Phospholipid Solubility Determined by Equilibrium Distribution between Surface and Bulk Phases

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A general strategy is proposed for determining the very low aqueous solubility limits of bilayer-forming phospholipids. The strategy exploits the inherent surface activity of phospholipids and has been termed EDSB, which stands for Equilibrium Distribution between Surface and Bulk phases. In this report, EDSB has been used to determine the critical bilayer concentration of dilauroylphosphatidylycholine (DLPC), a short-chain bilayer-forming phospholipid. At room temperature in neutral pH buffer, $CBC_{DLPC} = 2.5 \times 10^{-8}$ M. Using a mole fraction concentration scale, this corresponds to a standard-state free energy change of −12.8 kcal/mol for DLPC bilayer membrane formation.

Introduction

The fundamental structure underlying biomembranes is the phospholipid bilayer. Most natural phospholipids and their mixtures form bilayer membranes in an aqueous environment. Due to the amphiphilic nature of phospholipids, bilayer assembly is spontaneous in water and the process is driven by the hydrophobic effect:[1] all material exceeding a maximum soluble concentration will spontaneously assemble into bilayers. Most bilayer-forming phospholipid species are believed to have solubility limits $\leq 10^{-8}$ M.[2] This is in dramatic contrast to another class of amphiphiles, the detergents, for which solubilities typically range between 10^{-4} and 10^{-2} M.[3]

The solubility limit of a phospholipid, also known as its critical bilayer concentration (CBC), is a powerful thermodynamic parameter which serves as a measure of the standard-state free energy of formation of the bilayer structure[4]

$$\Delta\mu^{\circ}_{\text{formation}} = \mu^{\circ}_{\text{bilayer}} - \mu^{\circ}_{\text{soluble}} = RT\ln(\text{CBC})$$

(where $\mu^{\circ}_{\text{bilayer}}$ and $\mu^{\circ}_{\text{soluble}}$ are the standard-state chemical potentials of phospholipid in bilayer and water environments, respectively, and the ideal-dilute standard state has been chosen for the soluble species). For membrane species in multicomponent membranes, CBCs would provide insight into the thermodynamics of mixing between membrane components. Indeed, many studies of detergent mixtures have employed critical micelle concentrations (CMCs) to probe the physical-chemistry of mixing in multicomponent detergent micelles (see, for example, refs 5−8). The challenge in applying this strategy to phospholipid systems resides in the determination of the extremely low solubility limits which are typical of bilayer-forming phospholipids. While CBC estimates have been published for two particular phospholipids,[9,10] no general strategy has so far been described for the evaluation of phospholipid solubility limits.

In this work, we present a novel strategy for the determination of phospholipid CBCs based on the inherent surface activity of phospholipids. By incubating aqueous DLPC vesicle suspensions in Teflon vessels, partition equilibrium develops between bulk aqueous phases (suspended bilayer vesicles and soluble phospholipid) and interfacial phases (gas−water and Teflon−water monolayers). By gradually increasing the amount of DLPC in these Teflon vessels, the soluble aqueous and monolayer phases are gradually saturated until the chemical potential of DLPC in these phases reaches that of bilayer phase DLPC. At this point, all phospholipid in excess of the critical bilayer concentration remains in the bilayer state, suspended in aqueous buffer, so that the CBC can be estimated from the inflection point in an equilibrium plot of $[\text{DLPC}]_{\text{buffer}}$ vs $\text{DLPC}_{\text{total}}$.

Experimental Section

Materials. DLPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Purity (>99.5%) was confirmed by thin-layer chromatography on washed, activated silica gel plates (Alltech Associates, Inc., Deerfield, IL), developing with $CHCl_3$/MeOH/H_2O 65:25:4. ^{14}C-labeled lauric acid was purchased from American Radiochemicals, Inc. (St. Louis, MO). CDI (1,1′-carbonyldiimidazole) and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) were from Aldrich Chemical Corp. (Milwaukee, WI). PIPES (piperizane-1,4-bis(2-ethanesulfonic acid) buffer and disodium EDTA were purchased from Fluka Chemie AG (Switzerland). NaN_3, MeOH, and 20 mL low-potassium glass scintillation vials were obtained from Fisher Scientific (Fair Lawn, NJ). $CHCl_3$ and DMSO were from Mallinckrodt, Inc. (Paris, KY). Bio-Sil A silica (Bio-Rad Laboratories, Richmond, CA) was washed with $CHCl_3$/MeOH 1:1, activated by heating at 200 °C overnight, and stored over P_2O_5. Aqueous buffer (5 mM PIPES pH 7.0, 200 mM KCl, 1 mM EDTA, 1.5 mM NaN_3) was prepared from purified water (Milli-Q system, Millipore Corp.) and filtered through a 0.1 μm filter before use. FEP Teflon is tetrafluoroethylene−

