# Detection of Phase Separation in Fluid Phosphatidylserine/Phosphatidylcholine Mixtures

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ABSTRACT The nonideal mixing of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, (16:0,18:1)PS, and 1,2-didodecenoyl-*sn*-glycero-3-phosphocholine, (12:1,12:1)PC, in fluid lamellar model membranes was studied by measuring binding of aqueous $Ca^{2+}$ ions and by x-ray diffraction. A region of two-phase coexistence was found by invariance of the aqueous concentration and by the appearance of two sets of lamellar spacings. The phases were identified as fluid from the diffuse x-ray diffraction in the wide-angle region. The width of the two-phase coexistence region was greater at higher ionic strength. In 800 mM KCl, the phase boundaries were at PS mole fraction 0.5 and 0.8. In 100 mM KCl, the phase boundaries were at PS mole fraction 0.52 and 0.62. Monte Carlo simulations of the lateral distributions of these PS/PC mixtures show pronounced clustering of the lipids.

## INTRODUCTION

To understand the diversity of lipid types in biomembranes, many kinds of measurements have been made on real and model systems. In favorable cases, a lipid in a homogeneous phase can be described in terms of the state of local molecular motion of each part of the molecule, as well as the overall rates of diffusion (see Bloom et al., 1991, for a review). For single-component or binary mixtures, the thermal phase behavior can be mapped if the transitions involve sufficiently large enthalpy changes or motion changes (Tenchov, 1985; Caffrey, 1993).

It is likely that comparison of different lipid types involves some crucial differences in motional state or enthalpy that are too small to detect reliably. Furthermore, it is difficult to known whether differences that can be measured have significance. We approach this problem by measuring the thermodynamic activity of the lipids, to find the deviation from random mixing behavior. Significance, i.e., predictive power, might be found in a systematic, quantitative description of how the lipid activity coefficient depends on properties of the molecules of interest, and on composition of the mixture.

There have been few reports of attempts to measure activity coefficients of lipids in bilayers. Freed and coworkers describe how to use the orientational order parameter of spin-labeled phosphatidylcholine (PC) or cholestane to find activity coefficients (Shin et al., 1993). We have developed an indirect method (Feigenson, 1989; Huang et al., 1993) based on measuring the concentration of aqueous $Ca^{2+}$ in equilibrium with the stoichiometric chemical reaction

$$Ca^{2+}(aq) + 2PS^{-} \rightleftharpoons Ca(PS)_2 \qquad (1)$$

This method involves a large correction of the measured parameter, $[Ca^{2+}]_{aq}$, to take account of the increased thermodynamic activity of $Ca^{2+}$ at the membrane surface caused by the negative surface potential. The procedure we have used to make this correction involves Monte Carol simulation to examine explicitly the relation between the surface potential and the phosphatidylserine (PS)/PC distribution. In Huang et al. (1993) we worked with samples of the lowest mole fraction of PS for which we could obtain good data, to minimize the large correction required to find $[Ca^{2+}]_{surf}$. In that study, we found that the PS-PS electrostatic repulsion is overwhelmed by other interaction(s), giving rise to clustering of PS and of PC in mixtures of (16:0,18:1)PS with three different PCs. We predicted that the nonideal mixing was so pronounced that phase separation should occur over some range of PS mole fractions. Herein, we report finding this fluid-fluid lamellar phase separation.

## MATERIALS AND METHODS

### Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine, (16:0,18:1)PS, was from Avanti Polar Lipids, Inc. (Birmingham, AL) and was greater than 99% pure when 0.1 mg was chromatographed on Adsorbosil plus TLC plates (Alltech Associates, Inc., Deerfield, IL) using chloroform/methanol/water (65/25/4, vol/vol) or chloroform/methanol/concentrated ammonium hydroxide (65/25/5, vol/vol); the chromophoric calcium chelators 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and 1,2-bis-(*o*-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BrBAPTA) were from Molecular Probes (Junction City, OR); piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (Pipes) was BioChemika MicroSelect grade and the ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was of puriss grade from Fluka Chemical Corp. (Ronkonkoma, NY); calcium ion and phosphate standards were of analytical grade from J. T. Baker, Inc. (Bricktown, NJ); 1,1′-carbonyldiimidazole (CDI) and 1,8-diaza-bicyclo-[5.4.0]undec-7-ene (DBU) were from Aldrich Chemical Co. (Milwaukee, WI); 1-α-glycerophosphorylcholine (GPC·$CdCl_2$) was Grade 1 from Sigma Chemical Co. (St. Louis, MO); 11-dodecenoic acid (C12:1) was from Nu-Chek-Prep, Inc. (Elysian, MN); Chelex-100 ion-exchange resin and Bio-Sil A were from Bio-Rad Labs (Rockville Center, NY). Water was purified with a Milli-Q system (Millipore Corp., Bedford, MA). Chloroform and methanol were high-performance liquid chromatography grade, and the potassium chloride was of analytical reagent grade from Mallinkrodt, Inc. (Paris, KY).

