

Electrostatic Control of Phospholipid Polymorphism

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ABSTRACT A regular progression of polymorphic phase behavior was observed for mixtures of the anionic phospholipid, cardiolipin, and the cationic phospholipid derivative, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine. As revealed by freeze-fracture electron microscopy and small-angle x-ray diffraction, whereas the two lipids separately assume only lamellar phases, their mixtures exhibit a symmetrical (depending on charge ratio and not polarity) sequence of nonlamellar phases. The inverted hexagonal phase, H_{II}, formed from equimolar mixtures of the two lipids, i.e., at net charge neutrality (charge ratio (CR_(+/-)) = 1:1). When one type of lipid was in significant excess (CR_(+/-) = 2:1 or CR_(+/-) = 1:2), a bicontinuous cubic structure was observed. These cubic phases were very similar to those sometimes present in cellular organelles that contain cardiolipin. Increasing the excess of cationic or anionic charge to CR_(+/-) = 4:1 or CR_(+/-) = 1:4 led to the appearance of membrane bilayers with numerous interlamellar contacts, i.e., sponge structures. It is evident that interactions between cationic and anionic moieties can influence the packing of polar heads and hence control polymorphic phase transitions. The facile isothermal, polymorphic interconversion of these lipids may have important biological and technical implications.

INTRODUCTION

Phase transitions are intriguing properties of phospholipids that have attracted attention for many years. The polymorphic structural character of lipids may represent a fundamental characteristic that is essential for cellular function (Hyde et al., 1997; Landh, 1995; Lewis et al., 1998; Lindblom and Rilfors, 1989; Luzzati, 1997). Depending upon temperature, pH, ionic strength, hydration, etc. lipids may assume different structures such as micelles, bilayers, hexagonally packed cylinders, or one of several cubic phases (Briggs and Caffrey, 1994; Larsson, 1994; Lewis et al., 1998). The molecular basis of lyotropic lipid phase stability can be understood on the basis of shapes of lipid molecules (Israelachvili and Mitchell, 1975) or spontaneous (intrinsic) monolayer curvature (Gruner et al., 1985). The spontaneous curvature depends upon the effective cross-sectional area of the polar and nonpolar regions of the molecule. These, in turn, depend upon the nature of the interactions of the molecules with their neighbors; especially well known are hydration and temperature effects, which affect headgroup size or hydrocarbon chain areas (Eppand and Eppand, 1994; Leventis et al., 1991; Lewis et al., 1998; Koynova and Caffrey, 1994).

This study was based on the concept that packing of polar heads of charged lipids could be controlled through a wide range by composing lipid mixtures of different proportions

of cationic and anionic lipids. Thus, in equi-charge mixtures, attractive forces will dominate, and average headgroup area will be minimal, whereas when one lipid is much in excess, repulsive forces will dominate and headgroup area will be maximal. Intermediate compositions would provide access to intermediate areas per molecule. Given a large enough difference between the extreme situations, most lyotropic phases could be accessible with a single binary mixture. To test this concept, we examined mixtures of the cationic phospholipid derivative, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), bearing one positive charge per molecule and the anionic lipid cardiolipin (CL) with two negative charges per molecule. Both lipids, individually (in the absence of di- or trivalent counterions), produce lamellar phases, i.e., bilayer membrane structures.

Evidence that electrostatics can affect lipid phases has been clear from the profound effects counterions and pH can have. For example, cardiolipin can be converted from the lamellar to H_{II} phase in the presence of divalent metal cations (de Kruijff et al., 1982; de Kruijff et al., 1985). The polymorphic potential of cardiolipin is of particular interest given that the cellular organelles where this lipid is prominent are capable of assuming cubic phases under certain environmental conditions (Deng and Mieczkowski, 1998; Gunning, 1965; Williams et al., 1998). Other phospholipids can be converted to hexagonal or cubic phases in the presence of fatty acids (Seddon et al., 1997; Winter et al., 1999), and the relative stability of cubic phases of monoglycerides can be modulated by fatty acids (and additionally by pH and ionic strength) (Aota-Nakano et al., 1999).

