Equilibrium and Dynamic Interfacial Tension Measurements at Microscopic Interfaces Using a Micropipet Technique. 2. Dynamics of Phospholipid Monolayer Formation and Equilibrium Tensions at the Water−Air Interface

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Phospholipid monolayers at the water−air interface have been used extensively as models approximating one-half of the biological bilayer membrane and are of particular interest as stabilizers of microbubbles and emulsions. Interfacial tension is an important measure of adsorption and monolayer formation, but for macroscopic interfaces, equilibrium conditions have been difficult if not impossible to reach, especially over the wide range of temperatures necessary to study homologous series of lipids such as the phosphatidylcholines (C12−C18). By use of a new micropipet technique, however, clean, freshly prepared interfaces on the scale of microns can be rapidly and repeatably produced and exposed to monolayer-forming materials (such as phospholipids and surfactants), the equilibrium condition can be quickly achieved in minutes, and changes in the surface tension by introducing new solutions to the interface (and hence, adsorption dynamics) can be accurately measured and tracked. We have used this technique to study the formation of monolayers of various insoluble surfactant systems (pure and mixed phospholipids including cholesterol and charged lipids) at the water−air interface by measuring equilibrium and dynamic surface tensions. We show that liquid-phase lipid systems spread rapidly (50 mN/m min^{-1}) from vesicle suspensions to form monolayers and reach the same equilibrium surface tension of 25 mN/m. The incorporation of cholesterol to create a liquid ordered phase that dramatically changes the molecular packing, elastic modulus, and tensile strength of bilayers has no effect on the rate or equilibrium surface tension of the monolayers. From the limiting surface tension of 25 mN/m for all liquid lipid monolayers (including cholesterol rich), it appears that the liquid state of the lipid acyl chains is the major influence in determining a monolayer surface tension that is similar to the bulk liquid hydrocarbon/gas surface tension. For single-component lipids in their gel phase, the equilibrium surface tension and rate of monolayer formation decreases as the temperature is lowered below T_m, consistent with a reduced spreading pressure for gel-phase lipids. Finally, unscreened interfaces in deionized water were not readily coated by DOPC/DOPG lipids, while in salt solution, monolayers were readily formed, showing that even in the presence of large amounts of lipid as vesicles in suspension, the water−air interface can remain clean when electrostatically stabilized.

1. Introduction

The formation and properties of phospholipid monolayers at the water−air interface have long been the subjects of theoretical and experimental studies in which the monolayer has been treated as a model system approximating one-half of the biological bilayer membrane.[1−9] Phospholipids as monolayers are also of particular interest as stabilizers in formulations of microbubbles and emulsions where the stability of these lipid-stabilized colloidal systems is strongly dependent on the degree of interfacial coverage by the monolayer.[10−15]

Phospholipids form insoluble monolayers at the water−air interface because of their negligible solubility in the aqueous phase (on the order of 10^{-10} M for DPPC and 10^{-16} M for DSPC).[16] It has long been appreciated that the amphiphilicity of the phospholipid anchors the molecule at the interface such that the hydrophobic aliphatic chains are oriented toward the air side and the hydrophilic headgroup is in contact with the water side.[17,18] The

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Table 1. Phospholipids Used in This Study

full name	abbreviation	carbon:double bonds per chain	transition temp (°C)
1,2-dilauroyl-*sn*-glycero-3-phosphocholine	DLPC	12:0	-1 ± 0.8
1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	DMPC	14:0	23.5 ± 0.4
1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	DPPC	16:0	41.4 ± 0.5
1,2-distearoyl-*sn*-glycero-3-phosphocholine	DSPC	18:0	55.1 ± 1.5
1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	SOPC	18:0−18:1	6
1,2-dioleoyl-*sn*-glycero-3-phosphocholine	DOPC	18:1	−19
1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol	DOPG	18:1	−18

transfer of phospholipid bilayers in the form of vesicles to monolayers at the water−air interface results in a change in surface pressure of the interface as a consequence of monolayer formation. Among various experimental techniques for studying monolayers, surface pressure measurements using the Langmuir trough and its variants have been the earliest and most widely used. In many cases, the reported surface pressure and kinetics established by the monolayer vary according to the source of material, the spreading procedure, and time allowed for the experimental measurement.[7,19−21]

Previous measurements of the equilibration time for monolayers of various saturated diacyl phospholipids ($(C_n)_2$PC, $6 \leq n \leq 16$) at the water−air interface using the Wilhelmy plate method suggest that for phospholipids with longer acyl chains (i.e., each containing more than 12 carbons), monolayer equilibration required more than 1 day[10] and so for longer chains, it becomes impractical to reach equilibrium. Therefore, discrepancies among literature values for "equilibrium surface tensions", particularly for lipid monolayers formed at a temperature below their gel-to-liquid crystalline phase transition temperature (T_m), arise from the fact that true equilibrium was not obtained because of the slow adsorption process.[7,10] In addition to difficulties in satisfying equilibrium conditions, another experimental limitation of previous measurements has been the inability to maintain the temperature of lipid suspensions above their T_m for the required long equilibration time without allowing significant sample evaporation. This has been one of the major limitations of the classical trough-based surface tension method, designed for macroscopic interfaces, in studying phospholipids with higher T_m, such as DSPC ($T_m = 55$ °C). By use of a new micropipet technique, however, clean, new interfaces on the scale of microns can be rapidly and repeatably produced and exposed to monolayer-forming materials (such as phospholipids and surfactants). In addition, the equilibrium condition can be quickly achieved in minutes, and changes in the surface tension by introducing new solutions to the interface (and hence, adsorption dynamics) can be accurately measured and tracked. Throughout this procedure, temperature is strictly controlled, and a complete time history of monolayer formation at the interface is obtained.

