

Hydrophobic Moiety of Cationic Lipids Strongly Modulates Their Transfection Activity

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Abstract: Synthetic cationic lipids are widely used components of nonviral gene carriers, and the factors regulating their transfection efficiency are the subject of considerable interest. In view of the important role that electrostatic interactions with the polyanionic nucleic acids play in formation of lipoplexes, a common empirical approach to improving transfection has been the synthesis and testing of amphiphiles with new versions of positively charged polar groups, while much less attention has been given to the role of the hydrophobic lipid moieties. On the basis of data for ~20 cationic phosphatidylcholine (PC) derivatives, here we demonstrate that hydrocarbon chain variations of these lipids modulate by over 2 orders of magnitude their transfection efficiency. The observed molecular structure–activity relationship manifests in well-expressed dependences of activity on two important molecular characteristics, chain unsaturation and total number of carbon atoms in the lipid chains, which is representative of the lipid hydrophobic volume and hydrophilic–lipophilic ratio. Transfection increases with decrease of chain length and increase of chain unsaturation. Maximum transfection was found for cationic PCs with monounsaturated 14:1 chains. It is of particular importance that the high-transfection lipids strongly promote cubic phase formation in zwitterionic membrane phosphatidylethanolamine (PE). These remarkable correlations point to an alternative, chain-dependent process in transfection, not related to the electrostatic cationic–anionic lipid interactions.

Keywords: Cationic phospholipid; phosphatidylethanolamine; hydrophobic chain; lipoplex; cubic phase; hexagonal phase; transfection

Introduction

As nonviral carriers of nucleic acids into cells, synthetic cationic lipids are being developed for tasks of utmost importance in the laboratory and clinic, such as gene

transfection and gene silencing.^{1–4} They readily form complexes (lipoplexes) with the polyanionic nucleic acids, protecting the latter until entry into cells. However, a critical obstacle for application of lipid-mediated DNA delivery (lipofection) is its unsatisfactory efficiency for many cell types. Since the specific delivery routes by cationic lipid vectors are mostly unknown, the efforts to improve lipoplex transfection have been largely empirical. In view of the important role of electrostatic interactions with DNA taking place at the level of the lipid headgroups, most of these efforts have been focused on synthesis of amphiphiles with

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new versions of positively charged polar groups.^{5,6} Moreover, it is known that cationic lipids interact also with anionic membrane lipids and, in certain cases, their mixtures form nonlamellar phases that may be involved in lipoplex fusion with cellular membranes and release of encapsulated nucleic acid.^{7–10} Much less attention has been given to investigating the role of the hydrophobic portion of the lipid molecules.^{11–13} Our studies on a specific class of cationic lipids, cationic phosphatidylcholine (PC) derivatives, indicate, however, that substantial enhancement of lipoplex gene delivery could be attained by variations in the lipid hydrocarbon chains. These studies also provided evidence for a relationship between transfection success and lipid phase behavior.^{10,14–17} As is well-known, the latter is strongly modulated by the hydrophobic portions of the lipid molecules.

Here we report data demonstrating that a certain relationship exists between cationic lipid hydrocarbon chain molecular structure and transfection activity. It is of particular importance that high transfection rates correlate with enhanced cubic phase formation in cationic PC mixtures with membrane phosphatidylethanolamine (PE). This behavior indicates that enhanced transfection may be mediated by cationic lipid interactions with the zwitterionic membrane PEs in an alternative transfection pathway that does not involve interactions with anionic membrane lipids.

Experimental Methods

Lipids and DNA. The cationic phospholipids, triflate derivatives of phosphatidylcholines (Figure 1), were synthesized in house as previously described,^{18,19} or were custom synthesized by Avanti Polar Lipids, Inc. (Alabaster, AL), and were stored in chloroform solution at -20°C . Dipalmitoleoylphosphatidylethanolamine (DPOPE) was from Avanti Polar Lipids. All lipids were checked for purity by mass spectroscopy and TLC and found to contain no detectable impurities. Herring sperm DNA was from Invitrogen. CMV- β -galactosidase plasmid was purchased from Clontech Laboratories Inc. (Palo Alto, CA) and propagated and purified by Bayou Biolabs (Harahan, LA). HBSS (HEPES buffered saline solution) and Opti-MEM were obtained from Cambrex (Walkersville, MD) and Invitrogen, respectively.

