Detection of Coexisting Bilayer Gel and Fluid Phases by Equilibrium Surface Pressure Analysis

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A study of bilayer phase behavior is presented for the phospholipid mixture di12:0-PC/di16:0-PC, based on an analysis of equilibrium surface pressure values which develop at the gas–water interface above suspensions of bilayer vesicles. At 22 ± 1 °C, the fluidus and solidus bilayer phase boundaries are determined to be 32 and 85 mol % di16:0-PC, respectively, consistent with literature values obtained through calorimetry. Evidence is presented which suggests that Teflon substrates are better suited than high-energy substrates for surface pressure measurements involving insoluble monolayers.

Introduction

The phase behavior of lipid mixtures has long been the subject of study, either to provide models for biological membranes1 or as interesting states of matter in their own right.2,3 The liquid crystalline, fluid-phase bilayer is the predominant physical state in biological membranes, but the gel-phase bilayer state has also been observed.4 Even nonlamellar lipid phases have been suggested to play important roles in nature.5 Model biomembrane phase behavior has been studied extensively as a function of temperature and pressure. But in addition, much published research has been devoted to the construction of phase diagrams in composition space.6,7 Efforts have sought insight into the fundamental interactions between biomembrane lipid species through the systematic exploration of lipid mixture phase behavior. Of particular importance to such studies have been the techniques of X-ray diffraction8 and differential scanning calorimetry, or DSC,9 as well as a variety of spectroscopic techniques.10

The most fundamental physical property of biomembrane lipids is their tendency to self-assemble into lamellar structures in an aqueous environment, and this property is reflected in their surface activity.11 Indeed, much research has focused on lipid mixtures at the air–water interface. These interfacial monolayers have been treated as models of biomembrane leaflets and have generally been prepared by the direct application of lipid to a gas–water interface.11 For many decades, these so-called “insoluble” monolayers have been the object of considerable research.12 In addition, a few workers have studied the development of these monolayers by a more gradual method: deposition from an aqueous lipid suspension beneath the interface.13–15

In this work, we pursue a strategy for the study of model biomembrane phase behavior which exploits the surface activity of membrane lipids.16 Rather than focusing on the gas–water monolayer itself as an object of study, we use our experimental access to the monolayer in order to probe the phase behavior of bilayer membranes in the subphase. We refer to this approach as equilibrium surface pressure analysis (ESPA). First, a series of unilamellar bilayer suspensions is prepared, spanning the composition range of interest. Each suspension is then allowed to come to equilibrium with its gas–water interface, forming an interfacial monolayer in which the chemical potential of each lipid species is the same as in the bilayer suspension. Finally, the equilibrium surface pressure (σ_eq) of each fragile interfacial film is determined by a low-perturbation technique which employs a substrate made of Teflon. A profile of σ_eq values is presented for a binary lipid mixture which is known to manifest coexisting gel–bilayer and fluid–bilayer phases. This profile reveals the compositional phase boundaries between fluid-phase bilayer, gel-phase bilayer, and the regime of two-phase coexistence.

Experimental Section

Materials. di12:0-PC and di16:0-PC were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Purity (>99.5%) was confirmed by thin-layer chromatography (TLC) on washed, activated silica gel plates (Alltech Associates, Inc., Deerfield, IL), developing with chloroform/methanol/water (99.5:25:4). Triton X100, PIPES buffer, and disodium EDTA were all purchased from Fluka Chemie AG (Switzerland). Platinum wire (1 mm diameter), methanol, SDS, and NaN3 were obtained from Fisher Scientific (Fair Lawn, NJ). All solvents used were of HPLC grade. TLC plates were quantitated by charring and densitometry. Phospholipid stock solutions were quantitated by phosphate assay.17 Aqueous buffer (5 mM PIPES pH 7.0, 200 mM KCl, 1 mM EDTA, 1.5 mM NaN3) was prepared from purified water (Milli-Q system, Millipore Corp.) and filtered through a 0.1 μm filter before use. Polycarbonate membranes (0.1 μm) were the product of Costar Corp. (Cambridge, MA). Polonium foil was prepared according to a procedure described elsewhere.18

