Published in final edited form as:

J Phys Chem B. 2007 July 12; 111(27): 7786–7795. doi:10.1021/jp071286y.

Synergy in lipofection by cationic lipid mixtures: Superior activity at the gel-liquid crystalline phase transition

Rumiana Koynova^{*}, Li Wang, and Robert C. MacDonald

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208, USA

Abstract

Some mixtures of two cationic lipids including phospholipid compounds (*O*-ethylphosphatidylcholines), as well as common, commercially available cationic lipids, such as dimethylammonium bromides and trimethylammonium propanes, deliver therapeutic DNA considerably more efficiently than do the separate molecules. In an effort to rationalize this widespread “mixture synergism”, we examined the phase behavior of the cationic lipid mixtures and constructed their binary phase diagrams. Among a group of more than 50 formulations, the compositions with maximum delivery activity resided unambiguously in the solid-liquid crystalline two-phase region at physiological temperature. Thus, the transfection efficacy of formulations exhibiting solid-liquid crystalline phase coexistence is more than 5× higher than that of formulations in the gel (solid) phase, and over twice that of liquid crystalline formulations; phase coexistence occurring at physiological temperature thus appears to contribute significantly to mixture synergism. This relationship between delivery activity and physical property can be rationalized on the basis of the known consequences of lipid phase transitions, namely the accumulation of defects and increased disorder at solid-liquid crystalline phase boundaries. Packing defects at the borders of coexisting solid and liquid crystalline domains, as well as large local density fluctuations, could be responsible for the enhanced fusogenicity of mixtures. This study leads to the important conclusion that manipulating the composition of the lipid carriers so their phase transition takes place at physiological temperature can enhance their delivery efficacy.

Keywords

lipoplex; phase diagram; phase coexistence; fusogenicity; DNA release; gene therapy

Introduction

Although cationic lipids are promising non-viral gene carriers, a major handicap to their clinical application is unsatisfactorily low efficiency. Thus far, the primary approaches to improving transfection properties of cationic lipids has been the synthesis of new kinds of cationic amphiphiles or the inclusion of non-cationic helper lipids (e.g., DOPE or cholesterol). Although both approaches have produced considerable improvement in the transfection properties of cationic lipid carriers, their transfection activity is still rather low relative to that of viral vectors, and they are currently not efficient enough to be generally useful in the clinic.

An effective alternative strategy for improving lipofection efficiency emerged recently: combining two cationic lipid derivatives can synergistically enhance transfection¹. This

Corresponding author: Rumiana Koynova, Dept Biochemistry, Molecular Biology & Cell Biology, Northwestern University, 2205 Tech Drive, Evanston, IL 60208, USA, tel 847-864-6923, e-mail: r-tenchova@northwestern.edu.

intriguing synergy was first observed with cationic phospholipids having the same headgroup but different hydrocarbon chains; it was found that the optimal combination of the long chain and medium chain lipoids delivered DNA into human umbilical artery endothelial cells (HUAEC) more than 30× more efficiently than did either compound separately¹. This finding highlighted the importance of the hydrophobic portions of cationic lipids and suggested that certain properties of the non-polar parts are critical for the effectiveness of these transfection agents.

This phenomenon of synergy is not restricted to cationic phospholipids derivatives, for other cationic lipids, including compounds frequently used in research and in the clinic, such as dimethylammonium-bromides and trimethylammonium-propane, have also been found to exhibit synergy in binary mixtures. In an attempt to rationalize this finding, we performed structural and thermodynamic analyses of those mixtures. Besides forming the physiologically relevant liquid crystalline phase, lipid-water systems also assume the lamellar gel (solid) phase, and the gel-liquid crystalline (chain melting) phase transition is a major thermal event ('main' transition). Most of the compounds mentioned above have saturated hydrocarbon chains, and they, or their mixtures, exhibit solid-liquid crystalline phase transitions at temperatures relevant to many practical (clinical) applications. The chain-melting transition influences a host of bilayer properties, a number of which, including permeability, compressibility, fusogenicity, structural fluctuations, leakiness, etc., exhibit maxima that coincide with the transition temperature²⁻⁸. These are the kinds of properties that should be critical for gene delivery. Indeed, we have verified such an influence and report here a correlation between the transfection efficacy of the cationic lipid mixtures (including saturated-chain compounds), their fusability at physiological temperature, and their solid-liquid crystalline phase transition; without exception within a group of >50 formulations, compositions that fall within the two-phase transition region at physiological temperature exhibit maximal transfection activity.

Experimental Methods

Lipids

Ethylphosphatidylcholines (1,2-diacyl-*sn*-glycero-3-ethylphosphocholines) with oleoyl, myristoyl, and palmitoyl chains – EDOPC, EDMPC, EDPPC – were synthesized as previously described⁹. Ditetradecyldimethylammonium-bromide (diC14DAB) (Aldrich), dioctadecyldimethylammonium-bromide (diC18DAB) (Sigma, St. Louis, MO), dimyristoyltrimethylammonium-propane (DMTAP), egg phosphatidylcholine and dioleoylphosphatidylglycerol (DOPG) (Avanti Polar Lipids, Alabaster, AL) were used without further purification. The phospholipids migrated as a single spot by thin-layer chromatography.

Sample preparation

Chloroform solutions of cationic lipids were mixed in glass vials at the desired ratios, solvent was removed with a N₂ stream and, subsequently, high vacuum. The lipid mixtures were hydrated in PBS (50 mM phosphate buffer, 100 mM NaCl, pH 7.2) overnight at room temperature and vortex-mixed at a temperature above their gel-liquid crystalline phase transition. Samples were subsequently equilibrated overnight at room temperature before measurement.

