Interfaces

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Behavior of an Adsorbed Phospholipid Monolayer Submitted to Prolonged Periodical Surface Density Variations**

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The adsorption of phospholipids at interfaces determines many biological phenomena, from cell life to lung-surfactant dynamics. The lung surfactant reduces the surface tension in the alveoli and ensures normal breathing. Although the dynamic adsorption and interfacial rheology of dipalmitoylphosphatidylcholine (DPPC), the main component of the native lung surfactant, has been extensively investigated to understand the mechanism of respiration,[1–8] this quest has met some serious difficulties.

Many studies have focused on DPPC monolayers spread as insoluble films on water.[9–12] One serious limitation of these studies is that the DPPC monolayer was not in contact with DPPC vesicles and/or aggregates in the aqueous phase. These studies thus overlook the role that the vesicles present in the mucus that lines the alveoli do play in the relaxation processes. It was shown indeed that phospholipid aggregates released from the alveolar type II cells diffuse to, come in contact with, and spread on the interface, thereby resulting in surface-associated phospholipid reservoirs.[13]

The attempts made so far to investigate DPPC films adsorbed at the air/water interface while in coexistence with dispersions of vesicles, by using the Langmuir balance,[14] Wilhelmy plate,[15] or static captive bubble methods,[16,17] met another severe shortcoming: the equilibrium interfacial tension γ_{eq} could not be attained owing to exceedingly slow phospholipid adsorption. Further studies, using a pulsating bubble surfactometer, submitted DPPC bubbles to large surface variations (typically 50%), and hence to very strong constraint,[16,18,19] preventing linear responses. The lowest interfacial tension values were only collected when the surface of the bubble was minimum, and hence when constraint was the largest. These tensions increased again during the expansion of the oscillation, thus showing that the system was out of equilibrium. Moreover, no studies investigated the system once the constraint was removed.

We have now removed these roadblocks by studying a DPPC monolayer adsorbed at the interface with an aqueous dispersion of DPPC vesicles when submitted to prolonged periodical perturbations of low amplitude. We found that under these conditions the adsorption rate of the phospholipid at the interface is strongly accelerated, and the interfacial tension γ is strongly lowered (by up to $20\,mN\,m^{-1}$). We show that an equilibrium state is reached. We also show that the tension remains at this low value after the constraint applied on the bubble surface is removed. A sudden increase in adsorption rate was observed for a γ value that corresponds to the value at which the liquid expanded (LE)/liquid condensed (LC) phase transition occurs in a Langmuir DPPC monolayer.

The adsorption dynamics of DPPC at the interface between air and an aqueous dispersion of phospholipid vesicles was studied by using bubble profile analysis tensiometry. The DPPC concentration was set to $1\times10^{-3}\,mol\,L^{-1}$, a value that allows convenient and precise measurement of the adsorption kinetics, as shown in a previous static-bubble study.[17] The bubble was submitted to a series of oscillations with a period T varying from 3 to 100 s (0.01 to 0.2 Hz). This range is close to that of human respiration, which is from 0.2 Hz on. To establish the general character of the reported phenomena, we investigated a broad range of frequencies. The amplitude ΔA of the surface area of the bubble was varied from 5 to 20%, which is close to the respiration conditions. The chosen constraint ($\Delta A = 15\%$) allowed a linear response and equilibrium to be reached. The bubble was submitted to the oscillatory regime for long times (12–22 h), in contrast with earlier reports in which the oscillations were applied for seconds only.[16,18] All experiments were conducted at 37°C and repeated three to five times.

When DPPC is adsorbed from vesicles at the surface of a resting bubble, the interfacial tension γ starts decreasing monotonously from approximately $70\,mN\,m^{-1}$ (Figure 1). Adsorption is very slow,[17] because DPPC is in the P_β' ripple phase at 37°C ($T_m = 41.5$°C).[20] After 22 h, the interfacial tension γ is still approximately $50\,mN\,m^{-1}$ and far from equilibrium.