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hexafluoropropylene copolymer, a form of Teflon which is thermoplastically moldable. PTFE Teflon is poly(tetrafluoroethylene), a commonly used machinable form of Teflon. FEP tubing and a PTFE stopcock valve were obtained from Berghof America, Inc. (Concord, CA). FEP Oak Ridge tubes were from Nalge Co. (Rochester, NY).

Liquid Scintillation Counting (LSC). A Beckman TD 5000 was used for liquid scintillation counting, interfaced via an RS232 port to a Macintosh computer for data collection and storage. Three channels were recorded, and quenching was monitored in each sample by recording the shift in the Compton edge for an external ^{137}Cs source.[11] Samples were counted with a 1% 2σ limit for a maximum of 10 min, and counts were always repeated through 10 cycles. Data files were processed by averaging all cycles and subtracting background. The scintillation cocktail was Scinti Verse I (Fisher Sci.), and the composition of all LSC samples was 10 mL buffer + 10 mL cocktail. After adding cocktail, scintillation vials were sealed and inverted 20 times to form a stable emulsion. Data were collected under identical conditions for all LSC samples, so that differential quenching effects were minimized.

Synthesis and Purification of ^{14}C-Labeled DLPC. ^{14}C-labeled DLPC was prepared using the fatty acid imidazolide essentially as described by Boss et al.,[12] except that DBU was used as the base catalyst[13] and DMSO as the solvent (to facilitate sub-milligram synthesis). The synthetic reaction mixture was initially purified by thin-layer chromatography, developing with $CHCl_3$/MeOH/H_2O 65:25:4, and scraping the PC product from the plate while working inside a glovebag to contain radioactive silica dust.

A highly purified DLPC working stock, necessary for these measurements, was prepared by combining an appropriate quantity of the crudely purified ^{14}C-DLPC (~95% pure) with ~5 mg of unlabeled DLPC to yield a stock with a specific activity of $\sim 2 \times 10^3$ dpm/nmol. This DLPC stock material was then purified by silica column chromatography on a small column (~3 g silica), loading in $CHCl_3$/MeOH 65:25 and eluting with a linear gradient from $CHCl_3$/MeOH 65:25 to $CHCl_3$/MeOH/H_2O 65:25:4. Residual fatty acid and lyso-PC were well separated from PC by this strategy, and purity of the isolated material was determined according to the following procedure.

Approximately 50 μg of purified PC was spotted on a strip (1.5 cm × 10 cm) cut from a washed and activated aluminum-backed silica gel plate. The strip was developed with $CHCl_3$/MeOH/H_2O 65:25:4 and then sectioned lengthwise by cutting with a scissors. The strip was sectioned into three pieces: one portion below the PC spot (including any lyso-PC), another portion with the PC spot only, and a third portion containing the rest of the strip above the PC spot (including any fatty acid). The labeled material on each section was quantified by LSC, and the purity of the highly purified DLPC stock was determined to be >99.99%. Control samples containing known amounts of labeled lyso-PC, PC, and fatty acid were analyzed in parallel to validate the procedure.

Preparation and Specific Activity of Stock Solutions. Using this highly purified material, a DLPC stock solution was prepared in absolute ethanol and all subsequent manipulations were performed in the low-dust environment of a Cleansphere CA100 (SafeTech Ltd.). The solution was passed through a 0.22 μm filter to remove dust and then resealed inside a clean, dust-free vial. To determine the precise concentration and specific activity of this stock, aliquots were taken for phosphate assay[14] and for LSC. The concentration of the ^{14}C-DLPC stock solution was 1.58 ± 0.02 mM, and the specific activity was 2385 ± 30 dpm/nmol.

An unlabeled DLPC stock solution (1.5 ± 0.1 mM) was also prepared in ethanol for the preparation of samples for surface tension and nile red experiments (see below).