DSC measurements were performed using a VP-DSC Microcalorimeter (MicroCal Inc., Northampton, MA) at 30°C/h. Thermograms were analyzed using OriginLab (Northampton, MA) software. The onset and the completion temperatures of the phase transitions needed for the construction of the phase diagram were determined by the intersections of the peak slopes with the baseline on the thermograms. The maxima of the heat capacity curves were taken as the phase transition temperatures of the pure components. For asymmetric or split line shapes

of the composite aggregates, the transition temperature was defined as that temperature at which the area of the endotherm was divided into equal halves.

Synchrotron small-angle X-ray diffraction (SAXD)

Measurements were performed at Argonne National Laboratory, Advanced Photon Source, DND-CAT (beamline 5-IDD) and BioCAT (beamline 18-ID), using 12 keV X-rays, as previously described¹⁰. The lipid concentration of the dispersions was 20 wt %. Samples were filled into glass capillaries (Charles Supper Co., Natick, MA) and flame-sealed. A Linkam thermal stage (Linkam Sci Instruments, Surrey, England) provided temperature control. Linear heating and cooling scans were performed at rates of 1-5°C/min. Exposure times were typically ~0.5 sec. Data were collected using a MAR-CCD detector. Sample-to-detector distance was 1.8-2 m. Diffraction intensity vs. Q plots were obtained by radial integration of the 2D patterns using the interactive data-evaluating program FIT2D¹¹. Some samples with longer exposure time were checked by thin layer chromatography after the experiments. Products of lipid degradation were not detected in these samples and radiation damage of the lipids was not evident from their X-ray patterns.

Fluorescence resonance energy transfer (FRET) experiments were previously described^{1;12}. Briefly, cationic liposomes were prepared containing 0.5% NBD-PE and 0.5% rhodamine-PE (Rh-PE) (Molecular Probes, Eugene, OR). The lipid concentration of the dispersions was 0.1mM. Negatively charged liposomes were prepared at the same lipid concentration, without fluorescent labels. Labeled cationic liposomes were placed in an AlphaScan fluorometer (Photon Technology International, Princeton, NJ), and treated with unlabeled negatively charged lipids at 37°C. Fluorescence intensity was recorded as a function of time with Ex=320nm, Em=535nm, at 37°C. Fluorescence was also measured of samples in which negatively charged lipids were mixed directly with cationic lipids in chloroform and then liposomes were prepared as above; this intensity was then used for normalization of measurements.

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 under 5% CO₂. At confluence, the cells were passaged using 0.25 mg/ml Trypsin/EDTA (Cambrex). Passages 5-10 were used 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. CMV- β -galactosidase plasmid was purchased from Clontech Laboratories Inc. (Palo Alto, CA) and propagated and purified by Bayou Biolabs (Harahan, LA). Cationic lipid mixtures were hydrated in HBSS at a temperature higher than the phase transition of the higher-melting component and diluted in Opti-MEM, then pipetted at that temperature into an equal volume of plasmid DNA solution (also diluted in Opti-MEM) as previously described¹. The resultant DNA-lipid complexes were equilibrated for 15 min and then added to cells. At 2 h after their addition, the lipoplexes were removed and fresh medium was added. Cells were assayed for β -galactosidase activity 24 h after transfection using a microplate fluorometric assay¹³.

Results

EDMPC/EDPPC

A selection of calorimetric thermograms of aqueous dispersions of mixtures of EDMPC and EDPPC at different mole ratios is shown in Fig. 1A. Pure hydrated samples of EDMPC and EDPPC exhibited melting transitions (T_m) at 24°C and 42°C, respectively, as reported earlier¹⁴⁻¹⁶. The phase diagram (Fig. 1B) of this mixture was constructed based on the DSC data¹⁶. According to this diagram, EDMPC/EDPPC mixtures with up to 60 mol% EDPPC are in the liquid crystalline (L) phase at physiological temperature; mixtures with ≥ 90 mol% EDPPC are in the solid (S) phase. Mixtures with 70-80 mol% EDPPC reside within the two-phase region of the phase diagram, between the solidus and liquidus lines, and are thus expected to exhibit solid-liquid crystalline (S+L) phase coexistence at 37°C (Fig. 1B). We tested the transfection activity of EDMPC/EDPPC mixed lipoplexes with HUAEC cells *in vitro* at the normal growth temperature of 37°C (Fig. 1C). EDMPC lipoplexes are better transfection agents than EDPPC, an observation that is not unexpected since the former lipid is in the liquid crystalline phase, while the latter is in the solid phase at physiological temperature. It is noteworthy that the solid-phase EDPPC still transfects DNA into cells, provided the lipoplexes are formed at $T > T_m$. The mixed EDMPC/EDPPC lipoplexes exhibit a pronounced maximum in their transfection activity at 70-80 mol% EDPPC – precisely the compositions that are characterized by solid-liquid crystalline phase coexistence at 37°C. We compared the transfection efficacy of each sample to the average efficacy typical for this mixture (the red line in Fig. 1C) calculated from all the formulations that were examined (Fig. 1B, columns). Conspicuously higher-than-average activity is seen for the 70-80 mol% EDPPC mixtures that exhibited solid-liquid crystalline phase coexistence at 37°C.

diC14DAB/diC18DAB

A selection of calorimetric thermograms of aqueous dispersions of mixtures of diC14DAB and diC18DAB at different mole ratios is shown in Fig. 2A. Pure hydrated diC14DAB exhibited a melting transition at $T_m = 26^\circ\text{C}$ and that of diC18DAB was at $\sim 50^\circ\text{C}$, as reported¹⁷. The melting transition of diC14DAB/diC18DAB mixtures at 10-75 mol% diC18DAB starts at $\sim 20^\circ\text{C}$, i.e., at a lower temperature than that of the pure compounds. The phase diagram constructed on the basis of the calorimetric results is depicted in Fig. 2B. It is a eutectic type, with the eutectic point at $\sim 10\%$ diC18DAB. The horizontal portion of the solidus line indicates a phase separation in the solid phase for samples of 10-75 wt% diC18DAB – i.e., two solid phases of different composition coexist in these mixtures at $T < 20^\circ\text{C}$. Upon heating, the samples enter an extended solid-liquid crystalline phase coexisting region; for diC18DAB contents between ~ 45 mol% and 90 mol%, it extends to above physiological temperature.

