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Modeling Success and Failure of Langmuir-Blodgett Transfer of Phospholipid Bilayers to Silicon Dioxide

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ABSTRACT Formation of planar phospholipid bilayers on solid and porous substrates by Langmuir-Blodgett transfer of monolayers from the air-water interface could be of much greater utility if the process were not irreproducible and poorly understood. To that end the energetics of transferring two phospholipid monolayers to a hydrophilic surface has been examined. An approximate mathematical relationship is formulated that relates the surface pressure of the precursor monolayers to the tension within the bilayer created. Data are presented that demonstrate that bilayer transfer can be carried out reproducibly even with refractory phospholipids such as phosphatidylcholine, but only over a very narrow range of precursor monolayer surface pressures. This range is related to the lysis tension of the bilayer. The morphology of films formed within and below the successful range of surface pressures are examined by fluorescence microscopy, and the observed features are discussed in terms of the relationship above. These results provide practical guidelines for successful formation of lipid bilayers on hydrophilic surfaces; these guidelines should prove useful for research into the properties of biomembranes and for development of bilayer-based biosensors.

INTRODUCTION

The planar phospholipid bilayer has an important role as a model cell membrane. The primary use of such planar bilayers is in fundamental studies of ion transport and membrane protein function. Planar bilayers have also been considered as the basis of a number of applications, including receptor-based bioanalytical sensors (Arya et al., 1985; Yager, 1986, 1987; Hongyo et al., 1987; Ligler et al., 1988), vapor sensors for anesthetics and other organic compounds (Krull et al., 1988), and photocells based on the light-transducing ability of rhodopsin (Skulachev, 1976). Common to all of these uses of planar bilayers is the necessity of forming them on solid supports, in most cases with some portion of the bilayer freely suspended between two aqueous compartments so that the electrical properties of the membrane may be monitored. The ability to reliably and controllably deposit bilayers is critical to these endeavors.

The Langmuir-Blodgett (L-B) method of building up multilayer films from monolayers at the air-water interface has certain advantages over other methods of forming lipid bilayers. First, the L-B technique is generally applicable to the formation of bilayers on structures of highly variable shape and size, unlike very specific methods such as the Montal-Mueller (Montal and Mueller, 1972) or micropipette tip-dip (Coronado and Latorre, 1983) techniques. Second, the L-B technique does not require the presence of an additional solvent, as in the Montal-Mueller technique. Third, the L-B technique should in theory allow the formation of bilayers free from inclusions of three-dimensional lipid microstructures, which may occur when liposomes are adsorbed onto surfaces. Finally, the L-B method allows control of monolayer (and hence bilayer) lipid density, allowing the investigator an experimental variable unavailable with other methods.

However, L-B formation of phospholipid multilayers from monolayers has been found to be difficult, as described in a recent review by Swart (1990). Good results have been achieved for gel-phase amine-terminated phospholipids on platinum (Fare, 1990) or for negatively charged phospholipids in the presence of divalent cations (Cui et al., 1990), but the transfer of gel-phase neutral phospholipids to hydrophilic glass or silica has met with problems and liquid-crystalline-phase phospholipids have received little attention. Transfer of the initial monolayer is relatively easy, but transfer of successive monolayers has proven more challenging, prompting some investigators to use L-B deposition for the initial monolayer, but horizontal insertion of the substrate for the second (Kossi and LeBlanc, 1981; Tamm and McConnell, 1985). In general, normal Y-type L-B transfer of neutral phospholipids to hydrophilic glass or silica has been successful for at most three monolayers (Honig et al., 1973; Hasmonay et al., 1979) and has been studied only for gel phase monolayers. Further deposition has been achieved by using X-type transfer (Green et al., 1973; Albrecht et al., 1982), prepolymerized monolayers of diacetylenic phospholipids (Ligler et al., 1988), or a subphase containing uranyl acetate (Peng et al., 1987). However, all of these means of improving transfer compromise the model membrane qualities of the films. X-type transfer is geometrically incompatible with the formation of a bilayer over a fluid-filled aperture. The use of heavy metal salts such as uranyl acetate to improve transfer is incompatible with proper ion channel function due to the problems of poisoning the channel and rigidification of the membrane. Rigidification is also a liability of polymerized lipids. A somewhat fluid membrane is necessary for normal ion-channel protein function (Fong

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and McNamee, 1986) and provides a far more realistic model cell membrane.

The L-B formation of phospholipid bilayers is not well understood, due perhaps in part to the difficulty generally observed in forming such bilayers. Studies of L-B multilayer formation (Petrov and Radoev, 1981; Saint Pierre and Dupeyrat, 1983; Buhaenko and Richardson, 1988) have focused on the energetics of the interactions between layers, as opposed to the interactions between the layers and the substrate, and have dealt mainly with fatty acids and other single-chain amphiphiles. Nevertheless, certain useful points have been exposed that may prove generally applicable. For example, Petrov and Radoev (1981) have successfully modeled L-B transfer as a quasi-equilibrium process. As might be expected, transfer of the initial layers differs significantly from that of the remainder from an energetic standpoint (Robinson and Sambles, 1990), and further transfer can affect ordering in the primary layers (Duschl and Knoll, 1988). Given that L-B transfer of more than three monolayers of phospholipid has not generally been successful, we focus first on understanding the factors influencing success or failure of transferring the initial few layers. More specifically, as we are interested mainly in the creation of model cell membranes, we concern ourselves here only with the transfer of the first two monolayers, i.e., bilayer formation. As we are interested in bilayer formation, observations on non-L-B bilayer formation have some intuitive value. The necessity of a high lipid areal density or surface pressure for successful bilayer lipid membrane formation has been well documented (Schindler, 1980; Tancrède et al., 1983). It is reasonable to presume, then, that lipid density would have an impact on L-B bilayer formation and/or stability, especially when the substrate interacts only weakly with the lipid film, as in that circumstance it should behave more like a free-standing bilayer.

