phosphates to a higher-field environment (Figure 7). This is characteristic of an $L_\alpha \rightarrow H_{II}$ lipid phase change on the basis of previous reports[39,48] and thus demonstrates the ability of ortho ester hydrolysis to induce lipid phase changes. Similar ^{31}P NMR studies were carried out on **2**/DOPE liposomes but failed to generate reliable data because of the fast phase change and aggregation of **2**/DOPE liposomes. Nonetheless, because **2** is hydrolyzed into the same conically shaped lipid DOG, the same $L_\alpha \rightarrow H_{II}$ lipid phase change is expected. No shift of the ^{31}P NMR signal was observed in the pH-insensitive DOTAP/DOPE (1:1 molar ratio) liposomes over 11 h of incubation at pH 5.5 (data not shown). Taken together, our observations strongly

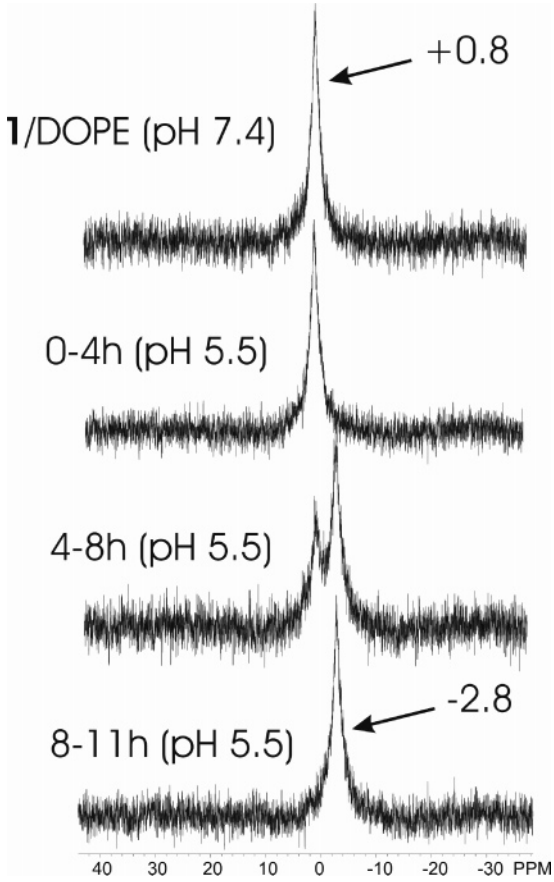


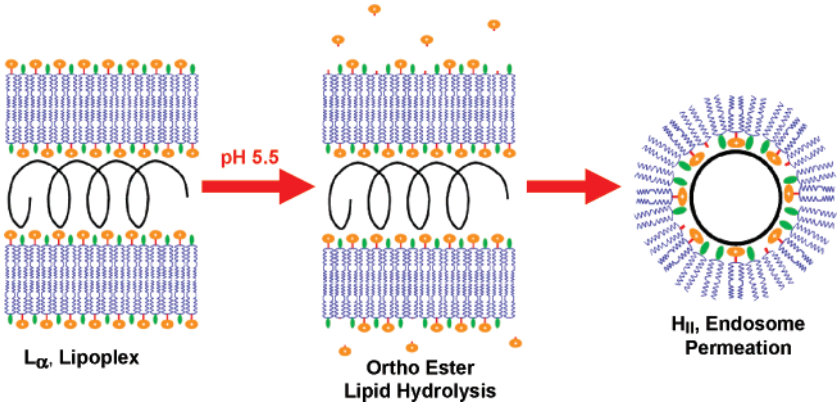
Figure 7. ^{31}P NMR of **1**/DOPE liposomes. The arrows label the signals of the DOPE phosphates with the chemical shift values. The labeled hours designate the time period after the initiation of incubation during NMR data acquisition. Each spectrum represents averaged data of 4500−7000 acquisitions over 3−4 h.

support the initial concept of design (Scheme 4) in which the lipoplexes **1**/DOPE/DNA and **2**/DOPE/DNA convert from predominantly the lamellar (L_α) phase to the fusogenic H_{II} phases in response to the low-pH-catalyzed hydrolysis of the ortho ester linkers in **1** and **2**.