Having established the validity and accuracy of the micropipet-based interfacial tension measurement on soluble surfactant systems in part 1,[22] we have used the technique with pure and mixed systems of insoluble phospholipid to study how lipids spread from a bilayer vesicle suspension to initially clean microscopic water−air interfaces and form monolayers. In this study, we measured the dynamic and equilibrium surface tensions of water−air interfaces bearing phospholipid monolayers for a range of common phospholipids, varying phase state via changes in temperature (liquid or gel), composition (pure or mixture with cholesterol), and electrostatics (negatively charged lipids with and without screening salt solution). Also, a comprehensive set of results on the temperature dependence of the equilibrium surface tension and the monolayer formation rate was obtained for the homologous series of saturated diacyl phospholipids from DLPC to DSPC.

2. Experimental Section

2.1. Materials. The phosphate buffered saline (PBS) aqueous solution contained 121.5 mM NaCl, 25.2 mM Na_2HPO_4, and 4.8 mM KH_2PO_4 (pH 7.36−7.44), and all components were products of Fisher Scientific. All lipids (see Table 1), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and cholesterol (CHOL), were obtained from Avanti Polar Lipids, Inc. as lyophilized powder or chloroform stock solution of greater than 99% purity and used without further purification.

2.2. Preparation of Lipid Suspensions. For measurements of the surface tension of water−air interfaces bearing spread phospholipid monolayers, 1.0 mM lipid was present as a lipid vesicle suspension in PBS buffered solution. For phospholipid/cholesterol mixtures, 1.0 mM total lipid concentrations were prepared. Phospholipid suspensions with and without cholesterol were prepared by first mixing appropriate amounts of the respective lipid stock solutions in chloroform in a glass vial. After the solvent was completely evaporated to deposit a thin film, the film was rehydrated with PBS and the suspension was placed in a Branson 1200 bath sonicator for approximately 30 s to reclaim lipid from the vial walls and promote dispersion of lipid. To fully hydrate the lipid, the suspension was incubated for 2−3 h in an oven at a temperature approximately 5 °C above the lipid bilayer gel-to-liquid crystalline phase transition temperature (T_m) of the main lipid component. Following incubation, probe sonication (XL2020 Misonix Inc.) with an output frequency of 20 kHz and output power of 82.5 W was used for 3 min to disperse the sample as multilamellar vesicles at a temperature above the appropriate phase transition temperature. This procedure created a suspension of vesicles ranging in diameter from 100 to 150 nm as determined from dynamic light scattering measurements (Brookhaven model BI-MAS instrument). For studies of the role of electrostatics on monolayer formation, a film of a DOPC (95 mol %) and DOPG (5 mol %) mixture was prepared and rehydrated with either PBS or water. The total lipid concentration of the DOPC/DOPG mixture was 1.0 mM. All pure phospholipids and lipid mixtures were prepared under the same conditions and examined on the day of their preparation.

2.3. Surface Tension Measurements. All surface tension measurements were made by the micropipet technique. The details of this new technique and measurement were described in part 1.[22] Briefly, a single measuring micropipet (tapered with tip diameter of ~10 μm) was used for dynamic and equilibrium surface tension measurements, and the temperature-controlled

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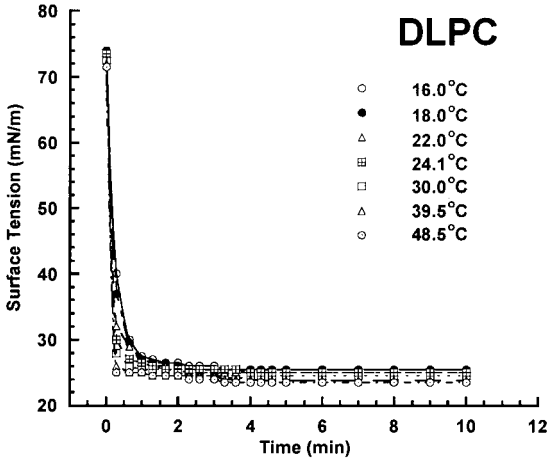

Figure 1. Effect of temperature on adsorption kinetics at the water−air interface, as monitored by change in surface tension with elapsed time of exposure of the interface to DLPC lipid aqueous suspensions (1.0 mM) at various temperatures. At all temperatures of study, the surface tension rapidly decreased within 1−2 min, approaching an equilibrium value of 25 mN/m.