We have chosen to study and model the L-B formation of fluid phase phospholipid bilayers on oxidized silicon. The surfaces of oxidized silicon wafers are extremely pure SiO_2, providing a well characterized (Iler, 1979) model hydrophilic surface. Furthermore, oxidized silicon wafers have recently been reported to be extraordinarily flat and smooth as determined by atomic force microscopy (Ducker et al., 1992), with root mean square surface roughness of 0.2 nm over a 0.45 μm^2 scan area, making them excellent substrates for the L-B technique. In addition, silicon and semiconductor batch-processing methods have been advocated for the development of bioanalytical sensors employing bilayer membranes (Ligler et al., 1988; Osborn et al., 1989).

We begin by examining the energetics of transferring the initial two phospholipid monolayers to a hydrophilic surface. We then formulate an approximate mathematical relationship between the surface pressure of the precursor monolayers and the tension within the bilayer thus created, arguing that the phospholipid bilayer must generally be a kinetically trapped form. Next, we present data demonstrating that successful bilayer transfer may be conducted only over a very narrow range of surface pressures. This range is related to the lysis tension of the bilayer. The morphologies of films formed within and below the successful range of surface pressures are examined by fluorescence microscopy, and the observed features are discussed in terms of the mathematical relationship above.

THEORY

Transfer of the initial and second monolayers

L-B transfer of insoluble amphiphiles to hydrophilic surfaces is generally pictured as shown in Fig. 1. The surface pressure, π, of the monolayer film is defined as:

$$\pi = \gamma_{H_2O} - \gamma_{monolayer} \quad (1)$$

where γ_{H_2O} is the surface tension of clean subphase in air and $\gamma_{monolayer}$ is the surface tension of the subphase bearing the lipid monolayer. The surface pressure is maintained at a value adequate to keep the film in a relatively condensed state by adjusting the film area. The initial monolayer is transferred during the first upward pass of the substrate and subsequent layers are transferred as the substrate direction is successively reversed. This process is the so-called Y-type deposition sequence and is the most common.

Transfer of the initial monolayer is depicted in Fig. 1 *A*. Depending on the amphiphile, the zipper angle, θ, may range from in excess of 40° to approximately zero. Following the example of Petrov we treat the monolayer as a separate

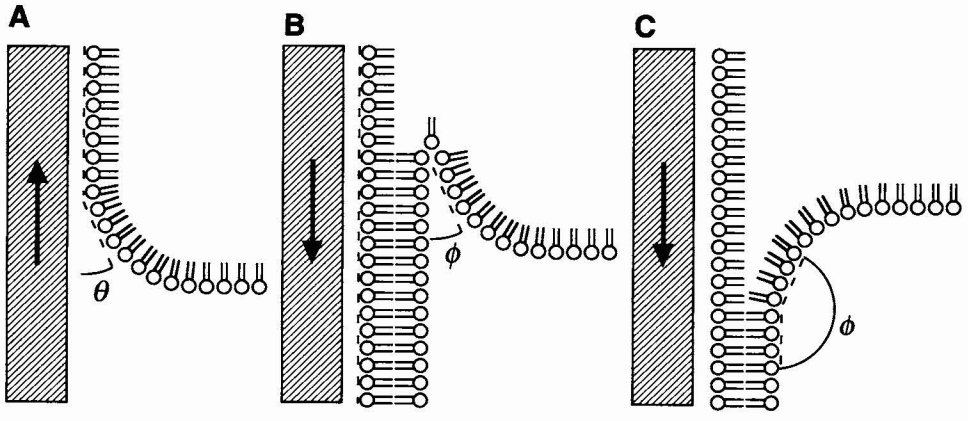

FIGURE 1 Schematic diagram of bilayer formation by the typical Y-type L-B transfer process (not to scale). Arrows show the direction of substrate motion. Macroscopically observable contact angles are designated. (*A*) Transfer of the initial monolayer occurs during the first upward pass of the substrate. Angle θ is the zipper angle. Transfer of the second monolayer occurs during the next downstroke. (*B*) Transfer geometry observed for condensed phospholipids. (*C*) Commonly observed transfer geometry. The space between the initial monolayer and the substrate represents the hydration layer.

phase. The energy of adhesion between the monolayer and the substrate is then given by the contact angle of the monolayer on the substrate through the Young-Dupré equation, modified here as θ, the subphase contact angle, is the supplement to the monolayer contact angle:

$$\Delta W = (1 - \cos\theta)\gamma_{\text{monolayer}}. \quad (2)$$

As θ approaches 0°, the energy of adhesion, ΔW, also approaches zero. One consequence of this result is that the energy available for expelling the aqueous subphase from between the monolayer and the substrate is greatly reduced. Therefore, in general, films having a small zipper angle must be transferred relatively slowly to ensure smooth transfer (Petrov et al., 1980). Nevertheless, transfer of the initial monolayer is generally successful even for films with a near-zero zipper angle because the monolayer film greatly reduces the surface energy of the hydrated substrate in air.

The deposition of a second monolayer may occur under favorable conditions when a monolayer-bearing substrate is lowered into the subphase. Transfer of the second monolayer results in apposition of the lipid tailgroups of the initial monolayer with more lipid from the air-water interface. This process is shown for condensed phospholipid layers at low transfer speeds in Fig. 1 *B*. The more familiar L-B transfer of fatty acids tends to follow the geometry shown in Fig. 1 *C*, although acute ϕ have been observed for single-chain amides (Buhaenko and Richardson, 1988). The tailgroup-air interface may be regarded as similar to an oil-air interface. Bilayer formation thus results in the apposition of two interfaces, each having a surface energy of $\geq$20 mN/m to form an interface having an energy of <20 mN/m for most stable phospholipid bilayers (Bloom et al., 1991). Therefore, provided the initial monolayer is tightly bound to the substrate, the creation of a bilayer is favorable by at least 20 mN/m.