We found that when a bubble is submitted to prolonged controlled sinusoidal oscillations, four phenomena occur: First, the adsorption of DPPC becomes considerably faster than when the bubble is static. Second, the minimum reachable surface tension is greatly lowered. Third, a transition is consistently seen in the tension-versus-time curve at approximately $52\,mN\,m^{-1}$ (ca. 1 h in Figure 1). Fourth, the γ value

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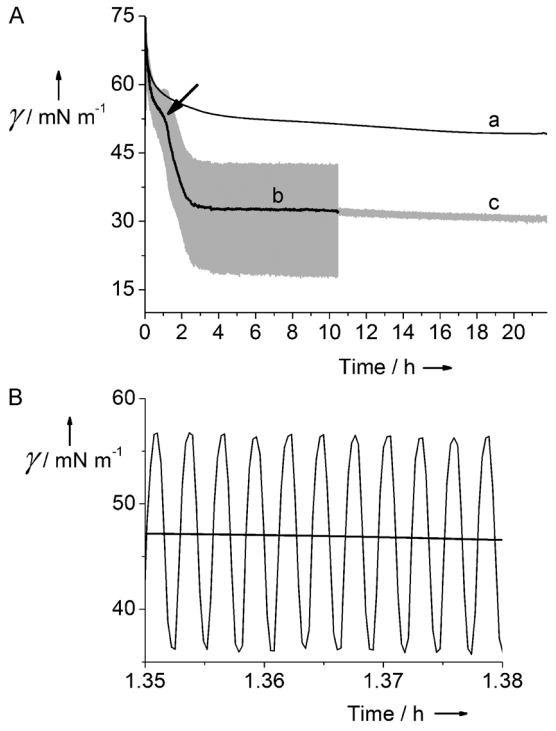

Figure 1. A) Kinetics of adsorption of DPPC (10^{-3} mol L^{-1}, provided in the form of a dispersion of vesicles) at the surface of an air bubble at 37 °C. The bubble was a) static; b) submitted to oscillations ($T = 10$ s, $\Delta A = 15\,\%$). The oscillations were applied for ca. 10 h and then stopped, while γ was subsequently monitored for another 12 h (c). The arrow points to an increase in the adsorption rate. The gray area represents the fluctuations in interfacial tension associated with the oscillations. B) Magnification of a part of (A.b) that shows the oscillations. The line corresponds to mean values obtained by treating the data with a low-pass digital filter.

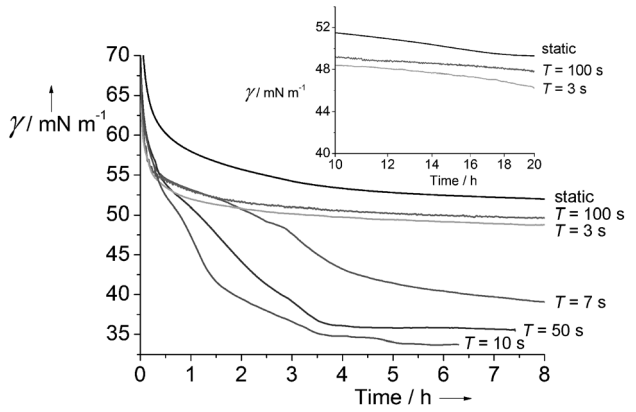

Figure 2. Effect of the oscillation period (T) on the kinetics of adsorption of DPPC (10^{-3} mol L^{-1}, as a dispersion of vesicles) at the surface of an oscillating air bubble (37 °C, $\Delta A = 15\,\%$). The case of the static bubble has been added for comparison. Inset: Representation in log scale showing the kinetics over 20 h.

remains at its minimum value after the oscillations have been removed.[21]

Although DPPC is one of the most abundant natural phospholipids and has been extensively investigated, there appears to be no previous report of these phenomena.

Concerning the γ-lowering effect, γ is, for example, lowered from 70 mN m^{-1} to approximately 33 mN m^{-1} after 3 h when the bubble is submitted to oscillations ($T = 10$ s and $\Delta A = 15\,\%$, Figure 1). This γ value is in good agreement with that determined by Needham and co-workers at 37 °C[22] in a study where the interfacial tension was measured by using a micropipette technique that allows equilibrium measurements. This confirms that our experimental conditions allow studying a DPPC film at equilibrium with the aqueous phase.

Concerning the transition (arrow on Figure 1 A), it depends directly on the oscillation period (Figure 2). For $T \leq 3$ s and $T \geq 100$ s, the adsorption profiles feature monotonous decays during the observation period (22 h). By contrast, for $4\text{ s} \leq T < 90$ s, a second regime sets in at approximately 50 mN m^{-1}, in which the phospholipid adsorption accelerates and the γ value drops more rapidly. This happening reflects a transition in the DPPC film between a low-density phase and a higher-density phase. When the period T increases, the coexistence plateau between the two phases becomes shorter and the interfacial tension γ decreases faster until the period T reaches approximately 50 s, after which the adsorption rate slows down again.