The cationic phospholipid derivative used in this study was developed originally as a DNA transfection agent (MacDonald et al., 1999c). It forms liposomal dispersions in which the bilayers are indistinguishable by x-ray diffraction from those of natural phospholipids (MacDonald et al.,

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1999a). These dispersions interact with DNA to form a transfection complex (MacDonald et al., 1999a) much like a number of other cationic amphipaths (Felgner et al., 1987; Legendre and Szoka, 1992; Farhood et al., 1992; Gao and Huang, 1995; Scherman et al., 1998; Sorgi et al., 1997). Such agents have attracted attention as potential delivery vehicles in gene therapy (Farhood et al., 1994; Templeton and Lasic, 1999). Electrostatic forces are responsible for formation of DNA-cationic lipid complexes (Kennedy et al., 2000; Zuidam and Barenholz, 1998) and the interaction of complexes with the cell surface (Miller et al., 1998) and are probably responsible for the release of DNA before its migration into the nucleus where it is expressed (Ashley et al., 1996; Bhattacharya and Mandal, 1998; Kinnunen et al., 1993; Xu and Szoka, 1996).

The binary mixture of phospholipids described here generates a complete array of phases—lamellar, sponge, inverted hexagonal, and cubic—that had heretofore been accessible only by varying water content or temperature. This system could provide a model for cellular membrane polymorphism and may also have applications in biotechnology.

MATERIALS AND METHODS

Materials

Beef heart cardiolipin was obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), as the trifluoromethylsulfonate salt, was synthesized according to the procedure described previously (MacDonald et al., 1999a,c). The compound is also available from Avanti Polar Lipids (currently as the chloride salt). Most other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared with double-distilled or Milli-Q purified water.

Sample preparation

Samples were prepared by dissolving the appropriate amount of lipid in chloroform:methanol (2:1). The organic solvents were evaporated under a stream of argon and then residual solvent was removed under high vacuum for at least 2 h. The lipids were hydrated in 150 mM NaCl, 1 mM EDTA, 20 mM Pipes (pH 7.4) for 4–8 days at room temperature in closed glass ampules filled with argon.

It was assumed that one molecule of cardiolipin bears two negative charges, which should be an excellent approximation as the neutral pH at which the experiments were performed is far removed from the pK values. The quaternary ammonium of EDOPC has one positive charge at all pH values.

Freeze-fracture electron microscopy

The hydrated sample, prepared as described above, was applied to 400-mesh palladium electron microscope grids. The samples were then sandwiched between two 50- μ m-thick copper plates and quenched in a mixture of liquid propane and liquid ethane (1:1, v/v). Fracturing was performed with a laboratory-constructed double-replica device in a Balzers 340 freeze-etching unit at a vacuum of 3×10^{-6} to 5×10^{-6} Torr and shadowed with a platinum-carbon mixture. Replicas were cleaned in a chloroform-methanol mixture (2:1) and examined on a JOEL 1200EX

transmission electron microscope at magnification of 50,000 \times and 100,000 \times .

Image analyses

The unidirectionally shadowed freeze-fracture replicas were inspected for the ordered domains. Areas of interest from these negatives were digitized and then analyzed using fast Fourier transformations (FFTs) implemented on an IBAS (Kontron, Eching, Germany) image analysis system. From each FFT spectrum, selected maxima were used to create a mask that was applied to the transform and then inverse transformed to obtain an FFT-filtered image (IFFT) that comprised just the selected maxima.