Cell Culture. Human umbilical artery endothelial cells (HUAECs) were obtained from Cambrex (Walkersville, MD) and maintained in EGM-2 MV containing 5% fetal bovine serum (FBS) (Cambrex) at 37°C with 5% CO_2 . At confluence, the cells were passaged using 0.25 mg/mL trypsin/EDTA (Cambrex) and were used at passages 5–10 for these experiments.

Transfection. The cells were seeded in 96-well plates at 24 h before transfection at densities appropriate to give about 80% confluence at the time of transfection. Chloroform solutions of cationic lipids were mixed in glass vials at the desired ratios, after which the chloroform was removed under an argon or N_2 stream. The vials were placed under high vacuum for several hours to remove the last traces of solvent. The lipid mixtures were hydrated in HBSS at 1 mg/mL to form liposomes. Liposomes and plasmid DNA were each diluted in OptiMEM to 160 $\mu\text{g/mL}$ for lipid and to 40 $\mu\text{g/mL}$ for DNA, and liposomes were pipetted into an equal volume of plasmid DNA solution at a 4:1 weight ratio and mixed gently. The resultant plasmid–lipid complexes were incubated at room temperature for 15 min, after which 50 μL per well was added to cells that were either in medium

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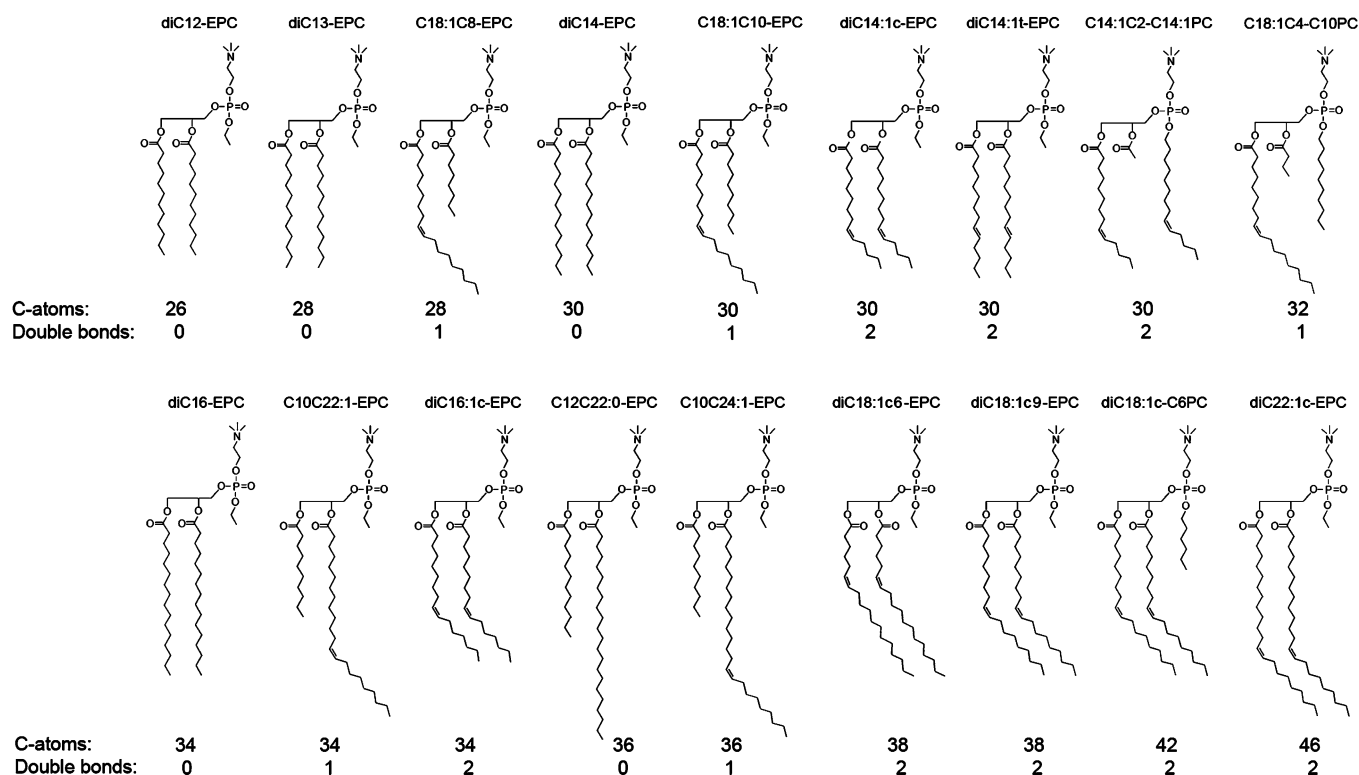