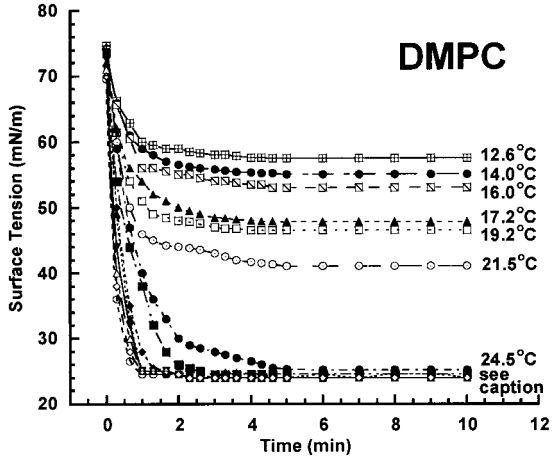

Figure 2. Effect of temperature on adsorption kinetics at the water−air interface, as monitored by change in surface tension with elapsed time of exposure of the interface to DMPC lipid aqueous suspensions (1.0 mM) at various temperatures. The surface tensions at 26.3 °C (■), 28 °C (♦), 34 °C (▲), 39 °C (▼), 43 °C (△), 46 °C (◊), and 52 °C (○) all rapidly reached an equilibrium value of 25 mN/m within 1−2 min.

microchamber was thermostated via an external circulating water bath. A clean interface for these experiments is readily established by applying a positive pressure to the pipet via an attached syringe, which forces the air column through the pipet toward the pipet mouth. When the air column in the pipet reaches the mouth, an additional increment of applied pressure causes the column to emerge from the pipet mouth and into the aqueous micropipet chamber medium, at which point the advancing column pinches off and forms a small (tens of microns) bubble that floats away to the chamber ceiling. What remains in the pipet is a new column front, which is then allowed to recede into the pipet upon removal of the forcing positive pressure. This new front in contact with fresh bathing solution constitutes the new and clean test interface for the experiment. This procedure can be repeated as many times as desired. Calibration of the instrument was made each day using pure liquids. All reported surface tension values are mean values of at least three measurements.

3. Results

3.1. Adsorption of Single Phospholipids at the Liquid−Air Interface. First, the formation of lipid monolayers from single-component lipid vesicles (DLPC, DMPC, DPPC, and DSPC at 1.0 mM concentration) in PBS (121.5 mM NaCl) at the water−air interface was considered. The time-dependent change in surface tension of a new and clean gas−solution interface was measured when a suspension of sonicated lipid vesicles was drawn into the pipet at the desired temperature. Before the surface tension measurements were made, the lipid vesicle suspension was pre-equilibrated at each temperature, continually monitored by means of a thermocouple directly inserted into the lipid suspension. Figures 1−4 represent the adsorption behavior of a series of lipid suspensions at different temperatures monitored by measuring the reduction in surface tension. As expected for this series of lipids with increasing gel-to-liquid crystalline phase transition temperatures (T_m), the time course for the change in surface tension of spread monolayers of the lipids varied considerably for the different temperatures examined. For DLPC, liquid L_α at all temperatures of study (Figure 1), the surface tension rapidly decreased within 1−2 min, approaching an equilibrium value of 25 mN/m. For DMPC, DPPC, and DSPC suspensions at various temperatures (Figures 2, 3, and 4, respectively), the

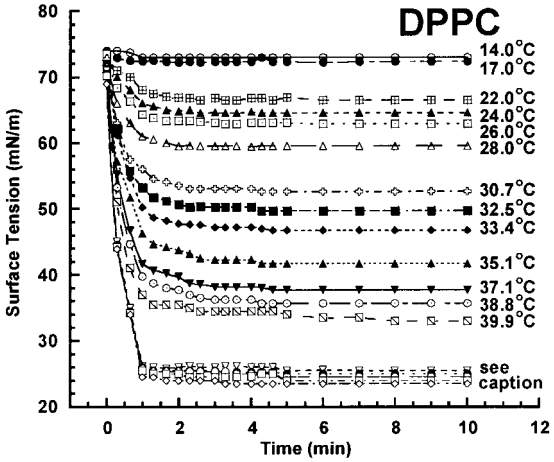

Figure 3. Effect of temperature on adsorption kinetics at the water−air interface, as monitored by change in surface tension with elapsed time of exposure of the interface to DPPC lipid aqueous suspensions (1.0 mM) at various temperatures. The surface tensions at 43 °C (▽), 45 °C (●), 47 °C (○), 51.1 °C (□), and 59.6 °C (◊) all rapidly reached an equilibrium value of 25 mN/m within 1−2 min.

resulting equilibrium surface tension values of the phospholipid monolayer are strongly dependent on the temperature at which the experiment was performed in relation to the T_m of the lipid. In general, at all temperatures an initial drop in surface tension (dependent on relative temperature below T_m) from the clean value of ∼73 mN/m was followed by a much slower decline in tension. For DMPC (Figure 2), an equilibrium surface tension value of 25 mN/m was obtained only for the temperatures above its T_m (>24 °C), with higher equilibrium tensions, ranging from 40 to 60 mN/m, attained at lower temperatures. Equilibrium tensions were observed to decrease monotonically with increasing temperature. Similarly, for DPPC (Figure 3) and DSPC (Figure 4), equilibrium tensions monotonically decreased with increasing temperature, with 25 mN/m measured only at temperatures above the T_m of the lipid. For all lipids studied, the equilibrium surface tension value for the liquid state of 25 mN/m did not vary significantly with T above the T_m of the lipid (i.e., this was the minimum attainable value).