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## Lipid synthesis

(12:1,12:1)PC was synthesized by a modification of the procedure of Boss et al. (1975). Dodecenoic acid and GPC·$CdCl_2$ were dried over $P_2O_5$ for several days. Each was then dissolved in a minimal amount of dry DMSO. The imidazolide was prepared by adding a 5% excess of CDI to the fatty acid. The organic base catalyst DBU was added in equimolar quantity to the GPC·$CdCl_2$. The two solutions in DMSO were then combined, and the reaction carried out at 45°C for 24 hours with stirring in the dark. The PC was purified from the crude reaction mixture by silicic acid chromatography on a Bio-Sil A column, eluting stepwise from chloroform/methanol, 65/25, vol/vol, to chloroform/methanol/water, 65/25/3, vol/vol. The product (12:1,12:1)PC was obtained in 65% purified yield, and was found to contain <0.5% impurity based on thin-layer chromatography of 0.1 mg.

## Measurement of $Ca^{2+}$ binding

The preparation of samples was essentially as described by Swanson and Feigenson (1990), except that the removal of contaminating $Ca^{2+}$ from the buffer (100 mM KCl, 5 mM Pipes, pH 7.0 or 800 mM KCl, 5 mM Pipes, pH 7.0) was effected by passing the buffer through a column of Chelex, rather than by batchwise equilibration with Chelex. Multilamellar dispersions of PS/PC were prepared from lipid mixtures that had been lyophilized from benzene/methanol, 19/1 vol/vol, and $[Ca^{2+}]_{aq}$ was measured using the $Ca^{2+}$ chelator/indicators BAPTA and BrBAPTA, as described (Swanson and Feigenson, 1990) where $[Ca^{2+}]_{aq}$ was measured by a spectroscopic assay. The basis of the assay is the measurement of the absorbance at the peak of the difference spectrum at a fixed wavelength for the calcium-bound and unbound chelators. The aqueous calcium ion concentration was calculated from

$$[Ca^{2+}]_{aq} = K_D \frac{[Ca^{+2} - \text{chelator}]}{[\text{chelator}]} = K_D \frac{A' - A_0'}{A_1' - A'} \tag{2}$$

where the $K_D$ is the dissociation constant of the chelator for $Ca^{2+}$, $A_0$ is the absorbance of the unbound chelator, $A_1$ is the absorbance of the $Ca^{2+}$-chelator complex, and $A$ is the absorbance of the chelator at a given $[Ca^{2+}]$. The prime indicates division by the absorbance at the isosbestic point. The $K_D$ of BAPTA in 100 mM KCl, 5 mM Pipes, pH 7.0 was previously measured as 0.14 $\mu$M (Dibble, 1993). The $K_D$ of BAPTA and BrBAPTA were measured by titrating the chelators with $Ca^{2+}$ in 800 mM KCl, 5 mM Pipes, pH 7.0. The absorbance at the peak of the difference spectrum was measured as a function of the total $Ca^{2+}$ concentration and the data fit to the relationship derived from Eq. 2 and mass balance:

$$[Ca^{2+}]_{total} = K_D \frac{(A' - A_0')}{(A_1' - A')} + [\text{chelator}]_{total}\left\{\frac{(A' - A_0')}{(A_1' - A')} \Big/ \left[\frac{(A' - A_0')}{(A_1' - A')} + 1\right]\right\} \tag{3}$$

where the $K_D$, the single adjustable parameter, is obtained by optimizing the fit to the titration curve (Yeager, 1989). The average $K_D$ calculated for BrBAPTA was 10.5 ± 0.5 $\mu$M and for BAPTA was 1.4 ± 0.1 $\mu$M.