Thus, according to the temperature-composition phase diagram (Fig. 2B), at physiological temperature (37°C), diC14DAB/diC18DAB mixtures with ≤ 45 mol% diC18DAB are in the liquid crystalline phase whereas mixtures with > 90 mol% diC18DAB are in the solid (gel) phase. Mixtures with 45-90 mol% diC18DAB reside within the solid-liquid crystalline phase coexistence region, between the solidus and liquidus lines of the phase diagram (Fig. 2B); thus, solid and liquid crystalline domains are expected to coexist in the lipid bilayers of these samples at 37°C.

We also did small-angle X-ray diffraction (SAXD) measurements on diC14DAB, diC18DAB, and the composition of maximum transfection activity (Fig. 2B, inset), namely, diC14DAB/diC18DAB 26:74 (mol/mol). The diffraction profiles recorded for heating scans from 20 to 90°C are presented in Fig. 2C. At 20°C, diC18DAB forms a lamellar solid phase with a rather short lamellar repeat distance, $d = 3.63$ nm; the short repeat has previously been ascribed to molecular tilt with respect to the bilayer surface^{18;19}. Upon heating, at 47°C it undergoes a

transition to another lamellar phase with a larger lamellar period, $d=4.2$ nm. Based on the magnitude of the transition enthalpy as measured by DSC, this corresponds to the solid-liquid crystalline transition. The shorter chain compound, diC14DAB, also arrange in the lamellar phase with a short repeat period, 3.17 nm, at 20°C. At 25°C, it undergoes a transition to another lamellar (liquid crystalline) phase with $d=4.53$ nm. The diC14DAB/diC18DAB 26:74 mol/mol mixture exhibits evidence of phase coexistence at low temperature, as expected from the binary phase diagram: two diffraction peaks at 3.3 nm and 3.5 nm. A structural rearrangement takes place at about physiological temperature, between 35–45°C, to a homogeneous lamellar (liquid crystalline) phase with $d=4.02$ nm. At still higher temperatures, ~67–70°C, additional reflections appear at rather small angles; at ~85°C the major peak spacings are 7.04 nm and 6.10 nm and in the ratio $1/\sqrt{6}:1/\sqrt{8}$, characteristic of the Ia3d cubic phase.

Maximum transfection activity for this cationic lipid mixture was at diC14DAB/diC18DAB 26:74 mol/mol (Fig. 2B, inset). We compared the transfection efficacy of each sample to the average efficacy typical for this mixture calculated from all the formulations that were examined (Fig. 2B, columns). The distinctly higher-than-average activity is exhibited by the 60–90 mol% diC18DAB mixtures, for which solid-liquid crystalline phase coexistence was observed at 37°C.

EDOPC/diC14DAB

A selection of thermograms of aqueous dispersions of mixtures of EDOPC and diC14DAB at different mole ratios is shown in Fig. 3A. No thermal events were recorded in the thermogram of EDOPC in the range 0–100°C. Our experience indicates that cationic ethyl derivatives of the phosphatidylcholines exhibit solid-liquid crystalline transitions at temperatures coinciding with those for the parent phosphatidylcholines^{14;15;20}. Thus, EDOPC is expected to have a $T_m \sim -20^\circ\text{C}$, similarly to DOPC^{21;22}. Although EDOPC almost certainly has a low transition temperature, addition of EDOPC to diC14DAB results in the appearance of a high-temperature component of the diC14DAB transition endotherm. At ~40 mol% diC14DAB, a single endotherm is observed at 53°C, with an enthalpy comparable to that of the melting transition of pure diC14DAB. At lower diC14DAB content (15–25 mol%), only low-enthalpy endotherms are recorded at high temperatures in the 0–100°C range, while at <15 mol% there were no observable thermal events in the thermograms.

Based on the DSC data, we constructed a partial temperature-composition phase diagram of the mixture (Fig. 3B). Since the phase transition of the pure EDOPC and presumably those of the samples in which EDOPC predominates (especially the temperatures of the transition onset) are outside the temperature range accessible by high-sensitivity DSC, the phase lines are only tentatively extrapolated (dotted lines) for those compositions. The phase diagram is apparently of the high-temperature azeotropic (isoconcentration point) type. Azeotropic phase diagrams have been previously reported for other lipid mixtures containing charged lipids^{23–26}. As in the case reported here, these are usually of the upper isoconcentration point type, in which the transition temperatures of the mixtures at particular compositions are higher than those of the pure components and exhibit a maximum. Analysis of phase diagrams in general shows that the presence of an upper isoconcentration point typically reflects higher values of the excess free energy of mixing (“nonideal” energy) in the liquid than in the solid state at those particular compositions²⁷. Such a combination of nonideal energies is easily conceivable in mixtures with negative nonideal energy in the solid state, in which contacts between unlike molecules are preferred (for, e.g., steric reasons) to those between like ones, i.e., when the nearest-neighbor pairs tend to be made up of unlike molecules. The horizontal portion of the solidus line at compositions dominated by diC14DAB indicates phase separation in the solid phase at temperatures below 20°C.

We also performed small-angle X-ray diffraction (SAXD) measurements on samples containing EDOPC and diC14DAB. The structural data for the pure diC14DAB were presented above – it undergoes a solid-liquid crystalline phase transition at $\sim 26^{\circ}\text{C}$. Pure EDOPC has been previously reported to arrange into a lamellar phase with a spacing of $\sim 4.8\text{ nm}$ ²⁰. The composition of maximum transfection activity (Fig. 3B, inset), EDOPC/diC14DAB 20:80 mol/mol, is characterized by coexistence of a lamellar phase ($d=3.17\text{ nm}$) and a cubic mesomorphic phase of the Im3m topology (indexed by 8 observable diffraction peaks) at 20°C (Fig. 3C). As expected from the phase diagram and as judged by its lamellar spacing, one of the coexisting phases is composed of virtually pure diC14DAB, equivalent to that of the solid-phase diC14DAB. The solid-liquid crystalline phase coexistence is observed up to $\sim 40^{\circ}\text{C}$, i.e., at physiological temperature the sample is characterized by extensive solid-liquid crystalline phase coexistence.