Stability analysis of the supported bilayer

If we assume that the initial monolayer is fixed, then bilayer formation is a straightforward, favorable process. This assumption is reasonable for monolayers that bind to the substrate with a high energy of adhesion, as is commonly observed for fatty acids. However, monolayers that have a near-zero zipper angle with the substrate also have low adhesive energy and will not necessarily remain attached. The supported bilayer must be energetically stable with respect to the uncoated substrate to remain on that substrate at equilibrium.

To proceed with the stability analysis of the supported bilayer a few assumptions are needed. The initial monolayer is assumed to remain fixed as long as the resultant bilayer is stable. If the zipper angle between the initial monolayer and the substrate is small ($\theta \leq 15°$), the energy of adhesion between a condensed phospholipid monolayer ($\gamma_{\text{monolayer}} \approx 25$ mN/m) and the substrate is less than 1 mN/m. The energy of adhesion between the second monolayer and the substrate is far smaller because of the greater distance between them. Therefore we assume that the energy of adhesion between the bilayer and the substrate is less than or approximately equal to 1 mN/m. Given such a weak interaction between the bilayer and substrate, we assume that the lateral stress in the bilayer is not significantly affected by the proximity of the substrate. We also assume that the critical stress of the bilayer at failure is approximately the same as that found for large liposomes of the same constitution. Accurate direct experimental comparison between the area per lipid molecule of monolayers and that of bilayers has not been possible because of large variation in the measurement of area per molecule in the bilayer form (Gruen and Wolfe, 1982). Nevertheless, MacDonald and Simon have convincingly argued that the area per molecule of the lipid monolayer at its collapse pressure (π_c, defined as the pressure at which the monolayer begins to collapse into three-dimensional forms) is identical to the area per molecule of unstressed liposomes (MacDonald and Simon, 1987). We therefore assume that a bilayer formed from monolayers at the collapse pressure bears no residual tension.

However, L-B transfer is generally carried out at surface pressures below the collapse pressure to avoid incorporating three-dimensional lipid formations in the transferred layers, thereby ensuring their uniformity. For this reason it is convenient to define the monolayer excess tension as:

$$\tau_M = \pi_c - \pi. \quad (3)$$

Presuming that the elastic area expansion modulus, K_M, of the monolayer at the air-water interface is half that of the bilayer, K_B, the tension of a bilayer formed from two such monolayers will be double the excess tension of the monolayer. If the elastic expansion modulus of the monolayer is not equal to half that of the bilayer, the more general relation is:

$$\tau_B = \frac{K_B}{K_M}\tau_M. \quad (4)$$

Here we have ignored the lower bilayer modulus seen at bilayer tensions <0.5 mN/m that results from the flattening out of membrane undulations (Evans and Rawicz, 1990) as this effect will lead to minor perturbations in bilayer areal strain at high tensions. Given that the energy of adhesion between the substrate and the bilayer is ~1 mN/m or less, a bilayer formed from a monolayer is not energetically stable if the excess tension of the monolayer exceeds 0.5 mN/m, provided the elastic modulus of the monolayer is about half that of the bilayer. This result is significant because it indicates that the bilayer is not thermodynamically stable on silica at the surface pressures commonly used ($\leq$40 mN/m). Consequently, except when formed from monolayers at unusually high surface pressures, the bilayer must be a kinetically trapped form that will contract when possible to lower its free energy. We may readily predict that cracks or other defects should develop in the bilayer if it is created at or above its critical

tension at failure. This situation exists when the monolayer excess tension is:

$$\tau_M \geq \frac{K_M}{K_B}\tau_{B,crit} \quad (5)$$

where $\tau_{B,crit}$ is the critical tension of the bilayer.

MATERIALS AND METHODS

The lipids 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL) and cholesterol (Chol) was purchased from ICN Biomedical (Cleveland, OH). The purity of the lipids was verified to within 1% by thin layer chromatography on silica gel using a solvent system of 65:25:4 $CHCl_3$:CH_3OH:H_2O by volume and I_2 detection. The solvents and I_2 were all of reagent grade or better. Buffer was prepared from 18 MΩ semiconductor grade deionized water and reagent grade chemicals. The buffer was 100 mM NaCl, 10 mM TRIS, adjusted to pH 7.4 with HCl and filtered to 0.2 μm.

Contact angle measurements between collapsed lipid monolayers and silica were performed on an optically smooth 8-mm diameter fused silica rod held vertically in a hole in a polytetrafluoroethylene (PTFE) block. A small chip of PTFE was placed in proximity to the rod. The apparatus was rinsed in methanol and then cleaned in an O_2 plasma for 10 min. Approximately 1 cm^3 of buffer was dropped at the base of the rod and 5 μl of lipid in $CHCl_3$ (10 mg/ml) were dropped on the side of the PTFE chip to create a collapsed monolayer, with the lipid on the chip providing a reservoir. The ability of this technique to create a collapsed monolayer was verified by the Wilhelmy method. The buffer contact angle with the silica rod was observed by using a contact angle goniometer.

A commercial L-B trough (Langmuir Trough 4, Joyce-Loebl, Tyne and Wear, UK) was used for measuring monolayer collapse pressure and performing π-A isotherms and monolayer transfers. The trough maintained the working monolayer area within a constant-perimeter tape held in position by PTFE rollers. The subphase was held at 20 ± 1°C. All lipids and mixtures were spread from 10 mg/ml solutions in $CHCl_3$ by dropping from a 10 μl syringe at a height ≤5 mm. Two to five minutes were allowed for solvent evaporation before compression. Monolayer collapse pressures were determined by compressing monolayers of the desired lipid to an area of less than half the critical area at collapse. Two minutes were allowed for stabilization before reading the surface pressure. Three measurements were taken for each lipid or mixture and averaged. The π-A isotherms used for determination of the monolayer elastic moduli were performed at a barrier compression rate of 0.61 ± 0.01 cm^2/s from an initial area of ~900 cm^2. Transfer ratios of the lipids to SiO_2 were determined by using plasma-cleaned 5.2-cm-wide square silicon wafers with a surface oxide layer ≥60 nm. Transfers were performed at a dipping speed of 59 ± 1 μm/s. Significant loss of material from the monolayer was observed during the initial downward excursion of the wafers into the subphase when the surface pressure exceeded 45 mN/m. Consequently, an oval PTFE barrier was positioned at the water line to provide a monolayer-free surface for the initial downward pass of the substrate. The barrier was submerged after the initial downward pass.