X-ray diffraction

After preparation as described above, the adhesive lipid material was scraped from the wall of glass tube and carefully transferred to x-ray glass

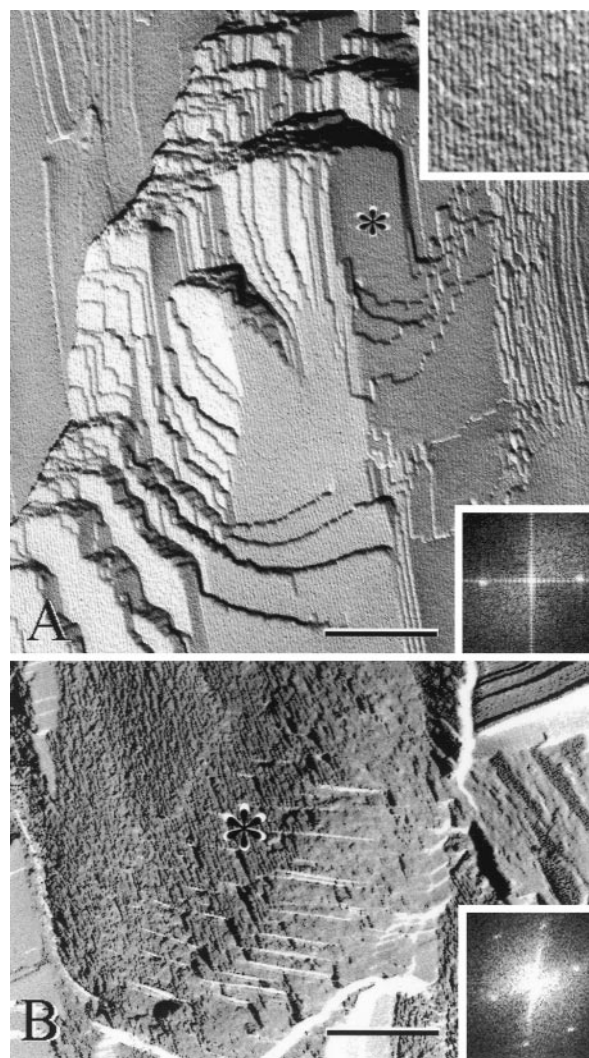


FIGURE 1 Freeze-fracture surface of the hexagonal H_{II} phase of a mixture of E-DOPC and cardiolipin, MR = 2:1, charge ratio $CR_{(+/-)} = 1:1$. (A) Longitudinal and (B) transverse fracture surfaces of lipid tubes. Asterisks represent regions chosen for FFT analysis (insets in lower right of each panel). That region in A chosen for analysis is shown at higher magnification in the upper right corner. Bar, 200 nm

capillaries along with excess buffer. The capillaries were sealed and mounted in a mirror-mirror point-focus x-ray camera. Diffraction patterns were recorded at ambient temperatures on Kodak DEF x-ray film, as described previously (McIntosh et al., 1987).

RESULTS

An inverted hexagonal phase is formed by an equimolar mixture of cationic and anionic lipids.

Electron microscopy and x-ray diffraction both revealed that the neutral mixture, EDOPC/CL, with $CR_{(+/-)} = 1:1$, represented a typical inverted hexagonal (H_{II}) phase. By freeze-fracture electron microscopy, this phase is quite distinctive, as seen in Fig. 1. The longitudinal fracture surfaces (Fig. 1 A) contained bundles of long parallel-oriented rods having a pronounced periodicity. The transverse sections (Fig. 1 B) of the bunches were less obviously ordered, but Fourier analyses demonstrated the existence of hexagonal areas. The planes of the tubes were on a 58-Å repeat spacing, according to Fourier transforms of the images (Table 1, row 4) as seen in the insets at the lower right side of each panel in Fig. 1.

Small-angle x-ray diffraction also revealed the presence of maxima with spacings in the ratio $1, \sqrt{3}, \sqrt{4},$ and $\sqrt{7}$, corresponding to Miller indices (10), (12), (22), (32) of a hexagonal H_{II} phase with reciprocal spacing $S_{(h, k)} = (2/d\sqrt{3})(h^2 + k^2 + hk)^{1/2}$ where h and k are Miller indexes, and d is the lattice constant. The indexing of the data is shown in Fig. 2 B, where it can be seen that the agreement between experimental and predicted reciprocal spacing is excellent. The distance between diffracting

planes was 58.2 Å. The lattice spacing (center-to-center distance between cylinders) derived from the slope of the plot was 67.2 Å (Table 1, row 4), a typical value for inverted hexagonal phases.