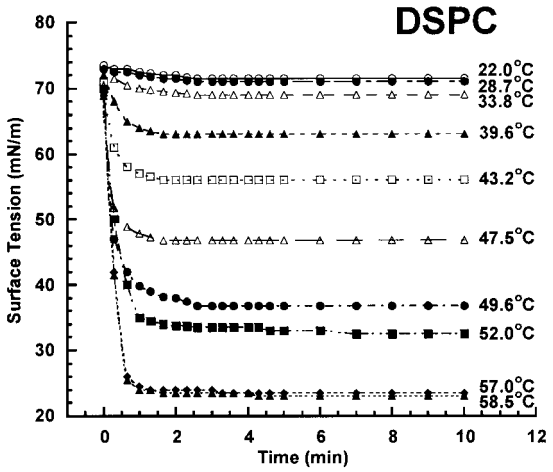

Figure 4. Effect of temperature on adsorption kinetics at the water−air interface, as monitored by change in surface tension with elapsed time of exposure of the interface to DSPC lipid aqueous suspensions (1.0 mM) at various temperatures.

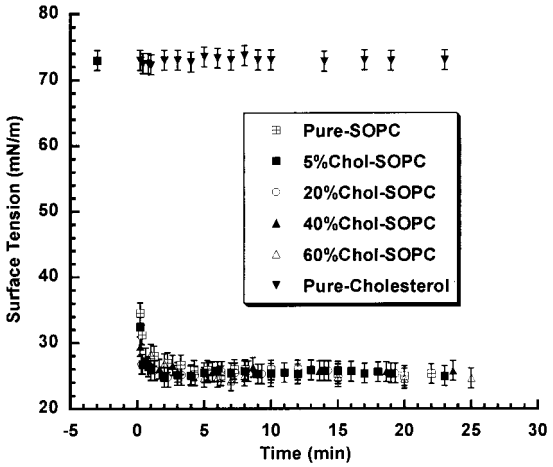

Figure 5. Change in surface tension with exposure time of water−air interface to vesicles composed of SOPC/cholesterol mixtures in PBS solution (total lipid concentration of 1.0 mM). Also shown are plots for exposure to pure SOPC vesicles and pure cholesterol crystallites. As the graph indicates, the surface tension reached the equilibrium values of ~25 mN/m within 2 min and does not change regardless of the concentration of cholesterol in SOPC vesicles. Pure cholesterol of the same concentration in the aqueous phase did not reduce the surface tension over the period of 25 min. All tension measurements were made at 22 °C.

3.2. Adsorption of Phospholipid−Cholesterol Mixtures at the Liquid−Air Interface. The results for four different mixtures of SOPC/cholesterol (at 1.0 mM concentration) are shown in Figure 5 plotted together with pure SOPC (at the same 1.0 mM concentration) and pure cholesterol. The interface in contact with pure SOPC vesicles in aqueous suspension reached an equilibrium value of 25 mN/m in 1−2 min at 22 °C, while pure cholesterol at the same concentration in the aqueous phase did not reduce the surface tension at all over a period of 25 min. The inclusion of 5, 20, 40, and 60 mol % cholesterol in the SOPC vesicles each reduced the surface tension of the monolayer in PBS solution to around 25 mN/m, the same value obtained for the pure SOPC suspension. Monolayer formation was also studied for mixtures of saturated lipids (DLPC, DMPC, DPPC, and DSPC) with cholesterol (1:1 mol ratio) in the PBS solution by monitoring the change in surface tension as a function of time at 22 °C as shown in Figure 6. The equilibrium tension of

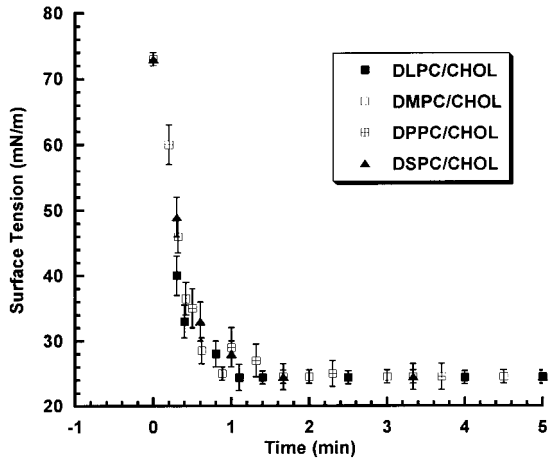

Figure 6. Change in surface tension with time of the water−air interface following exposure to various lipid/cholesterol mixtures (1:1 mol ratio, total concentration of 1.0 mM). Equilibrium surface tension of ~25 mN/m was reached within 2 min for all lipid mixtures and remained constant over the period of 4 h. All tension measurements were made at 22 °C.