Figure 1. Structures of the studied cationic PC derivatives.

lacking serum or in medium containing serum. Two hours after their addition, the lipoplexes were removed and fresh medium containing serum was added. Cells were assayed for β -galactosidase activity 24 h after transfection using a microplate fluorometric assay. Briefly, the latter procedure was as follows: Subsequent to aspiration of the medium from each well, the cells were washed once with PBS and then lysed by addition of 100 μ L of lysis buffer (0.03% Triton X-100 in 100 mM HEPES, 8 mM MgSO_4 , pH 7.8). Ten microliters of 100 μ M FDG (fluorescein di- β -D-galactopyranoside) was added into each well, and the fluorescence that had developed at 20 h was measured with a microplate fluorometer (model 7620, Cambridge Technology Inc.).

X-ray Diffraction Measurements. Pure cationic lipid dispersions, lipoplexes and mixtures with DPOPE were prepared using previously described procedures.^{17,20,21} Briefly, lipids were dissolved in chloroform, the chloroform was removed with a stream of argon, and the mixtures were subjected to high vacuum for 24–36 h. The dispersions were prepared by hydrating and vortexing at room temperature, followed by 5 freeze–thaw cycles between dry ice and room temperature, all accompanied by vortexing during the thaw-

ing steps. Measurements were carried out on dispersions with lipid contents of 10% (w/v) for the DPOPE/cationic lipid mixtures and 40% (w/v) for pure cationic lipids. The dispersion medium used was Dulbecco's phosphate-buffered saline (PBS, Gibco; pH 7.2). The samples were loaded into X-ray capillaries and flame-sealed before measurement. Low-angle X-ray diffraction patterns were recorded at stations 5IDD, DND-CAT and 18D, BioCAT, APS, Argonne, using 2-D 2048 \times 2048 MAR detectors at a sample-to-detector distance of \sim 200 cm, as described earlier.¹⁷

Results

Transfection Activity. The cationic lipids examined are phosphatidylcholine (PC) derivatives, in which the PC phosphate is esterified, generating triesters and converting the zwitterionic headgroup to a cation. The hydrophobic moieties of the lipids synthesized thus far vary in the range of medium chain lengths and 0–1 double bond per lipid chain (Figure 1). Lipids with more than one double bond per chain (linoleoyl, arachidonoyl) have been also tested, but found to exhibit unsatisfactory transfection (data not shown). The transfection activities of the cationic PC lipoplexes are shown in Figure 2 as a function of lipid chain length and unsaturation. To ensure compatibility, these

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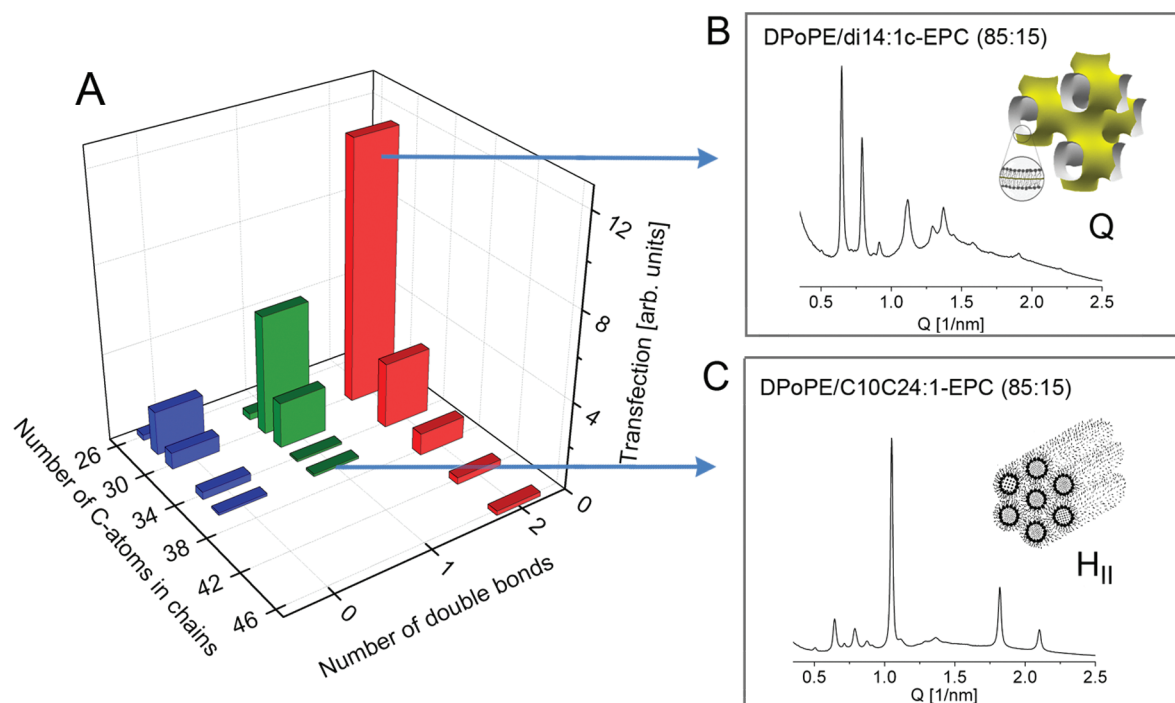