Monolayer elastic moduli were calculated by linear least-squares fitting to the π-A isotherms in the region of $40 \leq \pi \leq 46$ mN/m. The fits were projected back to the collapse pressure of the lipid to estimate the limiting area of the monolayer. The elastic compressibility modulus was then calculated as the product of the slope of the fit and the limiting area. The results reported herein are the average of three separate measurements for each lipid or mixture. Transfer ratios were calculated from linear fits to the monolayer area versus dipper position data using the central 20 mm of 30-mm excursions to exclude end effects.

Bilayers were prepared for morphological observations by transferring as above, except that 1 mass % of the fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate, Molecular Probes, Eugene, OR) was mixed with the lipid. After bilayer transfer the substrate was placed in a submerged clear polystyrene box. At low magnification the bilayer was observed through the box with a Zeiss ICM 405 fluorescence microscope (Oberkochen, Germany) using a mercury arc lamp and a Zeiss filter set (450–490, FT 510, and LP 520). The substrate was then sandwiched with a coverslip and observed through the coverslip at high magnification using an oil immersion lens. Images were recorded with a DAGE-MTI (Michigan City, IN) model 66 SIT camera and transferred directly to a Macintosh II computer equipped with a Data Translation (Marlboro, MA) QuickCapture frame grabber. Images were processed using the public domain Image software package (Version 1.47) from the National Institutes of Health.

RESULTS

Contact angles of lipids on silica

The supplementary contact angles on silica of collapsed monolayers of the six lipids and mixtures studied in this work were measured optically and are presented in Table 1. The observed angles did not change over a 5-min time span. The collapse pressures of the monolayers are also given. From these data the surface tensions of the collapsed monolayers were calculated and the work of adhesion of each of the monolayers to silica were determined by using Eq. 2. Because of the extreme difficulty of accurately measuring contact angles below 20° using a goniometer, these data should be viewed cautiously. Nevertheless, assuming that the measured angles represent the equilibrium contact angles reasonably well, the adhesion energies are all small, in the neighborhood of 1 mN/m or below, with a definite downward trend for SOPC/Chol mixtures as the cholesterol concentration is increased. These data support the assumption (made above) of low adhesive energy between condensed phospholipid monolayers and silica. As the adhesion energy between the substrate and the monolayer increases with monolayer density (Sato and Kishimoto, 1979), and monolayer density is maximal at the collapse pressure, it is reasonable to presume that the monolayer-substrate (hence, bilayer-substrate) adhesion energy is small at all surface pressures the film will bear.

Monolayer elastic moduli

The elastic area expansion moduli for bilayers of SOPC and mixtures of SOPC and cholesterol have been published

TABLE 1 Adhesive energy between lipid monolayers and silica

Lipid	π(mN/m)	γ(mN/m)	θ	ΔW(mN/m)
SOPC	48.7 ± 0.1	24.2 ± 0.1	15 ± 2°	0.8 ± 0.2
SOPC/Chol 86:14	50.2 ± 0.1	22.7 ± 0.1	14 ± 2°	0.7 ± 0.2
SOPC/Chol 67:33	50.6 ± 0.1	22.3 ± 0.1	10 ± 2°	0.3 ± 0.2
SOPC/Chol 50:50	50.6 ± 0.1	22.3 ± 0.1	9 ± 2°	0.3 ± 0.1
DOPE	48.3 ± 0.1	24.6 ± 0.1	10 ± 2°	0.3 ± 0.2
DPhPC	48.9 ± 0.1	24.0 ± 0.01	<5°	<0.1

The surface tension of 100 mM NaCl at 20°C is 72.92 mN/m according to the CRC handbook, and this value was used to find surface tension from surface pressure measurements. The adhesive energy was calculated by the Young-Dupré equation as modified for the supplement to the traditionally defined contact angle.

(Needham and Nunn, 1990). To relate bilayer tension with monolayer excess tension via Eq. 4, the elastic moduli of the monolayers in the vicinity of their collapse pressures are necessary. The elastic modulus of SOPC and the three mixtures of SOPC and cholesterol used in this study were measured from their pressure-area isotherms. A π-A of the mixture SOPC/Chol 67:33 is shown in Fig. 2 *A*. Considerable hysteresis is observed between the advancing and receding curves. Such hysteresis is usually indicative of monolayer collapse, yet the measured collapse pressure (Table 1) was not reached. An extremely slow π-A of the same mixture is shown in Fig. 2 *B*. One-third of the monolayer area is lost during compression at well below the collapse pressure. On the other hand, an area-versus-time plot for SOPC/Chol 67:33 near the collapse pressure shows the monolayer to be stable at this pressure for ~1 h, followed by a gradual reduction in area that could easily result from oxidation of the lipid (Fig. 2 *C*). A similar apparent collapse of the monolayer was observed by Hildebran et al. (1979) in experiments with dipalmitoylphosphatidylcholine on a sliding-barrier PTFE trough. They found that consistent isotherms were obtained after pretreating the trough by compression of a monolayer to extremely high surface pressures (near 70 mN/m). We believe that the observed hysteresis is not the result of a natural tendency of these monolayers to collapse at lower pressures. Rather it appears that lipid is temporarily transferred from the monolayer onto the barrier and that this transfer is exacerbated by motion of the constant-perimeter barrier and rollers of the Joyce-Loebl trough. Based on these results, measurement of the elastic moduli of the monolayers was carried out using least-squares fits to the data in the range of surface pressures between 40 and 46 mN/m, as π-A curves taken at a compression rate of 0.61 cm^2/s were not noticeably affected by hysteresis in this range. The expansion curves were not used because a break point representing complete return of the lipid from the barrier to the air-water interface could not be identified. Rather, return appears to be gradual. Monolayer elastic moduli are presented in Table 2 for SOPC and the SOPC/Chol mixtures. The monolayer/bilayer modulus ratio has generally been assumed to be approximately 0.5. The measured modulus ratio varies considerably, from 0.32 to 0.72 depending on the lipid composition. The SOPC monolayer is stiffer than expected, whereas the SOPC/Chol 50:50 monolayer is less stiff than predicted.