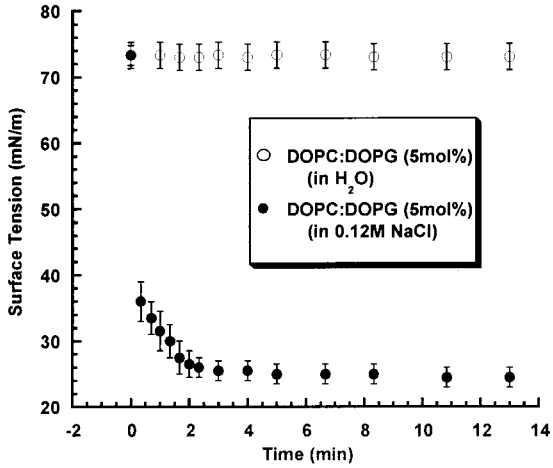

Figure 7. The effect of solution environment is shown for DOPC (95 mol %)/DOPG (5 mol %) lipid mixtures in pure water (○) and in salt solution (●). The surface tensions shown remained constant over the period of 1 h. Experiments were conducted at 22 °C.

~25 mN/m was again quickly reached for these liquid (liquid ordered) systems on the order of 1−2 min for all lipid monolayers and remained stable over a 4 h period.

3.3. Adsorption of DOPC/DOPG Mixtures at the Liquid−Air Interface. To introduce a known amount of negative charge into the vesicles, DOPC (95 mol %) was mixed with DOPG (5 mol %), which has one net negative charge per molecule. The surface tension of the DOPC/DOPG system (at 1.0 mM concentration) was measured in pure water and in salt solution (0.12 M NaCl) at 22 °C as shown in Figure 7. For the DOPC/DOPG system in pure water, the surface tension remained unchanged from the clean water−air interface value for the duration of the experiment (1 h). When salt solution was present, however, the surface tension decreased to 25 mN/m within 2 min for these liquid-phase monolayers, commensurate with the behavior and values of neutral lipids as liquid-state membranes and monolayers.

4. Discussion

4.1. Effects of the Phase State on Monolayer Formation. In this section, we discuss how the equilibrium surface tension and the formation rate of lipid

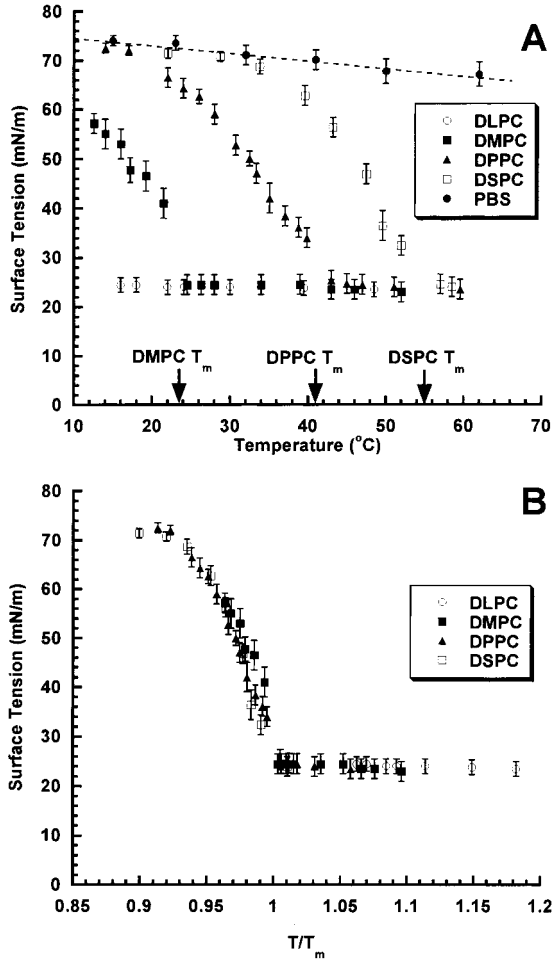

Figure 8. (A) Plots of the equilibrium surface tension obtained at different temperatures (the data in Figures 1−4) are summarized as DLPC (○), DMPC (■), DPPC (▲), and DSPC (□). Arrows mark the gel-to-liquid crystalline phase transition temperature (T_m) for DMPC, DPPC, and DSPC at 23.5, 41.5, and 55.1 °C, respectively. As a calibration, the change in surface tension of the PBS solution without lipid (●) was tested over the same temperature range and the temperature coefficient was found to be ca. −0.16 mN m^{-1} °C^{-1} which is the value reported for the bare water−air interface (ref 23). (B) The equilibrium surface tension values are plotted as a function of their relative phase transition temperature (T/T_m), and the behavior is collapsed to a single curve.

