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Cationic Phospholipids Forming Cubic Phases: Lipoplex Structure and Transfection Efficiency

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Abstract: The transfection activity and the phase behavior of two novel cationic *O*-alkyl-phosphatidylcholines, 1,2-dioleoyl-*sn*-glycero-3-hexylphosphocholine (C6-DOPC) and 1,2-dierucoyl-*sn*-glycero-3-ethylphosphocholine (di22:1-EPC), have been examined with the aim of more completely understanding the mechanism of lipid-mediated DNA delivery. Both lipids form cubic phases: C6-DOPC in the entire temperature range from −10 to 90 °C, while di22:1-EPC exhibits an irreversible lamellar−cubic transition between 50 and 70 °C on heating. The lipoplexes formed by C6-DOPC arrange into hexagonal phase, while the lipoplexes of di22:1-EPC are lamellar. Both lipids exhibit lower transfection activity than the lamellar-forming 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC). Thus, for the studied cationic phospholipid-DNA systems, the lipoplex phase state is a factor that does not seem to correlate with transfection activity. The parameter that exhibits better correlation with the transfection activity within the present data set is the phase state of the lipid dispersion prior to the addition of DNA. Thus, the lamellar lipid dispersion (EDOPC) produces more efficient lipoplexes than the dispersion with coexisting lamellar and cubic aggregates (diC22:1-EPC), which is even more efficient than the purely cubic dispersions (C6-DOPC; diC22:1-EPC after heating). It could be inferred from these data and from previous research that cubic phase lipid aggregates are unlikely to be beneficial to transfection. The lack of correlation between the phase state of lipoplexes and their transfection activity observed within the present data set does not mean that lipid phase state is generally unimportant for lipofection: a viewpoint now emerging from our previous studies is that the critical factor in lipid-mediated transfection is the structural evolution of lipoplexes within the cell, upon interacting and mixing with cellular lipids.

Keywords: Cationic phospholipid; *O*-alkyl-phosphatidylcholine; lipoplex; cubic phase; hexagonal; lamellar; transfection

Introduction

Positively charged lipid-like compounds form complexes (lipoplexes) with the polyanionic DNA. They can be used to deliver DNA to cells and are consequently considered to hold promise as nonviral gene carriers. A critical obstacle for clinical application of lipid-mediated DNA delivery (lipofection) is its unsatisfactory efficiency. Progress in enhancing lipofection efficacy has been impeded because its mechanism is still largely unknown.[1–7] In order to shed some light on the mechanism(s) of transfection and hence better understand what phenomena underlie efficiency differences between different agents, we examined some relevant physi-

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cal properties of the lipoplexes, with special focus on their phase behavior.

Polar lipids are known for their ability to form an impressive variety of polymorphic and mesomorphic phases—lamellar and nonlamellar—when dispersed in aqueous media.[8,9] Phase state has been recognized as potentially important for the transfection activity of lipoplexes.[10–13] Although the majority of cationic lipids used for transfection arrange into lamellar phases, it seems more than a coincidence that most of these compounds are more efficient transfection agents when mixed with certain neutral lipids (so-called helper lipids) and that such mixtures are more prone to form the inverted hexagonal phase.[10,12] Efforts have previously been made to correlate nonbilayer-forming propensity of the lipid transfection agents with their delivery activity, but the results are ambiguous and often contradictory.[10–19]

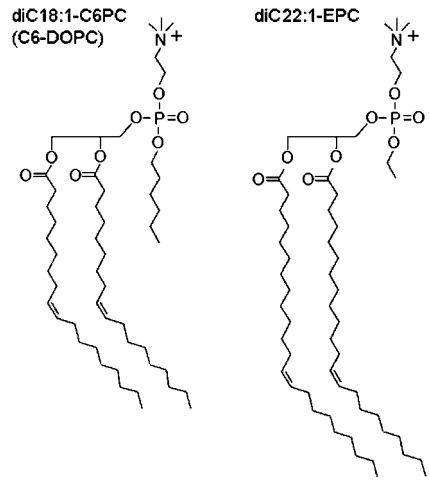

Figure 1. Structures of the studied cationic phospholipids, C6-DOPC and diC22:1-EPC.