These observations suggest that this transition, observed here in an adsorbed (Gibbs-like) film, is an LE/LC first-order phase transition similar to that observed for DPPC Langmuir monolayers. The value of the LE/LC coexistence γ (52 mN m^{-1}) is comparable to that found for a DPPC Langmuir monolayer ($\gamma = 45$–55 mN m^{-}1, 40 °C).[10] Visual observation of condensed domains on phospholipid microbubbles has been reported;[23] but the variation of the interfacial tension γ was not measured.

The rates of adsorption of DPPC were quantified by determining the characteristic times t_1 and t_2 of the two regimes found in the adsorption profiles (see the Experimental Section). The time t_1 corresponds to the adsorption of the DPPC molecules at the interface and the time t_2 to the transfer of DPPC molecules from the LE phase to the LC phase.

First, it is seen that the oscillatory regime has a strong impact on t_1 (Table S1 in the Supporting Information). Second, the oscillation period has little or no effect on t_1 (Table S1). Moreover, we checked that the rate of stirring of the test sample has no effect on the rate of adsorption of the phospholipid. This means that convection has no significant effect and that the overall phenomenon is not controlled by diffusion through the bulk phase or time-limited by formation of the LE phase. This led us to hypothesize that the phenomenon is directed by mechanisms occurring during the LE/LC transition.

To verify this hypothesis, the effect of the oscillatory regime on t_2, the final relaxation time of the system, was investigated. We found indeed that t_2 is largely impacted by both the period and amplitude of the deformation applied to the bubble. The effect of the oscillation period on t_2, at 37 °C and for $\Delta A = 15\,\%$, is reported in Figure 3. It is found that t_2 first decreases sharply with the period T, attains a minimum for $10\text{ s} < T < 30$ s, and subsequently increases again.

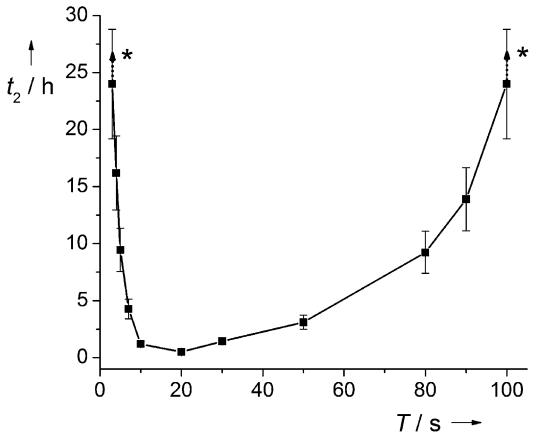

Figure 3. Characteristic time t_2 of the second regime of the DPPC adsorption at 37 °C, as a function of the bubble's oscillation period T for $\Delta A = 15\,\%$. * These t_2 values are higher than 24 h and could therefore not be determined precisely.

This means that for periods comprised between 7 and 50 s the characteristic time of the monolayer t_2 matches that of the oscillation, thereby allowing an increase of DPPC adsorption. In this situation, the oscillatory regime facilitates the recruitment of DPPC molecules at the interface during the expansion phase and their organization in LC domains during the compression phase.

For smaller periods ($T \leq 3$ s), the characteristic time of the DPPC film transition is longer than the oscillation period. Consequently, the oscillatory regime has no effect on DPPC adsorption during expansion or on the building up and growth of LC domains during compression. The bubbles oscillating at such low periods behave essentially like static bubbles, and t_2 values remain high. The same result is seen for periods larger than 50 s, for which the characteristic time of the transition is small relative to that of the deformation. The DPPC monolayer can relax more freely and hence respreads easily, leading again to high t_2 values.

The effect, for a given period ($T = 10$ s), of the amplitude of the oscillation-driven bubble deformation on the γ-lowering kinetics is shown in Figure 4 (the γ variation versus time is given in Figure S2; see also Table S2 in Supporting Information). The 10 s period is the lowest T value that leads to accelerated DPPC adsorption (Figure 3).