Polarization microscopy also revealed a pronounced anisotropy of the lipid material. Visually, the sample contained cloudy material that adhered to the wall of glass tubes (Table 1, row 4).

Cubic phases form when there are twice as many charges of one polarity as of the other.

In samples with a two-fold excess of positive or negative charges ($CR_{(+/-)} = 2:1$ or $CR_{(+/-)} = 1:2$), the macroscopic appearance of the sample was not greatly different from that of the hexagonal, neutral phase; these lipid mixtures were also rather adhesive, transparent ($CR_{(+/-)} = 2:1$), or slightly cloudy ($CR_{(+/-)} = 1:2$) gels that tended to remain on the surface of glass tubes. Under the polarizing microscope, however, these samples were devoid of birefringence and hence definitively isotropic (Table 1, rows 3 and 5).

The organization of these lipid mixtures was recognizable by freeze-fracture analysis. As is demonstrated in Fig. 3, the appearance of the mixtures with both $CR_{(+/-)} = 2:1$ and $CR_{(+/-)} = 1:2$ were quite similar and both were characterized by the presence of numerous ordered arrays. The dimensions of ordered areas were usually smaller than 1 μm . The most frequently observed domains were tetragonal (left panels) or hexagonal (right panels). Lattice spacings were about twice as large as those for the hexagonal phase (Table 1).

TABLE 1 Structure and macroscopic appearance of liposomes composed of different mixtures of EDOPC and cardiolipin based on x-ray diffraction, freeze-fracture, polarized light microscopy, and visual evaluation

| Row | EDOPC/CL molar ratio | (+/-) ratio | Phase | Appearance | Freeze-fracture analysis* | X-ray diffraction analysis |
|-----|----------------------|-------------|--------------------|--|---|--|
| 1 | 0:1 | 0:1 | Lamellar | Turbid suspension | Unilamellar vesicles | |
| 2 | 1:2 | 1:4 | Sponge | Transparent, nonbirefringent droplets, in suspension or attached to tube | Unordered granular and reticulated structure | |
| 3 | 1:1 | 1:2 | Cubic | Transparent, nonbirefringent adhesive gel | Hexagonal array spacing = 100 ± 4.5 Å (Fig. 2 A); tetragonal array spacing = 129 ± 3.4 Å (Fig. 2 B) | Primitive cubic lattice spacing = 124 Å (Fig. 4 A) |
| 4 | 2:1 | 1:1 | Inverted hexagonal | Slightly cloudy, birefringent very adhesive gel | Longitudinal fracture spacing = $58 + 2$ Å (Fig. 1 A); cross fracture spacing = 60.5 ± 1 Å (Fig. 1 B) | Hexagonal lattice spacing = 67.2 Å; plane-to-plane spacing = 58.2 Å (Fig. 4 B) |
| 5 | 4:1 | 2:1 | Cubic | Slightly cloudy, somewhat adhesive gel with some birefringent inclusions | Hexagonal array spacing = 96 ± 3.5 Å (Fig. 2 C); tetragonal array spacing = 123 ± 3.5 Å (Fig. 2 D) | Primitive cubic lattice spacing = 128 Å (Fig. 4 C) |
| 6 | 8:1 | 4:1 | Sponge | Transparent, nonbirefringent droplets, in suspension or attached to tube | Unordered granular and reticular structure | |
| 7 | 1:0 | 1:0 | Lamellar | Turbid suspension | Unilamellar vesicles | |