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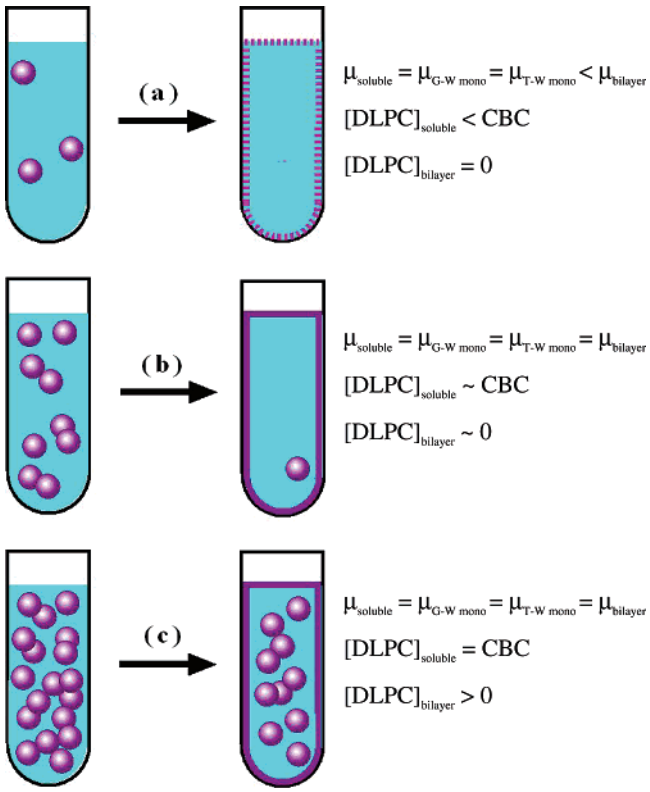

Figure 1. Equilibrium distribution of phospholipid between aqueous buffer volume and amphiphilic interfaces. When an aqueous suspension of bilayer vesicles is placed in a Teflon vessel, phospholipid redistributes between buffer phases (bilayer vesicles and soluble phospholipid) and surface phases (gas−water and Teflon−water monolayers) until its chemical potential is uniform throughout the system. In (a), the quantity of lipid added in the sample is insufficient to saturate the interfaces. At equilibrium, $\mu^{soluble} = \mu^{monolayers} < \mu^{bilayer}$ so that no bilayer phase remains; subsaturation monolayers coexist with soluble phospholipid. In (b), just enough lipid is present to saturate the interfaces, raising the chemical potential of surface phospholipid to that of bilayer phase phospholipid. In (c), a considerable excess of lipid is present and at equilibrium $\mu^{soluble} = \mu^{monolayers} = \mu^{bilayer}$. Most of the phospholipid remains suspended as vesicles in the buffer volume.

Sample Preparation and Incubation. Sets of DLPC vesicle suspensions were prepared by ethanol injection,[15] a procedure which produces small unilamellar vesicles that do not sediment. Clean FEP Teflon tubes were rinsed with filtered milliQ water inside the Cleansphere before receiving 25.0 mL of filtered buffer; to minimize any possible interference from trace suspended particulates, all sample buffer was pretreated overnight by continuous recirculation through a 0.1 μm depth filter. An appropriate volume of stock solution was injected into the buffer of each tube (with final ethanol concentrations never exceeding 0.1% v/v), so that a series of samples was prepared with identical solution volumes and increasing DLPC content. Teflon tubes were sealed under argon and placed upright in a rotating sample holder. This sample holder was slowly rotated in the horizontal plane during sample incubation, so that gentle mixing was promoted in the suspension without disturbing the interfaces. When the gas−water surface tension of any particular sample was to be determined, the sample was rotated into position beneath a surface tension balance. When an aliquot of the sample's bulk aqueous volume was to be taken, the sample was rotated into position beneath a Teflon siphon (see below).

During sample incubation, phospholipid redistributes between bulk phases and surface phases in the manner described by Figure 1. This redistribution proceeds until the chemical potential, μ_{DLPC}, is identical in all coexisting phases. Samples with smaller

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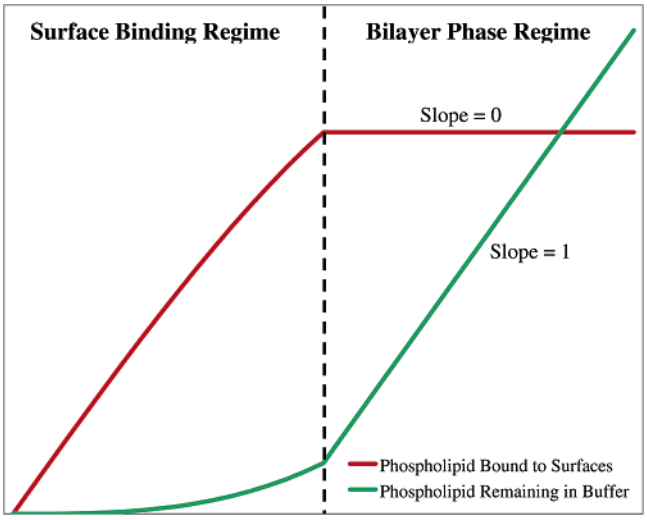