In Fig. 3D the fusion activity of the EDOPC liposomes is compared to that of the composition exhibiting solid-liquid crystalline phase coexistence and maximum transfection activity, EDOPC/diC14DAB 20:80 mol/mol, using a FRET assay^{14;28}, which is dependent on the dilution of fluorescent lipid probes in the participating membranes. For the fusion assay, we used as a target negatively charged liposomes, containing 80 wt% egg PC and 20 wt% DOPG, corresponding approximately to the amount of anionic lipid in cellular membranes²⁹. The extent of fusion of the coexisting-phase formulation was significantly higher than that of the liquid-phase EDOPC (Fig. 3D).

Fig. 3B (columns) compares the transfection efficacy of samples of different composition to the average efficacy typical for this mixture. The markedly higher-than-average activity of the formulations of 60-80 mol% diC14DAB precisely coincides with the region of the phase diagram in which solid-liquid crystalline phase coexistence occurs at 37°C .

EDOPC/diC18DAB

DSC thermograms of samples of different EDOPC/diC18DAB ratios are shown in Fig. 4A. The phase diagram constructed from these data is shown in Fig. 4B. Pure hydrated diC18DAB exhibited a melting transition at $\sim 50^{\circ}\text{C}$. Samples with $<90\text{ mol}\%$ diC18DAB exhibited low enthalpy thermal events that presumably indicate that a considerable part of the melting (solid $\rightarrow$ liquid crystalline) transition is carried out at the subzero temperatures of the EDOPC phase transition (see discussion above). We thus constructed only the liquidus line of the phase diagram, delimiting from above the region of solid-liquid crystalline phase coexistence. Since that liquidus line lies generally at $>37^{\circ}\text{C}$ (except for samples of very high ($\geq 90\text{ mol}\%$) EDOPC content), the EDOPC/diC18DAB mixtures are expected to exhibit solid-liquid crystalline phase coexistence at physiological temperature for a wide range of compositions. Since pure diC18DAB is in the solid phase at 37°C , it is also outside the coexistence region; the same is presumably true for mixed samples of very high content of that lipid ($\geq 95\%$).

The data from SAXD experiments on samples with compositions close to the ends of the expected phase coexistence region in the EDOPC/diC18DAB phase diagram, namely those of 15 mol% and 92 mol% diC18DAB, corresponded to the calorimetric data (Fig. 4C). Both samples show coexistence of a lamellar and a cubic phase at 37°C .

Given the shape of the phase diagram, solid-liquid crystalline phase coexistence would be expected at physiological temperature over a wide composition range, namely at almost any composition except for the pure compounds. The transfection efficacy of the mixed lipoplexes is thus above or close to the average for this mixture, and definitely higher than that of the pure compounds (Fig. 4B, right Y-axis).

EDOPC / DMTAP

DSC thermograms of samples of different EDOPC / DMTAP ratios are shown in Fig. 5A, and the corresponding phase diagram constructed from these data is shown in Fig. 5B. Because of the low T_m of EDOPC, the position of the solidus line is only tentatively drafted, especially at compositions in which EDOPC predominates (see discussion above). The phase diagram is presumed to contain a monotectic point at ~67mol% DMTAP and 20°C.

SAXD profiles of three different compositions, EDOPC/DMTAP 75:25, 33:67, and 15:85 (mol/mol), recorded on heating are shown in Fig. 5C. All three compositions exhibit phase separation at 20°C. The samples in which DMTAP predominates become homogeneously liquid crystalline at <37°C, however, the 75:25 mol/mol formulation—the composition of maximum transfection activity (Fig. 5B, inset)—falls within the transition region at 37°C. This composition becomes homogeneous only >50°C, in agreement with the phase diagram constructed from the DSC data.

Comparison of the fusion activity (using a FRET assay, see above) of the EDOPC/DMTAP 75:25 composition that resides inside the transition region, with that of the homogeneous liquid-phase EDOPC demonstrates significantly higher fusability of the phase coexistence formulation (Fig. 5D).

Comparing the transfection efficacy of the EDOPC/DMTAP mixtures with the average efficacy of the group (Fig. 5B, right Y-axis) reveals that the higher-than-average activity of the samples containing 20-60 mol% DMTAP precisely corresponds to the region in the phase diagram for which there is solid-liquid crystalline phase coexistence at 37°C.

Discussion

Understanding the mechanism of gene delivery by cationic liposomes is clearly of utmost importance, since it may well provide clues to improving lipofection activity, and consequently also lead to improving the success of gene therapy. A number of physical and physico-chemical factors have been suggested as lipofection modulators, but the specific route of DNA delivery by cationic lipid vectors is still mostly unknown and at present their efficiency of delivery is unsatisfactorily low for many applications.

DNA release from cationic lipoplexes is an essential step during lipofection. Cationic lipid-DNA interactions are strong³⁰ and the likely mechanism for release of DNA under cellular conditions is neutralization of the cationic lipid charge with cellular anionic lipids, as first demonstrated in model experiments³¹⁻³⁵. A similar process of lipid exchange between lipoplexes and cytoplasmic membranes resulting in DNA release into the cytoplasm was later observed in cultured cells³⁶⁻³⁸. Thus, intermixing of membrane lipids with lipoplex lipids is a necessary step in transfection. Currently, such intermixing of lipids is thought to occur by fusion of the lipoplex lipid with cell membranes and, as will be discussed below, fusogenicity is presumed to be an important characteristic of cationic lipids used for transfection.