Figure 2. Correlations between transfection efficiency, hydrocarbon chain structure and phase behavior of the cationic PC derivatives. (A) Transfection activity as a function of the total number of carbon atoms and double bonds in the lipid chains (the three lipids with 30 C atoms and 2 double bonds, diC14:1c-EPC, diC14:1t-EPC, and C14:1C2-C14:1PC, as well as the two lipids with 34 C atoms and 2 double bonds, di18:1c9-EPC and di18:1c6-EPC (Figure 1), exhibit virtually identical transfection activity and are represented by a single bar on the plot). (B) Cubic phase formed in mixture of DPOPE with a superior transfection agent, diC14:1c-EPC (85:15 molar ratio). (C) Hexagonal H_{II} (with a small amount of cubic phase) formed in a mixture of DPOPE with a weak transfection agent, C18:1C4-C10EPC (85:15 molar ratio). The phase behaviors shown in (B) and (C) are representative for the strong and weak transfection agents, respectively.¹⁷

activities are given in units of the activity obtained for dioleoyl ethylphosphatidylcholine (diC18:1-EPC; EDOPC), the best studied and historically the first representative of the cationic PCs.^{18,22} According to early studies, EDOPC transfection activity for BHK cells is similar to that of Lipofectamine.²² As seen from Figure 2, transfection varied in a range of over 2 orders of magnitude and displayed a clearly expressed maximum for lipids with total of 30 C atoms and 2 double bonds in the chains. This behavior was represented by three lipids, each comprising two 14:1 (myristoleoyl or myristelaidoyl) hydrocarbon chains: diC14:1c-EPC, diC14:1t-EPC and C14:1C2:0-C14:1PC. Increasing the chain length and/or decreasing the number of double bonds away from this maximum resulted in strongly reduced transfection activity. Introducing chain asymmetry also diminished transfection activity at the same total C atom number $C = 28$ (cf. diC13-EPC vs C18:1C8:0-EPC, Figure 3A), or $C = 34$ (cf. diC16-EPC vs C10C22:1-EPC, Figure 3B); a specific three-chain configuration is also typified by weaker activity than its two-chain counterpart with the same overall number of carbon atoms (Figure 3A).

Structural Studies. In order to gain insight into the mechanism underlying the observed dependence of transfection efficiency on the hydrocarbon chain structure, we used X-ray diffraction to characterize the physical properties of the lipids. Studies on pure cationic PCs showed however

that most of the cationic PCs, in particular, all ethylphosphatidylcholines (EPCs), arrange into bilayers and readily form dispersions of uni- or oligolamellar liposomes in water. At higher electrolyte concentrations at which electrostatic repulsion is largely screened, e.g., in physiological solutions, and at sufficiently high lipid concentrations that prevent unbinding and disordering of the lipid phase caused by electrostatic repulsion (>30 – 40 wt % of lipid), lipids of low, moderate and high transfection were found to typically form well-correlated liquid crystalline lamellar L_{α} phases. This behavior is illustrated in Figure 4A (bottom five patterns). Some lipids may form nonlamellar phase, e.g., a cubic phase (Figure 4A, top pattern); however, in the presence of DNA all these lipids formed lamellar lipoplexes (Figure 4B). In summary, the highly efficient transfection agents diC14:1c-EPC, diC14:1t-EPC and C14:1C2:0-C14:1PC, as well as most lipids of low and moderate transfection activity such as C18:1C2-C12PC and EDOPC, respectively, formed lamellar L_{α} phases of similar structure, and the same was valid also for their lipoplexes (Figure 4). Such measurements have been carried out for a large number of other cationic