Both ortho-ester-based lipoplexes (**1**/DOPE/DNA and **2**/DOPE/DNA) improved the gene transfection in cultured cells compared to the pH-insensitive control DOTAP/DOPE/DNA. However, the improvement by **2**/DOPE/DNA is more significant. This may be attributed to more prompt endosome membrane permeation by **2**/DOPE/DNA in response to mildly acidic pH. The intracellular processing of endosomes following endocytosis occurs in 30−60 min, after which the endosomal contents are delivered to the lysosome.[8,23,24] At pH 5.5, **2**/DOPE/DNA was able to cause more than 60% contents leakage of the model liposome within 30 min, whereas **1**/DOPE/DNA caused extensive model liposome leakage (50%) only after 140 min. Thus, our studies suggested the importance for pH-sensitive lipoplexes to quickly destabilize the endosome membranes upon a small drop of pH in order to minimize the degradation in the lysosome.

Because the hydrolysis of the ortho ester lipids removes excessive positive charges from the lipid bilayers, it would weaken the electrostatic interaction between the lipid bilayers and DNA and eventually facilitate the release of free plasmid DNA from the lipoplexes for transgene expression. Furthermore, Figure 6B suggests that, under certain conditions, the improved gene transfection by the ortho-ester-based lipoplexes could also be attributed in part to their lower toxicity compared to the pH-

Scheme 4



insensitive lipoplexes. However, more detailed cytotoxicity studies are needed to confirm this hypothesis.

The differences between the transfection profiles (gene transfection levels, optimal doses, etc.) of the two cell lines are not fully understood but may be attributed at least in part to their differences in morphology. The square-shaped HTB-129 cells form a thicker monolayer of a larger number of cells per well than the spindle-shaped CV-1 cells at confluence, as reflected by the significantly higher total cellular protein levels in HTB-129 cells (Figures 5B vs 6B). This would contribute to generally higher luciferase gene expression per well by HTB-129 cells than CV-1 cells. The lower CV-1 cell density would also subject each CV-1 cells to more lipoplexes during transfection and make the CV-1 cells more sensitive to lipoplex toxicity at higher doses (1.0 and 3.0 μg DNA/well), where both the luciferase gene expression and the total cellular protein decreased. The lower transfection levels in CV-1 cells could also be attributed to the presence of other significant barriers of gene delivery, including trafficking in the cytosol[9] and cell nuclear entrance.[49]

The hydrolysis of **1** and **2** was quantified as in liposomes and lipoplexes. At constant pH, apparent zero-order kinetics was observed, in contrast to the apparent first-order hydrolysis kinetics of a previously reported ortho ester lipid POD grafted in freeze−thawed liposomes.[20,21] Such a difference probably resulted from the different colloidal environments of the ortho ester lipids. Liposomes prepared by freeze−thawing are mostly in single lamellar structure,[50,51] where at least 50% of the ortho ester linkers are exposed to the bulk aqueous medium. In contrast, cationic liposomes constructed by agitation in aqueous buffer[52] and lipoplexes thereby prepared[53] possess multilayer lamellar structures, where hydrolyzes are more likely to be controlled by surface erosion. Indeed, apparent zero-order hydrolysis kinetics and surface erosion were both exhibited by aggregates of hydrophobic polyortho esters in aqueous media.[19,54] Surface erosion would also explain, at least in part, why the hydrolysis rates of **1** or **2** in its lipoplexes are similar to those in the corresponding liposomes. The ζ-potential measurements (Table 1) indicate that, despite of the presence of polyanionic DNA, the surface of the lipoplexes carry a high density of positive charges, similar to that of the corresponding liposomes. This suggests that the lipoplexes would impose similar field effects on the proton-catalyzed ortho ester hydrolysis reaction compared to the corresponding liposomes.

The difference between the hydrolysis rates of the open chain ortho ester **1** and that of the cyclic ortho ester **2** promises the flexibility of designing ortho ester-based lipids and lipidic colloids of various pH-sensitivity. Deslongchamps and associates have carried out extensive experimental and computational studies on ortho ester hydrolysis, which highlighted the influence of stereoelectronic and steric effects on the reaction mechanism. Although the detailed mechanisms that contributed to the difference in the hydrolysis rate of **1** and **2** is beyond the scope of this investigation, our data suggest that, in the case of ortho-ester-based lipids, the hydrolysis rate can also be affected by the nature of their colloidal assemblies. The considerable hydrolysis of **1** and **2** even when incubated in a pH 7.4 buffer (20−50% in a few days) would raise the concern about the shelf life of ortho-ester-based transfection reagents if they are to be developed into viable pharmaceutical products. One practical approach to address this issue is to lyophilize the preparations for storage and to reconstitute them in aqueous media at the time of administration.[55]