Here we examine the transfection activity of two novel cationic phospholipids, 1,2-dioleoyl-*sn*-glycero-3-hexylphosphocholine (C6-DOPC) and 1,2-dierucoyl-*sn*-glycero-3-ethylphosphocholine (di22:1-EPC) (Figure 1), as well as their phase behavior, in an effort to contribute to the understanding of the mechanism of lipid-mediated DNA delivery.

Experimental Methods

Lipids and DNA. The cationic phospholipids, triflate derivatives of 1,2-dioleoyl-*sn*-glycero-3-hexylphosphocholine (C6-DOPC) and 1,2-dierucoyl-*sn*-glycero-3-ethylphosphocholine (di22:1-EPC) (Figure 1), were synthesized as previously described[20,21] and were stored in chloroform solution at −20 °C. For X-ray diffraction sample preparation, aliquots were transferred to vials where the bulk of the solvent was removed under argon, and the residual solvent was eliminated under high vacuum. Next, PBS (50 mM phosphate buffer,

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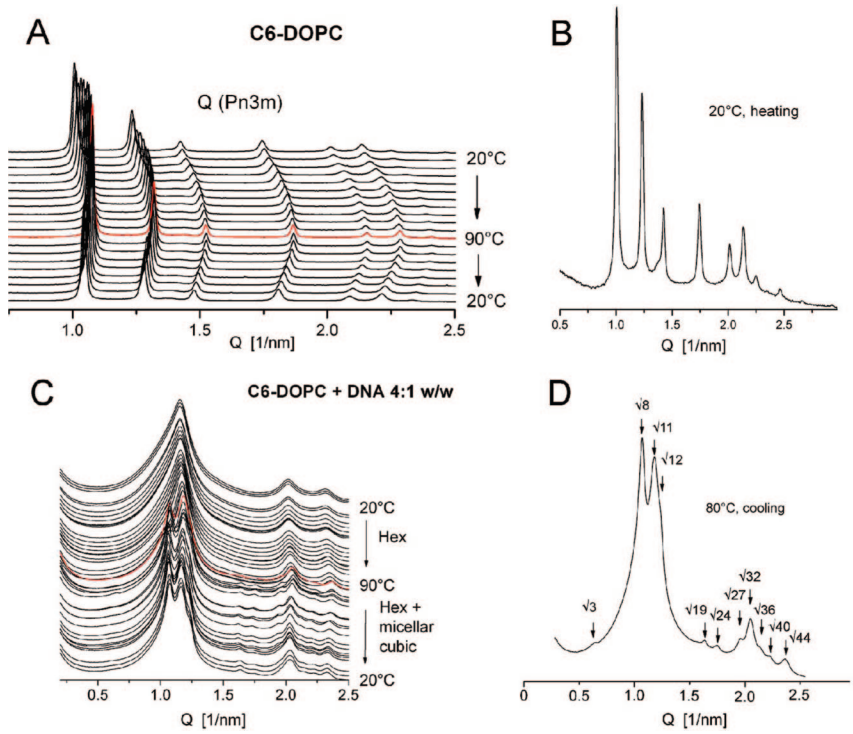

Figure 2. SAXD patterns of C6-DOPC dispersions (A) and lipoplexes (C) recorded on heating−cooling scans (the pattern in red was taken at the maximum temperature). (B) Diffraction pattern of C6-DOPC dispersion recorded at 20 °C at the beginning of the heating scan. (D) Diffraction pattern of C6-DOPC/DNA 4:1 w/w lipoplexes recorded at 80 °C on cooling.

100 mM NaCl, pH 7.2) was added. The dispersions were hydrated overnight at room temperature and then vortex-mixed for several minutes. Thereafter, several cycles of freezing−thawing were applied for homogenization. Herring sperm DNA (Invitrogen, Carlsbad, CA) was used for preparation of lipoplexes for X-ray diffraction experiments. DNA/lipid dispersions were prepared by adding an aqueous DNA solution to the dry lipid film and immediately vortexing,[22] unless otherwise indicated.

