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Electrostatic Stitching in Gel-Phase Supported Phospholipid Bilayers

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We show that mixing zwitterionic lipids with up to 20% mole % cationic lipids produces gel-phase supported lipid bilayers that are morphologically free of defects detectable using noncontact mode atomic force microscopy (AFM). This contrasts with the observation of massive defects when anionic lipid was added, and also when no charged lipid was added. Infrared measurements of headgroup orientation in the presence of cationic lipid show that the mean headgroup orientation changes only minimally when temperature is lowered from the fluid phase to the gel phase. This is consistent with a tentative explanation, based on simple electrostatic arguments, in which cationic lipids "stitch" the bilayers together. On the functional side, this study demonstrates a simple method by which to minimize defects in gel-supported phospholipid bilayers.

Introduction

Lipid bilayers supported on planar substrates find widespread application[1−3] in part because they provide model systems to study different physical phenomena on 2-D surfaces with the advantage of a well-defined planar geometry, and on the practical side because they provide a useful environment in which to embed polypeptides and membrane proteins.[4,5] One of their most interesting properties concerns phase transitions; most prominent is the "fluid-to-gel" phase transition, where the gel state reflects crystallization of previously fluid lipid carbon chains.[6,7] At relatively high temperatures, "liquid-crystalline" (LC) phase lipids display fluidity similar to that found in free-standing liposomes, owing to the presence of a thin, lubricating water layer between the bilayers and the hard solid substrate,[8] but this switches to crystalline order when temperature is lowered. Quantitatively best-studied are single-component bilayers consisting of zwitterionic phospholipids, but they have been problematic to study below the phase transition because of a high density of defects in the gel phase. This has been believed to be inevitable due to the considerable shrinkage of the area per lipid molecule in gel phase: 15−20% less than in the fluid phase.[9,10]

How to minimize these defects? It is a known fact that the P−N (phosphorus−nitrogen) dipole in zwitterionic phosphatidylcholine (PC) is nearly parallel to the local bilayer plane with an average angle of 0∼3° in fluid phase but normally with a tilt angle of about 30° in the gel phase.[11,12] If the zwitterionic lipid were mixed with a cationic lipid, could this be modified? Bearing in mind that the cationic lipid is expected to mix well owing to electrostatic repulsion, a simple electrostatic argument suggests that its presence might "stitch" together the structure, even when the temperature is lowered below the gel-to-fluid phase transition. The geometrical relation between the P−N dipole of the zwitterionic lipid headgroup, and the cationic lipid that would reside preferentially near the negative side of the dipole, would encourage this. The main idea is sketched in Figure 1, panels A and B. Since completing this study, we have learned that computer simulations had anticipated a similar argument.[13]

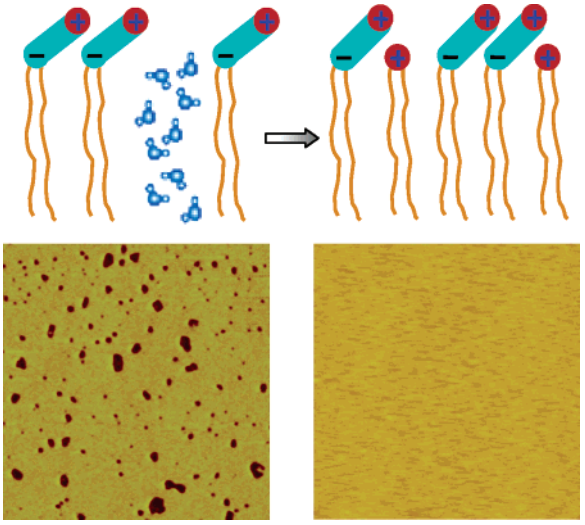

Figure 1. Distinction between gel-phase morphologies of single-component zwitterionic lipid bilayers (A and C) and two-component lipid bilayers, zwitterionic lipids mixed with up to 20% cationic lipid (B and D). (A) Schematic illustration that in the single-component bilayers, mutual repulsion between P^--N^+ headgroup dipoles in the headgroups facilitates defect formation. (B) Schematic illustration that electrostatic interaction of zwitterionic with cationic lipid headgroups favors a compact structure. (C) Representative MAC-mode AFM images of single-component zwitterionic (DMPC) bilayers; see text for details. (D) Representative MAC-mode images of bilayers containing 10% cationic lipid (DMTAP). In panels C and D, the temperature is 15 °C and the image area is 5 μm × 5 μm.