Conclusion

Unsaturated, ortho-ester-containing cationic lipids and their corresponding lipoplexes have been devised, where both the structure of the cationic lipids and the composition of the lipoplexes were optimized for pH-triggered endosome permeation. We propose that the cyclic ortho ester lipid **2** significantly improved the gene transfection compared to the pH-insensitive lipid DOTAP by promptly destabilizing the endosome membrane via pH-triggered hydrolysis coupled with an $L_\alpha \rightarrow H_{II}$ phase change of its lipoplex. The unique pH-sensitivity of the lipoplexes containing unsaturated ortho ester cationic lipids may grant them significant advantages for applications in gene therapy. Further mechanistic and gene transfection studies on ortho-ester-based cationic lipids and lipoplexes are underway and will be disclosed in due course.

Experimental Section

General Methods and Materials. All chemicals from commercial suppliers were used without further purification unless indicated otherwise. The lipids were from Avanti Polar Lipids (Alabaster, AL). ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and DPX (*p*-xylene-bis-pyridinium bromide) were from Molecular Probes (Eugene, OR). 1,2-Dioleylglycerol was prepared as previously described.[33] Other reagents and solvents were from Sigma-Aldrich or Fisher. Solvent compositions of chromatography mobile phases are in volume ratios. ^{1}H NMR (300 MHz), ^{31}P NMR (75 MHz), and ^{13}C NMR (75 MHz) were recorded on a Varian Mercury 300 MHz NMR spectrometer and chemical shifts were calibrated against residual solvent signals of $CDCl_3$ (δ 7.26 for ^{1}H NMR, 77.16 for ^{13}C NMR) or TMS (δ 0.00 for ^{1}H NMR) and reported in ppm. ESIMS studies were performed on a Varian 1200L triple-quadruple mass spectrometer, which recorded averaged *m*/*z* values. Elemental analyses were performed at the UC Berkeley Micro-Mass Facilities. The pH measurements were made with a Fisher Scientific Accumet model 15 pH meter. Colloidal size and

ζ-potential were measured with a Malvern Zetasizer $3000HS_A$ dynamic light scattering instrument. Fluorescence spectra were obtained using a Quatamaster fluorometer (Photon Technology International, Lawrenceville, NJ).

2-[Methoxy-(1,2-dioleylglyceroxy)methoxy]-*N*,*N*-dimethylethanamine (4). To a solution of 1,2-dioleylglycerol (570 mg, 0.96 mmol) and *N*,*N*-dimethylethanolamine (0.68 mL, 6.72 mmol) in dry THF (40 mL) under N_2 was added sodium hydride (60% dispersion in mineral oil, 338 mg, 8.45 mmol). The mixture was stirred at room temperature for 30 min. α,α-Dichloromethyl methyl ether (0.34 mL, 3.84 mmol) was then injected in one bolus and the mixture was stirred at 45 °C for 10 h. Triethylamine (1 mL) was added to stop the reaction and to stabilize the product. The reaction mixture was partitioned between CH_2Cl_2 (30 mL) and saturated aqueous sodium carbonate (30 mL). The organic layer was washed sequentially with saturated sodium carbonate (2 × 30 mL) and water (30 mL) and then dried over sodium carbonate/magnesium sulfate (1:1, w/w). The solution was filtered and rotoevaporated. The resultant residue was loaded onto a silica gel column and eluted with CH_2Cl_2/MeOH/NEt_3 (100:2:0.5). Fractions corresponding to the product were pooled and rotoevaporated to yield a clear viscous oil (148 mg, yield 21%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (t, 7 Hz, 6H, $(CH_2)_7CH_3$), 1.28 (m, 44H, $(CH_2)_6CH_3$ and $OCH_2CH_2(CH_2)_5$), 1.51 (m, 4H, $OCH_2CH_2(CH_2)_5$), 2.0 (m, 8H, $CH_2CH{=}CHCH_2$), 2.26 (s, 6H, $N(CH_3)_2$), 2.53 (t, 5.9 Hz, 2H, NCH_2), 3.32 (s, 3H, OCH_3), 3.40−3.70 (m, 11H, OCH_2 and $OCHCH_2$), 5.14 (s, 1H, ortho ester O_3CH), 5.35 (m, 4H, $CH{=}CH$); ESIMS calcd for $[M + H]^+$ $C_{45}H_{90}NO_5$ 725.2, found, 724.8.