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Table 1. Surface Tension Measurements on Pure Liquids, Insoluble Films, and Soluble Films: Comparison of Teflon and Platinum Substrates

<table>
<thead>
<tr>
<th>sample</th>
<th>γfilm</th>
<th>γads</th>
<th>γTeflon</th>
<th>γPlat</th>
<th>γTeflon - γPlat</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer</td>
<td>73.0</td>
<td>72.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>22.5</td>
<td>22.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X16 = 0</td>
<td>24.0</td>
<td>26.3</td>
<td>49.0</td>
<td>46.6</td>
<td>2.4</td>
</tr>
<tr>
<td>X16 = 0.50</td>
<td>26.1</td>
<td>31.3</td>
<td>46.9</td>
<td>41.6</td>
<td>5.3</td>
</tr>
<tr>
<td>X16 = 0.98</td>
<td>36.5</td>
<td>45.5</td>
<td>36.5</td>
<td>27.4</td>
<td>9.1</td>
</tr>
<tr>
<td>Triton X100</td>
<td>30.9</td>
<td>34.1</td>
<td>42.1</td>
<td>41.5</td>
<td>0.6</td>
</tr>
<tr>
<td>SDS</td>
<td>37.1</td>
<td>37.4</td>
<td>35.9</td>
<td>35.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a Experimental results obtained with either a Teflon or a platinum rod are shown for pure liquids (buffer, MeOH), insoluble films (0.5 mM di16:0-PC/di16:0-PC LUV; X16 = 0.50, 0.98), and soluble films (0.5 mM Triton X100, SDS at 22 ± 1 °C). Values are expressed in units of mN/m. Tabulated data are averaged from four replicate samples, and the uncertainties are ± 0.1 mN/m for surfacetensions and ±0.2 mN/m for surfacepressures. Sub-atmospheric micelle concentration.

obtained from NRD, Inc. (Grand Island, NY). FEP Teflon is polytetrafluoroethylene—hexafluoropropane copolymer, a thermoplastic melt-form of Teflon. FEP rod stock was purchased from Berghof America, Inc. (Concord, CA), and FEP Teflon Oak Ridge tubes were the product of Nalge Co. (Rochester, NY). PTFE Teflon is poly(tetrafluoroethylene), a commonly used machinable form of Teflon produced from powdered material by a pressing process. PTFE materials were machined in a facility maintained by the Cornell Laboratory of Atomic and Solid State Physics.

Sample Preparation. Membrane suspensions were prepared by Rapid Solvent Exchange. This method was developed specifically for the preparation of homogeneously mixed, multi-component membrane suspensions and has recently been described in detail. Briefly, lipids were codissolved in 80 μL of chloroform (0.1% MeOH, 0.05% H2O) and then sprayed into a vortexing, high-surface-area cylindrical shell of buffer at reduced pressure. This rapidly vaporizes the organic solvent and pre-cipitates the lipid mixture in an aqueous environment. Large unilamellar vesicles (LUVs) were produced by extruding the suspensions at 50 °C (10 passes through two stacked 0.1 μm polycarbonate membranes) using a modified extruder apparatus (Lipex Biomembranes, Vancouver, BC). The suspensions were then sealed under argon and annealed in a 50 °C water bath which was cooled to room-temperature overnight.

After being annealed, room-temperature samples were transferred to clean FEP Teflon tubes with a siphon, which was rinsed with buffer between transfers. Samples which have been transferred in this way are free of initial surface pressure (data not shown). The tubes were sealed under argon and placed upright in a rotating sample holder (50 tube capacity). This sample holder can be slowly rotated in the horizontal plane during sample incubation, so that gentle mixing is promoted in the suspension can be slowly rotated in the horizontal plane during sample incubation, driving surface pressures to subequilibrium values. The procedure is outlined below.