Here, systematic experiments were designed to test this hypothesis. In-situ AFM (atomic force microscopy) was employed to characterize the morphology of solid-supported bilayers of zwitterionic lipids mixed with a small concentration of cationic lipid. These experiments validated the hypothesis

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and found no detectable defects in the gel-phase bilayers. To test this hypothesis further, direct measurements were made of phospholipid headgroup orientation (using Fourier transform infrared spectroscopy in attenuated total reflection, FTIR-ATR). These showed only a small change in headgroup orientation between the fluid and the gel phase, suggesting a minimal change in the area per headgroup, and thus afforded additional confirmation of the hypothesis.

Materials and Methods

For study, we selected the zwitterionic phospholipid DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine, because its main phase transition at 23−24 °C affords convenient study of bilayers in the gel state in the vicinity of room temperature. It was mixed with quantities up to 20 molar % of the cationic lipid DMTAP, 1,2-dimyristoyl-3-trimethylammonium-propane. Both lipids were obtained from Avanti Polar Lipids, Inc. For AFM experiments, the supported bilayers were formed on a freshly cleaved sheet of muscovite mica placed at the bottom of an AFM fluid cell made of Teflon. Temperature was controlled using a cryogenic temperature regulator and an ice−water reservoir to respectively gradually heat and cool the AFM chamber with 0.1 °C resolution. For FTIR-ATR experiments, the supported bilayers were formed on a single crystal of germanium.

AFM experiments were performed in the magnetic acoustic code (MAC) mode,[14] a noncontact mode ideal for imaging delicate structures, using a PicoSPM 300 (Molecular Imaging) device controlled with a Nanoscope E controller (Digital Instruments). The resonance frequency of the cantilever was 22−25 kHz and the spring constant was 2.8 N m^{-1}. Infrared spectra were collected using a BioRad FTS-60 Fourier transform infrared spectrometer equipped with a broadband mercury−cadmium−telluride detector. A wire-grid polarizer (Graesby/Specac) was used to discriminate infrared absorption in p- and s-polarization. All measurements were made in PBS buffer (10 mM, pH = 6.0).

Results and Discussion

Bilayers of single-component DMPC and of DMPC/DMTAP mixtures were prepared using the vesicle fusion method described elsewhere.[14,15] Figure 1C shows a typical AFM image of the gel phase DMPC bilayer at 15 °C. The defects are obvious and are of the type studied previously.[9,10] In this image, the dark spots represent naked mica surfaces, and the comparison of dark and light spots shows the thickness of the DMPC bilayer to be 4.5±0.5 nm, agreeing with literature values. The contrasting image in Figure 1D was obtained for a mixture of DMPC with 10% molar mass DMTAP; these images were highly reproducible in multiple independent experiments.

As a control experiment, the thickness of the mixed bilayer, DMPC/DMTAP, was measured by using the AFM tip to dig a hole in the bilayers; within experimental uncertainty the thickness was the same as that of a pure DMPC bilayer. The phase transition temperature of the mixed bilayers was slightly elevated, as found previously by Rädler and co-workers.[16] Furthermore, both DMPC and mixed bilayers displayed within experimental uncertainty the same thickness increase upon cooling, 0.4 nm. In principle, some thickness difference might be anticipated because of the different headgroup tilt angle when cationic lipid is present. However, the estimated length of the PC headgroup is only about 0.4 nm. Although there is a significant change in headgroup tilt angle (from 6° to 28°) when the cationic lipid is present, the associated bilayer thickness change would be less than 0.2 nm, which is within the experiment uncertainty of 0.5 nm. Actually, the bilayer thickness is mainly dominated by its hydrophobic tailgroup length.

To test the hypothesis that morphological homogeneity in the gel phase stemmed from favorable electrostatic interactions of zwitterionic with cationic phospholipid headgroups (Figure 1B), further control experiments were performed in which DMPC was mixed with an anionic phospholipid instead. The scheme in Figure 1B predicts that a mixture of zwitterionic with anionic lipid would be electrostatically unfavorable. As predicted, when DMPC was mixed with 10% anionic lipid DMPA, 1,2-dimyristoyl-sn-glycero-3-phosphate, defects in the gel phase were invariably observed. This showed self-consistency of the hypothesis.