2-[Methoxy-(1,2-dioleylglyceroxy)methoxy]-*N*,*N*,*N*-trimethylethanammonium Iodide (1). To a suspension of **4** (135 mg, 0.19 mmol) in dry THF (3 mL) were added sodium carbonate (121 mg, 1.14 mmol) and iodomethane (0.059 mL, 0.95 mmol). The suspension was stirred under N_2 at room temperature for 8 h. The reaction mixture was filtered and the precipitate washed with CH_2Cl_2. The filtrate was pooled and rotoevaporated. The residue was dried in high vacuo to give a yellow solid (165 mg, yield 100%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (t, 6.6 Hz, 6H, $(CH_2)_7CH_3$), 1.28 (m, 44H, $(CH_2)_6CH_3$ and $OCH_2CH_2(CH_2)_5$), 1.53 (m, 4H, $OCH_2CH_2(CH_2)_5$), 1.98 (m, 8H, $CH_2CH{=}CHCH_2$), 3.36 (s, 3H, OCH_3), 3.38−3.70 (m, 11H, NCH_2 and dioleylglyceroxy OCH_2/OCH), 3.50 (s, 9H, $N(CH_3)_3$), 4.01 (m, 2H, OCH_2CH_2N), 5.18 (s, 1H, ortho ester O_3CH), 5.35 (m, 4H, $CH{=}CH$); ESIMS calcd for $[C_{46}H_{92}NO_5]^+$ 739.2, found 739.1. Anal. $C_{46}H_{92}NO_5I$: C, H, N.

***N*-(2-Methyl-2-ethoxydioxolan-4-yl)methyl-*N*,*N*-dimethylamine (5).** Triethyl orthoacetate (1.82 mL, 10 mmol), 3-dimethylamino-1, 2-propanediol (1.19 mL, 10 mmol), and TsOH (13.6 mg) were added under N_2 into a dry 50 mL round-bottom flask equipped with a magnetic stirring bar. The reaction mixture was vigorously stirred under N_2 for 1.5 h at 100−110 °C, after which 5 mL of cyclohexane was injected into the mixture. The azeotrope of byproduct ethanol and cyclohexane was distilled at 55−64 °C in 1.5 h. Another aliquot of cyclohexane (3 mL) was injected and gradually distilled at temperature rising from 55 to 110 °C in 3 h.

Triethylamine (0.1 mL) was then added to stop the reaction and to stabilize the product. The reaction mixture was partitioned between CH_2Cl_2 (30 mL) and saturated aqueous sodium carbonate (30 mL), and the organic layer was washed sequentially with saturated sodium carbonate (2 × 30 mL) and water (30 mL). The solution was dried over sodium carbonate/magnesium sulfate (1:1, w/w) and filtered. The filtrate was rotoevaporated and the resultant residue was separated on a silica gel plug (5 cm diameter × 3 cm length) using CH_2Cl_2/MeOH/NEt_3 (100:2:0.5) as the eluting solvent. Fractions containing the product were pooled and rotoevaporated to afford the product as a clear oil (684 mg, yield 36%): ^{1}H NMR (300 MHz, $CDCl_3$) (mixture of cis- and trans-diastereomers) δ 1.18 (t, 7.1 Hz, 3H, OCH_2CH_3), 1.55 and 1.57 (s, 3H, O_3CCH_3), 2.30 and 2.32 (s, 6H, $N(CH_3)_2$), 2.36−2.68 (m, 2H, NCH_2), 3.47−3.70 (m, 3H, OCH_2CH_3 and one proton of endocyclic OCH_2), 4.15 (dt, 7.7 Hz, 21.5 Hz, 1H, one proton of endocyclic OCH_2), 4.35 (m, 1H, endocyclic OC*H*); ESIMS calcd for $C_9H_{20}NO_3$ $[M + H]^+$ 190.3, found 190.0.

