Role of Lipid Polymorphism in Pulmonary Surfactant

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The development of artificial surfactants for the treatment of respiratory distress syndrome (RDS) requires lipid systems that can spread rapidly from solution to the air-water interface. Because hydration-repulsion forces stabilize liposomal bilayers and oppose spreading, liposome systems that undergo geometric rearrangement from the bilayer (lamellar) phase to the hexagonal II (H_{II}) phase could hasten lipid transfer to the air-water interface through unstable transition intermediates. A liposome system containing dipalmitoylphosphatidylcholine was designed; the system is stable at 23°C but undergoes transformation to the H_{II} phase as the temperature increases to 37°C. The spreading of lipid from this system to the air-water interface was rapid at 37°C but slow at 23°C. When tested in vivo in a neonatal rabbit model, such systems elicited an onset of action equal to that of native human surfactant. These findings suggest that lipid polymorphic phase behavior may have a crucial role in the effective functioning of pulmonary surfactant.

Surfactant protein A (SP-A) and phosphatidylglycerol are key missing elements of the pulmonary surfactant system of infants with RDS (*1*, *2*). Their absence curtails the rapid recruitment of the essential lipid dipalmitoylphosphatidylcholine (DPPC) to the air-water interface. Although production of these materials begins as early as 6 hours after delivery, the degree of lung compliance, or the ease of breathing, during those first several hours is critical (*3*). Exogenous surfactant systems have been developed as replacement therapies (*4*). Because clinical results for completely synthetic formulations have been mixed (*5*), we endeavored to design an alternative artificial formulation solely on the basis of considerations involving lipid polymorphic phase behavior.

Any effective surfactant formulation must transfer lipid rapidly to the air-water interface and must considerably reduce surface tension. Because the hydration-repulsion forces that stabilize liposomal bilayers oppose rapid spreading, we focused on mechanisms that lead to bilayer destabilization. Unlike the gel-to-liquid crystalline phase transition, in which the liposomal structure is maintained, the lamellar-to-H_{II} transition rearranges the lipids into a new geometry (Fig. 1, A and B). This rearrangement could facilitate lipid transfer to the air-water interface through nonequilibrium processes that would expel lipid into the aqueous phase above its critical micellar concentration (*6*).

We examined the spreading of lipid assemblies to the air-water interface under conditions that favored the H_{II} phase transition (Fig. 1C). Surface pressure (Π) at the interface is related to surface tension (γ) by $\Pi = \gamma_o - \gamma$, where γ_o is the surface tension of pure water. When cardiolipin liposomes were injected into buffer devoid of calcium, the spreading was slow, with a surface pressure change ($\Delta\Pi$) of <2 mN/m at 14 min. However, when liposomes were injected into buffer containing 50 mM $CaCl_2$ [calcium drives the lamellar-to-H_{II} transition of cardiolipin (*7*)], the spreading was rapid ($\Delta\Pi$ = 44 mN/m within 10 s). We next examined the spreading of dioleoylphosphatidylethanolamine (DOPE) liposomes (Fig. 1D). At pH 9.6, DOPE forms stable liposomes; at neutral and lower pH values, DOPE adopts the H_{II} phase (*8*). The transformation to the H_{II} phase upon injection into pH 4.5 buffer was accompanied by rapid spreading of lipid to the air-water interface. Equilibrium Π's were reached quickly (<30 s). Thus, transformation to the H_{II} phase allows rapid spreading to the air-water interface.

Natural lung surfactant is predominantly composed of DPPC, a disaturated lipid capable of low γ upon monolayer compression (*9*); the other lipids in surfactant may only facilitate rapid spreading and would then be "squeezed out" (*10*). With this in mind, we designed a stable liposome system that not only could be triggered to form the H_{II} phase but also included DPPC, a lipid that normally only adopts a lamellar arrangement. Our approach was to formulate DPPC into DOPE-cholesterol systems that would remain lamellar at room temperature but would adopt the H_{II} configuration at 37°C. DOPE alone readily forms a H_{II} phase, but we expected it to phase separate from DPPC in any mixture capable of the lamellar-to-H_{II} transition between 23° and 37°C. Inclusion of cholesterol, however, would allow the two lipids to remain dispersed within the same membrane and would also act to modulate the lamellar-to-H_{II} phase transition temperature (T_h).