monolayers formed at temperatures below and above their T_m depend on their relative phase transition temperature (T/T_m). Plots of the equilibrium surface tension obtained at different temperatures (the data in Figures 1−4) are summarized in Figure 8A,B. It is clear from Figure 8A that for each phospholipid, the equilibrium surface tension values decreased with increasing temperature until T_m (23.5, 41.4, and 55.1 °C for DMPC, DPPC, and DSPC, respectively, flagged by arrows in Figure 8A), at which temperature the surface tension values reached their minimum values at ∼25 mN/m. As a calibration, the change in surface tension of the PBS solution without lipid was tested over the same temperature range (shown in Figure 8A) and the temperature coefficient was found to be ca. −0.16 mN m^{-1} °C^{-1} which is the same as previously reported values for the bare water−air interface[23] and is therefore negligible compared to lipid effects. Because measurements were carried out with a series of

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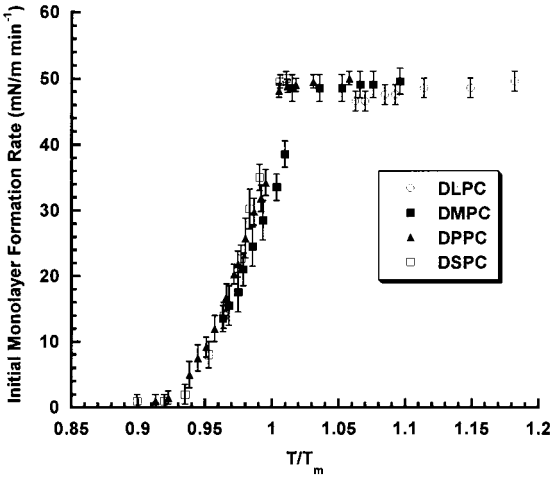

Figure 9. The initial monolayer formation rates of lipid suspensions at their water−air interfaces are given as a function of their relative phase transition temperature (T/T_m). The monolayer formation rates are determined from the initial drop of the surface tension in Figures 1−4. Again, behavior for all systems collapsed to a single curve.

lipids that cover a wide range of T_m, for purposes of comparison it is useful to compare the behaviors at their relative phase transition temperature (T/T_m) which indicates how far the system is above or below its T_m at the temperature at which the adsorption experiment was performed (T). It has been consistently observed that systems which are below T_m (with a relative phase transition temperature of less than 1) exhibit more gel-like character. This character is evidenced by higher surface yield shear and shear viscosity compared to systems which are closer to or above their T_m, as measured in another experiment in our laboratory.[12] Therefore, the relative phase transition temperature provides a useful standard of comparison for lipids with different T_m and is plotted for the equilibrium surface tension values in Figure 8B. This collapses all the data onto a single curve, and for each of the various lipid systems, the same equilibrium surface tension value is obtained at the same T/T_m. Figure 9 shows a plot of the initial monolayer formation rate as a function of T/T_m. The rate of formation of a monolayer at the water−air interface of a vesicle suspension was obtained from the initial slope of the surface tension versus time plots (Figures 1−4). The monolayer formation rate increased with increasing temperature until T_m, at which point the initial monolayer formation rate reached the maximum of around 50 mN/m min^{-1}. Above T_m (where the equilibrium surface tension is independent of temperature) in this relative temperature plot, the initial monolayer formation rate increases only slightly with temperature. Again, the data collapses onto a single curve and the same monolayer formation rate is obtained at the same T/T_m for each of the various lipid systems. The data summarized in Figures 8 and 9 then indicate the thermotropism of lipid monolayer formation at water−air interfaces, that is, lipid vesicles must be in the liquid state for rapid spreading on the clean interface to occur, and the equilibrium surface tension and the monolayer formation rate depend on the relative phase transition temperature of the lipid. For DLPC monolayers, no significant variations were observed in either surface tension or the monolayer formation rate, because all experimentally achievable temperatures are above the gel-to-liquid crystalline phase transition temperature of the lipid (T_m = −1 °C). In the experiment,

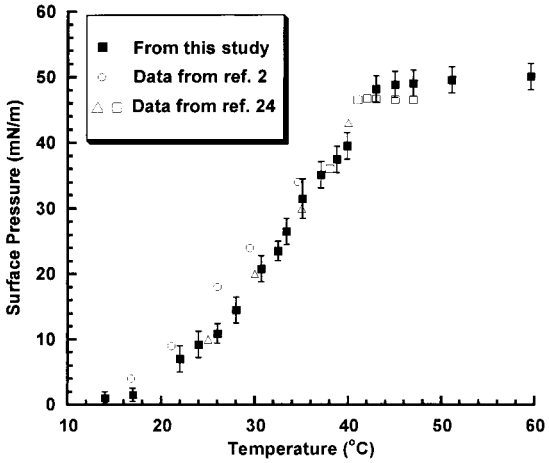

Figure 10. Surface pressure (converting the surface tension values (γ) into surface pressure values (Π) via the relation $\Pi = \gamma_0 - \gamma$) of DPPC monolayers at the water−air interface as a function of temperature are plotted together with the equilibrium spreading pressures (Π_e) and the transition pressures (Π_c) measured on a Langmuir trough reported in the literature (refs 2 and 24) (○ and △, the transition pressures (Π_c) from refs 2 and 24; □, the equilibrium spreading pressures (Π_e) from ref 24).