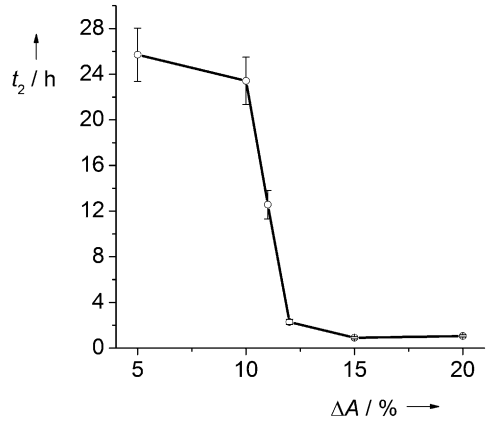

Figure 4. Characteristic time t_2 of the adsorption of DPPC in the second regime as a function of the variation of the surface area of the bubble, ΔA. The oscillation period T was 10 s. Temperature was 37 °C.

Figure 4 shows that t_2 is high, that is, the adsorption rate is slow, until the amplitude ΔA of the variation of the surface of the bubble reaches approximately 10 %, after which t_2 decreases sharply and levels off when ΔA attains approximately 15 %.

The Gibbs viscoelastic moduli E of the DPPC shell were determined for both the static and oscillating bubbles. In the former case, it was important to minimize the perturbation of the phospholipid film during the measurement. Therefore, the bubble was only submitted to a short series of five oscillations with a deformation amplitude ($\Delta A = 1$ and 2 %) well below the amplitude that causes the drop seen on Figure 4, and for T values ranging from 3 to 20 s. Under these conditions, after 12 h ($\gamma = 51$ mN m^{-1}), E was (28 ± 1.5) mN m^{-1}. By contrast, the E value was much higher (70 mN m^{-1}) when the bubble was submitted to the above oscillatory regime, that is, the bubble is perturbed immediately after formation ($T = 10$ s, $\Delta A = 15\,\%$). The fact that, after the same time span (12 h), the E value increased to such a large extent confirms the reorganization of the DPPC film toward a more organized state. The E value increases with time up to a point at which it levels off (Figure S3 in the Supporting Information). This point corresponds to the limit of compressibility of the LE phase, which occurs when the LC domains are percolated and somewhat before the LE/LC transition is completed.

To examine whether or not the γ value that is obtained under oscillations is an equilibrium value, the bubble was monitored for 12 h after the oscillations had been stopped. Figure 1 shows that the interfacial tension γ stays at its minimum value, thus indicating that equilibrium is attained. Likewise, the E value remained constant at 70 mN m^{-1}.

The exchange of phospholipids between vesicles and an adsorbed monolayer occurs predominantly between the outer lipid layer of the vesicles and the monolayer.[14,24] This exchange proceeds through diffusion collisions that involve individual molecules with regained mobility of their hydrophobic tails owing to transient abolishment of hydrophobic interactions. The fact that the kinetics of equilibration of vesicle-forming phospholipids at the air/water interface is highly sensitive to the chain length of the phospholipid (a decrease of ca. one order of magnitude per two CH_2 groups) also supports that diffusion of individual molecules (rather than fusion of large assemblies such as bilayer fragments) is at work in the process.[15]

When oscillations are applied, our observations are consistent with a mechanism in which the unexpected permanent lowering of the γ value is due to a coupling between the adsorption of DPPC molecules present in vesicles at the interface and the relaxation of the interfacial Gibbs-like DPPC film. During the expansion phase, the relative surface occupied by the LE phase increases, thus providing a driving force for recruitment of additional DPPC molecules from the phospholipid reservoir (Figure 5 a, b). The concentration of DPPC molecules forming the LE phase is increased, which in turn shifts the equilibrium of the phases toward formation of the LC phase (Figure 5 c). The compres-

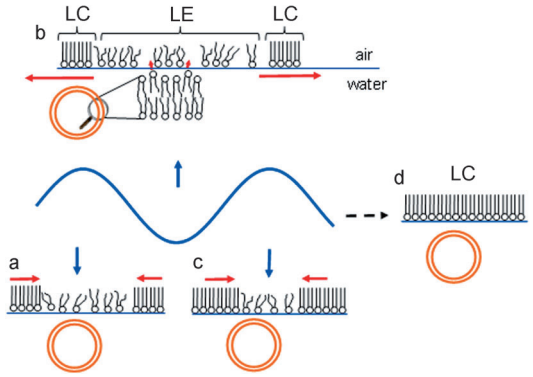

Figure 5. Schematic representation of the coupled film relaxation/phospholipid adsorption mechanism leading to a permanent decrease of the interfacial tension under the effect of oscillations: a) compression, b) expansion, c) recompression, d) equilibrium state after 24 h. Vesicles not on scale. Red arrows indicate compression and expansion episodes of the oscillatory regime represented by the blue line.

sion phase favors the formation of the LC phase. The LC domains become larger and more numerous and eventually cover large portions of the interface (Figure 5 d), which leads to the lowering of the interfacial tension.