***N*-[2-Methyl-2-(1′,2′-dioleylglyceroxy)dioxolan-4-yl]methyl-*N*,*N*-dimethylamine (6).** To a suspension of 1,2-dioleylglycerol (296.5 mg, 0.5 mmol) in dry cyclohexane (12 mL) under N_2 were added **5** (378.5 mg, 2 mmol) and pTsOH (20.4 mg). The reaction mixture was vigorously stirred under N_2 for 1.5 h at 100−110 °C. Dry cyclohexane (10 mL) was then injected and the reaction mixture was heated for an additional 1.5 h to codistill the byproduct ethanol. Triethylamine (0.5 mL) was then added to stop the reaction and to stabilize the product. The reaction mixture was partitioned between CH_2Cl_2 (50 mL) and saturated aqueous sodium carbonate (50 mL), and the organic layer was washed sequentially with saturated aqueous sodium carbonate (2 × 50 mL) and water (50 mL). The organic solution was then dried over sodium carbonate/magnesium sulfate (1:1, w/w), filtered, and rotoevaporated. The resultant residue was loaded on top of a small Amberlite XAD-7 plug and eluted with hexane/ether/NEt_3 (80:20:0.5). Fractions containing the product were pooled, concentrated, and further purified by a Sephadex LH-20 column (mobile phase: CH_2Cl_2/NEt_3 = 100/0.5) to give the pure product (362.7 mg, yield 98%) as a clear viscous oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 7.1 Hz, 6H, $(CH_2)_7CH_3$), 1.31 (m, 44H, $(CH_2)_6CH_3$ and $OCH_2CH_2(CH_2)_5$), 1.55 (m, 4H, $OCH_2CH_2(CH_2)_5$), 1.58 (s, 3H, ortho ester O_3CCH_3), 2.0 (m, 8H, $CH_2CH{=}CHCH_2$), 2.28 (s, 6H, $N(CH_3)_2$), 2.42 (m, 2H, NCH_2), 3.40−3.69 (m, 10H, OCH_2/OC*H* of dioleylglyceroxy group and one proton of endocyclic OCH_2), 4.19 (m, 1H, one proton of endocyclic OCH_2), 4.40 (m, 1H, endocyclic OC*H*), 5.35 (m, 4H, $CH{=}CH$); ESIMS calcd for $[M + H]^+$ $C_{46}H_{90}NO_5$ 737.2, found 737.1.

***N*-[2-Methyl-2-(1′,2′-dioleylglyceroxy)dioxolane-4-yl]methyl-*N*,*N*,*N*-trimethylammonium Iodide (2).** To a suspension of **6** (181.4 mg, 0.25 mmol) in dry THF (4 mL) under N_2 were added sodium carbonate (159 mg, 1.5 mmol) and iodomethane (0.078 mL, 1.25 mmol). The suspension was stirred under N_2 at room temperature for 8 h. The suspension was then filtered and sodium carbonate residue was washed with CH_2Cl_2. The organic solutions were pooled and rotoevaporated, and the resultant residue was dried in vacuo to give a yellowish solid (220 mg, yield 100%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (t, 6.7 Hz, 6H, $(CH_2)_7CH_3$), 1.2−1.4 (m, 44H, $(CH_2)_6CH_3$ and $OCH_2CH_2(CH_2)_5$), 1.55 (m, 4H, $OCH_2CH_2(CH_2)_5$), 1.62 (s, 3H, ortho ester O_3CCH_3), 1.99 (m, 8H, $CH_2CH{=}CHCH_2$), 3.48−3.65 (m, 10H, OCH_2/OC*H* of dioleylglyceroxy group and one proton of endocyclic OCH_2), 3.52 (s, 9H, $N(CH_3)_3$), 3.75 (m, 1H, one proton of NCH_2), 4.40 (m, 1H, one proton of NCH_2), 4.59 (m, 1H, one proton of endocyclic OCH_2), 4.82 (m, 1H, endocyclic OC*H*), 5.33 (m, 4H, $CH{=}CH$); ^{1}H NMR (300 MHz, CD_3COCD_3) δ 0.89 (t, 6.7 Hz, 6H, $(CH_2)_7CH_3$), 1.26−1.4 (m, 44H, $(CH_2)_6CH_3$ and $OCH_2CH_2(CH_2)_5$), 1.5−1.64 (m, 7H, $OCH_2CH_2(CH_2)_5$) and ortho ester O_3CCH_3), 1.95−2.15 (m, 8H, $CH_2CH{=}CHCH_2$), 3.40−3.65 (m, 10H, OCH_2/OC*H* of dioleylglyceroxy group and one proton of endocyclic OCH_2), 3.54 (s, 9H, $N(CH_3)_3$), 3.7−4.0 (m, 1H, one proton of NCH_2), 4.1−4.6 (m, 2H, one proton of NCH_2 and one proton of endocyclic OCH_2), 4.72 and 5.06 (two groups of m, 1H, endocyclic OC*H*, abundance ratio ∼ 9/1), 5.27−5.45 (m, 4H, $CH{=}CH$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.32, 15.49, 15.77, 21.25, 21.80, 21.85, 22.12, 22.89, 26.28, 26.32, 27.43, 29.40, 29.52, 29.70, 29.73, 29.76, 29.89, 29.91, 29.98, 30.30, 30.41, 32.11, 32.82, 55.33, 58.98, 59.05, 63.45, 63.56, 67.04, 67.33, 68.79, 70.26, 70.38, 70.47, 70.72, 70.90, 71.99, 72.02, 123.30, 130.04, 130.15; ESIMS calcd for $[C_{47}H_{92}NO_5]^+$ 751.2, found 750.7. Anal. $C_{47}H_{92}NO_5I$: C, H, N.