Relaxation of Surface Pressures from Superequilibrium Values. As a test of equilibrium, some monolayers were driven to higher values of surface pressure in order to see whether they would relax to their original values. Lipid associated with the Teflon–water interface along the sample tube walls was redistributed to the gas–water interface, driving surface pressures to subequilibrium values. The procedure is outlined below.

For each composition examined (0, 50, and 98 mol % di16:0–PC), the tube was rotated into position for surface tension measurement. Just before measurement began, a long stainless steel needle (21 gauge, 10 cm in length) was gently guided down through the gas–water interface, and 5 mL of the suspension was delivered to each sample tube during measurement. The Teflon rod was cleaned between samples using a series of solvents. The platinum rod was cleaned between samples by heating the tip in a blue flame until it glowed. Because of its small diameter, the platinum rod cooled very quickly, but the measurement procedure provided more than 10 min between flaming and commencing a measurement.

Production of Teflon and Platinum Rods. The Teflon rod was produced from 2 mm diameter FEP stock which had been softened in a heated air stream before being drawn out to a neck of <0.9 mm diameter. After cooling, the neck was cut with a razor and the thin end of the stock was then sealed through a 0.940 mm hole at the bottom of a socket (5.13 mm i.d.), in a piece of PTFE. The socket holds a flat shoulder seated for the barrel, and the 0.940 mm hole opens onto a flat cutting face along which a thin razor blade can be moved. This PTFE assembly serves to hold the FEP rod perpendicular to the cutting face as the rod stock is turned (~100 rpm), and the blade is slowly advanced toward the rod axis on a threaded drive. This cutting procedure produces a flat-bottomed FEP rod with a defined cutting edge, which is easier than the grinding process we have described previously.

Some tooling grooves are evident on the rod bottom, but these are of such a scale that they do not amount to surface roughness and the wetting characteristics are that of a polished rod bottom. To orient the FEP rod perpendicular to the sample surface, a stainless steel hanging hook is centered on axis at the other end of the PTFE barrel. A sleeve of polonium foil surrounds the bottom end of the barrel, to reduce the end charge near the sample surface.

The platinum rod was produced from 1 mm diameter wire stock. To ensure zero contact angle, the platinum stock was roughened with abrasive paper (no. 1200 corundum) before being straightened by gentle rolling between flat-faced blocks of stainless steel. The straightened stock was then fit into a PTFE Teflon barrel, as above. The rod bottom was then sealed under argon and annealed in a 50 °C water bath which was cooled to room-temperature overnight.

After being annealed, room-temperature samples were transferred to clean FEP Teflon tubes with a siphon, which was rinsed with buffer between transfers. Samples which have been transferred in this way are free of initial surface pressure (data not shown). The tubes were sealed under argon and placed upright in a rotating sample holder (50 tube capacity). This sample holder can be slowly rotated in the horizontal plane during sample incubation, so that gentle mixing is promoted in the suspension without disturbing the developing interfacial films. When the surface tension of any particular sample was to be determined, the sample was rotated into position beneath the balance. The force curve analysis employed by this variation makes it possible to use a low-energy substrate, such as Teflon, but a high-energy substrate like platinum can still be used. Rods of both Teflon and platinum were produced (see below), and comparative measurements were carried out with a rod bottom diameter of 1 mm and a 0.940 mm diameter of Teflon, but these are of such a scale that they do not amount to surface roughness and the wetting characteristics are that of a polished rod bottom. To orient the FEP rod perpendicular to the sample surface, a stainless steel hanging hook is centered on axis at the other end of the PTFE barrel. A sleeve of polonium foil surrounds the bottom end of the barrel, to reduce the end charge near the sample surface.