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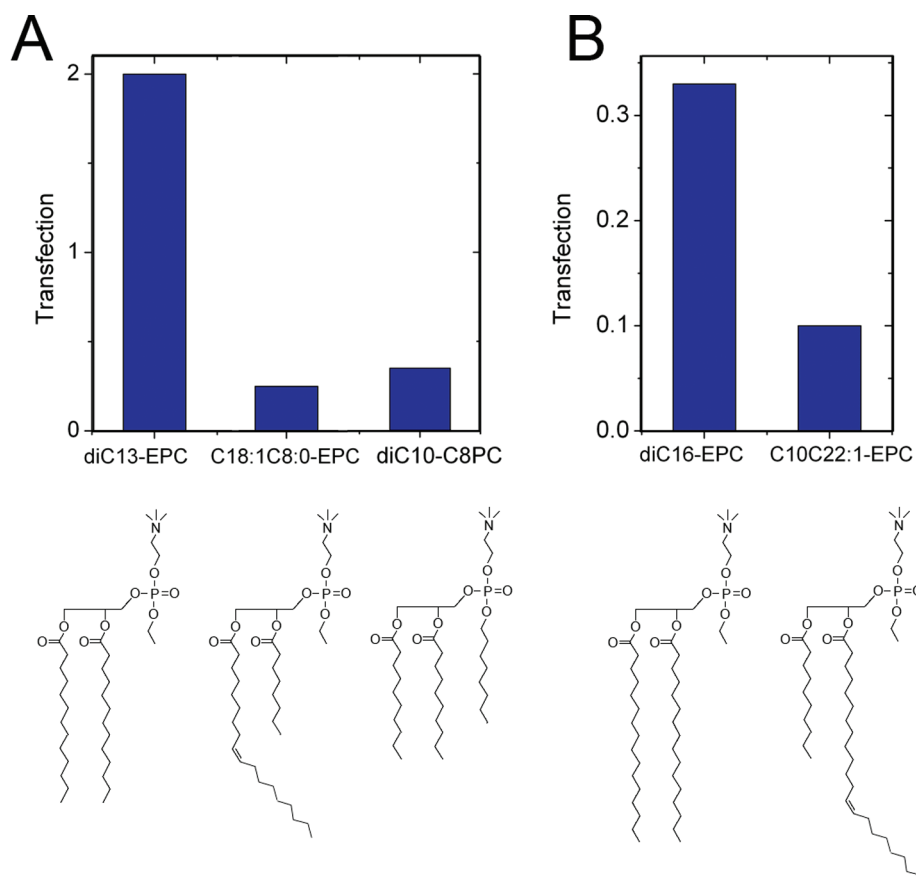


Figure 3. Effect of lipid molecular shape on transfection activity as demonstrated by three lipids with total of 28 C atoms in the chains (A) (diC10 C8PC, which is effectively triple-chained, is not included in Figures 1 and 2), and two lipids with 34 C atoms in the chains (B).

PCs.^{19,22–25} Similarly to the results shown in Figure 4, the structural data obtained from these studies revealed no correlations between transfection activity and structural parameters of both lipoplexes and pure cationic lipid phases.

Based on the premise for the important role of lipoplex interactions with cellular membranes, a different approach to clarifying the underlying structural mechanism is provided by X-ray studies on cationic PC mixtures with membrane lipids and membrane-mimicking lipid compositions.^{10,15–17,20,21,24,26} No correlations of the mixture phase behavior with the cationic PC hydrocarbon chain structure have been sought in previous work; however, such correlations appear to exist, in particular, in mixtures with nonlamellar membrane lipids. Figure 5 presents the effects of cationic PCs with different chains and transfection efficiency on the

lamellar–inverted hexagonal (L_{α} – H_{II}) transition of DPOPE (see also ref 17). Pure DPOPE dispersions are known to exhibit a direct L_{α} – H_{II} transition at 42 °C (Figure 5A).²⁷ Noteworthy, strong transfection agents such as diC14:1c-EPC eliminated the direct L_{α} – H_{II} phase transition and promoted formation of inverted cubic phase of Pn3m type between the L_{α} and H_{II} phases, almost eliminating the formation of the H_{II} phase (Figure 5C); similar is the effect of other strong transfection agents, e.g., diC14:1t-EPC and C14:1C2:0-C14:1PC. By contrast, weak transfection agents retained the L_{α} – H_{II} transition (Figure 5B; the same holds also for other weak transfection agents, e.g., C18:1C2-C12PC and C18:1C4-C10PC). Plots of H_{II} phase diffraction intensity and temperature range of existence clearly illustrate the extent of H_{II} phase suppression by the high-efficiency cationic lipids and clearly distinguish between strong and weak transfection agents (Figure 5D).