The $[Ca^{2+}]_{aq}$ was measured repeatedly over the course of the experiment. The first part of the assay involves a binding period of 1–2 weeks, which was sufficient for 5–10% of the PS to be incorporated into the $Ca(PS)_2$ phase. $[Ca^{2+}]_{aq}$ decreases as $Ca(PS)_2$ forms. The second part of the assay is to find the equilibrium value, $[Ca^{2+}]^*_{aq}$, by reducing $[Ca^{2+}]_{aq}$ below its equilibrium value by addition of chelator to the supernatant. The amount of chelator to add was calculated to dissolve 10–20% of the $Ca(PS)_2$ phase. The dissolving $Ca(PS)_2$ releases PS to fluid PS/PC, and $Ca^{2+}$ to the aqueous phase, until equilibrium is reached. The equilibrium value $[Ca^{2+}]^*_{aq}$ did not vary with further addition of chelator, or with time. The entire procedure takes 40–50 days.

The experimental objective is to find the activity coefficient, $\gamma_{PS}$, in the fluid bilayer mixture of PS/PC. The measured value of $[Ca^{2+}]_{aq}$ is used to find the concentration of $Ca^{2+}$, $[Ca^{2+}]_{surf}$, that reacts with the PS, as described in Huang et al. (1993). With a standard state of $\gamma_{PS} = 1$ for mole fraction $\chi_{PS} = 1$, the definition of PS thermodynamic activity, $a_{PS} = \gamma_{PS}\chi_{PS}$, and following Feigenson (1989):

$$\gamma_{PS} = \left(\frac{[Ca^{2+}]_{surface,\chi_{PS}=1}}{[Ca^{2+}]_{surface,\chi_{PS}}}\right)^{1/2} \Big/ \chi_{PS} \tag{4}$$

## Low-angle x-ray diffraction

(16:0,18:1)PS/(12:1,12:1)PC dispersions were prepared from mixtures that had been lyophilized from benzene/methanol, 19/1, vol/vol, or else from mixtures that had been dried directly from chloroform solution. After drying under mechanical vacuum pumping overnight, the 3 $\mu$M total lipid samples were hydrated with 0.5 ml of buffer containing 20 mM Pipes, 1 mM EDTA, pH 7.0, and either 100 or 800 mM KCl. Thus, these samples were not identical to those examined by means of the $Ca^{2+}$ binding assay, because we did not want to form any of the $Ca(PS)_2$ phase with the attendant numerous Bragg reflections in both low and wide-angle regions of the diffraction pattern. After a hydration period, under argon and in the dark, of 2 hours to 8 days, each sample was transferred to a 0.8 mm diameter thin-walled glass capillary (Charles Supper Co., Natick, MA). Lipid was concentrated by centrifugation for several minutes at ~2000 × $g$. X-ray diffraction measurements were made using wiggler-enhanced monochromatic, focused x-rays at the Cornell High-Energy Synchrotron Source (CHESS). Both sample-to-film distance and wavelength were measured for each run. X-ray diffraction patterns were recorded on x-ray-sensitive film (Eastman Kodak Co., Rochester, NY). For some samples, wide-angle diffraction was also collected, using film and image plates (Fuji Photo Film Co., Japan).

## $^{31}$PNMR spectroscopy

Samples containing about 3 $\mu$M total lipid were briefly centrifuged, supernatant removed, and the pellet solubilized in 1 ml of a solution of 10% $K^+$ cholate, 10% $D_2O$, 0.1 M EDTA, pH 7.7. The sample solution was transferred to a 5 mm diameter NMR tube. Proton noise-decoupled free induction decays were collected at 20°C using a Varian VXRS-400 spectrometer operating at 161.9 MHz.