According to our recent investigations, such intermixing of the cationic lipids with negatively charged liposomes takes place considerably more readily with cationic lipid *mixtures* than with single-component cationic liposomes³⁹. This phenomenon was attributed to nonideal mixing, formation of domains of different composition (*compositional domains*), and/or higher probability for packing defects in the cationic lipid mixtures relative to single component bilayers.

While it is obvious that two different lipids in the same lipid bilayer represent a two-dimensional mixture, it is also the case—one especially relevant to the present discussion—

that a bilayer undergoing a phase transition also represents a functional mixture from the physical point of view, whether or not it consists of one or several lipid species. This is true because, even when composed of a single lipid component, such a bilayer contains domains of two coexisting phases (*phase domains*). As mentioned above, domain formation facilitates lipid exchange, which is required for successful DNA delivery.

Among the five binary cationic lipid mixtures studied here, the high-efficiency formulations for each mixture correlate unambiguously with the physical condition in which the lipid bilayers reside within the phase transition (two-phase) region, i.e., they correlate with solid-liquid crystalline phase coexistence. These data are summarized and compared in Fig. 6: the transfection efficacy (TE) of each composition studied was compared to the average efficacy for the given mixture and plotted against the difference between the temperature of the phase line (liquidus or solidus) and 37°C; otherwise stated, $100(TE - \langle TE \rangle) / \langle TE \rangle$ (the 100 is for an expression in %), was plotted against $[T(\text{liquidus}) - 37^\circ\text{C}]$ or $[37^\circ\text{C} - T(\text{solidus})]$ for each composition. As may be seen, higher-than-average transfection activities correlate with lipid compositions that fall within the transition region. There is no apparent correlation, however, between how distant the position of the phase line is from 37°C at given composition, and the transfection efficacy of that composition. It is thus clear that the increased activity is not a simple temperature effect, but actually a transition region (two-phase) effect.

In order for DNA to enter the cell nucleus where it is transcribed, it must escape from the lipoplex. As discussed above, neutralization of cationic lipid carriers by anionic membrane lipids, generally accepted to be required for DNA release, presupposes lipid exchange between cationic lipoplexes and negatively charged membranes of cytoplasm, most likely by fusion of cell membranes with lipoplexes. It has thus been proposed that fusion between cationic bilayers and endosome membrane (or other cellular membrane) leads to displacement of DNA from the lipoplexes^{14;31;32;40}.

Indeed, we found that the highly efficient cationic lipid formulations studied here, such as EDOPC/diC14DAB (20:80, mol/mol) (Fig. 3D) and EDOPC/DMTAP (75:25, mol/mol) (Fig. 5D), also exhibit remarkably higher fusion ability with negatively charged membrane-mimicking liposomes than do pure EDOPC lipoplexes. Given the structural, thermodynamic, and fusion data reported here, release of DNA from the lipoplexes of superior efficacy can be confidently attributed to some characteristic that plays a role in transfection, the rate of which is affected by the solid-liquid crystalline phase transition taking place at the temperature of transfection. We suggest this characteristic is *fusogenicity*. This suggestion is based in part on a well-recognized relationship between propensity of lipid vesicles for fusion and their phase transition temperature; a number of publications have reported a dramatic (by up to two orders of magnitude) increase in the rate and extent of vesicle fusion at the temperature of the gel-liquid crystalline phase transition for a variety of neutral and charged lipids and lipid mixtures^{3-5;41-43}. Indeed, as discussed above, solid and liquid crystalline domains of different compositions coexist within the transition region²⁷. Similar to the situation involved in transfection synergy of mixtures^{1;39;44}, this heterogeneity inevitably generates packing defects. Such packing defects certainly facilitate the fusion of bilayers, because bilayer fusion, at some stage in the process, must involve merging of the hydrophobic cores of the two participating membranes. Furthermore, the bilayer bending constant is known to be reduced in mixed bilayers, because such bilayers may have different compositions in the two opposing monolayers⁴⁵. Thus, mixed bilayers are expected to exhibit an unusual tendency to curve, and therefore to fuse. As alluded to above, high fusogenicity is likely to accompany superior transfection activity.

In the present study we find that the solid-liquid crystalline (“chain melting”) phase transition of cationic lipid carriers is apparently relevant to the process of DNA release and also promotes

transfection when it occurs at the temperature of transfection. During such phase transitions, solid and liquid crystalline phase domains coexist in the bilayer. The melting transition is accompanied by changes in area (about 25%) and volume (about 4%), as well as in enthalpy (20-40 kJ/mol)⁴⁶. Enthalpy fluctuations in membranes are maximal in the chain melting region and result in a pronounced heat capacity maximum. Fluctuations in volume are also high near phase transitions and, accordingly, a dramatic increase of the isothermal compressibility of the bilayer occurs at chain melting transition temperatures⁴⁷. Volume and area fluctuations are related to the bilayer elastic constant, and because bending elasticity increases dramatically at the melting transition, the likelihood of structural transitions is increased; as a consequence, large curvature undulations are predicted⁴⁸ and observed⁴⁹. Charged lipid dispersions undergo structural transitions from a vesicular state to a continuous extended bilayer network of different mean curvature and back to vesicles at the melting phase transition⁵⁰ (since such morphological transitions involve the spontaneous and reversible fusion of vesicles, they may be significant for membrane fusion processes in general).

In the case of binary lipid mixtures, two-dimensional membrane structure within phase-separated regions has recently been extensively investigated with various experimental techniques as well as by Monte-Carlo simulations^{51;52}. These studies of lipid mixtures revealed a complex, highly heterogeneous membrane structure of phase domains of different composition within the solid - liquid phase coexistence regions of the phase diagrams⁵³. Monte-Carlo simulations have shown that local fluctuations, accompanied by an increase in elastic constants, are especially strong at domain interfaces, thus give rise to distinctly different physical properties at these domain boundaries, which are typically very extensive⁵⁴.