Discussion

The cationic PCs are attractive drug and gene delivery carriers because they are biodegradable and have remarkably low toxicities.^{18,22} We have tested their potency to transfect the β -galactosidase reporter gene most frequently with

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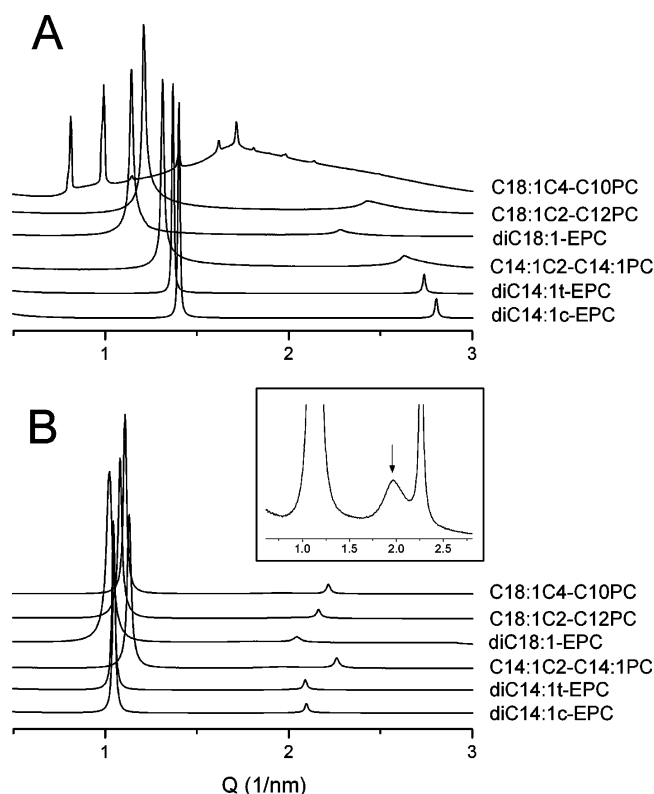


Figure 4. Small-angle X-ray diffraction patterns of cationic PC dispersions (A), and lipoplexes prepared with herring sperm DNA (B) at 20 °C. The inset shows an enlarged view of an example of the DNA diffraction peak in the C18:1C2-C12PC lipoplexes.

HUAEC.^{16,17,20,24,28} This choice of cell culture was influenced by the fact that the vascular endothelial cells are of interest as a gene therapy target. They act as an organ and tissue interface with circulating blood and, even though readily accessible, their gene therapy with nonviral vectors has been hampered because these cells are very difficult to transfect.²⁹ The present results, which show strong increase of HUAEC transfection caused by specific variations of the hydrophobic moieties of the cationic PC carriers, can be of considerable interest.

The data presented in Figures 2 and 3 demonstrate that the hydrocarbon chain structure of the cationic PCs is an important determinant of their transfection activity. Figure 2 shows well-expressed dependences of activity on two important molecular characteristics: chain unsaturation and total number of carbon atoms in the 3 lipid chains. The chain unsaturation is given by the number of double bonds per lipid and assumes typical for phospholipids values of 0, 1, and 2 double bonds, and the total number of carbon atoms in the chains is representative of the lipid hydrophobic

volume. Because the cationic PCs have identical hydrophilic groups, the latter parameter may be considered also as a measure of the cationic PC hydrophilic–lipophilic balance (HLB). In addition to these two parameters, another characteristic of importance appears to be related to the lipid molecular shape, which may be varied by varying the chain asymmetry at constant values of HLB. Examples of the large effect that variations in molecular shape have on transfection are shown in Figure 3. However, the limited number of such examples available at present precludes further analysis and establishment of quantitative representations of this effect.