Transfer ratio versus excess tension

The transfer ratios of all the lipid systems studied here were measured versus excess tension. Transfer ratios of the initial monolayers were close to unity in all cases and appeared to have no systematic variation with excess tension or lipid. Taken together, the average initial monolayer transfer ratio was 1.02 ± 0.11 ($n = 29$), which is very close to the theoretical ideal of unity. The transfer ratios of the second monolayers varied considerably with excess tension, from near unity to ~ -0.7. A negative transfer ratio indicates that net removal of lipid has occurred during the dipping motion,

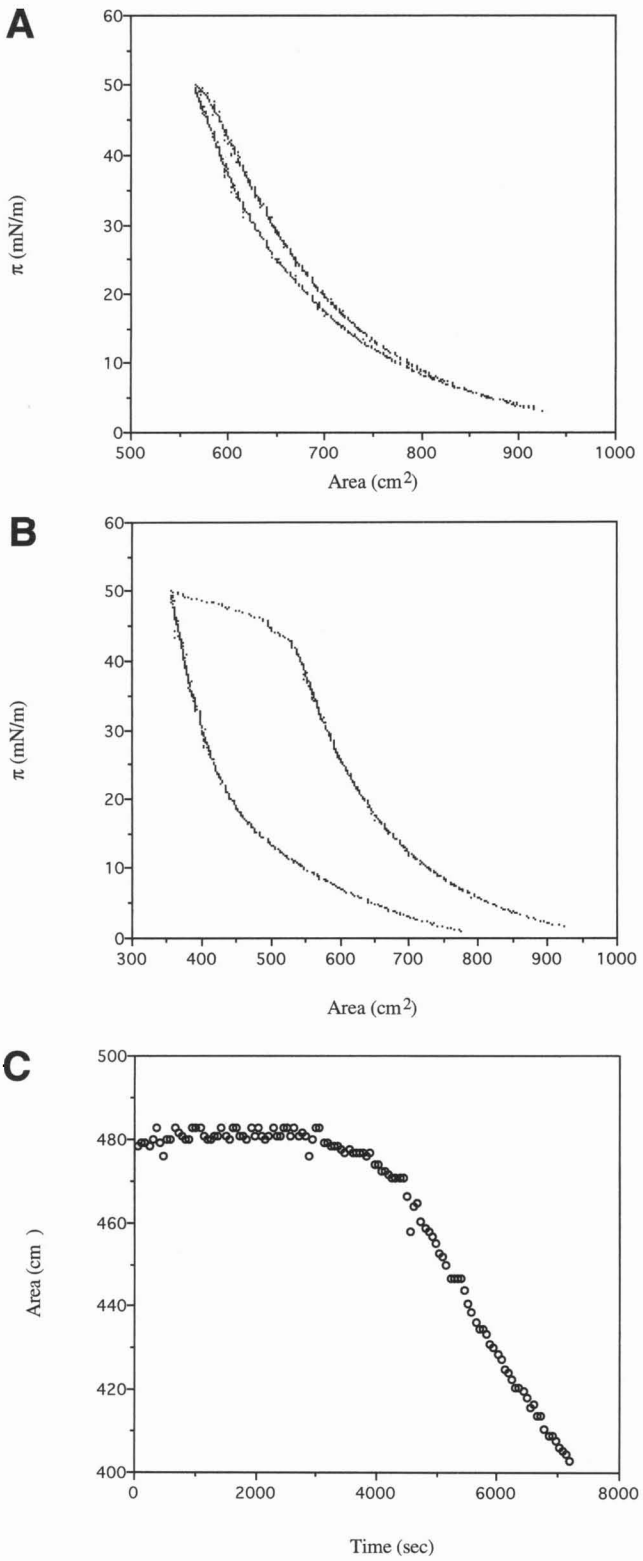

FIGURE 2 Evidence that hysteresis in the π-A isotherm is not the result of collapse of the monolayer at the air-water interface. (*A*) The π-A isotherm of SOPC/Chol 67:33 shows visible hysteresis when measured at a compression rate of 0.61 cm^2/s. (*B*) When compressed at 0.017 cm^2/s in the range from 40–50 mN/m, the hysteresis is marked, showing a loss of approximately one-third of the film area. The remainder of the isotherm was performed at 3 cm^2/s. (*C*) The area-versus-time response of the film at $\pi = 49.2$ mN/m shows essentially no loss in film area for ~1 h after spreading and compression when the barrier position is held steady.

TABLE 2 Compressibility moduli of monolayers at the air-water interface

Lipid	Monolayer area compressibility modulus (mN/m)	Bilayer area compressibility modulus* (mN/m)	Monolayer/ bilayer modulus ratio
SOPC	139 ± 2	193 ± 20	0.72 ± 0.07
SOPC/Chol 86:14	143 ± 7	216 ± 12	0.66 ± 0.06
SOPC/Chol 67:33	163 ± 10	289 ± 18‡	0.56 ± 0.05
SOPC/Chol 50:50	250 ± 15	781 ± 45	0.32 ± 0.03

*Bilayer expansion modulus data are from Needham and Nunn (1990).
‡No data were available for this lipid ratio, so the modulus was interpolated from data for the nearest available concentrations.

meaning that part of the initial monolayer was removed. Transfer ratios of SOPC and SOPC/Chol mixtures are plotted in Fig. 3 *A*. Three observations may be readily made regarding this plot. First, the transfer ratio declines with increasing excess tension. Second, the decline in transfer ratio is initially gradual but eventually becomes more abrupt, especially for the films containing a large fraction of cholesterol. Third, the low cholesterol mixtures exhibit relatively poor transfer even at low excess tension. The decline in transfer ratio with increasing tension supports the expectation that as the bilayer is inherently unstable on silica at excess tensions >0.5 mN/m, transfer of the second monolayer should become more difficult as excess tension increases beyond that point. The final abrupt failure of transfer indicates, however, that the trapped bilayer form is untenable beyond some threshold tension.