To further test the hypothesis sketched in Figure 1B, FTIR-ATR measurements were performed to quantify the mean orientation of the headgroup dipole. Dichroic ratio was defined as the ratio of IR absorptivities for p- and s-polarization, $D \equiv A_p/A_s$. If the P−N dipole in the DMPC headgroup rotates freely in the X−Y plane with a constant angle θ toward the local bilayer surface, it is known that the dichroic ratio can be expressed as

$$D \equiv \frac{A_p}{A_s} = \frac{E_x^2 + 2E_z^2 \times tg^2\theta}{E_y^2} \quad (1)$$

where E_x, E_y, and E_z are the electric field amplitudes of the evanescent wave in the plane of and normal to the interface where the bilayer resides, respectively.[17,18] In the present experiments using a germanium crystal in an aqueous environment, with IR incident angle 45°, the "thin film approximation" gives $E_x = 1.40$, $E_y = 1.50$, and $E_z = 1.38$. The dichroic ratio of an isotropic sample is 1.72, which is the same as for a dipole aligned on the bilayer surface with a "magic angle" of $\theta = 35.3°$. It is known that the value of dichroic ratio decreases with alignment of the P−N dipole preferentially parallel to the bilayer surface and increases gradually when this dipole prefers vertical orientation.[19]

Figure 2 shows IR spectra of symmetric (920 cm^{-1}) stretch vibrations of $-N^+(CH_3)_3$ taken with p- and s-polarization in fluid phase (40 °C) and gel phase (15 °C) of pure DMPC and mixed bilayers. As explained in the figure, the transition moment of the choline symmetric stretch is considered to reflect the P−N dipole orientation of the phospholipid headgroup. The dichroic ratio of the pure DMPC bilayer was 0.89±0.01 in the fluid phase, corresponding to $\theta = 6 \pm 2°$, meaning that the PC headgroup was almost parallel to the bilayer surface, which agrees with known behavior for this single-component bilayer,[11,12] but rose to 1.30 ± 0.01 in the gel state, a mean tilt angle of 27±1°.

This contrasts sharply with behavior of the mixed bilayers containing 10% molar mass of DMTAP. Here the dichroic ratio in the fluid phase was 1.36 ± 0.02, corresponding to a mean tilt angle of 28±1°. It was 1.53 ± 0.02, corresponding to $\theta = 32 \pm 1°$, in the gel state. Reflection shows that quantification is slightly more subtle because the measurements comprise the average value of two populations: 90% from the symmetric stretch of choline from the PC group and 10% from the TAP headgroup, the latter being more vertical than the former. The main point is that numbers changed so little between the fluid and gel phases. Obviously, this implies a minimal change in area per phospholipid headgroup. Since completing this study, we have learned that computer simulations had anticipated a similar result;[13] these experiments can be viewed as a successful

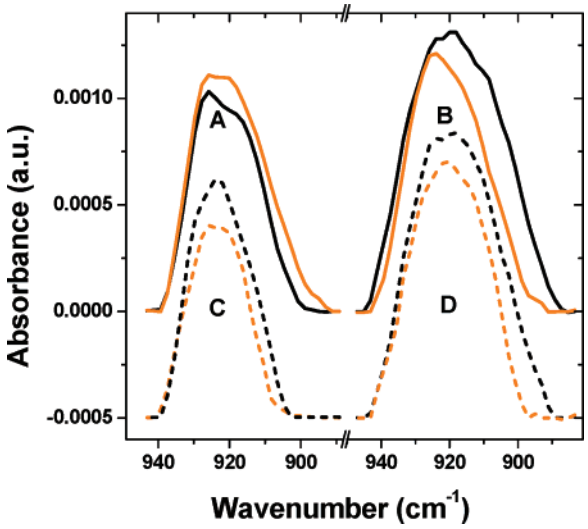

Figure 2. Representative infrared spectra of symmetric (920 cm^{-1}) stretch vibrations of $-N^+(CH_3)_3$ in the gel state at 15 °C (dotted lines) and in the fluid state at 40 °C (solid lines). The ratio of absorbance measured in p- and s- polarizations (black and orange lines, respectively) reflects the mean angle with respect to the surface normal of the choline headgroup. The data contrast single-component zwitterionic DMPC bilayers (A and C) with mixed bilayers of DMPC and 10% cationic DMTAP (B and D). For clarity, spectra in the gel state are vertically offset by a constant amount. Upon lowering temperature into the gel phase the mean tilt angle changed significantly for the single-component bilayers but not for bilayers mixed with cationic lipid; see text for details.

test of the hypothesis presented by this theory. It is remarkable that this simple picture is also consistent with the AFM experiments in Figure 1.

Conclusion

In summary, the presence of cationic lipids is found to minimize differences in headgroup orientation between the fluid and gel phases. This in turn minimizes density mismatch between the fluid and gel phases, tending to stabilize the bilayer structure against defects when the bilayer passes from the fluid to the gel phase. If this observation generalizes to cellular environments, it is possible that this could have bearing on understanding the action of cationic liposomes in molecular cell biology. For example, cationic liposomes can be employed for plasmid DNA delivery into eukaryotic cells and for gene transfer in procedures for potential human gene therapy.

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