The mechanism underlying the prominent effects of the lipid chain structure is of considerable interest. It is clearly not of electrostatic nature because the cationic PCs studied here have identical surface choline groups and cannot be distinguished at the level of electrostatic interactions. Factors related to the phase behavior of the pure lipids can also be excluded from consideration on the basis of the results shown in Figure 4. As seen in Figure 4, both high- and low-transfection lipids as well as their lipoplexes form similar, well-correlated lamellar liquid crystalline phases and the structural parameters of these phases appear to display no correlations with the lipid transfection activity. This behavior gives no grounds to expect relationships with the properties of the lipoplexes per se but rather shifts the attention to the role of their interactions with the intracellular membrane structures. These interactions are long considered an important stage of transfection, for example, it is commonly accepted that lipoplex interactions with anionic intracellular lipids are essential for lipoplex disassembly and DNA release. Along this line of reasoning, a number of model studies on cationic/membrane lipid mixtures have been carried out in recent years in order to shed light on the mechanisms of intracellular lipoplex disassembly.^{10,14–16,21} With respect to the very strong hydrocarbon chain effects demonstrated in the present work, of particular interest become studies on model systems, in which electrostatic cationic–anionic lipid interactions are not present. The binary mixtures of the zwitterionic phospholipid DPOPE with a set of high- and low-transfection cationic PCs are actually the first example of such a system (Figure 5). DPOPE represents the most abundant class of nonlamellar-forming membrane lipids, the zwitterionic phosphatidylethanolamines (PEs), which are believed to be involved in lipidic fusion events. It is of significant interest that the effects of the cationic PCs on the DPOPE L_{α} – H_{II} transition correlate very well with their transfection efficiency. X-ray diffraction measurements showed that high-transfection agents eliminated the direct $L_{\alpha} \rightarrow H_{II}$ phase transition and promoted formation of an inverted cubic phase between the L_{α} and H_{II} phases, which

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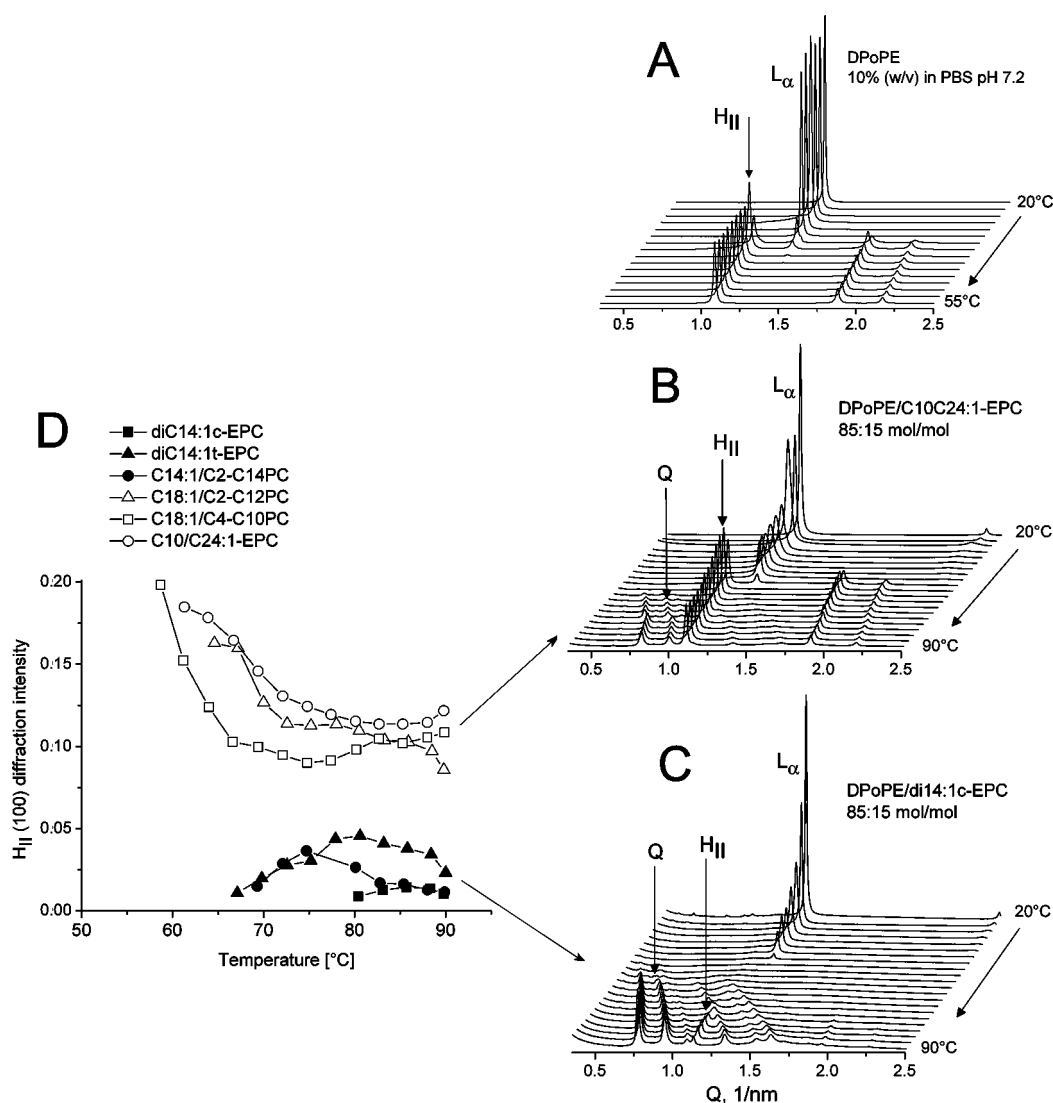