Figure 2. Schematic plots illustrating the surface-binding and bilayer-phase regimes. The redistribution of phospholipid between buffer and surface phases implies that, as a function of total phospholipid content, equilibrium plots of surface lipid (red line) or buffer lipid (green line) will reveal two regimes. At lower total lipid contents, a large fraction of the phospholipid will bind to the amphiphilic gas−water and Teflon−water interfaces. For example, a sample like that illustrated in Figure 1a would fall within this surface-binding regime. At higher total lipid contents, however, all phospholipid in excess of that required to saturate the interfaces will remain suspended in the bilayer phase. Samples such as that depicted in Figure 1c would fall in this regime. The sample described by Figure 1b would be on the boundary between the two regimes.

quantities of total phospholipid (Figure 1a) retain no bilayer phase phospholipid at equilibrium. Instead, the phospholipid redistributes to form subsaturation monolayers which are in equilibrium with a sub-CBC concentration of soluble DLPC. Samples with larger quantities of total phospholipid (Figure 1c) do retain bilayer vesicles at equilibrium, and bilayer phase coexists with saturated monolayer and soluble aqueous phases. A sample with just enough total phospholipid to saturate the monolayer and soluble phases (Figure 1b) will retain perhaps a few vesicles at equilibrium.

With respect to total phospholipid content, the redistribution of phospholipid between bulk and surface phases is manifest as two equilibrium regimes, as illustrated by Figure 2. Equilibrated samples within the surface-binding regime contain coexisting, subsaturation monolayer and soluble phases. In this regime, most of the total phospholipid partitions onto amphiphilic surfaces, while very little remains in the buffer. In contrast, samples in the bilayer-phase regime will retain bilayer vesicles at equilibrium, coexisting with saturated monolayer and soluble phases. In this regime, all "excess" phospholipid remains suspended in the buffer as bilayer vesicles and no further surface binding takes place.

Siphoning Procedure to Obtain Aliquots of Sample Buffer. Due to the presence of a gas−water monolayer phase, we employed a siphoning strategy to obtain aliquots of sample buffer which are free from surface-associated lipid. The siphon we used is made of FEP Teflon tubing with a PTFE Teflon stopcock valve. The siphon is filled with buffer and kept full by closing the stopcock. When an aliquot of bulk buffer volume is needed from a particular sample, the sample is placed beneath the siphon and raised until the inlet tube penetrates the sample surface to within ~2 cm from the bottom of the sample tube. The stopcock is then opened, allowing sample buffer to flow through the siphon. For each sample, the siphon is flushed with three siphon volumes (~3.6 mL) in order to discard any surface-derived lipid before the desired volume of buffer is collected in a scintillation vial. The weight of collected buffer is recorded via a top-loading digital balance.

On the time scale of aliquot collection (~2 min) and under the conditions of continuous flow which persist during siphoning, the Teflon siphon appears to bind no detectable lipid. This was checked by siphoning as much as possible of several 35 mL samples with sub-CBC concentrations of ^{14}C-DLPC (data not shown). For each sample, a spike of ^{14}C-DLPC due to surface-associated lipid was detected in the initial flushing buffer. However, following this spike, the measured lipid concentration in the flow-through was constant, from the first aliquot collected (~3 mL post-flush) to the last (~30 mL post-flush).

Surface Tension Measurements. Surface tension values were determined as previously described.[16]

Nile Red Assay for the Presence of Bilayer Phase. Nile red is a lipophilic dye which has little fluorescence in water. However, in a nonpolar environment, the excitation and emission maxima of nile red are blue-shifted and fluorescence is enhanced.[17] For this reason, enhanced fluorescence of nile red can serve as a sensitive indicator of the presence of bilayer-phase phospholipid.