Rod bottom diameters were determined using the surface tension value of hexadecane, as previously described. Diameters obtained in this manner are consistent with measurements on microscopy, but are more precise. The Teflon and platinum rod bottoms were determined to be 0.929 and 0.915 mm in diameter, respectively.

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was carefully removed with a syringe. This volume reduction decreased the sample's Teflon–water interface by more than 40% (from 19.3 to 11.3 cm²), causing a redistribution of some interfacial lipid to the gas–water interface. The effect of this volume reduction was to cause the gas–water surface pressure to rise, approaching the collapse value. Immediately afterward, the sample was brought into contact with the Teflon rod and force curve collection began, without detachment, at increasing time points. In this way, the surface pressure of a single sample could be followed in time, as it relaxed back to its original value.

Results

Comparative Measurements with Teflon or Platinum Rods. An interesting pattern is present in the comparative surface tension data presented in Table 1. For aqueous buffer or methanol, both the Teflon and platinum rod yield the same experimental value of surface tension. In contrast to these pure liquids are the comparative results for “insoluble” films developed from suspensions of di12:0-PC/di16:0-PC unilamellar vesicles. For these latter samples, there is a marked difference in the experimental surface tensions, such that the Teflon rod consistently yields higher apparent surface pressures. The difference between δ_{Teflon} and δ_{Plat} is considerable, ranging from 2.4 to 9.1 mN/m, which amounts to 5–30% of the total apparent surface pressure. Furthermore, δ_{Teflon} − δ_{Plat} increases with the di16:0-PC content of the suspension. Such a large discrepancy between comparative surface pressures is not evident in measurements made on soluble film samples, though. For Triton X100 and SDS, δ_{Teflon} and δ_{Plat} agree within 1.5%. The Teflon rod was used for all subsequent measurements on insoluble films (Figures 1–3).

Development of Surface Pressure and Demonstration of Equilibrium. Time courses of surface pressure development are shown in Figure 1 for LUV suspensions of di12:0-PC/di16:0-PC unilamellar vesicles. Two different lipid suspension concentrations were examined at 22 ± 1 °C: 0.5 mM LUV (●) and 5.0 mM LUV (○). Panels a–d represent different compositions of the subphase bilayer phase: (a) $\chi_{120} = 0$ (pure di12:0-PC); (b) $\chi_{120} = 0.50$; (c) $\chi_{120} = 0.98$; (d) $\chi_{120} = 1.0$ (pure di16:0-PC). Samples in panels a–c came to a constant surface pressure which was stable indefinitely (>7 days).
Suspensions reach the same constant value of surface pressure within 24 h. A pure di16:0-PC suspension, however, is slow to approach a constant surface pressure, even at 5.0 mM lipid. For this reason, we limited our studies to compositions between 0 and 98 mol % di16:0-PC. As is evident from Figure 1, the rate of surface pressure development decreases with increasing di16:0-PC content.

Figure 2 demonstrates the relaxation of gas-water monolayers after being driven to superequilibrium values of surface pressure. These experiments show that, for compositions between 0 and 98 mol % di16:0-PC, the surface pressures return to their original values (cf. Figure 2 and Figure 1a–c). As with the rate of surface pressure development, the rate of relaxation decreases with increasing di16:0-PC content.

Discussion

Teflon vs Platinum Substrate. When seeking to determine the $\pi_{eq}$ of a fragile interfacial film that is slow to approach equilibrium, it is important to consider any perturbation which may be associated with the measurement itself. The high-energy substrates that are normally used for force-based methods of surface tension measurement are chosen to ensure strong wetting of the sample, so that a vanishing contact angle can be assumed. Our recently published variation on Padday's rod-pull method requires only that the contact angle be less than 180°. This allows us to make use of a low-energy substrate, Teflon.