## RESULTS

The approach to equilibrium is shown in Fig. 1. The starting value of $[Ca^{2+}]_{aq}$ exceeds the equilibrium value by about 25-fold, so $Ca^{2+}$ binds to PS during this initial period of

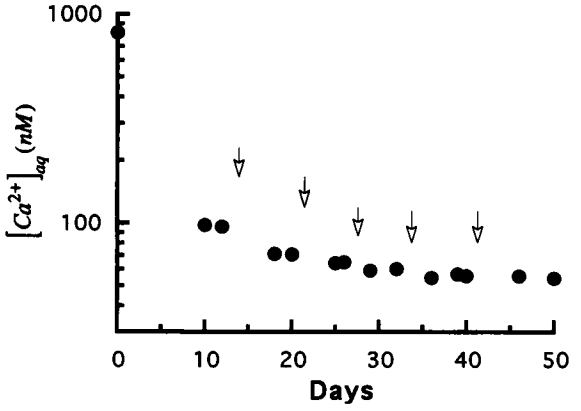


FIGURE 1 Semilog plot of the approach of $[Ca^{2+}]^*_{aq}$, to the value in equilibrium with a PS/PC mixture of $\chi_{PS} = 0.56$. The approach to equilibrium followed much the same course for the other samples. Arrows indicate addition of chelator. Temperature 20°C, buffer 100 mM KCl, 5 mM Pipes, pH 7.0.

10–14 days, as shown by the loss of aqueous $Ca^{2+}$. The value of $[Ca^{2+}]_{aq}$ is then gradually reduced by addition of chelator, until time and more chelator still yield the same value over a period of 1–2 weeks.

The duration of the binding experiment is so long that we must test for any breakdown of the chelator or the lipids. Changes in the absorbance spectra of the chelators upon exposure to light and/or oxygen cause the calculated values of $[Ca^{2+}]_{aq}$ to vary significantly with wavelength. This proved to be a reliable and sensitive way to monitor stability of the chelator. We found BrBAPTA and BAPTA to be stable over the entire course of our experiments. Two methods were used to test for lipid breakdown. By use of $^{31}$PNMR spectroscopy of PC/lysoPC standards, as little as 0.5% lysolipid could be detected (London and Feigenson, 1979). For PS/PC mixtures examined after the 7-week $Ca^{2+}$ binding experiment, no resonances corresponding to lysolipids were detected. Thin-layer chromatography of the centrifuged, dried pellet after the 7-week experiment showed low levels of fatty acid and lysolipid breakdown products, estimated to be about 0.5%.

Fig. 2 shows the variation in equilibrium aqueous $Ca^{2+}$ concentration as a function of the PS mole fraction. To facilitate comparison of different experiments, to show the full range of concentrations, and also to clearly show the interesting regions, the $Ca^{2+}$ concentration is normalized to the value that is in equilibrium with pure PS, and the logarithm is plotted. Instead of a monotonic decrease in $[Ca^{2+}]^*_{aq}$ with increasing PS concentration, as we have observed for other PS/PC mixtures (Feigenson, 1989; Swanson and Feigenson, 1990), there are three distinct regions: 1) an initial steep drop in $[Ca^{2+}]^*_{aq}$ at lower PS mole fractions; 2) a virtually flat region (in 100 mM KCl) from $0.52 \leq \chi_{PS} \leq 0.62$ wherein $[Ca^{2+}]^*_{aq} = 55$ nM; and 3) a gradual drop in $[Ca^{2+}]^*_{aq}$ until PS mole fraction = 1.0. The values of $\chi_{PS}$ at the boundaries, which are uncertain by about $\pm 0.02$, were determined by the intersections of straight lines, with the entire isotherm approximated simply as three straight lines. Since we suspected that the flat region corresponded to extreme demixing resulting in phase separation, and since the PS-PS electrostatic repulsion favors mixing, the experiment was repeated at 800 mM KCl to reduce the mixing and thereby to expand the phase coexistence region. Again, there are three distinct regions of $Ca^{2+}$ binding, with the flat region at $0.50 \leq \chi_{PS} \leq 0.80$ (both boundaries having $\chi_{PS} \pm 0.05$) and $[Ca^{2+}]^*_{aq} = 750$ nM.