A nile red stock was quantified by absorption spectroscopy in MeOH ($\epsilon_{636} = 4.5 \times 10^4$ M^{-1} cm^{-1} [18]), and a 1.0 mM solution was prepared in EtOH. This solution was used for all subsequent assays, which were performed as follows. Two milliliters of a bulk buffer sample was added to a 3 mL quartz fluorescence cuvette containing a micro stir bar. The cuvette was placed into the fluorometer, and the background signal was recorded (λ_{ex} = 540 nm, λ_{em} = 615 nm). The chamber door was then opened, 2 μL of nile red solution was added to the sample, and the door was closed again. After the fluorescence signal had stabilized, the value was recorded. The time scale of the assay, from transferring the sample into the cuvette to recording the stabilized fluorescence signal, was about 2 min.

Results

Time scale of Surface-Buffer Partitioning for DLPC. Figure 3 illustrates the time scale of DLPC redistribution between sample buffer and interfaces. Note that 15 min after introducing the phospholipid to the buffer, all the material remains in the bulk aqueous buffer volume. After 1 day, much of the material has left the bulk and has bound to the interfaces. By 3 days, surface binding seems to be at equilibrium, and no significant increase is evident at either 6 or 11 days. This surface binding is reversible, however: bound phospholipid can be released to the buffer again simply by vortexing (data not shown).

Saturating Molecular Areas of Surface-Bound DLPC. Equilibrium quantities of surface-bound lipid are consistent with the formation of monolayer phases at the gas−water and Teflon−water interfaces. The surface area (gas−water + Teflon−water) of a 25.0 mL sample is about 48 cm^2, and ~15 nmol of DLPC are needed to saturate these interfaces (Figure 3). Assuming that the lipid is uniformly distributed over the sample surface, this implies an interfacial molecular area of 56 ± 1 Å^2/lipid, which is comparable to that of spread monolayers at maximum density.[19] By comparing the saturating quantity of surface-bound lipid for two different sample volumes, 25.0 and 35.0 mL, it was possible to make separate estimates of lipid density at each amphiphilic interface. This experiment was performed (data not shown), and the estimated saturating molecular areas were 58 Å^2/lipid at the gas−water interface and 55 Å^2/lipid at the Teflon−water interface.

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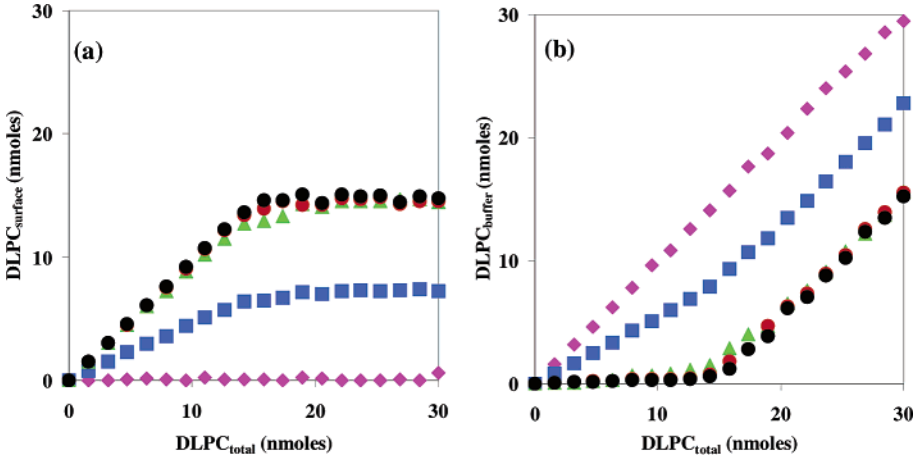

Figure 3. Approach to equilibrium: experimental data illustrating the surface-binding and bilayer-phase regimes. Five replicate sets of ^{14}C-labeled DLPC vesicle suspensions were prepared, each containing 20 samples with between 0 and 30 nmol DLPC. The sample sets were incubated for increasing periods of time, after which the buffer was sampled and its phospholipid content was analyzed by liquid scintillation counting. Surface-associated phospholipid was computed from the difference between $DLPC_{total}$ and $DLPC_{buffer}$. Surface lipid is plotted in (a); buffer lipid is plotted in (b). The redistribution of DLPC can be followed in time: 15 min (□); 1 day (■); 3 days (▲); 6 days (red dot); 11 days (black dot).