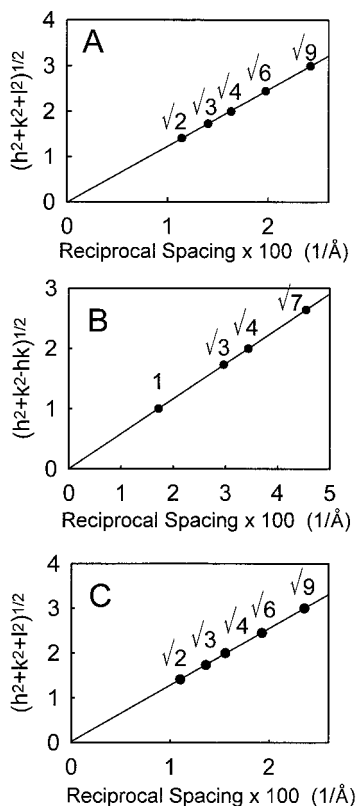


FIGURE 2 Plots of reciprocal spacings of observed x-ray reflections versus Miller indices (h , k , and l) for 1:1 EDOPC/CL in the cubic phase, 2:1 EDOPC/CL in the hexagonal phase, and 4:1 EDOPC/CL in the cubic phase. The solid lines indicate the least squares fits to the data points ($R^2 > 0.999$ for all cases). The slopes of the lines give the fundamental repeating units of: (A) $d = 124.0 \text{ \AA}$ for the cubic phase of 1:1 EDOPC/CL ($CR_{(+/-)} = 1:2$); (B) $d = 58.2 \text{ \AA}$ for the hexagonal phase of 2:1 EDOPC/CL ($CR_{(+/-)} = 1:1$); (C) and $d = 126.4 \text{ \AA}$ for the cubic phase of 4:1 EDOPC/CL ($CR_{(+/-)} = 2:1$).

X-ray diffraction analyses of these samples also revealed the presence of a cubic phase. In this case, the reciprocal spacing is related to the lattice spacing, d , according to the relationship, $S_{(h, k, l)} = (1/d)(h^2 + k^2 + l^2)^{1/2}$, where l is a Miller index. As shown in Fig. 2, the experimental maxima were in the ratio $\sqrt{2}$, $\sqrt{3}$, $\sqrt{4}$, $\sqrt{6}$, $\sqrt{9}$, corresponding to the (110), (111), (200), (211), and (221) planes for an array belonging to the cubic space group $Pn3m$ (Q^{224}). Although diffraction intensity at spacing $\sqrt{8}$ (index 220) was not detectable, the absence of this reflection is not unusual for lipid cubic phases of this space group (Aota-Nakano et al., 1999). Spacings determined by x-rays and electron microscopy were quite similar (Table 1). There was no indication of lamellar or hexagonal phase lipid in these samples by x-ray diffraction, although some lamellar and hexagonal phase regions were detected by electron microscopy.

Close inspection of freeze-fracture electron micrographs of the ordered cubic phase surfaces revealed that the ordered domains were often mosaically organized and composed of

smaller subdomains. All subdomains belonging to the same domain had similar lattice parameters but were distinguished by the presence of a marked discontinuity between arrays.

A larger charge imbalance leads to the disorganization of the cubic array into a sponge phase

When the net charge was increased to the point where only 1/5 of the molecules were neutralized, an additional structure was found. The samples with the charge ratios $CR_{(+/-)} = 1:4$ and $CR_{(+/-)} = 4:1$ were highly transparent and exhibited no anisotropy under polarized light (Table 1, rows 2 and 6). The material was less adhesive to test tube walls than the cubic-phase material and it could be dispersed into the buffer solution. When so dispersed, some of this material comprised very large, up 1 mm diameter, transparent spheres that were visible with a hand lens. Freeze-fracture electron microscopy revealed no regular order in these samples (Fig. 4 B); most common were large multilamellar structures with numerous and randomly distributed stalk-like contacts between membranes. These structures evidently represent examples of sponge organization of lipid bilayers (Noordam et al., 1980). These samples also contained some granular material as well as uni- or multilamellar vesicles. X-ray diffraction was not done on these compositions.

Component cationic and anionic lipids form lamellar phases

As expected, the pure lipid materials, in which molecules of a single charge are represented, were found to be lamellar by light and electron microscopy. Fig. 4 A shows an EDOPC sample and Fig. 4 C shows the cardiolipin dispersion. Both are composed entirely of vesicular structures. The macroscopic behavior of these samples was typical for liposome dispersions (Table 1, rows 1 and 7).