The second monolayer transfer ratios of DOPE and DPhPC are plotted in Fig. 3 *B*. Transfer of DOPE was poor at all excess tensions. On the other hand, the lipids with two unsaturated chains measured thus far have been found to form relatively weak bilayers. Furthermore, DOPE is not stable in the bilayer form at the temperature used (20°C). Transfer of DPhPC was similar to that of SOPC/Chol mixtures. This similarity is reasonable given that the archaebacterial lipids that DPhPC resembles must provide stable cell membranes under extreme conditions without the benefit of the condensing and membrane-toughening effects of cholesterol.

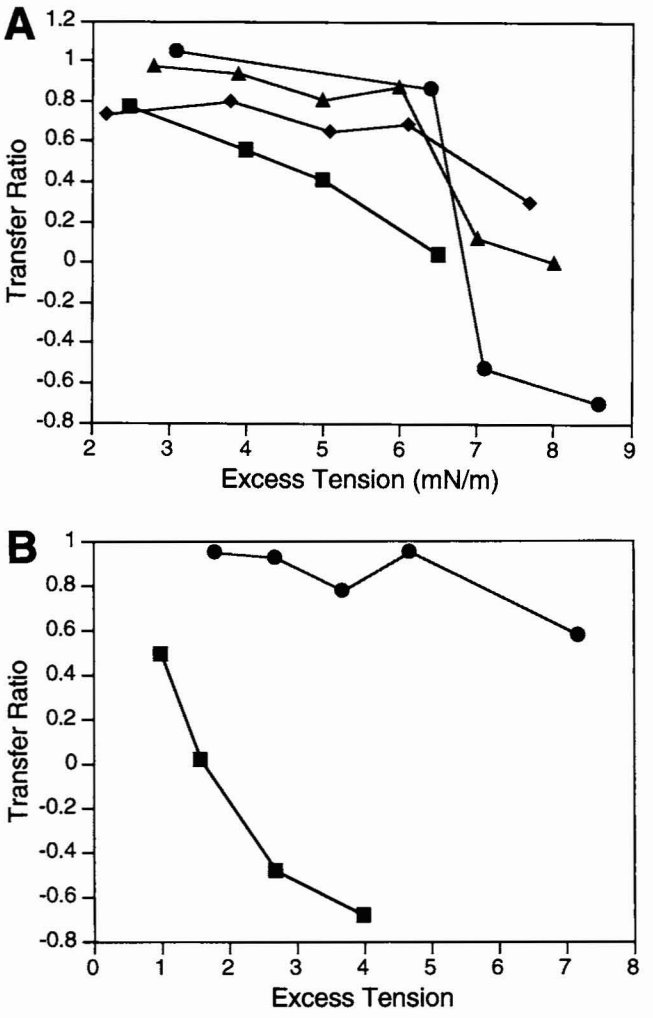

FIGURE 3 Second monolayer transfer ratios as a function of monolayer excess tension. Negative ratios indicate partial removal of the initial monolayer. (*A*) Mixtures of SOPC (■) and SOPC/Chol 86:14 (♦), 67.33 (▲), and 50:50 (●). (*B*) DPhPC (●) and DOPE (■). Transfer ratios are to within ±0.11 and excess tensions are to within ±0.2 mN/m.

Correspondence between bilayer and monolayer critical tensions

The data in Fig. 3 *A* show that, beyond a threshold level, transfer of the second monolayer decreases rapidly with excess tension, especially for mixtures having a high cholesterol concentration. In the theoretical section we discussed the proclivity of the bilayer toward defect formation once the bilayer critical tension at failure is met or exceeded. Along with formation of defects we may expect a decrease in the transfer ratio of the second monolayer, provided contraction of the bilayer in the vicinity of the defects occurs during the transfer process. By using Eq. 5 with the monolayer elastic moduli of Table 2 and published data for the bilayer elastic moduli and critical tensions (Needham and Nunn, 1990), an estimate for the maximal allowable excess tension consistent with good transfer of the second monolayer is found as shown in Fig. 4 (gray bars). The threshold transfer ratio is somewhat arbitrary; however, a transfer ratio of 0.5 or less is generally considered poor. Using a threshold of 0.5, the excess tension at bilayer transfer failure is determined (black bars). Excellent agreement between the estimated and measured excess tension limit is found. This agreement is especially meaningful for the high cholesterol mixtures, as they are less sensitive to the choice of threshold.

Fluorescence microscopy of bilayers

A low magnification fluorescence micrograph of an SOPC/Chol 67:33 (1 mass % DiI) bilayer formed from a monolayer at an excess tension of 7.5 mN/m shows striations in the direction of transfer (Fig. 5 *A*). A high magnification micrograph of this bilayer (Fig. 5 *B*) shows that the striations

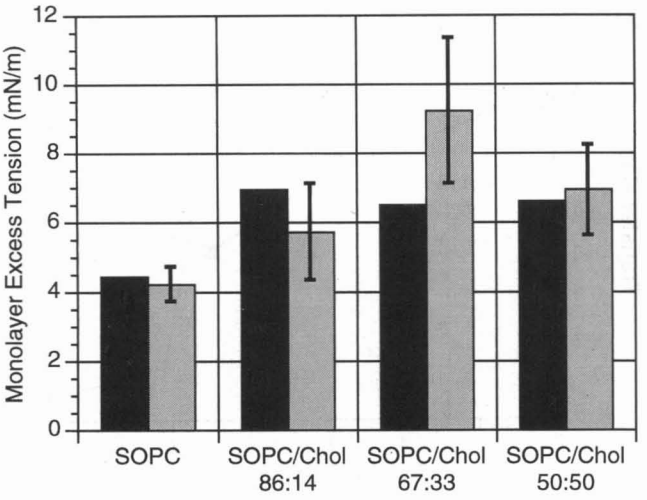

FIGURE 4 Comparison between theoretical excess tension limit for transfer and limits determined by thresholding. A threshold transfer ratio of 0.5 was taken as the lower limit for successful transfer. *Gray bars*, theoretical expectation calculated with data of Needham and Nunn (1990). *Black bars*, limits determined by thresholding.