DLPC vesicles are in a liquid state and therefore spread rapidly at the water−air interface.

To make comparisons with previously reported data, our results from surface tension measurements on DPPC monolayers are plotted in Figure 10 as a function of temperature after converting the surface tension values (γ) into surface pressure values (Π) via the relation $\Pi = \gamma_0 - \gamma$. Data from an earlier study by Yamanaka[24] regarding the temperature dependence of the equilibrium spreading pressures (Π_e) and the transition pressures (Π_c) of DPPC monolayers is also plotted in Figure 10 for comparison. The transition pressures (Π_c) were determined from the point at which the two-dimensional condensation from liquid expanded to solid occurs in the $\Pi-A$ isotherm. A nearly linear increase in equilibrium spreading pressure was observed as the temperature was raised to the transition temperature, and this linear relationship was found to be identical with the relationship describing the temperature dependence of the transition pressure.[24] Figure 10 also includes data from an earlier Langmuir trough based study by Phillips and Chapman[2] on the temperature dependence of the $\Pi-A$ isotherm for a DPPC monolayer, and the Π_c obtained from the $\Pi-A$ isotherm is shown as a function of temperature. As Figure 10 illustrates, the surface pressure values determined from the micropipet technique for a DPPC monolayer at the water−air interface as a function of temperature are consistent with the equilibrium spreading pressure and the transition pressure of the monolayer obtained from the Langmuir trough experiment.

As mentioned earlier, at $T < T_m$ for several other systems of lipids, discrepancies in surface tension values in the literature are mostly due to the required long equilibration times for such macroscopic (tens of square centimeters) surfaces. However, the micropipet technique allows fast and accurate examination of various lipid monolayer systems over a practical range of temperatures (~15−60 °C), including the typically slow times associated with temperatures below the T_m. The measured equilibrium surface tension of 25 mN/m for all liquid-phase lipid monolayers (at $T > T_m$) is equal to typical values for the hydrocarbon−air interface (20−25 mN/m). This implies that these systems reach a limiting surface pressure of ~ 50 mN/m (i.e., from the equation $\Pi = \gamma_0 - \gamma$; ~73 mN/m − 25 mN/m = 48 mN/m), equivalent to the limiting surface pressures in Langmuir trough experiments.[6,7] It seems then that there is little contribution from the headgroup side of the monolayer to the surface tension. Interestingly, the measured tension is almost solely due to the liquid acyl chains making an interface with the air that is similar to liquid bulk hydrocarbon/air interfaces.

4.2. Monolayer Formation Rate and the Equilibrium Surface Tension Compared with Bilayer Area Dilation Modulus and Tensile Strength. From earlier studies on cholesterol in bilayers by Needham et al.,[25] it is known that the incorporation of cholesterol into SOPC bilayers dramatically increases the elastic modulus and tensile strength of the bilayer by promoting intermolecular interactions that condense the bilayer interface. The area dilation modulus increases with added cholesterol, reaching a maximum 6-fold increase at ~60 mol % cholesterol, while the area per lipid molecule decreases from 65 Å^2 for a pure SOPC bilayer (0 mol % CHOL) to 41.9 Å^2 at 58 mol % cholesterol.[25−27] It was therefore of interest, and a goal of this study, to determine whether the incorporation of cholesterol in lipid monolayers at the water−air interface had any effect on the surface tension. That is, would it lower the surface tension in a manner consistent with the reduction in area per molecule and possibly retard monolayer formation in accordance with the increasing tensile strength and work to expand the bilayer interface? Our results in Figure 5 show that there were in fact no significant differences in the monolayer surface tensions for the different cholesterol concentrations, even though the bilayer membrane area dilation modulus and molecular area have been shown to vary widely with cholesterol concentration. These results indicate that the surface tension reaches a minimum (commensurate with other liquid-phase systems) for this mixture, for all concentrations of cholesterol, and the rate of formation at the water−air interface was independent of the modulus and the area per lipid molecule in the bilayer. The absence of a change in surface tension for an aqueous phase containing pure cholesterol shows that this insoluble molecule does not transfer through the aqueous phase on a time scale of tens of minutes, nor do its crystallites readily spread at the water−air interface.