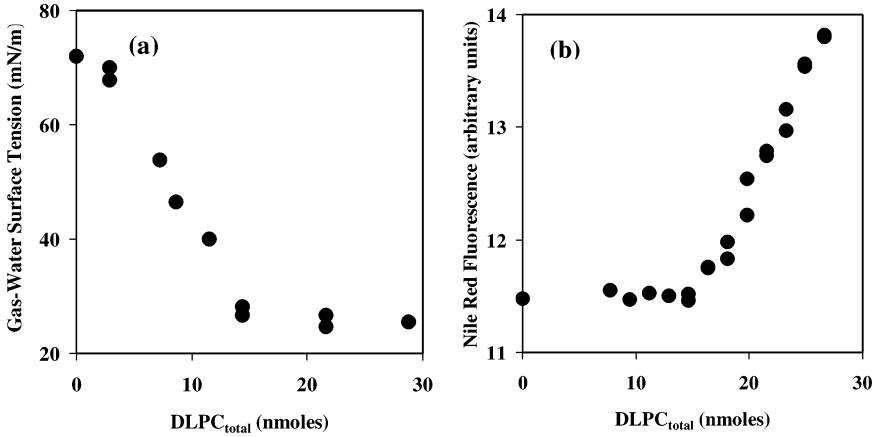

Figure 4. Verification of the surface-binding and bilayer regimes by surface tension and fluorescence measurements. In (a), the equilibrium surface tension of the gas−water interface decreases as a function of $DLPC_{total}$ throughout the surface-binding regime, as expected. Surface tension appears to be invariant in the bilayer-phase regime. In (b), no enhancement of nile red fluorescence is evident in the surface-binding regime. However, fluorescence is enhanced throughout the bilayer regime, consistent with the presence of bilayer phase DLPC.

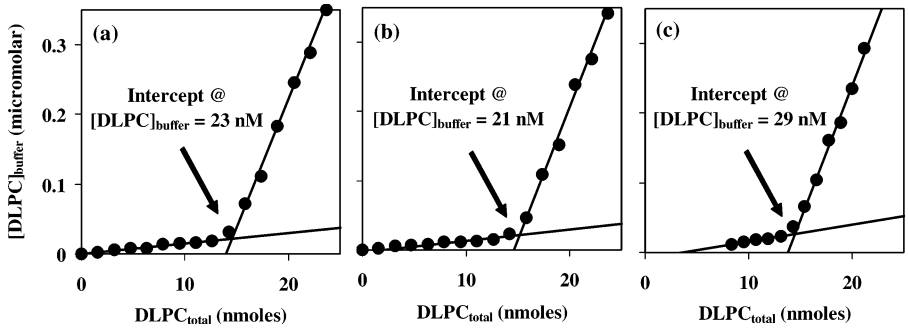

Figure 5. Estimation of the CBC for DLPC. Equilibrium data are presented for three independent sample sets, each incubated for a different period of time. $[DLPC]_{buffer}$ is plotted as a function of $DLPC_{total}$, and linear fits to data on either side of the boundary aid in estimating the critical concentration that marks the transition between regimes. The estimates are consistent, averaging about 2.5×10^{-8} M, and indicate stable equilibrium: 6 (a), 11 (b), and 25 days (c).

Surface-Binding and Bilayer-Phase Regimes. Figure 4a demonstrates independently that a surface-binding regime persists over the appropriate range of DLPC sample content. The tension of the gas−water interface decreases between 0 and 15 nmol $DLPC_{total}$, but is invariant for $DLPC_{total}$ > 15 nmol. Nile red fluorescence (Figure 4b) indicates that an aggregated form of DLPC (i.e., bilayer phase) is present only for $DLPC_{total}$ > 15 nmol, whereas none is detected between 0 and 15 nmol $DLPC_{total}$.

Estimation of CBC_{DLPC}. Equilibrium plots of $[DLPC]_{buffer}$ vs $DLPC_{total}$ are shown in Figure 5, representing three separate sample sets which were analyzed at either 6, 11, or 25 days of incubation. Estimates of CBC_{DLPC} are obtained by noting the aqueous concentration

(i.e., $[DLPC]_{buffer}$) at which the plots reveal a transition between regimes, indicating the onset of bilayer stability. These data suggest 25 (± 5) nM for the CBC of DLPC, at room temperature.

Discussion

Verification of Surface Binding and Bilayer Regimes. All of the results presented here are consistent with the redistribution of phospholipid between aqueous buffer volume and amphiphilic interfaces, in the manner that is illustrated by Figures 1 and 2. As the amount of total phospholipid is increased from very low initial values, samples in Teflon vessels clearly pass from a surface-binding regime into a bilayer-phase regime.