At a minimum, the use of a low-energy substrate decreases the adhesion forces that could locally deplete the interfacial film during measurement. In addition, it is worth noting that monolayers are stable at a Teflon—water interface, just as at a gas—water interface. It seems plausible, therefore, to consider the possibility that a Teflon substrate could be less perturbing to a gas—water monolayer than conventional substrates. In contrast to Teflon, a strongly wetting substrate like platinum could deplete a monolayer on contact, and recovery of the surface film by deposition from the suspension would be very slow for an “insoluble” lipid suspension.

The data in Table 1 are consistent with this view. The Teflon and platinum rods produce comparable values of surface pressure for either of two dissimilar liquids, aqueous Teflon and platinum rods produce comparable values of surface tension for an “insoluble” lipid suspension.

The observations imply that the Teflon rod is less perturbing to surface films than conventional, strongly wetting substrates. Therefore, the Teflon rod has become our substrate of choice for surface pressure measurements on insoluble films. We are not the first group to note the advantage of a Teflon substrate for measurements involving insoluble monolayers. In a 1979 paper on phospholipid monolayer—vesicle fusion, Ohki and Duzgunes emphasized the superior performance of a Teflon Wilhelmy plate, reporting that the standard glass Wilhelmy plate produced erratic output at the microbalance.

Theoretical Basis for ESPA. That coexisting phases in the bilayer vesicle suspension should be manifest as a regime of invariant surface pressure follows from the fact that the chemical potentials of all components are constant throughout the system at equilibrium. In a system with two surface active components, the isothermal Gibbs relation for surface pressure may be written

$$d\tau = \Gamma_1 d\mu_1 + \Gamma_2 d\mu_2$$

where $\tau$ is the surface pressure, $\Gamma_i$ is the surface excess, and $\mu_i$ is the chemical potential of component $i$. If we limit our consideration to a system at equilibrium, we need not distinguish between $\mu_i$ for different phases (e.g., bilayer vs monolayer), since they are equal throughout the system. To see how the equilibrium surface pressure should depend on bilayer composition, we then write

$$d\tau_{eq}/d\chi_{bil} = \Gamma_1 (d\mu_1/d\chi_{bil})_{eq} + \Gamma_2 (d\mu_2/d\chi_{bil})_{eq}$$

where $\chi_{bil}$ is the composition of the bilayer suspension. If the system is in a regime of coexisting bilayer phases, then the chemical potentials of all components will be independent of bilayer composition (i.e., $(d\mu_1/d\chi_{bil})_{eq} = 0$, $(d\mu_2/d\chi_{bil})_{eq} = 0$), implying that

$$d\tau_{eq}/d\chi_{bil} = 0$$

throughout the regime. Tajima and Gershfeld have obtained this same conclusion from an argument based on the phase rule.

Experimental Strategy of ESPA. The strategy presented in this work is the same as that followed by Tajima and Gershfeld in 1978. According to this approach, a surface monolayer is allowed to develop and come to equilibrium with an aqueous bilayer suspension, which is actually the object of interest. The equilibrium surface pressure of this monolayer can then provide information on the phase behavior of the bilayer suspension. A regime of invariance with respect to bilayer composition ($d\tau_{eq}/d\chi_{bil} = 0$) indicates bilayer phase coexistence.

It is certainly essential to this strategy that the measured surface pressures be equilibrium values. In this work, we have made an effort to establish rigorously the equilibrium nature of our interfacial films. We have attempted to do this in three ways. First, for $0 < \chi_{bil} < 0.98$, we have shown that surface pressure develops spontaneously at the gas—water interface and reaches a stable value (Figure 1a—c). This process is slow, but not prohibitively so, for the above range of compositions; the timescale is on the order of $10^2$—$10^3$ min. Second, we have shown that suspensions differing 10-fold in lipid concentration reach the same stable value of surface pressure, for the indicated range of compositions (Figure 1a—c, cf. 0 and O). The only notable difference between these samples appears to be that surface pressure develops more rapidly above the more concentrated suspension. Third, we have shown that these stable surface pressures return to their original value when driven to higher values (Figure 2a—c), as expected for an equilibrium quantity. For these three reasons, we conclude that the stable surface pressures may be considered to be equilibrium values.