The ^{31}P nuclear magnetic resonance (NMR) spectra of a DOPE-DPPC-choles-

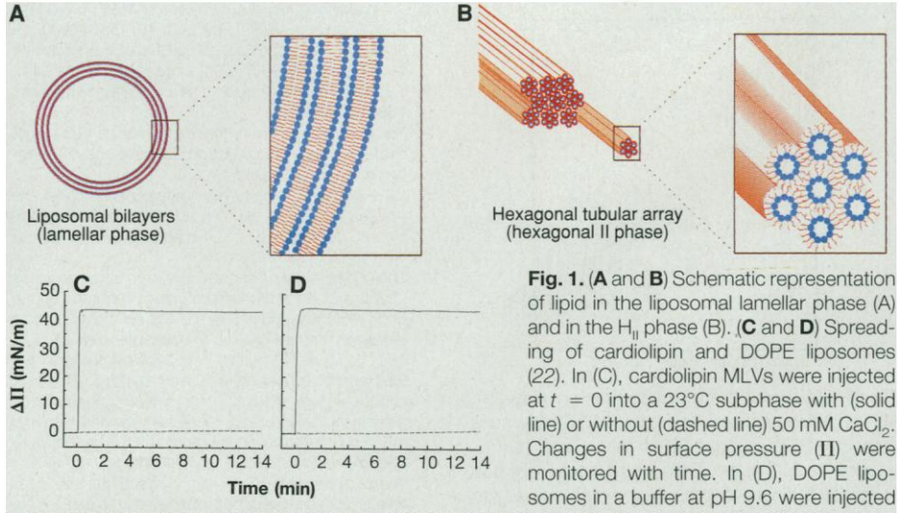

Fig. 1. (**A** and **B**) Schematic representation of lipid in the liposomal lamellar phase (A) and in the H_{II} phase (B). (**C** and **D**) Spreading of cardiolipin and DOPE liposomes (*22*). In (C), cardiolipin MLVs were injected at t = 0 into a 23°C subphase with (solid line) or without (dashed line) 50 mM $CaCl_2$. Changes in surface pressure (Π) were monitored with time. In (D), DOPE liposomes in a buffer at pH 9.6 were injected into a subphase at pH 4.5 (solid line) or pH 9.6 (dashed line). In both cases, rapid spreading occurred only under conditions that favored the lamellar-to-H_{II} phase transition.

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terol mixture (molar ratio 7:3:7) equilibrated to different temperatures are shown in Fig. 2A. The line shapes indicate that only the lamellar phase existed up to 30°C. Above 35°C, a mixture of lamellar and H_{II} phases was apparent. The small-angle x-ray diffraction pattern of a similarly made sample of 7:3:7 DOPE-DPPC-cholesterol (Fig. 2B) showed spacings indicative of H_{II} formation at 37°C but not at 23°C.

To delineate the phase behavior of DOPE-DPPC-cholesterol mixtures, we next examined the ^{31}P NMR spectra of samples formulated with molar ratios of 7:3:5 and 7:3:10 (Fig. 2C). For the 7:3:10 mixture, the H_{II} phase was observed at both 25° and 37°C. For both the 7:3:5 and 7:3:7 mixtures, there appeared to be only a bilayer signal at 25°C, with the H_{II} phase apparent at 37°C. Although the temperature at which the H_{II} phase was completely established for the 7:3:7 mixture was not determined, the pure H_{II} phase was observed for the 7:3:5 mixture (≥60°C) and the 7:3:10 mixture (≥51°C) (*11*).

After the lamellar-to-H_{II} transition for the 7:3:7 DOPE-DPPC-cholesterol mixture was established by NMR, we examined the spreading rate to the air-water interface as a function of temperature. Liposomes were injected into a subphase equilibrated either at 23°C, where lamellar structure would be maintained, or at 37°C, where the H_{II} phase would form. The changes in Π with time for both cases are shown in Fig. 3A. Spreading at 23°C was slow and produced only a modest ΔΠ of 4 mN/m after 14 min. Injection of the 7:3:7 mixture into buffer at 37°C yielded a marked change (Π = 46 to 49 mN/m or γ ≈ 24 to 27 mN/m), which, at 50 μg of lipid per milliliter, was complete in <3 min. At the same final lipid concentration of 50 μg/ml, the spreading rate for the 7:3:7 formulation was similar to that for Curosurf, a surfactant extracted from porcine lungs (*12*). The concentration of material dosed to preterm infants would be much higher at 5 to 10 mg/ml, assuming a functional residual lung capacity of 10 to 20 ml/kg (*13*) and a dose of 100 mg per kilogram of body weight. At such concentrations, we expect that spreading in vivo would occur essentially instantaneously.