are actually bundles of thinner striations and that a large number of spot defects are present in addition to the line defects. The low fluorescence intensity of these narrow striations and spots indicates that little if any lipid remains within them. Fewer striations were seen at an excess tension of 6.5 mN/m, and at 4 mN/m no defects were visible. The appearance of this profusion of defects at excess tension beyond the limits determined in Fig. 4 is in agreement with Eq. 5 and the results presented in Fig. 3, as the transfer ratio is expected to be well below unity when a considerable portion of the substrate is uncoated. Similar defects were seen for SOPC bilayers formed at 3 and 5 mN/m, with much wider striations at 5 mN/m. These results are in agreement with the generally poor transfer of SOPC at all excess tensions studied.

DISCUSSION

These results indicate that formation of phospholipid bilayers on silica by L-B transfer may be carried out with a high degree of success, but complete transfer of the second monolayer is only possible within a narrow window of surface pressures. This window is bounded by the collapse pressure and a pressure that is lower by approximately one-half of the liposomal bilayer lysis tension, depending on the monolayer/bilayer expansion modulus ratio. Incomplete transfer is manifested by point and line defects in the transferred bilayer within which the underlying substrate appears to be exposed to the subphase.

Given that the monolayer area expansion modulus has generally been assumed to be one-half that of the bilayer (Israelachvili, 1985; MacDonald and Simon, 1987), our observations to the contrary are worthy of further discussion, as they demonstrate that the correspondence between monolayer and bilayer states is inexact. This assumption follows

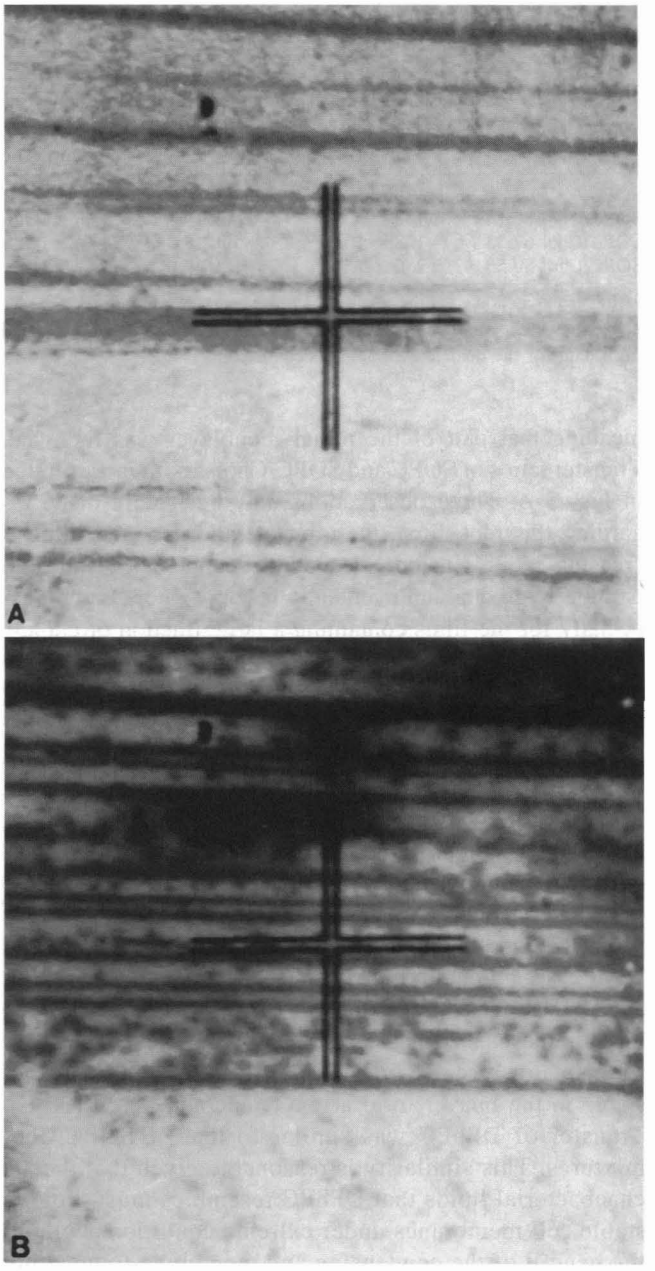

FIGURE 5 Fluorescence microscopy of bilayers on silicon. Monolayer excess tension = 7.5 mN/m. (*A*) Low magnification (crosshairs = 100 μm) showing parallel striations in the direction of substrate motion during dipping. (*B*) High magnification (crosshairs = 10 μm) showing that the striations as shown in *a* are composed of groupings of more narrow striations. A multitude of spot defects are visible, with sizes ranging from $\leq$0.5 μm to $\sim$2 μm.

from a model of the bilayer as consisting of two effectively independent monolayer leaflets. The alkyl chain termini of the lipid molecules at an air-water interface are in a considerably different milieu, however, than at an alkane-water interface or within a bilayer. The effects of this difference in environment on lipid behavior have been discussed by Gruen and Wolfe (1982). In the bilayer form, the lipid chain termini fluctuate above and below the midplane of the bilayer. These

fluctuations are roughly five times less likely for the chain termini of a monolayer at an air-water interface because of the Van der Waals energy cost associated with exposing the chain terminus to air (Gruen and Wolfe, 1982). The lipid chains of a condensed monolayer are relatively ordered, becoming less ordered as the monolayer undergoes expansion. Therefore, there is an increased energy required for expansion of a monolayer at an air-water interface relative to that required for expansion of a monolayer leaflet in a bilayer. This increased energy requirement should lead to an increased area expansion modulus for the monolayer at the air-water interface. Therefore, although we might expect the elastic area expansion modulus of a monolayer at an alkane-water interface to be one-half that of the bilayer, we do not expect the same behavior at the air-water interface.