ESPA Phase Boundaries: Comparison with Literature. Three regimes are evident in the equilibrium surface pressure profile for di12:0-PC/di16:0-PC, between 0 and 98% di16:0-PC. These regimes are clearly revealed in Figure 3, which shows $\tau_{eq}$ values for $0 < \chi_{bil} < 0.98$. This lipid mixture has long been known to manifest coexisting gel- and fluid-bilayer phases, with phase boundaries determined by DSC and reported by Van Dijck et al. These workers found the fluidus boundary to be around $\chi_{bil} = 0.30$ at room temperature, and the inferred solidus line implied a room-temperature solidus boundary near $\chi_{bil} = 0.80$—0.85. The phase boundaries that we have estimated from the $\tau_{eq}$ profile in Figure 3 are in good agreement with this earlier work: $\chi_{bil} = 0.32$ and $\chi_{bil} = 0.85$ for the fluidus and solidus boundaries, respectively.

ESPA Compared with X-ray Diffraction and DSC. For some studies of phase behavior, ESPA may offer advantages over more conventional methods like, for example, calorimetry or X-ray diffraction. Unlike calorimetry, the phase behavior need not entail an enthalpic transition. For instance, two coexisting fluid-bilayer phases could, in principle, be detected by $\tau_{eq}$ analysis. Or even if an enthalpic transition can be induced, there may be some reason the phase behavior should be studied at constant temperature. Unlike X-ray diffraction, the phase behavior need not entail a detectable change in a structural repeat. As long as an equilibrium monolayer can be developed from the aqueous membrane suspension, the
membrane phase behavior should be reflected in the \( \sigma_{eq} \) profile. However, with regard to the strategy of ESPA, it must not be forgotten that equilibrium between surface and suspension is absolutely necessary. Therefore, every effort should be made to establish rigorously this condition of equilibrium in an ESPA experiment.

**Mechanism of Lipid Transfer.** On the mechanism of lipid transfer between insoluble lipid suspension and surface monolayer, we can say but little. Some workers who have studied this question suggest that the transfer is dominated by the disruption and spreading of vesicles at the gas–water interface.\(^{15}\) However, if this were the only transfer mechanism at work, the composition of the interfacial lipid phase could not differ from that of the membrane suspension, and this would make it unlikely that equilibrium could be reached since the bilayer membrane and interfacial monolayer are distinct physical environments. For this reason, we believe that some chemical exchange occurs between monolayer and bilayer. This seems to be true, at least, for LUV suspensions of di12:0-PC/di16:0-PC with \( 0 < \chi_{16} < 0.98 \). The observed kinetics of both approach to equilibrium (Figure 1a–c) and return from superequilibrium (Figure 2) seem to be consistent with a role for chemical exchange via a soluble intermediate: the rates decrease as the content of the less soluble lipid species, di16:0-PC, increases.

**Summary and Future Plans.** In this work, it has been demonstrated that (i) a Teflon substrate seems to be more appropriate than a high-energy substrate for measurements involving insoluble monolayers, (ii) phospholipid monolayers deposited from bilayer vesicle suspensions of di12:0-PC/di16:0-PC can be shown to be at equilibrium with the subphase by three different measures, and (iii) the di12:0-PC/di16:0-PC \( \sigma_{eq} \) profile reveals the compositional phase boundaries between regimes of fluid-bilayer, gel-bilayer, and two-phase coexistence.

Equilibrium surface pressure analysis seems ideally suited for the detection of coexisting fluid-bilayer phases which might exist, for example, in some phosphatidylcholine/cholesterol mixtures. Our lab is undertaking to employ ESPA as part of a study of the phase behavior of phospholipid/cholesterol model membranes.

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