We reported recently that the cationic phospholipid oleoyldecanoylphosphatidylcholine (C18:1/C10-EPC) is remarkably effective as a DNA carrier for transfection. This molecule forms lamellar arrays alone and with DNA, and tends to form nonlamellar arrays when mixed with membrane lipids. Significantly, it undergoes the lamellar-nonlamellar phase reorganization precisely at physiological temperature⁵⁵. Because nonlamellar phase-forming compositions are frequently highly fusogenic, the obvious explanation of the superior efficacy of the C18:1/C10-EPC lipoplexes was that they easily fuse with membranes and, as a result of lipid mixing, the resultant aggregates should exhibit extensive lamellar-nonlamellar phase coexistence and heterogeneity, thereby facilitating DNA release and leading to superior transfection efficiency. The formation of nonlamellar arrays as a result of cationic-membrane lipid mixing has been reported to strongly promote DNA release^{12;56}.

It is important to point out that, for the mixtures undergoing solid-liquid crystalline phase transition at physiological temperature presented here, the transfection enhancement is not as large as that previously reported for lipids that undergo a curvature changing (lamellar-nonlamellar) transition. Indeed, we have previously done an extensive study of the transfection activity of cationic phospholipids, from which we were led to the conclusion that although lipoplex/membrane fusion is a necessary step in DNA delivery, some step *thereafter* – namely, release of DNA from the lipoplex, a step facilitated by nonlamellar phase formation – is commonly rate-limiting for transfection³⁹.

An important corollary of this study is that manipulating the composition of the lipid carriers so as to adjust their phase transition to take place at physiological temperature may constitute a valuable tool to enhance their delivery efficacy.

In summary, we believe that lipid phase transitions are critical for effective transfection of cells in more than one way. As shown previously, a lamellar-non lamellar transition seems to greatly facilitate DNA release. Here we describe an increased efficacy of transfection for binary mixtures of cationic lipid that exhibit solid-liquid crystalline phase transitions at physiological

temperature. Although it remains to be further verified experimentally, current knowledge of lipid physical properties suggests that the transition facilitates fusion of the lipoplex lipid with cellular lipid (partly anionic), accelerating the loss of electrostatic attraction between lipoplex lipid and DNA.

Acknowledgements

Supported by NIH grants GM52329, GM57305, and the Center for Cancer Nanotechnology Excellence (CCNE) initiative of the National Institutes of Health's National Cancer Institute under Award Number U54CA119341. The use of the high-sensitivity DSC instrument (VP-DSC) in the Keck Biophysics Facility at Northwestern University is acknowledged. BioCAT is a NIH-supported Research Center, through Grant RR08630. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research under Contract No. W-31-102-Eng-38. DND-CAT is supported by E.I. DuPont de Nemours & Co., The Dow Chemical Company, NSF Grant DMR-9304725, and the State of Illinois through the Department of Commerce and the Board of Higher Education Grant IBHE HECA NWU 96. Use of APS was supported by the U.S. DOE, Basic Energy Sciences, Office of Energy Research, Contract No. W-31-102-Eng-38.

References

1. Wang L, MacDonald RC. *Gene Ther* 2004;11:1358–1362. [PubMed: 15215886]
2. Mouritsen, OG.; Jorgensen, K.; Honger, T. *Permeability and Stability of Lipid Bilayers*. CRC Press; Boca Raton: 1995. p. 137-160.
3. Vanderbosch J, McConnell HM. *Proc Natl Acad Sci USA* 1975;72:4409–4413. [PubMed: 1060120]
4. Prestegard JH, Fellmeth B. *Biochemistry* 1974;13:1122–1126. [PubMed: 4814717]
5. Martin FJ, MacDonald RC. *Biochemistry* 1976;15:321–327. [PubMed: 1247519]
6. Papahadjopoulos D, Jacobson K, Nir S, Isac T. *Biochim Biophys Acta* 1973;311:330–348. [PubMed: 4729825]
7. Blok MC, Vandeenen LLM, Degier J. *Biochim Biophys Acta* 1976;433:1–12. [PubMed: 1260054]
8. Marsh D, Watts A, Knowles PF. *Biochemistry* 1976;15:3570–3578. [PubMed: 182212]
9. MacDonald RC, Rakhmanova VA, Choi KL, Rosenzweig HS, Lahiri MK. *J Pharm Sci* 1999;88:896–904. [PubMed: 10479351]
10. Koynova R, MacDonald RC. *Biophys J* 2003;85:2449–2465. [PubMed: 14507708]
11. Hammersley AP, Svensson SO, Hanfland M, Fitch AN, Hausermann D. *High Pressure Res* 1996;14:235–248.
12. Tarahovsky YS, Koynova R, MacDonald RC. *Biophys J* 2004;87:1054–1064. [PubMed: 15298910]
13. Rakhmanova VA, MacDonald RC. *Anal Biochem* 1998;257:234–237. [PubMed: 9514778]
14. MacDonald RC, Ashley GW, Shida MM, Rakhmanova VA, Tarahovsky YS, Pantazatos DP, Kennedy MT, Pozharski EV, Baker KA, Jones RD, Rosenzweig HS, Choi KL, Qiu RZ, McIntosh TJ. *Biophys J* 1999;77:2612–2629. [PubMed: 10545361]
15. Koynova R, MacDonald RC. *Nano Lett* 2004;4:1475–1479.
16. Koynova R, MacDonald RC. *Biochim Biophys Acta-Biomembranes* 2005;1714:63–70.
17. Schulz PC, Puig JE, Barreiro G, Torres LA. *Thermochim Acta* 1994;231:239–256.
18. Jung M, German AL, Fischer HR. *Colloid Polym Sci* 2001;279:105–113.
19. Okuyama K, Soboi Y, Hirabayashi K, Harada A, Kumano A, Kaziyama T, Takayanagi M, Kunitake T. *Chem Lett* 1984;2117–2120.
20. Koynova R, MacDonald RC. *Biochim Biophys Acta-Biomembranes* 2003;1613:39–48.
21. Lipid Data Bank. 2000. <http://www.lidb.chemistry.ohio-state.edu/>
22. Koynova R, Caffrey M. *Biochim Biophys Acta-Rev Biomembranes* 1998;1376:91–145.
23. Silvius JR. *Biochim Biophys Acta* 1991;1070:51–59. [PubMed: 1751538]
24. Zantl R, Baicu L, Artzner F, Sprenger I, Rapp G, Radler JO. *J Phys Chem B* 1999;103:10300–10310.
25. Linseisen FM, Bayerl S, Bayerl TM. *Chem Phys Lipids* 1996;83:9–23.
26. Garidel P, Johann C, Blume A. *Biophys J* 1997;72:2196–2210. [PubMed: 9129822]
27. Gordon, P. *Principles of phase diagrams in material systems*. McGraw-Hill; New York: 1968.