Synchrotron Small-Angle X-ray Diffraction (SAXD). SAXD measurements were performed at Argonne National Laboratory, Advanced Photon Source, DND-CAT and Bio-CAT, using 12 keV X-rays, as previously described.[23] The lipid concentration of the dispersions was 20 wt %. Samples were filled into glass capillaries and flame-sealed. A Linkam thermal stage (Linkam Sci Instruments, Surrey, U.K.) provided temperature control. Linear heating and cooling scans were performed at rates of 0.8−3 °C/min. Exposure times were typically ∼0.5−1 s. Data were collected using a MAR-CCD detector. Diffraction intensity vs Q plots were obtained by radial integration of the 2D patterns using the interactive data-evaluating program FIT2D.[24]

The lipid concentrations used in the SAXD experiments (∼50 mM) are certainly considerably higher than those used in transfection experiments, but still lower than the "excess water" limit, thus not expected to affect the lipid phase behavior.[9] In any case, their relevance to physiological concentrations with respect to phase data obtained has been repeatedly checked by control experiments at low concentrations (e.g., ref 25).

Dynamic Light Scattering (DLS). DLS measurements were performed with a Brookhaven Instruments BI-200SM goniometer and BI-9000 digital correlator (Brookhaven, NY). Cationic lipid dispersions in PBS were prepared at 50 μg/mL. DNA (1 mg/mL in PBS) was added to generate lipoplex samples at a 4:1 lipid/DNA weight ratio. Measurements were carried out at 37 °C. Borosilicate glass, 250 μL, 3 × 30 mm, flat bottom tubes were used. Delay times of 10 μs to 1 s were examined. The correlation data were fitted with quadratic cumulants, using the algorithm provided with the instrument.

Transfection. HUAEC cells were obtained from BioWhittaker, Inc. (Walkersville, MD), and were seeded in 96-well plates. For the lipoplex preparation, liposomes and plasmid

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DNA (β-galactosidase, purchased from Clontech Laboratories Inc., Palo Alto, CA, propagated and purified by Bayou Biolabs, Harahan, LA) were diluted in OptiMEM, and liposomes were pipetted into the plasmid DNA solution at a 4:1 weight ratio (the molecular weights of the two lipid derivatives are nearly the same, so the mole ratios of lipid to DNA in the lipoplexes do not differ significantly for the two lipids). The resultant DNA−lipid complexes were incubated at room temperature for 15 min, and then portions of 50 μL/well (1 μg of DNA/well) were added to the cells, either in medium alone or in medium containing 5% fetal bovine serum. At 2 h after addition of lipoplexes, the cells were washed with HBSS and fresh complete medium was added. Cells were assayed for β-galactosidase activity 24 h after transfection with a microplate fluorometric assay. Data present the mean ± SD of a representative experiment performed in quadruplicate.

Results

C6-DOPC Resides in the Cubic *Pn3m* Phase at All Temperatures in the Range −10 °C to 90 °C. Small-angle X-ray diffraction patterns recorded upon heating and cooling of an aqueous dispersion of C6-DOPC over the temperature range 20−90 °C are shown in Figure 2A. At 20 °C, up to twelve maxima were visible on the diffraction pattern (Figure 2B), indexing as the full set of the initial 12 reflections characteristic of the cubic *Pn3m* phase (cubic aspect #4),[26] with a ∼8.8 nm unit cell size. This highly ordered structure was retained on heating up to 90 °C (the unit cell of the cubic lattice had shrunk to 8.2 nm at that temperature) and subsequent cooling down to room temperature, and remained unchanged on storage during the time-course of the experiment (up to 24 h). It was also stable upon deep cooling to −10 °C.

C6-DOPC Lipoplexes Arrange into Hexagonal or Micellar Cubic Arrays. Diffraction patterns of C6-DOPC lipoplexes are shown in Figure 2C. The amount of added DNA (lipid/DNA 4:1, w/w) corresponded to ∼1.3:1 positive/negative charge ratio (assuming an average nucleotide mol wt 330) and was chosen to match the composition of the lipoplexes exhibiting maximum transfection activity.