Samples prepared at 100 mM KCl were examined by x-ray diffraction, but the low angle lines were too diffuse to permit analysis. In contrast, samples prepared at 800 mM KCl gave rise to much sharper diffraction. The wide-angle region showed the diffuse line centered at 4.6 Å, characteristic of fluid lipid. Samples that were equilibrated for 1 week before transfer to capillaries for measurement showed a set of low angle lines that indexed to a single lamellar repeat (data not shown; a faint line, sometimes appearing to be the edge of a more diffuse disk, was present in most samples, including pure PC or pure PS. This line corresponded to a spacing of about 50–55 Å with an intensity that seemed to depend on organic solvent and on hydration time. This line might result from diffraction by uni- or paucilamellar vesicles.) In contrast, some samples that were equilibrated for 2 hours or for 24 hours showed double lines in the low angle region. The lamellar repeat is plotted against $\chi_{PS}$ in Fig. 3. The region showing the additional lamellar repeat is from $\sim 0.5 \leq \chi_{PS} \leq 0.8$. The photographic film showed barely resolved doubling of the line at $n = 2$. Resolution of the doubling in the $n = 1$ line was even more difficult. Densitometer scans within and just outside this region are shown in Fig. 4.

The lateral distribution of PS/PC for a given value of $\chi_{PS}$ can be found by a Monte Carlo simulation in which the Hamiltonian of a PS/PC mixture is represented by an electrostatic

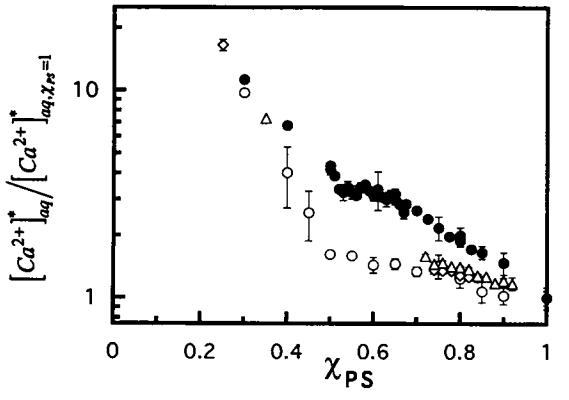


FIGURE 2 Semilog plot of the equilibrium calcium ion concentration as a function of $\chi_{PS}$ in the PS/PC mixture. Error bars not shown are smaller than the size of the symbol. The measured equilibrium value $[Ca^{2+}]^*_{aq}$ at a given $\chi_{PS}$ is normalized by division by the $[Ca^{2+}]^*_{aq}$ measured at $\chi_{PS} = 1.00$. 100 mM KCl, 5 mM Pipes, pH 7.0 (●). 800 mM KCl, 5 mM Pipes, pH 7.0 using BrBAPTA (○) or BAPTA (△).

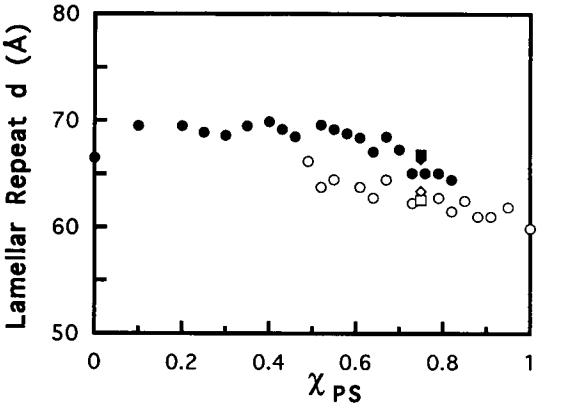


FIGURE 3 Lamellar repeat for (16:0,18:1)PS/(12:1,12:1)PC as a function of $\chi_{PS}$ in 800 mM KCl, 20 mM Pipes, 1 mM EDTA, pH 7.0. X-ray diffraction data were collected at the F1 station at CHESS using a 0.1-mm collimator, a wavelength of 0.908 Å, and a measured sample-to-film distance of 428.4 ± 0.2 (●, ○) or 689.4 ± 0.4 mm (■, □, ◆, ◇). Data were collected on x-ray-sensitive film at ambient temperature 22 ± 1°C. Where resolution permitted, the lamellar repeat was calculated as an average of the $n = 1$ and $n = 2$ spacings. Some samples were prepared by drying lipid mixtures from solution in chloroform (◆, ◇). Other samples were prepared from mixtures lyophilized from benzene/methanol.

