# Variety of Lamellar and Nonlamellar Phases in Mixtures of the Cationic Ethylphosphatidylcholine with Membrane Lipids: Temperature–Composition Phase Diagrams

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## Introduction

In recent years, it has become apparent that lipidlike compounds can be used to deliver DNA to cells, a procedure that is finding clinical applications. The molecules that are most efficient in delivering DNA to cells are positively charged amphiphiles [1], and such cationic compounds are now being intensively studied. Although the majority of cationic lipids used for transfection arrange themselves into lamellar phases, it has been shown that their mixtures with other lipids (so-called helper lipids, introduced to improve transfection efficiency) are also able to form an inverted hexagonal phase and even to incorporate DNA within it [2, 3]. Correlations between the mesomorphic phase state of the lipoplexes and their transfection activity have been sought, but so far, there is no clear consensus as to whether the nonlamellar phases *per se* are beneficial for transfection [2-4]. The topic of the phase behavior of the mixtures of cationic lipids with other polar lipids is important not only from the standpoint of the possible use of helper lipids and their effect on lipoplex structure and activity, but also with regard to attempts to assess the lipid phases that may arise when cationic lipids of the lipoplexes interact with cellular lipids during DNA transfection. It was recently shown that mixtures of cationic and anionic lipids form nonlamellar phases in the composition region close to charge neutrality, although the pure components form only lamellar phases [5, 6]. This propensity could be important in the process of DNA delivery by amphipathic cationic vectors [4].

By using differential scanning calorimetry and small-angle x-ray diffraction (SAXD), we have constructed detailed temperature-composition phase diagrams of mixtures of the cationic phospholipid *O*-ethyldipalmitoyl-phosphatidylcholine (EDPPC) with representatives of the major cellular lipid classes — phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylglycerol, and cholesterol. Corresponding phase diagrams of these mixtures in the presence of DNA were also created.

## Methods and Materials

Cationic EDPPC was synthesized as previously described [7, 8]. The dipalmitoylphosphatidylcholine (DPPC), dielaidoylphosphatidylethanolamine (DEPE), and dipalmitoylphosphatidylglycerol (DPPG) came from Avanti Polar Lipids, Inc. (Birmingham, AL), and the cholesterol came from Sigma (St. Louis, MO). Herring sperm DNA (Invitrogen, Carlsbad, CA) was used. High-sensitivity microcalorimetric measurements were performed with a VP-DSC microcalorimeter (MicroCal Inc., Northampton, MA), at scan rates of 0.2-0.5°C/min. SAXD measurements were performed at the DND-CAT beamline 5-ID-D and Bio-CAT beamline 18-ID at APS. At DND-CAT, 15-keV x-rays were used. Data were collected by using a marCCD detector (165-mm diameter, 2048 × 2048 pixels, 78.75-μm pixel size, 300 × 300-μm beam size). At Bio-CAT, 12-keV x-rays were used; 2-D diffraction patterns were recorded by using a high-sensitivity charge-coupled device (CCD) detector [9] (50 × 90 mm, 1028 × 1798 pixels, 48-μm pixel size, beam size at the CCD surface of 50 × 150 μm). The sample-to-detector distance was 1.8-2 m. Silver behenate (DuPont, Wilmington, DE) was used as a calibrant ($d_{001}$ = 58.376 Å). For temperature control, either a THMS600 thermal-stage (Linkam Sci Instruments, Surrey, England) or NESLAB programmable (Thermo NESLAB, Portsmouth, NH) water bath was used. Linear heating and cooling scans were performed at rates of 0.8-3°C/min. Exposure times were typically 0.5-2 s. The 2-D diffraction patterns did not show angular dependence of the scattered intensity for the phases studied. Diffraction intensity versus reciprocal space $\underline{s}$ plots were obtained by radial integration of the 2-D patterns done with the interactive data-evaluating program FIT2D [10]. Some samples with a 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 to the lipids was not evident from their x-ray patterns.

## Results and Discussion

The hydrated binary lipid mixture DPPC/EDPPC was found to be homogeneous at all compositions. In physiological saline, its temperature-composition phase diagram is of the lower isoconcentration point type. Simulation of the phase diagram within the regular solution approximation suggests a tendency to clustering in the gel phase and near-ideal mixing in the liquid crystalline phase. The major disparity in the gel phase packing arrangements of the two lipids — interdigitated versus noninterdigitated, tilted — is the

presumed reason for the clustering at low temperatures, in spite of the electrostatic repulsion. Chain interdigitation in the gel phase, characteristic of pure EDPPC, is preserved in mixtures with up to ~70 mol % DPPC. In the absence of electrolyte, the DPPC/EDPPC phase diagram exhibits lower and upper isoconcentration points, at ~20 and 65 mol % EDPPC, respectively. Its complicated shape likely reflects the delicate balance between electrostatic interactions (dominating at high charge density) that tend to separate the like molecules and steric interactions (dominating at low charge density) that tend to cluster them together.