Figure 5. Lamellar–nonlamellar phase transformations in pure DPOPE dispersion (A) and 85:15 (mol/mol) mixtures of DPOPE with C10C24:1-EPC (B) and diC14:1c-EPC (C). Diffraction patterns were recorded every minute during heating scans at 3 °C/min. (D) Diffraction intensity and temperature ranges of existence of the H_{II} phase in DPOPE/cationic lipid mixtures. The intensity of the H_{II} (100) reflection is normalized to the intensity of the initial lamellar phases of the mixtures.¹⁷

replaced the H_{II} phase in a wide temperature range. In contrast, moderate and weak transfection agents retained the $L_{\alpha} \rightarrow H_{II}$ transition. The hydrocarbon chain structures of cationic PCs exhibiting highest transfection thus appear to facilitate formation of inverted cubic phases in nonlamellar phase-forming lipid mixtures.

The phase behavior of the DPOPE/cationic PC mixtures is not unusual in principle, because both introduction of double bonds and variations in the chain lengths are known to have considerable effect on the lamellar–nonlamellar transitions in lipids. A specific structural characteristic of lipids that exhibits distinct change around the chain length of 14 carbons is the formation of bicontinuous inverted cubic phases Q_{II} . Such phases tend to be found with diacyl or dialkyl phospholipids and glycolipids that have chain lengths of C14 or shorter; the longer-chain lipids typically experience direct lamellar–inverted hexagonal $L_{\alpha} \rightarrow H_{II}$ phase

transitions.^{27,30,31} The rationale for this tendency is that longer hydrocarbon chains can relieve packing frustration by filling the “voids” between parallel adjacent H_{II} phase cylinders; consequently, lipids with longer chains are able to more easily form the H_{II} phase.^{30,32–34} In contrast, the H_{II} phase gives way to formation of inverse bicontinuous cubic phases in shorter-chain lipids because the latter lipids are unable to extend easily to fill the H_{II} voids.^{32,34} Although cationic lipids with even shorter chains (<14), respectively, with lower total number of carbon atoms in their hydrocarbon chains (<30), are expected to even more promote formation of cubic phases, we did not observe a corresponding high transfection efficiency with these lipids. The reason for diminished activity of these shorter-chain compounds is almost certainly related to their toxicity.²⁴

The relationship between the ability of the cationic lipids to induce cubic phase and their transfection activity may be

related to the molecular details of lipoplex fusion with cellular membranes. Indeed, multiple studies have demonstrated a relationship between transfection success and enhanced lipoplex–membrane fusion, the latter being required also for neutralization of the cationic lipid charge and subsequent DNA release.^{22,24,35,36} Although the mechanism of lipid membrane fusion is still not fully understood, there is now sound evidence that it is related to the mechanism of lamellar–cubic phase transitions.³⁷

Conclusions

The data presented here demonstrate that (i) a clearly expressed relationship between cationic PC hydrocarbon

chain structure and transfection efficiency manifests as transfection increase with decrease of chain length and increase of chain unsaturation; (ii) the hydrophobic lipid moiety modulates by over 2 orders of magnitude the transfection potency of cationic PCs; (iii) cationic PCs with two 14-C monounsaturated (myristoleoyl or myristelaidoyl) chains exhibit maximum activity; (iv) the superior transfection agents strongly promote formation of cubic phase at the expense of the inverted hexagonal phase in a membrane PE. The latter effect points to an alternative transfection process mediated by interactions with zwitterionic membrane PEs that does not originate from electrostatic interactions of the cationic lipids with anionic membrane lipids. Such effect is consistent with enhanced lipoplex–membrane fusion and provides a plausible physical explanation of the observed hydrocarbon chain effect on transfection.

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