As is obvious from the X-ray patterns, the addition of DNA caused a dramatic change in the organization of the lipid aggregates. At room temperature, the lipoplexes were organized in the hexagonal phase, with a lattice parameter $a = 2d/3 = 6.26$ nm. This kind of arrangement was retained upon heating to 90 °C. Upon subsequent cooling, additional reflexes appeared in the diffraction pattern and coexisted with those of the hexagonal phase. They index as micellar cubic phase of cubic aspect #15, *Fd3m*/*Fd3* (Figure 2D), with a ∼17.6 nm unit cell size.[26]

DiC22:1-EPC Forms Lamellar and Cubic Phases, and Fully Converts into a Cubic Phase after Heating. Small-angle X-ray diffraction patterns recorded upon heating and cooling of aqueous dispersion of diC22:1-EPC over the temperature range 20−90 °C are shown in Figure 3. The diffraction pattern at room temperature, before heating,

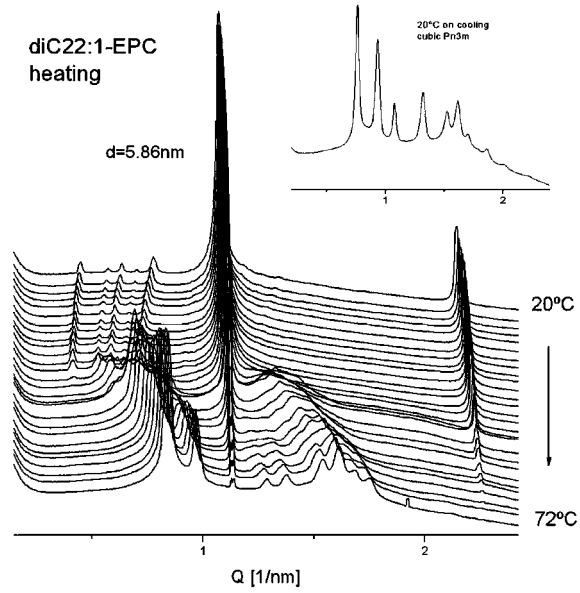

Figure 3. SAXD patterns of diC22:1-EPC dispersions recorded on heating. Inset: Diffraction pattern taken at 20 °C after cooling.

reveals a lamellar phase (repeat period $d = 5.86$ nm), coexisting with a mixture of cubic *Im3m* and *Pn3m* phases. Upon heating, conversion of the lamellar phase into the cubic *Pn3m* phase takes place at temperatures between 50−70 °C. This transition is not reversible: the cubic phase remains stable on cooling and on subsequent storage at room temperature during the time-course of the experiment, up to 24 h; it indexes as the full set of the initial 11 *Pn3m* reflections, with an 11.65 nm unit cell size (Figure 3, inset).

DiC22:1-EPC Lipoplexes Are Lamellar and Exhibit No Memory of the Thermal History of the Lipid Aggregates. Lipoplexes were prepared from diC22:1-EPC using two different protocols: (i) by adding DNA solution to a lipid dispersion prepared at room temperature; or (ii) by adding DNA solution to a lipid dispersion that had been preheated to 80 °C. Given our prior results, the organization of the lipid aggregates before addition of the DNA should be predominantly lamellar phase in the first case, while it should be fully converted into cubic phase in the second case. Thus, the hysteretic phase behavior of diC22:1-EPC provides a remarkable opportunity to examine, with minimal ambiguity, the effect of the initial lipid phase on lipoplex properties. Surprisingly, the lipoplexes prepared according to two protocols exhibited virtually identical X-ray diffraction patterns. They were lamellar, with a $d = 7.0$ nm lamellar repeat period and a DNA spacing of ∼3.7−3.8 nm (Figure 4). The increase of the lamellar repeat distance by ∼1.2 nm relative to that of the pure lipid, as a consequence of the DNA intercalation between the lipid lamellae, is typical for the lamellar liquid crystalline lipoplexes.[22,25,27]

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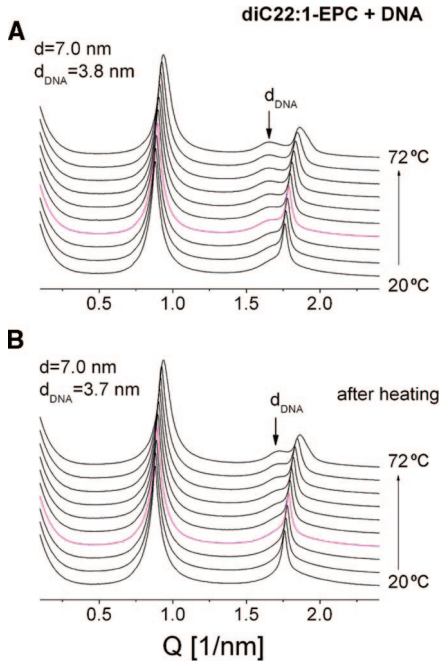

Figure 4. SAXD patterns of diC22:1-EPC lipoplexes prepared by adding DNA at room temperature to (A) lipid dispersion that has not been heated (lamellar phase) or (B) lipid dispersion preheated to 80 °C and then cooled to room temperature (cubic phase). The pattern in magenta was taken at 37 °C.