Having shown that the 7:3:7 DOPE-DPPC-cholesterol monolayer met the criterion of rapid spreading, we next examined the characteristics of this monolayer to determine the minimum attainable γ upon compression. We examined profiles of Π versus surface area (*A*) for a monolayer of the 7:3:7 mixture deposited onto the surface from solvent (Fig. 3B). Upon compression at 22°C, the monolayer achieved a Π value near 50 mN/m (γ = 23 mN/m), which remained constant with further compression. This value did not change with repeated compression-expansion cycling. As expected, material was lost from the monolayer with successive compressions, as indicated by the shift of the Π-*A* loops to lower *A* values. Although this collapse pressure was slightly lower at 37°C (48 to 49 mN/m), repeated cycling again did not exhibit an effect. The collapse point was also unaffected by a change in the rate of compression (15 to 120 cm^2/min), by repeated compression-expansion of the monolayer below the collapse point pressure before compressing to the collapse point, or by the presence of 5 mM $CaCl_2$. When multilamellar vesicles (MLVs) of the 7:3:7 mixture were injected into the subphase of the trough (at 37°C), an equilibrium Π of 48 mN/m was established. Upon compression, a plateau in Π was observed (48.5 mN/m), the same as that of the monolayer deposited from solvent at 37°C; this result suggested, but did not confirm, an equivalent composition. Under the conditions used, complete squeeze-out did not occur; pure DPPC exhibited a much higher collapse point, near 70 mN/m [consistent with previous reports (*14*)].

The concept that γ must be near zero was required to explain the classical alveolar continuous liquid lining theory, which is now disputed (*15*). Most recent alveolar models do not invoke a continuous liquid lining, and the ability of the Wilhelmy plate method to report low values of γ has been questioned (*16*). Given these concerns, we could not rule out the possibility that our formulations would function well in vivo [the minimum γ measured for Exosurf, a synthetic surfactant formulation, has been reported to be 25 mN/m (*17*)].