DISCUSSION

We found that the polymorphic variations of the cationic-anionic lipid mixture of cardiolipin and EDOPC followed the sequence: vesicles (pure cardiolipin) \Leftrightarrow sponge phase ($CR_{(+/-)} = 1:4$) \Leftrightarrow cubic phase ($CR_{(+/-)} = 1:2$) \Leftrightarrow hexagonal H_{II} phase ($CR_{(+/-)} = 1:1$) \Leftrightarrow cubic phase ($CR_{(+/-)} = 2:1$) \Leftrightarrow sponge phase ($CR_{(+/-)} = 4:1$) \Leftrightarrow vesicles (pure EDOPC). The H_{II} phase, the continuous lipid structure with maximal negative intrinsic curvature (inverted micellar phases may have higher curvature), was found at the charge ratio $CR_{(+/-)} = 1:1$, where condensation of the headgroups should be maximal and the cross-sectional area

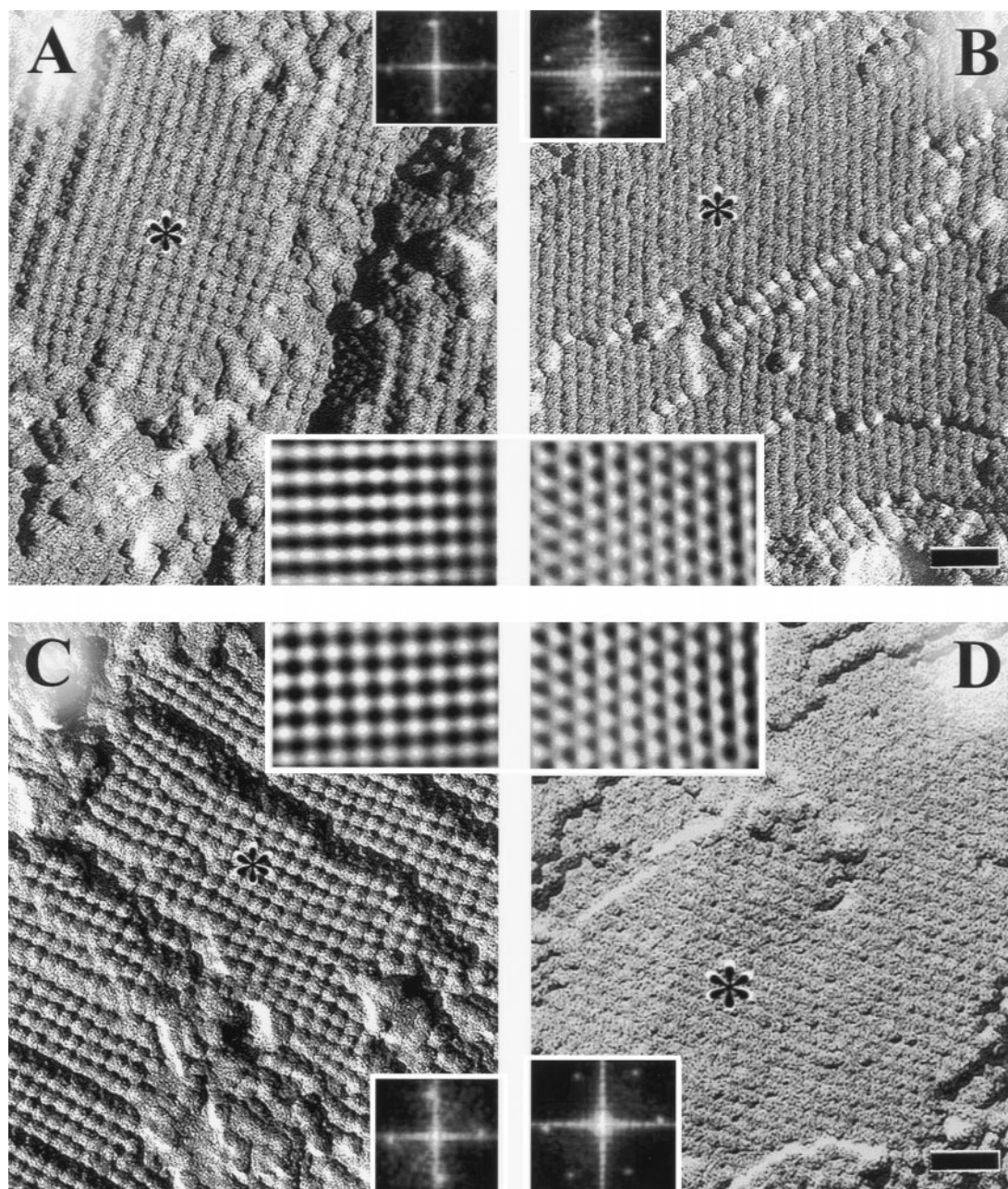


FIGURE 3 Freeze-fracture surface of cubic phases. The two upper panels are of ordered domains found in the mixture of E-DOPC and cardiolipin, mole ratio = 1:1 and $CR_{(+/-)} = 1:2$. Surfaces of fracture planes are shown with (A) tetragonal as well as (B) hexagonal arrays. The two lower panels are of ordered domains found in the mixture of E-DOPC and cardiolipin, mole ratio = 4:1 and $CR_{(+/-)} = 2:1$. Surfaces of fracture planes are shown with (C) tetragonal as well as (D) hexagonal arrays. Asterisks indicate regions chosen for Fourier transforms (*insets at top and bottom, center*) and the inverse Fourier transformed images are shown in the four insets in the center of the figure. Bars, 50 nm.