The temperature-composition phase diagram of the DEPE/EDPPC mixture in physiological saline is of eutectic type, with a eutectic point at 40 mol % EDPPC and 27°C. Phase separation between aggregates of 30 and 90 mol % EDPPC takes place in the gel phase at intermediate EDPPC contents. The reasons suggested for the phase separation are the major difference in the gel-phase packing arrangement of EDPPC and DEPE as well as the different headgroups. At high temperatures, cubic and inverted hexagonal mesomorphic phases form over a wide compositional range. Three different cubic topologies — Pn3m, Im3m, and Ia3d — were distinguished at different lipid compositions and temperatures. The propensity to form nonlamellar phases is highest at the eutectic composition, at which the lamellar-nonlamellar transition temperature displays a minimum of 33°C. The transformation follows a $L_\alpha$ – $Q_{II}$ (Pn3m) – $Q_{II}$ (Im3m) – $H_{II}$ sequence (Fig. 1). Addition of an isoelectric amount of DNA to the mixtures suppresses the formation of the Im3m cubic phase and also the formation of the $H_{II}$ phase at high EDPPC content. Bicontinuous lipid cubic phase formation in the presence of DNA is reported for the first time.

In the presence of PBS (pH 7.2), the phase diagram of the DPPG/EDPPC binary mixture is complicated, with several peculiar points. The two lipids mix well at low DPPG concentrations, up to ~25 to 30 mol %. At a DPPG content of >30 mol %, the system exhibits phase separation regions of the solid-solid, solid-liquid, and liquid-liquid types. Nonlamellar liquid crystalline phases (inverted hexagonal, cubic) form, with the lowest-temperature (47°C) lamellar-nonlamellar transformation occurring at 2:1 EDPPC/DPPG stoichiometry. In the absence of electrolytes, the phase separation in the mixture is not observed. Addition of an isoelectric amount of DNA does not change the DPPG/EDPPC phase diagram in water significantly. This is taken to indicate that the lipid-lipid affinity is stronger than the catatonic lipid-to-DNA affinity. Such interactions should be important for transfection and can explain DNA release upon contact of lipoplexes with cell membranes.

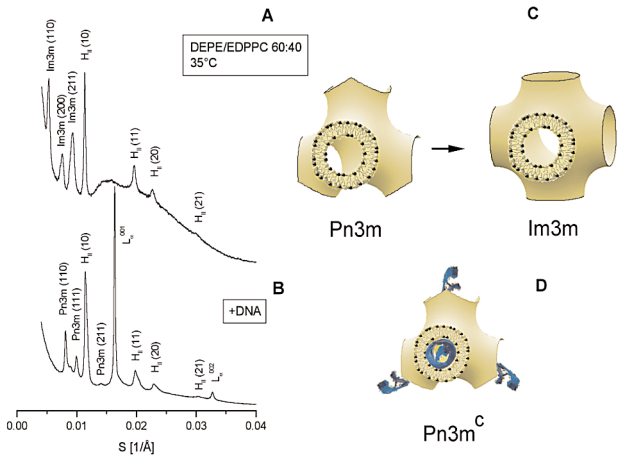


*FIG. 1. Cubic phases in the DEPE/EDPPC 60:40 sample (eutectic composition). A shows a diffraction pattern at 35°C exhibiting coexisting Q (Im3m) and $H_{II}$ phases. B shows a diffraction pattern from a sample containing an isoelectric amount of DNA, exhibiting coexisting $L_\alpha$, Q (Pn3m), and $H_{II}$ phases. C is a representation of the bicontinuous Pn3m and Im3m cubic phases. D is a representation of a complex Pn3m phase including DNA helices.*

The cholesterol/EDPPC phase diagram exhibits a eutectic point at 30 mol % cholesterol. Coexistence of interdigitated and noninterdigitated low-temperature lamellar phases is observed at as high as 30 mol % cholesterol. With the eutectic composition, the $L_\alpha$ phase is least stable — an $H_{II}$ phase forms at the lowest temperature of 41°C. Formation of a second $H_{II}$ phase with a slightly smaller structural unit is observed in the presence of DNA.

All four mixtures exhibit a tendency to molecular clustering in the gel phase, presumably due to the specific interdigitated molecular arrangement of the EDPPC gel bilayers. With the EDPPC/DEPE, EDPPC/DPPG, and EDPPC/cholesterol mixtures, this tendency results in phase separation in the gel phase and eutectic points in their phase diagrams. Clearly, the difference of the headgroups in the last three mixtures is an additional reason for the contacts between like molecules to be preferred. Marked enhancement of the affinity for formation of nonlamellar phases is observed in mixtures of the cationic EDPPC with DEPE and cholesterol as well as DPPG. Because of the potential relevance to transfection, it is noteworthy that such phases form at close to physiological conditions and in the presence of DNA. The composition with the strongest nonlamellar-phase-forming tendency was different for different systems. Cubic-phase-containing lipoplexes are reported for the first time; effects on transfection are yet to be established.

In the present study, a large variety of phases have been found to form in mixtures of the cationic ethylphosphatidylcholine with representatives of cellular lipid classes [11]. Although in multicomponent systems, such as biomembranes, the phase behavior could hardly be expected to match that of the binary mixtures studied here, it is still conceivable that a similarly broad array of lipid phases could arise in transfected cells. Their potential effect on the function of biomembranes needs to be carefully considered.

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