First, for 25.0 mL samples at equilibrium (Figure 3 ▲, red dot, black dot), a surface-binding regime is indicated between 0 and 15 nmol DLPC; all DLPC redistributes from the bilayer phase into either surface or soluble phases (i.e., $\mu_{G-W\ monolayer} = \mu_{T-W\ monolayer} = \mu_{soluble} < \mu_{bilayer}$). This surface-binding regime is confirmed by surface tension analysis, and measurements of surface-bound DLPC in the bilayer regime are consistent with the presence of densely packed monolayers at both gas−water and Teflon−water interfaces.

Second, in 25.0 mL samples with $DLPC_{total} > 15$ nmol, surface and soluble phases are saturated at equilibrium (i.e., $\mu_{G-W\ monolayer} = \mu_{T-W\ monolayer} = \mu_{soluble} = \mu_{bilayer}$), so that all excess phospholipid remains suspended in buffer as stable bilayer vesicles. The presence of an aggregated form of phospholipid is confirmed by the enhanced fluorescence of nile red.

Finally, the Phase Rule for nonreactive components

$$F = C + 2 - P$$

(where F, C, and P are the numbers of degrees of freedom, components, and phases, respectively[20]) indicates that a system must lose a degree of freedom accompanying the appearance of an additional phospholipid phase. Consistent with this constraint, surface tension becomes invariant as samples pass from the surface binding-regime into the bilayer regime.

The boundary between these regimes is marked by a critical $DLPC_{buffer}$ concentration. Our data seem to indicate that there is no thermodynamic evidence for more than one form of stable DLPC aggregate above this concentration. If there were to be some kind of equilibrium between aggregate forms (i.e., micelles ↔ bilayer), we would expect to see some evidence that the chemical potential of DLPC continues to change. However, our EDSB data (Figures 3 and 5) indicate that no further surface binding is detectable above this threshold, implying that μ_{DLPC} becomes fixed throughout the system. For this reason, we choose to refer to our result as a critical bilayer concentration.

We are aware that other workers have reported micelle formation by DLPC,[25] and we regret that we cannot fully resolve this discrepancy. We would like to emphasize, however, that all our EDSB experiments were carried out with an unusually pure DLPC preparation. It is conceivable that EDSB experiments based on less pure preparations could produce different results near the critical concentration; impurities could be concentrated in the first aggregates that form, especially if the impurities differ from DLPC in surface activity. Indeed, this concern was the motivation behind our exclusive use of highly purified DLPC. Perhaps it can help to explain the apparent contradiction between our work and earlier reports of DLPC micelles.

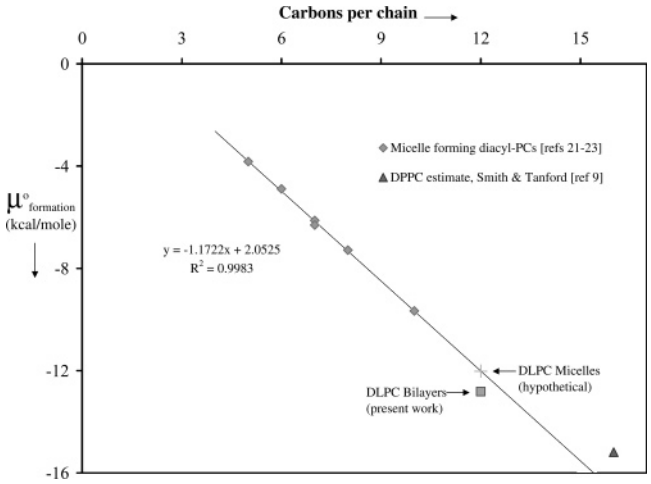

Figure 6. Standard-state free energies of formation for diacyl-PC bilayers and micelles. A linear fit describes the chain-length dependence for diacyl-PC micelle formation.[21−23] At room temperature, DLPC forms bilayers rather than micelles, but by extrapolation one may compare the hypothetical value for DLPC micelles (+) to the experimentally determined value for bilayers (■) reported here. For comparison, Smith and Tanford's 1972 estimate for DPPC is included (▲), although it seems likely that their experimental procedure underestimated the stability of DPPC bilayers (see Discussion).