Preparation of Cationic Liposomes and Lipoplexes.[35] A CH_2Cl_2 solution of a cationic lipid and DOPE (1:1 molar ratio) in a Pyrex glass test tube was rotoevaporated to form a lipidic film at the bottom of the test tube. The residual solvent of the lipidic film was removed under high vacuum at room temperature for 2 h. For lipid hydrolysis analyses, lipoplex aggregation assays, and model liposome leakage assays, the lipidic film was then hydrated by a pH 7.4 buffer (10 mM HEPES, 20 mM NaCl, 4% glucose) into a 7.5 mM (total lipid) suspension followed by 5 min agitation by a bench top vortex machine. For gene transfection and ^{31}P NMR experiments, the lipidic film was hydrated with a pH 7.4 buffer (10 mM HEPES, 20 mM NaCl) without 4% glucose. The hydrated

lipids were extruded 11 times through a 200 nm membrane to yield a cationic liposome preparation. The liposome preparation was left at room temperature for 30 min, after which an equal volume of a plasmid DNA solution (0.25 mg DNA/mL, pH 7.4, 10 mM HEPES, 20 mM NaCl) was added to the liposome preparation followed by brief agitation to form a lipoplex suspension at N/P = 5/1, where N/P is defined as the molar ratio of the quaternary ammonium of cationic lipids and the phosphate of DNA.

Hydrolysis of 1 and 2 in Liposomes and in Lipoplexes. To characterize the hydrolysis of **1** and **2** in liposomes at pH 7.4, a liposome suspension freshly prepared in a pH 7.4 buffer was incubated at 37 °C. Small aliquots (100 μL) of the liposome suspension were extracted at different time points and mixed with 900 μL of 90% (v/v) MeOH in water. An aliquot (100 μL) of each diluted sample was immediately analyzed by HPLC.

To characterize the hydrolysis of **1** and **2** in liposomes at pH 5.5, one volume of 20 mM NaOAc/HOAc buffer (initial pH 5.26) was mixed with four volumes of a liposome suspension freshly prepared in a pH 7.4 buffer. The final pH 5.5 of the mixture was confirmed with a pH meter. The mixture was incubated at 37 °C, and small aliquots (100 μL) of the liposome suspension were extracted at different time points and mixed with 900 μL of 90% MeOH in water. An aliquot (100 μL) of each diluted sample was immediately analyzed by HPLC.

To characterize the hydrolysis of **1** and **2** in cationic lipoplexes at pH 7.4, a lipoplex suspension freshly prepared in a pH 7.4 buffer was split into 100 μL aliquots and incubated at 37 °C. At different time points, one of the aliquots was sequentially mixed with 250 μL of methanol, 125 μL of CH_2Cl_2, another 125 μL of CH_2Cl_2, and 125 μL of 0.9% NaCl. The final mixture was centrifuged at 9300*g* RCF for 30 s to give a two-layer solution. The organic layer at the bottom was extracted and evaporated with a gentle nitrogen flow. The residue was dissolved in 500 μL of 80% MeOH in water. An aliquot (100 μL) of the solution was immediately analyzed by HPLC.