28. Struck DK, Hoekstra D, Pagano RE. *Biochemistry* 1981;20:4093–4099. [PubMed: 7284312]
29. Siakotos AN, Rouser G, Fleische S. *Lipids* 1969;4:234–239. [PubMed: 4306677]
30. Pozharski E, MacDonald RC. *Biophys J* 2003;85:3969–3978. [PubMed: 14645086]
31. Xu YH, Szoka FC. *Biochemistry* 1996;35:5616–5623. [PubMed: 8639519]
32. Zelphati O, Szoka FC. *Proc Natl Acad Sci USA* 1996;93:11493–11498. [PubMed: 8876163]
33. Szoka FC, Xu YH, Zelphati O. *Adv Drug Delivery Rev* 1997;24:291.
34. Bhattacharya S, Mandal SS. *Biochemistry* 1998;37:7764–7777. [PubMed: 9601037]
35. Harvie P, Wong FMP, Bally MB. *Biophys J* 1998;75:1040–1051. [PubMed: 9675205]
36. Wrobel I, Collins D. *Biochim Biophys Acta-Biomembranes* 1995;1235:296–304.
37. Noguchi A, Furuno T, Kawaura C, Nakanishi M. *FEBS Lett* 1998;433:169–173. [PubMed: 9738955]
38. Nakanishi M, Noguchi A. *Advanced Drug Delivery Rev* 2001;52:197–207.
39. Wang L, Koynova R, Parikh H, MacDonald RC. *Biophys J* 2006;91:3692–3706. [PubMed: 16935955]
40. Bhattacharya S, Mandal SS. *Biochemistry* 1998;37:7764–7777. [PubMed: 9601037]
41. Sun ST, Hsang CC, Day EP, Ho JT. *Biochim Biophys Acta* 1979;557:45–52. [PubMed: 549643]
42. Sun ST, Day EP, Ho JT. *Proc Natl Acad Sci USA* 1978;75:4325–4328. [PubMed: 279918]
43. Martin, FJ. PhD Thesis. Northwestern University; 1975. Model Systems Applied to Study of Membrane-Membrane Interactions.
44. Koynova R, Tarahovsky Y, Wang L, MacDonald RC. *Biochim Biophys Acta-Biomembranes* 2007;1768:375–386.
45. Bergstrom M. *J Colloid Interface Sci* 2001;240:294–306. [PubMed: 11446813]
46. Marsh, D. *Handbook of Lipid Bilayers*. CRC Press; 1990.
47. Schrader W, Ebel H, Grabitz P, Hanke E, Heimburg T, Hoeckel M, Kahle M, Wente F, Kaatz U. *J Phys Chem B* 2002;106:6581–6586.
48. Helfrich W. *Z Naturforsch A-A J Phys Sci* 1978;33:305–315.
49. Honger T, Mortensen K, Ipsen JH, Lemmich J, Bauer R, Mouritsen OG. *Phys Rev Lett* 1994;72:3911–3914. [PubMed: 10056328]
50. Schneider MF, Marsh D, Jahn W, Kloesgen B, Heimburg T. *Proc Natl Acad Sci USA* 1999;96:14312–14317. [PubMed: 10588702]
51. Heimburg T. *Current Opinion Colloid Interface Sci* 2000;5:224–231.
52. Sugar IP, Biltonen RL. *Energetics of Biological Macromolecules, Pt C* 2000;323:340–372.
53. Mouritsen OG, Andersen TL, Halperin A, Hansen PL, Jakobsen AF, Jensen UB, Jensen MO, Jorgensen K, Kaasgaard T, Leidy C, Simonsen AC, Peters GH, Weiss M. *J Phys Condensed Matter* 2006;18:S1293–S1304.
54. Seeger HM, Fidorra M, Heimburg T. *Macromol Symp* 2004;219:85–96.
55. Koynova R, Wang L, MacDonald RC. *Proc Natl Acad Sci USA* 2006;103:14373–14378. [PubMed: 16983097]
56. Koynova R, Wang L, Tarahovsky Y, MacDonald RC. *Bioconj Chem* 2005;16:1335–1339. [PubMed: 16287225]
57. Jorgensen K, Mouritsen OG. *Thermochim Acta* 1999;328:81–89.

Fig. 1.

(A) Heating thermograms of dispersions of EDMPC/EDPPC mixtures at different ratios (adapted from ref. 16, Fig.2, with permission from Elsevier). The thermograms of the mixtures with maximum transfection activity are shown in red. (B) Relationship of transfection activity to phase diagram. Left Y-axis and lines and symbols apply to the phase diagram, which was constructed from calorimetric data¹⁶; squares represent the transition onset and circles denote transition end; these were used to draw the solidus and liquidus lines, respectively, that outline the phase regions in the phase diagram. Below the solidus line the mixtures are in the solid phase (S) and above the liquidus line they are in the liquid crystalline (L) phase; between the two lines there is a region of solid-liquid phase coexistence (S+L). Right Y-axis and columns correspond to transfection efficacy TE of each composition compared to the average efficacy <TE> of all examined compositions: $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$. The shaded region indicates compositions that reside within the solid-liquid crystalline two-phase region at 37°C; (C) Transfection of HUAECs by EDMPC/EDPPC mixtures. Average efficacy <TE> is indicated with the red line.