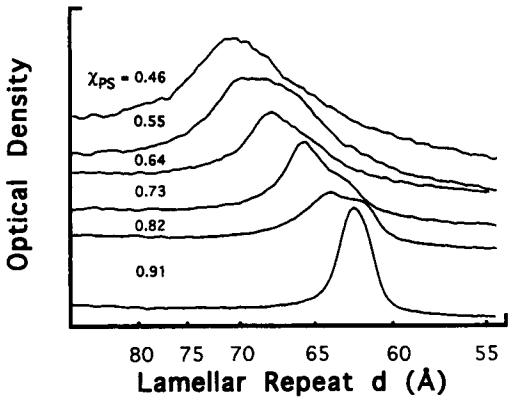


FIGURE 4 Optical density from densitometer scans of x-ray-sensitive film plotted against molecular spacing for PS/PC samples just outside as well as within the two-phase region. Optical densities were not normalized. Densitometer scans were radially averaged.

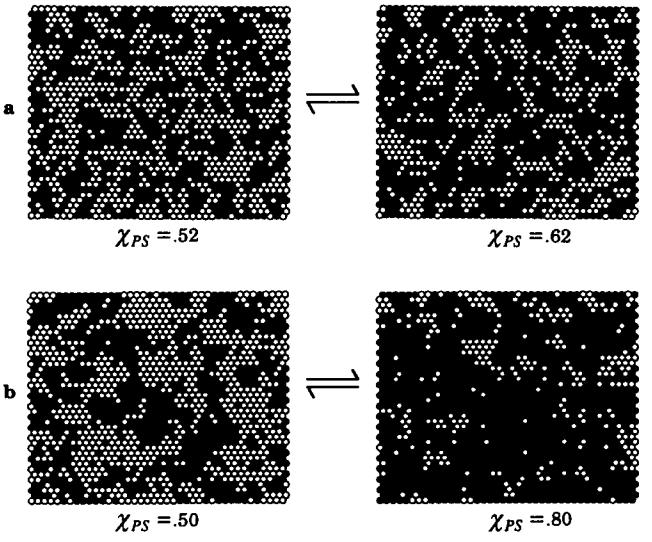


FIGURE 5 Snapshot pictures of the lateral distribution of PS/PC mixtures in equilibrium within the two-phase coexistence region for two different ionic strength buffers. 100 mM KCl (*a*); 800 mM KCl (*b*). Monte Carlo simulations were performed on 100 × 100 lipid arrays at the PS mole fractions $\chi_{PS}$ corresponding to the observed phase boundaries, and having $\Delta E_m$ giving the observed width of the two-phase region. For clarity, 40 × 40 lipid arrays are shown.

term, $U^{el}$, and one other term, $\Delta E_m N_{PS-PC}$, that includes all other interactions (Huang et al., 1993). Previously, we have shown how to choose the simulated distribution that corresponds in total Gibbs free energy, $\Delta G^T_{mix}$, to a measured value of $[Ca^{2+}]^*_{aq}$. For values of $\Delta E_m$ so large that phase separation occurs, the phase boundaries are determined by the double tangent method (i.e., $\partial \Delta G^T_{mix}(\chi = a)/\partial\chi = \Delta G^T_{mix}(\chi = b)/\partial\chi$, where $a$ and $b$ are phase boundaries; Guggenheim, 1952). Since Fig. 2 shows a reasonably well-defined width of the two-phase region, we can simulate the lateral distribution of PS/PC that corresponds both to the observed values of $\chi_{PS}$ at the boundaries, and to a value of $\Delta E_m$ that gives the observed width of the two-phase region. These simulated lateral distributions are shown in Fig. 5. More details of this simulation procedure are described in Huang and Feigenson (1993).

## DISCUSSION

### Experimental

A full description of the properties of a multicomponent model biomembrane should include the activity coefficients of the components. However, there are few reports of techniques to find the activity coefficients (Shin et al., 1993). The measurement of the aqueous $Ca^{2+}$ concentration that is in equilibrium with a fluid PS/PC mixture and with $Ca(PS)_2$ is such a technique (Feigenson, 1989; Huang et al., 1993).

We have previously shown that this $Ca^{2+}$ binding technique does not depend upon details of sample drying or sample support (Swanson and Feigenson, 1990). The chelator-indicator is chosen so that the measured $[Ca^{2+}]_{aq}$ is within a factor of ~10 of the chelator $K_D$. In this study, we have used two chelator-indicators, BrBAPTA and BAPTA, having $K_D$ values to cover the needed range.