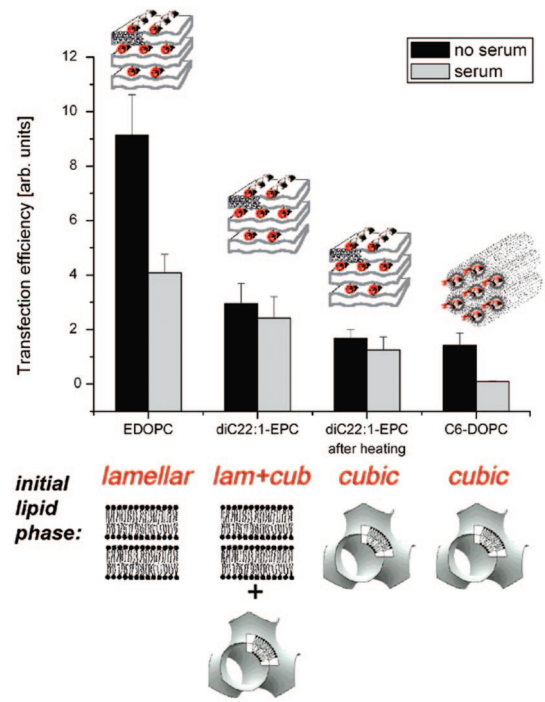

Figure 5. Transfection efficiency of diC22:1-EPC and C6-DOPC lipoplexes, as quantified by expression of β-Gal in HUAEC cells. For comparison, the figure also includes the transfection efficiency of ethyldioleoylphosphatidylcholine (EDOPC). The cartoons above the bars illustrate the lipoplex structure, while the initial lipid phase is illustrated below the graph.

Lipoplex Sizes. Particle size is important for transfection. Because both cubic and hexagonal phase cationic lipids are only rarely observed, and given that the relationship between particle size and their transfection activity is not understood, we used DLS to estimate the sizes of the C6-DOPC and diC22:1-EPC lipoplexes. The hexagonal phase C6-DOPC lipoplexes were large, in the size range 1500−2500 nm, several times larger than the usual lamellar lipoplexes formed by similar cationic phospholipids (*cf.* EDOPC lipoplexes have a mean size of ∼400 nm[18]). Brief sonication (∼30 s) reduces the C6-DOPC lipoplex size to ∼400−500 nm, with subsequent growth back to the initial size within several hours.

The diC22:1-EPC lipoplexes prepared from lamellar (nonheated) lipid dispersion were similar in size to the EDOPC lipoplexes, 350−400 nm, whereas the lipoplexes prepared from preheated lipid dispersion (cubic phase) were somewhat larger, 550−600 nm.

C6-DOPC and diC22:1-EPC Exhibit Lower Transfection Activity Than the Lamellar EDOPC. The transfection activity of C6-DOPC and diC22:1-EPC lipoplexes was tested *in vitro* using β-galactosidase expression in HUAEC cells. The results are presented in Figure 5. For reference, the figure also includes the transfection efficiency of ethyldioleoylphosphatidylcholine (EDOPC), an effective cationic phospholipid transfection agent that has already been extensively described.[14,28] Thus, the transfection potency of C6-DOPC and diC22:1-EPC lipoplexes is some 3−5 times lower than that of EDOPC. The hexagonal phase C6-DOPC lipoplexes exhibited the lowest efficiency of the three compounds, but it was not significantly different from that of the lamellar diC22:1-EPC lipoplexes prepared from either the lamellar or the cubic (preheated) lipid aggregates. Short sonication (∼30 s) of the C6-DOPC lipoplexes before transfection did not change significantly their activity. The transfection activities of the diC22:1-EPC lipoplexes prepared from lamellar or cubic lipid aggregates (see above) were virtually identical.