To characterize the hydrolysis of **1** and **2** in lipoplexes at pH 5.5, one volume of 20 mM NaOAc/HOAc buffer (initial pH 5.26) was mixed with four volumes of a lipoplex suspension freshly prepared in a pH 7.4 buffer. The final pH 5.5 of the mixture was confirmed with a pH meter. The mixture was immediately split into 100 μL aliquots and incubated at 37 °C. At different time points, one of the aliquots was sequentially mixed with 250 μL of methanol, 125 μL of CH_2Cl_2, another 125 μL of CH_2Cl_2, and 125 μL of 0.9% NaCl. The final mixture was centrifuged at 9300*g* RCF for 30 s to give a two-layer solution. The organic layer at the bottom was extracted and evaporated with a gentle nitrogen flow. The residue was dissolved in 500 μL of 80% MeOH in water. An aliquot (100 μL) of the solution was immediately analyzed by HPLC.

The lipids were separated at room temperature by a reversed-phase C_4 Dionex 214TP column (4.6 × 150 mm, flow rate = 0.5 mL/min) on a Beckman Coulter 125 HPLC system. The solvent gradient started from 80% MeOH in water to 100% MeOH in 10 min, followed by 100% MeOH for 30 min. The column was then re-equilibrated at 80% MeOH before the analysis of another sample. The lipids were detected by UV absorbance at 210 nm using a deuterium-lamp-based System Gold 166 detector. Under such chromatographic conditions, the cationic lipids **1** and **2** gave only broad streaking peaks, which were too weak for quantifications. Instead, the resolved peaks for the major hydrolysis product, dioleylglycerol (retention time = 15.4 min), and the stable helper lipid, dioleylphosphatidylethanolamine (DOPE, used as internal standard, retention time = 13.6 min), were monitored for quantifications. Dioleylglycerol was estimated by HPLC to be more than 85% of the hydrolysis products of **1** and more than 98% of the hydrolysis products of **2**.

The extent of hydrolysis is expressed as percentage of hydrolysis (H%), which is defined and calculated by the following equation

$$\mathrm{H\%} = \frac{\dfrac{A_{\mathrm{DOG}t}}{A_{\mathrm{DOPE}t}}}{\dfrac{A_{\mathrm{DOG100}}}{A_{\mathrm{DOPE100}}}} \times 100\%$$

where $A_{\mathrm{DOG}t}$ is the peak area of dioleylglycerol in a sample that has been incubated for a certain period of time (t), $A_{\mathrm{DOPE}t}$ is the peak area of DOPE in the same sample as that of $A_{\mathrm{DOG}t}$, A_{DOG100} is the peak area of dioleylglycerol in a control liposome preparation for which the ortho ester hydrolysis has been driven to completion (see below for details), and A_{DOPE100} is the peak area of DOPE in the same control liposome preparation as that of A_{DOG100}.

To prepare a control liposome in which the ortho-ester-based cationic lipids are completely hydrolyzed, 100 μL of a liposome preparation was mixed with 100 μL of glacial acetic acid followed by 2 h of incubation at 37 °C. The control liposome preparation was then diluted with 800 μL of MeOH, and an aliquot (100 μL) of the diluted sample was analyzed by HPLC. No significant degradation of DOPE was observed under this condition. The data of a control liposome preparation were used to normalize liposome and lipoplex samples of the same original lipid composition.

Lipoplex Aggregation Assay. Three volumes of a freshly prepared lipoplex suspension in a pH 7.4 buffer (10 mM HEPES, 20 mM NaCl, 4% glucose) was mixed with one volume of an acidic buffer (100 mM NaOAc/HOAc, 50 mM NaCl) of pH 4.69, 5.07, or 5.30 to obtain a mixture at a final pH of 5.0, 5.5, or 6.0, respectively. The final pH of each mixture was confirmed by a pH meter. To characterize the stability of lipoplex at pH 7.4, one volume of a freshly prepared lipoplex suspension was mixed with three volumes of a 100 mM HEPES buffer (pH 7.4, 50 mM NaCl). The mixture was incubated at 37 °C and a small aliquot (45 μL) was extracted at different time points and diluted into 2.5 mL of 100 mM HEPES buffer (pH 7.4) followed by an immediate measurement of the particle size on a Malvern light scattering instrument (Zetasizer 3000 HS_A) following the manufacturer's recommendations.