Because each experiment requires about 50 days, it is important to test for any breakdown of the chelator or the lipids. A spectrophotometric assay showed no breakdown (<0.5%) of the chelators. A $^{31}$PNMR spectroscopic assay and thin layer chromatography showed little breakdown (<0.5%) of the lipids.

### Prediction

In recent work (Huang et al., 1993) we found that PS/PC fluid lamellar mixtures of (16:0,18:1)PS with (14:1,14:1)PC, or (18:1,18:1)PC, or (16:0,18:1)PC all show positive deviations from ideality. That is, PS is clustered (as is PC), despite the electrostatic repulsion between PS headgroups. In that study, Monte Carlo simulations of the PS/PC distributions were performed to explore both the influence of PS clustering on the calculation of $[Ca^{2+}]_{surf}$ and also the effects on the lipid distributions of the electrostatic contribution to the excess energy, $U^{el}$, and all other contributions, $\Delta E_m$. In terms of the excess free energy of mixing, $\Delta G^E_{mix}$, the calculated deviations from ideality were quite large. For (14:1,14:1)PC and (16:0,18:1)PC at $\chi_{PS} = 0.5$, $\Delta G^E_{mix} = 0.25$–$0.4$ RT. For mixtures with (18:1,18:1)PC, $\Delta G^E_{mix} \approx 0.7$ RT at $\chi_{PS} = 0.5$. This latter value would require a phase separation at $\chi_{PS} = 0.5$, although we had not made measurements in this range of PS mole fraction for this lipid mixture.

In the present study, we sought to test the prediction of phase separation. We used (16:0,18:1)PS mixed with the shortest chain PC that would be in a lamellar phase, (12:1,12:1)PC. In this way the acyl chain mismatch would contribute to $\Delta E_m$ to enhance nonideality.

### Invariant $[Ca^{2+}]^*_{aq}$?

Fig. 2 shows a nearly constant value for $[Ca^{2+}]^*_{aq}$ over a range of PS mole fractions. A truly constant value, i.e., loss of one degree of freedom, would strongly support phase separation (Lewis and Randall, 1961). However, the observed $[Ca^{2+}]^*_{aq}$ values show a small increase toward the

lower $\chi_{PS}$ side of the putative two-phase region. One difficulty with this assay is that finite $Ca^{2+}$ binding to form $Ca(PS)_2$ decreases the actual value of $\chi_{PS}$ in a poorly characterized way: some lamellae, especially those nearer the outside, will be exposed to sufficient $Ca^{2+}$ during the time course of the experiment to enable $Ca(PS)_2$ formation, with consequent depletion of PS from the coexisting PS/PC in that region. Inner lamellae would hardly be exposed to $Ca^{2+}$ and thus would have the unperturbed, original PS/PC concentration. That is, the binding of $Ca^{2+}$ decreases the value of $\chi_{PS}$. For this reason, the amount of $Ca^{2+}$ binding is kept to a minimum, generally in the range of 0.02–0.07 of the total amount of PS. This effect is expected to be more pronounced at lower values of $\chi_{PS}$, where loss of PS could move the composition out of the phase coexistence region, and hence into a region of strongly changing $[Ca^{2+}]^*_{aq}$. However, we cannot, on the basis of this experiment alone, rule out the possibility that the nearly constant value of $[Ca^{2+}]^*_{aq}$ is, in fact, gradually changing, with the apparent boundary near $\chi_{PS} = 0.5$ actually part of a continuous change in properties of a single phase.

## X-ray diffraction

Coexisting fluid lamellar phases would in general have different bilayer thickness. If this characteristic thickness plus the interlamellar aqueous layer exist as stacks of this phase, then there is a possibility to detect separate lamellar repeats in the low angle region of the x-ray diffraction pattern. Working against detection are the following factors (see Blaurock, 1982, for a review): 1) ill-defined lamellar repeats, as are found for some negatively charged mixtures (Bach et al., 1992); 2) lamellar spacings that are too similar to be resolved by the experiment; and 3) coexisting phases that are not present in orderly stacks.