Fig. 2.

(A) Heating thermograms of dispersions of diC₁₄DA/diC₁₈DAB mixtures at different ratios. (B) Relationship of transfection activity to phase diagram. Left Y-axis and lines and symbols apply to the phase diagram, which was constructed from the calorimetric data. Squares denote transition onset and circles transition end. The right Y-axis and bars depict the transfection efficacy TE of each composition compared to the average efficacy $\langle TE \rangle$ of all studied compositions: $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$; the shaded region indicates compositions that reside within the solid-liquid crystalline two-phase region at 37°C. Inset: Transfection of HUAECs by diC₁₄DA/diC₁₈DAB mixtures; (C) SAXD patterns of the diC₁₄DAB, diC₁₄DAB/diC₁₈DAB 26:74 (mol/mol) and diC₁₈DAB samples, both recorded upon heating; the bolded pattern was recorded at 37°C.

Fig. 3.

(A) Heating thermograms of dispersions of EDOPC/diC₁₄DAB mixtures at different ratios. (B) Relationship of transfection activity to phase diagram. Left Y-axis and lines and symbols apply to the phase diagram, which was constructed from the calorimetric data. Squares denote transition onset and circles transition end. The left part of the diagram where EDOPC predominates is only tentatively indicated because the EDOPC transition at subzero temperature was outside the temperature range accessible by high-sensitivity DSC. The right Y-axis and bars depict the transfection efficacy TE of each composition compared to the average efficacy $\langle TE \rangle$ of all studied compositions: $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$; the shaded region indicates compositions that reside within the solid-liquid crystalline two-phase region at 37°C. Inset: Efficacy of transfection of HUAECs by EDOPC/diC₁₄DAB mixtures. (C) SAXD patterns of EDOPC/diC₁₄DAB 20:80 (mol/mol), recorded upon heating; the bolded pattern was recorded at 37°C. (D) Fusion of cationic liposomes (EDOPC and EDOPC/diC₁₄DAB 20:80) with negatively charged liposomes as assessed by FRET. Data are presented as normalized fluorescence recovery 7 min after addition of unlabeled egg PC/DOPG 80:20 liposomes to the cationic lipid dispersion labeled with 0.5% NBD-PE and 0.5% Rh-PE. 100% fluorescence recovery corresponds to complete mixing of the anionic and cationic lipids.

Fig. 4.

(A) Heating thermograms of dispersions of EDOPC/diC₁₈DAB mixtures at different ratios. (B) Relationship of transfection activity to phase diagram. Left Y-axis and lines and symbols apply to the phase diagram, which was constructed from the calorimetric data. Squares denote transition onset and circles transition end. The right Y-axis and bars depict the transfection efficacy TE of each composition compared to the average efficacy $\langle TE \rangle$ of all studied compositions: $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$; the shaded region indicates compositions that reside within the solid-liquid crystalline two-phase region at 37°C. Inset: Transfection of HUAECs by EDOPC/diC₁₈DAB mixtures. (C) SAXD patterns of EDOPC/diC₁₈DAB 92:8 and 15:85 (mol/mol) dispersions, recorded upon heating; the bolded pattern was recorded at 37°C.

Fig. 5. (A) Heating thermograms of dispersions of EDOPC/DMTAP mixtures at different ratios. (B) Relationship of transfection activity to phase diagram. Left Y-axis and lines and symbols apply to the phase diagram, which was constructed from the calorimetric data. Squares denote transition onset and circles transition end. The right Y-axis and bars depict the transfection efficacy TE of each composition compared to the average efficacy $\langle TE \rangle$ of all studied compositions: $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$; the shaded region indicates compositions that reside within the solid-liquid crystalline two-phase region at 37°C. Inset: Transfection of HUAECs by EDOPC/DMTAP mixtures. (C) SAXD patterns of EDOPC/DMTAP 75:25, 33:67, and 15:85 (mol/mol) dispersions, recorded upon heating; the bolded pattern was recorded at 37°C. (D) Fusion of cationic liposomes (EDOPC and EDOPC/DMTAP 75:25) with negatively charged liposomes as assessed by FRET. Data are presented as normalized fluorescence recovery 7 min after addition of unlabeled egg PC/DOPG 80:20 liposomes to the cationic lipid

dispersion labeled with 0.5% NBD-PE and 0.5% Rh-PE. 100% fluorescence recovery corresponds to complete mixing of the anionic and cationic lipids.

Fig. 6.

Transfection efficacy (TE) of all samples of the five mixtures studied. Data are shown for the solid (A), solid-liquid transition (B) and liquid (C) regions. For each region, TE, normalized to the average efficacy for each given mixture (calculated as $[(TE - \langle TE \rangle) / \langle TE \rangle] \times 100$), is plotted against the difference between the liquidus temperature for the given lipid composition and 37°C [$T(\text{liquidus}) - 37^\circ\text{C}$]. (In a few cases the given composition fell outside the transition region, being in the solid phase at 37°C – such as, e.g., EDMPC/EDPPC at EDPPC ≥ 90 mol %, etc.; for these compositions, the X-axis value was calculated as $[37^\circ\text{C} - T(\text{solidus})]$). (B') Illustrative snapshot of the microscopic lateral organization of an equimolar DMPC-DSPC binary lipid mixture in the solid-liquid phase coexistence region obtained by computer simulation of a bilayer system of 5000 lipid molecules; the symbols for the conformational states of the acyl chains are: gel-DMPC (light-gray), fluid-DMPC (blank), gel-DSPC (black), fluid-DSPC (dark-gray) (reproduced from ^{ref. 58}, with permission from Elsevier).