per headgroup at a minimum. Indeed, measurements on monolayers at the air/water interface revealed average molecular areas that gave a minimum for 1:1 cationic/anionic lipid mixtures (MacDonald et al., 1999b). Furthermore, there is calorimetric evidence for interactions of anionic lipids with other cationic amphipaths (Silvius, 1991).

Departures from the neutral mixture of lipids in both directions, i.e., either an excess of cationic or of anionic

component, were symmetrical and produced similar structures. These were both Q^{224} cubic phases with the space group $Pn3m$. This structure is of the bicontinuous type (Longley and McIntosh, 1983), which is one of the three bilayer-based cubic arrangements thus far described for lipids (Delacroix, 1998; Mariani et al., 1988). These arrays are thought to correspond to the mathematical construct of an infinite periodic minimal surface (Hyde, 1996).

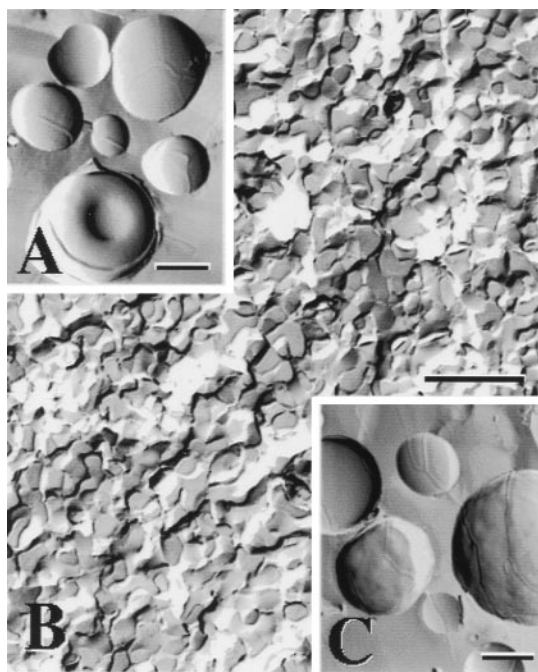


FIGURE 4 Freeze-fracture surfaces of lamellar phase-forming E-DOPC/cardiolipin mixtures. (A and C) Samples of E-DOPC and cardiolipin liposomes, respectively, showing lamellar-phase bilayer vesicles. (B) The mixture, $MR = 1:2$, $CR_{(+/-)} = 1:4$. This composition gave fracture surfaces that were indistinguishable from those of the mixture with the inverse charge ratio, $MR = 8:1$, $CR_{(+/-)} = 4:1$, and so the latter surfaces are not shown. Numerous intermembrane stalk-like contacts are indicative of the sponge structure of these two compositions. Bar, 200 nm.