Comparison of Estimated CBC_{DLPC} with Literature Data. The data in Figure 5 indicate that the room-temperature CBC for DLPC is about 2.5×10^{-8} M, with an estimated experimental uncertainty of $\pm 5 \times 10^{-9}$ M. Because it has only 12 carbons in each of its hydrocarbon chains, we expected DLPC to be among the most soluble of the bilayer-forming phospholipids. This is why we chose to use DLPC in our initial EDSB studies, and our results confirm that expectation.

However, it is worth noting that 2.5×10^{-8} M is almost 4-fold lower than an extrapolation estimate ($\sim 9.7 \times 10^{-8}$ M) based on the CMCs of shorter chain, *micelle-forming* saturated diacyl-PCs.[21−23] Although both micelle assembly and bilayer formation are driven by the hydrophobic effect, these are nonetheless distinct structures and the 4-fold discrepancy is a measure of the increased stability of diacyl-PC bilayers relative to diacyl-PC micelles. This appears to be just a manifestation of packing constraints for diacylphospholipids, which tend to favor extended bilayers over micellar structures for acyl-chain lengths in excess of 10 carbons.[24] In terms of standard state-free energies of formation ($\Delta\mu^\circ_{formation} = \mu^\circ_{bilayer} - \mu^\circ_{soluble}$; mole fraction scale), DLPC bilayers appear to be about 0.8 kcal/mol more stable than DLPC micelles (Figure 6), a difference that is well outside the range of experimental uncertainty (i.e., ±0.1 kcal/mol on the mole fraction scale).

It is worth noting that the Smith and Tanford's 1972 estimate of DPPC solubility seems to be anomalously high when compared to the rest of the diacyl-PC data presented in Figure 6. This is perhaps not surprising, when one considers their experimental procedure: filtration of a series of methanol−water ^{3}H-DPPC suspensions followed

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by phenomenological extrapolation of DPPC filtrate concentrations to zero methanol content. This procedure raises a number of experimental concerns (e.g., the likelihood of incomplete filtration; possible perturbations caused by the filtration itself; the arbitrary shape of the extrapolating curve), so it may be reasonable to hold their result in abeyance, pending an independent estimate of DPPC solubility.

Future Plans. One extension of these EDSB studies might be to evaluate the CBCs for other vesicular forms of DLPC bilayer phase. The vesicles studied here, which form by ethanol injection, are mostly small unilamellar vesicles, or SUVs.[15] SUVs are only about 25 nm in diameter and are considered to be sterically strained relative to larger vesicles.[4] For this reason, the CBC we have reported here may actually be an upper limit for DLPC bilayer formation at room temperature. It will be interesting to compare these results to data obtained with other vesicular forms, such as LUVs (large unilamellar vesicles, diameter > 80 nm) or MLVs (multilamellar vesicles, larger and polydisperse).

It will also be important to extend these studies to other phospholipids and lipid mixtures. For example, when modeling chemical interactions within a membrane mixture, a lipid's concentration is often assumed to be equivalent to its chemical activity (i.e., the ideal-mixture assumption). However, one could actually test this assumption by evaluating the free energies of formations for various bilayer mixtures; choosing, perhaps, lipid compositions that approximate a mammalian plasma membrane (either the outer or inner leaflet). In particular, we could learn how the activity of cholesterol is related to its concentration in a plasma membrane, a mixture that is almost certain to be highly nonideal.

To extend these studies to less-soluble phospholipids, we will have to deal with even longer equilibration times. This will make it difficult to study chemically unstable molecules, so we are currently building an inert atmosphere chamber (kept under positive pressure with HEPA-filtered nitrogen gas) in which to conduct future EDSB experiments. It may well prove difficult to study polyunsaturated lipids by EDSB, but only time will tell. The next phospholipids we plan to study will be monounsaturated (perhaps the POPC, POPS, POPE headgroup series) and our experience with them will inform any future attempts to study polyunsaturated compounds.

What is the theoretical detection limit for ESDB? In these experiments, we used a ^{14}C-labeled stock at about 0.1 mCi/mmol. To study less-soluble lipids, we will need to employ stocks with much higher specific activities. While it is true that ^{14}C-labeled lipids are not commercially available at much more than 100 mCi/mmol, for the least-soluble lipids, we plan to work with ^{3}H-labeled stocks. Tritiated compounds provide much higher count rates and are affordable in suitable quantities; for example, commercially prepared ^{3}H-labeled DPPC is available at 45 Ci/mmol. At this specific activity, the detection limit for an EDSB experiment would be about 5×10^{-14} M, or 500 000 times lower than the estimated CBC for DLPC.

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