Model Liposome Preparation. The model liposomes mimicking the lipid composition of biomembranes were prepared using the freeze−thaw method.[51] A chloroform solution of POPC (22.5 μmol, 50 mol %), POPE (9 μmol, 20 mol %), POPS (2.25 μmol, 5 mol %), L-α-PI (4.5 μmol, 10 mol %), and cholesterol (6.75 μmol, 15 mol %) was evaporated under reduced pressure to form a lipidic film. The residual chloroform in the lipidic film was removed in high vacuo overnight at room temperature. The lipidic film was hydrated with a 1.5 mL solution of 50 mM ANTS, 50 mM DPX, and 10 mM HEPES at pH 7.4 by 20 min of intermittent agitation to obtain a suspension at 30 mM total lipid concentration. The tube containing the lipid suspension was then filled with N_2 and sealed. The lipid suspension in the tube was rapidly frozen in dry ice/acetone for 5 min, followed by melting in water at room temperature for 5 min. The freeze−thawing was repeated for 10 times. The resultant liposome suspension was extruded 11 times through a polycarbonate membrane with pores 0.4 μm in diameter. The size of the extruded liposomes was 284.8 nm in diameter as measured by Malvern Zetasizer 3000 HS_A. The liposomes were separated from the unencapsulated fluorescent dyes with a Sephadex G-75 column eluted with an isotonic buffer (10 mM HEPES, 140 mM NaCl, pH 7.4). The lipid concentration of the eluted liposome was determined by a phosphorus assay.[56]

Model Liposome Leakage Assay. Lipoplex was mixed with the model liposome at a total lipid molar ratio of 1:1. To carry out the leakage assay at pH 5.5, one volume of an acidic buffer (pH 5.15, 100 mM NaOAc/HOAc, 50 mM NaCl) was mixed with three volumes of the lipoplex−model liposome mixture to obtain a final mixture of pH 5.5. To carry out the leakage assay at neutral pH, a HEPES buffer (pH 7.4, 100 mM HEPES, 50 mM NaCl) was used in place of the acidic NaOAc/HOAc buffer. The resultant mixture was incubated at 37 °C and a small aliquot was transferred into a cuvette containing 2 mL of a pH 7.4 buffer (100 mM HEPES, 50 mM NaCl) at different time points. The fluorescence (λ_{ex} = 370

nm, λ_{em} = 550 nm) of the diluted sample was measured on a Quatamaster fluorometer (Photon Technology International, Lawrenceville, NJ). The data were processed and expressed as percentage of leakage as previously reported.[38]

Gene Transfection in Cultured Cells. The gene transfection procedure is similar to a method described by Felgner and co-workers.[57] The cells were cultured in T25 flasks according to ATCC recommendations. CV-1 cells (a monkey fibroblast cell line, ATCC-CCL70) were cultured in MEM (Eagle) containing 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). HTB-129 cells (a human breast ductal carcinoma cell line) were cultured in MEM (Eagle) containing 10% FBS, 2 mM L-glutamine, 0.01% insulin, and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin).

Twelve hours prior to gene transfection, when cells were at approximately 90% confluence, the cells were seeded onto 24-well plates (3×10^4 CV-1 cells/well and 5×10^4 HTB-129 cells/well; cell counting performed with a Coulter Z1 particle counter; 1 mL complete growth medium per well) and cultured overnight at 37 °C in a humidified atmosphere containing 5% CO_2 to approximately 60% confluence. The complete growth medium was aspirated. The cells were washed once with FBS and antibiotics-free medium (150 μL/well) and supplemented with serum-free medium (500 μL/well). Appropriate volumes of freshly prepared lipoplexes (less than 50 μL/well) were added to the cells followed by mild agitation of the 24-well plate. After incubation at 37 °C, 5% CO_2 for 5 h, the serum free medium was aspirated, and the cells were washed three times with PBS (150 μL/well). The cells were resupplemented with complete growth medium (1 mL/well) and cultured for an additional 24 h before the assessment of reporter gene expression.

Reporter Gene Expression Assay. Reporter gene (firefly luciferase) activity was assessed with the luciferase assay system (Promega, E1500) following a modified protocol based on the manufacturer's recommendations. Briefly, the growth media were aspirated and the cells gently rinsed with PBS buffer (Mg^{2+}- and Ca^{2+}-free). To each plate well was added 150 μL of M-PER Reagent (Pierce) instead of the CCLR reagent recommended by the manufacturer. The M-PER Reagent (Pierce) minimized the background interference in the cellular protein assay. The luciferase activity was measured using a TD-20/20 luminometer (Turner Design) with a 2-s delay followed by a 10-s data acquisition. Luciferase activity is expressed as relative light units (RLU) per well. Cytotoxicity of the gene transfection was estimated by the decrease of total cellular protein, which was determined by the BCA protein assay (Pierce) following the manufacturer's recommendations.

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Supporting Information Available: NMR and elemental analyses of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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