The near-identity of the cubic phases is particularly noteworthy, given the opposite charge polarity and the complementary nature of the chemical compositions. The clear conclusion from these results is that electrostatic interactions between positive and negative charges promote the compaction of the polar heads of the constituent lipid molecules and decrease the polar surface area of membranes relative to that of the nonpolar chains. It is well known that the headgroup area relative to the tail area increases in the progression as follows: inverted hexagonal < bicontinuous cubic < lamellar (Larsson, 1994; Lewis et al., 1998); hence it appears that the increased repulsion among polar groups is the primary determinant, independent of polarity of charge, of the sequence of phases we observed.

An important implication of this work has to do with the ease with which lamellar and bicontinuous cubic phases are interconverted, a conclusion that follows from the fact that phase changes result from very modest changes in molecular areas and correspondingly in curvature energies (Chung and Caffrey, 1994). In this respect, the behavior of these lipid mixtures appears to be closely related to some basic cell biological phenomena. Of particular relevance, because they are organelles distinguished by their cardiolipin content, are mitochondria and chloroplasts. These structures

undergo cubic-lamellar transformations in response to environmental conditions. The mitochondria of amoebae transform from lamellar to cubic organization when their food intake is restricted and revert to the lamellar organization when food is supplied. The cubic phase has been analyzed in detail (Deng et al., 1999; Deng and Mieczkowski, 1998). Prolamellar bodies are a light-starved arrangement of chloroplast membranes that exhibit a response analogous to that of mitochondria; in the presence of light, these organelles rapidly transform into the familiar lamellar array characteristic of chloroplasts. Prolamellar bodies were identified long ago as interconnected tubules on a cubic lattice (Gunning, 1965) and more recently have also been shown to represent cubic membrane arrays by x-ray diffraction (Williams et al., 1998). Thus, it appears that both chloroplasts and mitochondria, in effect, store their membranes in a compact cubic array when they are inactive. Other cellular membrane systems also exhibit cubic symmetry (Landh, 1995) and it has been frequently suggested that lipid polymorphic potential could be taken advantage of by cells for specific purposes (Lindblom and Rilfors, 1989; Luzzati, 1997; Hyde et al., 1997; Mariani et al., 1988). Although we have emphasized mixtures with cardiolipin in this report, polymorphism is not to limited mixtures of this particular anionic lipid with EDOPC; cubic phases were also observed with phosphatidylglycerol as the anionic lipid. Also, polymorphism in EDOPC mixtures with other anionic lipids has recently been reported (Lewis et al., 2000).

Two connections of the present findings to biotechnology immediately present themselves. The first has to do with the fact that cationic phospholipids of the *O*-alkyl phosphatidylcholine type are potent DNA transfection agents, both in vitro (MacDonald et al., 1999c) and in vivo (Gorman et al., 1997). Transfection by such agents requires release of the DNA within the cell, a process that could involve displacement of the cationic lipid from the DNA by anionic cellular lipids (Ashley et al., 1996; Kinnunen et al., 1993; Xu and Szoka, 1996). Because the DNA cannot be released until the cationic lipid partner is at least partially neutralized, it is anticipated that a side product of transfection would be the generation in the cell of nonlamellar lipid. Indeed, clear indications of cubic membrane arrays are seen in transfected cells (see Fig. 7 of Zabner et al., 1995).

A potential second important application is to the crystallization of membrane proteins. Membrane proteins can be grown from cubic phases of glycerol monooleate (Landau and Rosenbusch, 1996; Rummel et al., 1998) and it may be that such crystals could also be grown from cubic phases such as we have described here. These not only consist entirely of phospholipids but also allow polar-polar interactions to be controlled, which may offer new and different opportunities for membrane protein crystallization.

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