The data in Table 2 show that the monolayer expansion modulus is indeed more than one-half that of the bilayer for SOPC, but the monolayer/bilayer modulus ratio decreases as cholesterol is added to the lipid composition, to the extent that at 50 mol % cholesterol the monolayer elastic modulus is actually much less than one-half that of the bilayer. This result seems contradictory to the explanation given above. Mixtures of cholesterol and phosphatidylcholine lipids containing an appreciable amount of cholesterol form a cholesterol-rich ordered fluid phase in coexistence with the usual liquid-crystalline phase of the pure lipid (Vist and Davis, 1990). The elastic properties of SOPC/Chol liposomes have been modeled well by treating the bilayer as a composite material (Needham and Nunn, 1990). Therefore, we presume that the lower than expected elastic modulus of the SOPC/Chol 50:50 monolayer is due to a reduced stiffness in the cholesterol-rich phase. The expansion modulus and packing density of steroid/phosphatidylcholine lipid monolayers varies considerably even among highly similar steroids (Yamauchi et al., 1993), for which cholesterol gives both the highest modulus and density. This variability implies that the packing is highly sensitive to the orientation of the steroid relative to the phospholipid. Given the well established position of cholesterol in the phosphatidylcholine lipid membrane with the 3 β-hydroxyl group of cholesterol hydrogen bonding to the phospholipid carbonyl (Franks, 1976; Huang, 1976; Worcester and Franks, 1976), cholesterol is too short to match the length of the stearic chain of SOPC (Yeagle, 1987). This mismatch may result in an unevenness of the surface at the bilayer midplane that would be suppressed in a monolayer at the air-water interface. Such suppression of the normal packing arrangement might interfere with the condensing effect of cholesterol, leading to a reduced area expansion modulus.

We have identified two possible causes of bilayer transfer failure: a limitation based on the thermodynamic instability of phospholipid bilayers on silica at all but the highest surface pressures and a limitation based on the lysis tension of the bilayer. The generally poor transfer of SOPC at all excess tensions studied may be explained by the former mechanism. The excellent transfer of SOPC/Chol 50:50 at low excess tension, followed by abrupt, complete failure on crossing a threshold tension may be explained by the latter mechanism. The question of why the failure mechanism should depend on lipid composition remains. We may note that defects in the bilayer form in SOPC even at tensions below that expected from the bilayer lysis tension, although spot defects were not observed. It may be that the greater mobility of SOPC allows portions of bilayer that do form to be removed by a conveyor belt action. In other words, we expect SOPC to have less viscous friction between monolayer leaflets at the bilayer midplane than we expect for mixtures of SOPC and cholesterol. This lower friction may allow the outer monolayer to slip off, with rolling up of the inner monolayer. On the other hand, the catastrophic failure mechanism appears to account for the results seen for SOPC/Chol 50:50 and 67:33. We then ask why the transfer ratio drops suddenly at the threshold tension. One explanation for the defects seen in Fig. 5 would be that, once the critical tension is reached, large numbers of defects form in the bilayer directly as it is formed. The material in the vicinity of the defects contracts to relieve stress, and some of it returns to the air-water interface. The remainder, having contracted sufficiently, is then relatively stable and remains on the substrate as isolated patches or as a continuum with patches missing. This explanation is consistent with the low intensity seen in the pinstripe defects that suggests a complete or near-complete absence of lipid material in the defects.

The development of the pinstripe defects is puzzling. Given that the defects are aligned with the direction of L-B transfer, and that only circular defects have been seen with horizontal transfer of the second monolayer (Tamm and McConnell, 1985), we presume that the dipping motion is a prerequisite for the formation of these linear defects. The pinstripe defects might form as elongations of spot defects, as straight bilayer edges are more stable than small circular edges due to the unsatisfactory packing of bilayer-forming lipids at circular edges (Israelachvili et al., 1976). The observed dimensions of the defects are sufficiently large that the effects of edge curvature are negligible. Therefore, the defects must form as described, and then the bilayer regions surrounding each defect must rapidly contract, creating larger defects. Contraction must be rapid for some lipid to return to the air-water interface, otherwise the transfer ratio should not decrease. As the development of these defects appears to be critical in bilayer transfer failure, there might be some value in observing bilayer transfer by in situ fluorescence microscopy to follow the time course of defect formation.

An understanding of the parameters influencing bilayer formation by the L-B technique is critical to the development of biophysical tools and sensors employing reproducibly formed, solvent-free lipid membranes. The primary intention of this work has been to develop an understanding of the effects of monolayer surface pressure on the formation of supported bilayers. It is worth noting that, although successful transfer has been demonstrated, the necessary surface

pressures (>42 mN/m) are well above the 35–40 mN/m generally used in the past. For most complete bilayer formation, the highest surface pressure that can be accurately maintained below collapse should probably be used. If the creation of a model membrane representative of animal cell membranes is desired, then so much the better, as incorporation of cholesterol leads to better transfer in general and a more forgiving system with respect to surface pressure fluctuations. Bilayers bearing a high cholesterol content also tend to be mechanically tougher than bilayers formed from pure phospholipids (Needham and Nunn, 1990), which may significantly reduce the effects of vibrations on the lifetime of freely suspended portions of the membrane. The development of long-lived membranes is vital to the implementation of sensors based on ligand-gated ion channel proteins. Adherence to these rules of thumb should improve the rate of successful model membrane formation and may increase the useful lifetimes of such membranes as well. We have already observed that the protocols developed allowed us to form long-lived bilayers spanning large holes in planar silicon supports (Osborn, 1994; Osborn and Yager, 1995).

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