In summary, we show that prolonged sinusoidal oscillations applied to a film of DPPC adsorbed on the surface of a bubble and in contact with a dispersion of phospholipid vesicles provoke accelerated phospholipid adsorption and permanent lowering of the interfacial tension. This phenomenon, which depends closely on the oscillation frequency and amplitude, is assigned to a coupling between the periodical variation of the surface density of the phospholipid at the air/water interface and its LE/LC transition. The phenomenon only occurs when the characteristic time of the periodic variation matches the characteristic time of the LE/LC transition. A possible mechanism is consistent with the oscillation-driven interplay of the phase equilibrium of the phospholipids at the air/water interface with the enrichment of the interface by phospholipid molecules coming from vesicles present at the interface. Interestingly, the oscillation period range is close to that of human respiration. The observed new phenomena should therefore be relevant to lung-surfactant film dynamics and respiration (not considering the key role of the specific lung surfactant proteins). It is also relevant to the engineering and control of phospholipid-coated bubbles for diagnostic and therapeutic purposes.[25] From a methodology standpoint, these findings open the possibility of studying the dynamic and transport properties of films of insoluble phospholipids, which has so far been hindered by the fact that, in the absence of the oscillations, equilibrium could not be reached within a practical time frame.

Experimental Section

Materials: L-α-1,2-Dipalmitoyl-*sn*-3-glycero-phosphatidylcholine (DPPC, 99% purity) was obtained from Sigma. A solution of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Sigma, 20 mM) in NaCl (150 mM) was prepared, and its pH value was adjusted to 7.4 with NaOH (0.1N). The equilibrium surface tension of the buffer was $(69.5 \pm 0.2)\,mN\,m^{-1}$ at 37 °C. Water was obtained from a MilliQ (Millipore) system (γ: $(71.7 \pm 0.2)\,mN\,m^{-1}$ at 20 °C; resistivity 18.2 MΩ cm). All measurements were made at 37 °C and repeated three to five times.

Phospholipid dispersions: Dispersions of DPPC ($10^{-3}\,mol\,L^{-1}$, 50 mL) required sonication (30 min) until they became transparent and contained vesicles with a mean diameter of approximately 80 nm. Vesicles of such small size are stable in buffers having a high electrolyte concentration,[26] which is the case for the HEPES buffer used. The size and size distribution of the utilized vesicles did not change significantly after 22 h at 37 °C (Figure S4 in the Supporting Information).

Dynamic light scattering (DLS): A Malvern Zetasizer Nano ZS was used at 25 °C for DLS measurements at a scattering angle of 90°. The *z*-averaged hydrodynamic mean diameters of the DPPC vesicles were determined using the Malvern software.

Bubble profile analysis tensiometry: Axisymmetric bubble shape analysis was applied to a rising air bubble formed in the DPPC dispersion. Only vesicle dispersions having close mean diameters (ca. 80 nm) and narrow size distributions were used. Time dependence of the interfacial tension during phospholipid adsorption at the gas/liquid interface was measured using a Tracker tensiometer (Teclis, Longessaigne, France).[27] The experiments lasted up to 24 h. A lid fitted on the measuring glass cell (10 mL) prevented water evaporation during these long equilibration times. The bubble (5 μL) was formed at the end of a stainless-steel capillary with a tip diameter of 1 mm. The interfacial tensions were means of values measured for various bubble surfaces. It was carefully determined that the systems were at equilibrium at the end of each experiment.

Oscillating-bubble measurements: The oscillations were produced by a position-encoded motor and transmitted through a piston coupled to the syringe carrying the capillary. The oscillatory regime was applied when the intended bubble volume had been attained. The bubble was maintained under harmonic oscillations with periods ranging from 1 to 100 s with surface amplitudes varying from 5 to 20% at (37 ± 0.5) °C. The given γ values are mean values obtained by treating the data with a digital low-pass filter (Order 3 Butterfly). The characteristic times t_1 and t_2 were determined by fitting the experimental curves with an exponential decay function (e.g. fit in Figure S1 and Tables S1,2 in the Supporting Information). The viscoelastic moduli were calculated as $E = d\gamma/d\ln A$.

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