Diffraction from all PS-containing samples that were prepared in buffer with 100 mM KCl showed very diffuse scattering in the low angle region. The lamellar repeat could not be determined. However, the samples prepared with 800 mM KCl showed low angle lines that were sufficiently narrow that at least two diffraction orders were detected, and the lamellar repeat could be measured. Samples of PS/PC that had been hydrated for 2 hours or for 24 hours before pipetting into thin-walled capillaries revealed two lamellar repeats over a range of PS mole fractions. These lamellar repeats are shown in Fig. 3. The scale of $\chi_{PS}$ is the same as that in Fig. 2, to facilitate comparison. There is agreement between the nearly constant region of $[Ca^{2+}]^*_{aq}$ and the region showing two lamellar repeats. The x-ray diffraction data recorded on film show a double line for $n = 2$. The densitometer tracings, shown in Fig. 4, are perhaps more ambiguous, although we have not attempted to deconvolute the curves. It is apparent that the lamellar repeats are not quite constant in the putative two-phase region. We would expect constant lamellar spacings only if the two coexisting phases were entirely separated, i.e., not stacked together. We are surprised that the phases should be stacked separately enough to give rise to resolved lamellar repeats.

Samples of PS/PC that had been hydrated for 1 week before pipetting into capillaries showed no evidence for more than one lamellar repeat. It is possible that regions of different lamellar spacing become mixed, so that stacks of a well-defined repeat disappear. For comparison, samples that were pipetted into capillaries 24 hours after hydration and did show two lamellar repeats when first examined, again showed the two lamellar repeats 6 months later (data not shown). The reason for this complex time and, perhaps, sample preparation dependence of the appearance of two lamellar repeats has not been established yet. We suggest that within the two-phase region of $\chi_{PS}$, stacks of each phase type form initially. These stacks might be pinched off during the process of pipetting into a capillary, and in this condition retain their well-defined lamellar repeat.

We cannot rule out entirely an influence of sample preparation on the observed phase separations. Gruner et al., (1985) showed that egg PC multilamellar liposomes, incubated for 2 hours in buffer, did not equilibrate the ions in the buffer across the lamellae. As a result, these liposomes were under osmotic compression. In our $Ca^{2+}$ binding experiments, we saw invariant behavior after about 40 days, but we could not detect whether the lipid mixing was influenced by the initial stages of hydration. However, the x-ray diffraction patterns obtained within 1 day of sample hydration showed evidence of phase separation consistent with that found by $Ca^{2+}$ binding measurements after much longer times.

What is the significance of our finding of fluid-fluid lamellar phase coexistence at equilibrium? Although there is overwhelming evidence for heterogeneity of composition in the plane of real biomembranes (see review by Edidin, 1990; Dupree et al., 1993; Gennis, 1989), as well as vastly different protein and lipid composition of the membranes of organelles in the same cell (Bretscher and Munro, 1993; review by Pagano, 1990), these membranes are not at equilibrium. Thus, kinetic or far-from-equilibrium factors might control the observed heterogeneity: 1) site-specific delivery of components (see review by Thompson et al., 1992); 2) facilitated or energy-dependent transbilayer diffusion (Connor et al., 1992); or 3) binding of membrane proteins to cytoskeletal or other cellular proteins (see review by Kinnunen, 1991). If fluid-fluid lamellar phase separation were to occur in real biomembranes, then proteins and lipids could distribute heterogeneously according to, e.g., charge, length, or hydrogen bonding. However, equilibrium fluid-fluid phase separation has not been proven in any biomembrane, and has been exceedingly difficult to detect even in chemically simple model membranes (Silvius, 1986). Webb et al. (1993) report lamellar fluid-fluid coexistence for partially dehydrated mixtures of (18:1,18:1)PE/(18:1,18:1)PC. However, only for saturated PC/cholesterol is there agreement among many different research groups for coexistence of liquid ordered-liquid disordered phases (Vist and Davis, 1990; Sankaram and Thompson, 1990; Recktenwald and McConnell, 1981).

Our view is that the thermodynamic activity coefficients of membrane components provide a type of information not previously available that has some power to enable prediction of the tendency of membrane components to cluster, to change phase, to chemically react, or to transfer to another membrane. The problem is to find a type of experimental result that can be interpreted to yield the thermodynamic activity coefficients. Detection of fluid-fluid lamellar phase coexistence is such a clear observation that the interpretation, strong positive deviation from ideal mixing, is rather insulated from errors in data analysis or defects in models of the interactions. We plan to use the compositions at the phase boundaries, together with computer simulations, to find relationships between the lipid thermodynamic activity coefficients and the structure of the molecules in the model membrane mixture.

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