Discussion

A variety of key parameters that are relevant to drug and gene delivery are quite clearly dependent upon the phase organization of delivery vehicles. These include encapsula-

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tion capacity, release kinetics, external charge density and interactions with target cells, permeability, susceptibility to fusion with cellular membranes and phase evolution thereafter, and ability to circumvent specific biological barriers and avoid capture by particular tissues. Thus, phase state has been recognized as potentially important for the transfection activity of lipoplexes.[10–13,19] An early investigation suggested that hexagonal phase lipoplexes are more efficient than the lamellar ones,[10] although no transfection data were presented in support of this claim. In later studies, estimates ranging from "significantly more efficient"[12] to "nonbeneficial"[14] or "noncorrelating",[16,29] or simply "poor",[17] have been published regarding the transfection efficiency of the hexagonal phase. The situation is further complicated by the diversity of lipid phases, which extends considerably beyond the lamellar/hexagonal alternatives and encompasses dozens of polymorphic and mesomorphic phase structures, particularly for lipid mixtures.

The data presented here illustrate that hexagonal C6-DOPC lipoplexes exhibit lower transfection potency than lamellar lipoplexes formed by a cationic phospholipid of a rather similar chemical structure (EDOPC). Likewise, low transfection activity was recently reported for two other cationic phospholipids forming hexagonal lipoplexes: stearyl-dioleoylphosphatidylcholine (SDOPC) and palmityl-diphytanoylphosphatidylcholine (PDΦPC).[18] Induction of hexagonal lipoplex formation by the addition of DOPE to cationic *O*-alkyl-phosphatidylcholines was also found generally nonbeneficial for transfection.[14]

The presented data demonstrate that the diC22:1-EPC dispersions exhibit large temperature hysteresis and could form either lamellar or cubic phases at room and physiological temperatures, depending on the thermal prehistory (Figure 3). This compound thus provides a remarkable opportunity to, with minimal ambiguity, examine the effect of the initial lipid phase on lipoplex properties.

Although hexagonal phase lipoplexes seem to be generally associated with low transfection activity, the organization of a lipoplex in the lamellar phase is no guarantee, *in itself*, of high transfection potency, for rather low activity was also observed with the lamellar lipoplexes formed by diC22:1-EPC (Figure 5). Taken together, the present and previously published data indicate that the lipoplex phase state is not likely to correlate directly with the transfection activity. The parameter that exhibits better correlation with the transfection activity within the present data set is the phase state of the lipid dispersion prior to the addition of DNA (Figure 5, bottom). Thus, the lamellar lipid dispersion (EDOPC) produces more efficient lipoplexes than the dispersion with coexisting lamellar and cubic aggregates (diC22:1-EPC), which is even more efficient than the purely cubic dispersions (C6-DOPC; diC22:1-EPC after heating).

Previous experiments have documented poor transfection potency of lipoplexes produced from other cubic lipid dispersion as well. Thus, EDOPC/SDOPC cationic lipid mixture arranges into lamellar phase at EDOPC contents >60 mol %, into hexagonal phase at EDOPC contents <40 mol %, and into cubic (*Pn*3*m*) phase at EDOPC contents between 40 and 60 mol % (Figure 5 in ref 18). Coincidently, these are precisely the compositions with the minimum transfection activity (40−60 mol % EDOPC, see Figure 2B in ref 18). Poor transfection activity of lipoplexes produced from cubic dispersion has been reported also in ref 14. We infer from these data that cubic phase lipid aggregates are unlikely to be beneficial to transfection.

We conclude on a note of caution: The lack of correlation between the phase state of lipoplexes and their transfection activity observed within the present data set does not mean that lipid phase state is generally unimportant for lipofection. A viewpoint now emerging from our previous research is that the critical factor in lipid-mediated transfection is the structural evolution of lipoplexes within the cell, upon interacting and mixing with cellular lipids. In particular, those studies showed that *the phase evolution of lipoplex lipids upon interaction with membrane lipids* appears to be decisive for transfection success: thus, lamellar lipoplex formulations, which were readily susceptible to undergoing lamellar−nonlamellar phase transitions upon mixing with cellular lipids, were found rather consistently associated with superior transfection potency, presumedly as a result of facilitated DNA release.[18,19,30,31]

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