The addition of cholesterol to the saturated diacyl lipid bilayers at ~50 mol % is known to completely abolish any gel-to-liquid crystalline phase transition for the saturated phospholipid[28−31] and produce a liquid-phase bilayer (liquid ordered[17]). These cholesterol-rich liquid bilayers are characterized by very high area dilation modulus and tensile strength values that are even greater than those of gel-phase ($L_{\beta'}$) DMPC bilayers.[28] The cholesterol-containing bilayers are therefore much less compressible relative to even the gel-phase bilayer. For these saturated lipid/cholesterol systems (1:1), a comparison of the initial reduction in surface tension for the different compositions was made, again to test if and to what extent these highly

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condensed interfaces might influence bilayer spreading and monolayer formation. The results once again showed that no significant variation appeared in the initial stage of adsorption as shown in Figure 6. The final surface tension achieved by the inclusion of cholesterol (up to 50 mol % in this study) at equilibrium was again unaffected by composition, reaching the same value of ~25 mN/m for all cholesterol-containing membranes, despite the fact that these lipid/cholesterol mixtures have areas per lipid molecule close to the fully extended chain of ~41 Å^2, and dilation moduli ranging from 2000 to 4000 mN/m and tensile strengths up to 40 mN/m, compared to pure SOPC bilayers with values of 200 and 6 mN/m, respectively.[32] Thus, somewhat surprisingly, our finding is that the addition of cholesterol to lipid systems produced the same equilibrium surface tension value of 25 mN/m at a rate of 50 mN/m min^{-1} that was observed for other more compressible and weaker liquid-phase bilayers. This is in strong contrast with the behavior of the same lipids as pure bilayers and monolayers in their gel state (same area per molecule and relatively high modulus and strength, but solid), which spread very slowly.

Two conclusions from these data then can be made:

1. The residual 25 mN/m of all liquid-phase monolayer systems showed that surface tension is largely determined by the acyl chain/air interface, and a condensed and stiff headgroup region does not increase an already high surface pressure beyond ~50 mN/m.

2. Bilayers must be in the liquid phase in order to spread quickly as monolayers, and the modulus and the area per molecule are not necessarily determining factors in the rate at which lipid transfers from a vesicle bilayer to form a monolayer.

4.3. The Effect of Electrostatics on Monolayer Formation. Last, we also addressed the role that electrostatics play in monolayer formation using both neutral and charged lipid mixtures by examining their formation in pure water (no ionic screening) and in salt solution (121.5 mM NaCl). In the absence of any lipid, salt solution alone slightly raises the surface tension of the otherwise clean water−air interface, an effect well-known in the literature.[10,33] For the DOPC/DOPG (95:5) system in pure water (Figure 7), the surface tension remained nearly unchanged from the clean interface value, indicating that the charged lipid vesicles did not spread on the water−air interface in a nonscreening environment despite the liquid-phase state of the lipid (neutral SOPC rapidly spreads). In contrast, when salt solution was present, lipid spreading occurred rapidly at the same rate as for SOPC, and a lowering of the surface tension to the same value of 25 mN/m was achieved due to monolayer formation. This is an important practical result since even in the presence of large amounts of lipid as vesicles in the suspension, the water−air interface remained completely clean and essentially no lipid (or other impurity) had transferred or accumulated at the interface over a period of 1 h. Here, the lipid vesicle suspension can actually act as a kind of scrubbing agent that removes any impurities from solution that might otherwise adsorb at the clean interface and reduce the water−air surface tension. In the presence of this lipid suspension, then, the interface is kept clean.

5. Concluding Remarks

This micropipet technique has made it possible to quickly and reliably study phospholipid monolayer formation at the water−air interface over a practical range of temperatures to obtain surface tension measurements on microscopic interfaces. By use of this technique, the equilibrium surface tension of insoluble lipid monolayers formed at $T < T_m$ can be reached within 10 min for lipid systems that require much longer equilibration times on the more macroscopic interfaces. We were able to obtain a comprehensive set of results on the temperature dependence of lipid monolayer formation for the homologous series of lipids from DLPC to DSPC. These results show that the equilibrium surface tension and the rate of formation of lipid monolayers depend strongly on their relative phase transition temperature (T/T_m). However, different lipid systems exhibit the same surface tension and formation rates at the same T/T_m. These results further support an earlier finding[2,3,6,7,24] that the lipids as vesicles must be in the liquid phase to readily and completely spread on the clean interface to form a monolayer. Equilibrium spreading pressure data that have been measured on the Langmuir trough for the DPPC monolayer system are consistent with our data on the same system.[2,24] This result confirms that the surface tension measured by the micropipet technique provides the true equilibrium value compared to other trough experiments that have allowed only relative measurements to be made because of long equilibrium times.[34]

With respect to the actual equilibrium surface tension value for all the liquid lipid systems studied (pure or mixture with cholesterol), it is clear that irrespective of lipid chain length or composition, lipid vesicles in the liquid state spread rapidly to form equilibrium monolayers with a surface tension of 25 mN/m and an initial monolayer formation rate of 50 mN/m min^{-1}. Although several material properties of lipid bilayers, such as the elastic area dilation modulus, tensile strength, and area per molecule, are dependent on cholesterol content of the bilayer, our results showed that there is no apparent corresponding effect on equilibrium surface tension and monolayer formation rate of the monolayer formed from the lipid suspensions at the water−air interfaces. From the limiting surface tension of 25 mN/m for the liquid lipid monolayers, it appears that the liquid state of the terminal lipid acyl chains is the major influence in determining the monolayer interfacial tension that is similar to the bulk liquid hydrocarbon−gas surface tension.

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