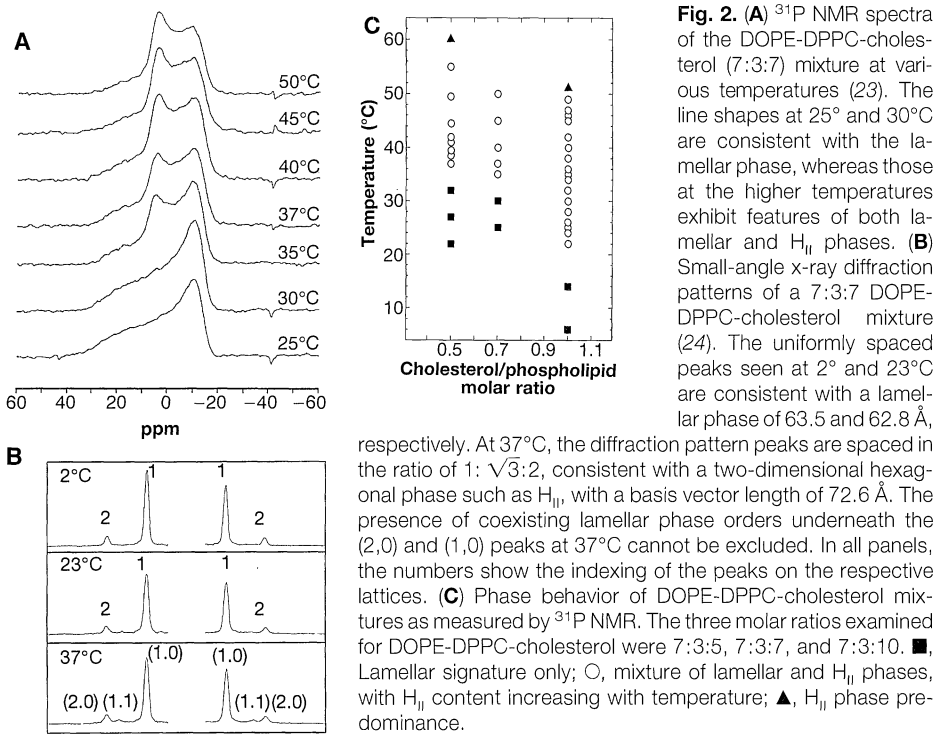

Fig. 2. (**A**) ^{31}P NMR spectra of the DOPE-DPPC-cholesterol (7:3:7) mixture at various temperatures (*23*). The line shapes at 25° and 30°C are consistent with the lamellar phase, whereas those at the higher temperatures exhibit features of both lamellar and H_{II} phases. (**B**) Small-angle x-ray diffraction patterns of a 7:3:7 DOPE-DPPC-cholesterol mixture (*24*). The uniformly spaced peaks seen at 2° and 23°C are consistent with a lamellar phase of 63.5 and 62.8 Å, respectively. At 37°C, the diffraction pattern peaks are spaced in the ratio of 1: $\sqrt{3}$:2, consistent with a two-dimensional hexagonal phase such as H_{II}, with a basis vector length of 72.6 Å. The presence of coexisting lamellar phase orders underneath the (2,0) and (1,0) peaks at 37°C cannot be excluded. In all panels, the numbers show the indexing of the peaks on the respective lattices. (**C**) Phase behavior of DOPE-DPPC-cholesterol mixtures as measured by ^{31}P NMR. The three molar ratios examined for DOPE-DPPC-cholesterol were 7:3:5, 7:3:7, and 7:3:10. ■, Lamellar signature only; ○, mixture of lamellar and H_{II} phases, with H_{II} content increasing with temperature; ▲, H_{II} phase predominance.

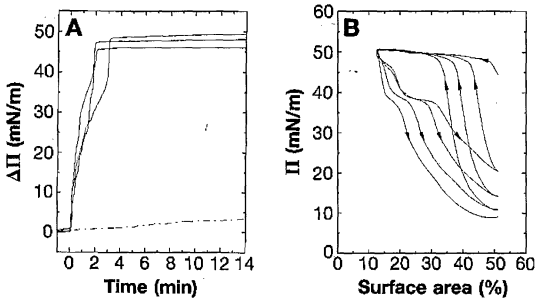

Fig. 3. (**A**) Spreading of 7:3:7 DOPE-DPPC-cholesterol liposomes (*25*). Liposomes were injected into subphases equilibrated at either 37°C (solid lines) or 23°C (dashed line). The equilibrium Πs were 46 to 49 mN/m. The final lipid concentration was 50 μg/ml. (**B**) Plots of Π versus surface area for the 7:3:7 DOPE-DPPC-cholesterol monolayer (*26*). The monolayer was formed by deposition of lipid in hexane-ethanol (9:1) onto the aqueous surface. Compressions and expansions were performed at 22°C with an area change of 120 cm^2/min. Arrows indicate the direction of barrier movement.

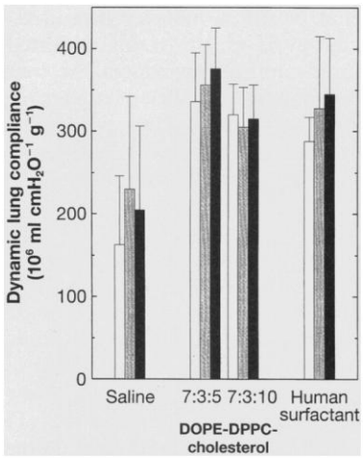

Fig. 4. Dynamic lung compliance in preterm rabbits (*27*). Compliance was measured over three time periods: 0 to 10 min (open), 11 to 20 min (gray), or 21 to 30 min (black). The number of rabbits in each group was $n = 5$ for the 7:3:5 DOPE-DPPC-cholesterol mixture and $n = 4$ for the 7:3:10 mixture, saline control, and human surfactant control. Bars represent SD. The rabbit model used is described in (*28*).

We tested our systems in vivo (*18*) in preterm rabbits (27th day of gestation) to compare the effectiveness of two of our DOPE-DPPC-cholesterol formulations (7:3:5 and 7:3:10) as well as saline (negative control) and natural human surfactant (positive control). The two DOPE-DPPC-cholesterol formulations, both driven to increasing lamellar-to-H_{II} conversions at physiological temperature, elicited an onset of action equal to that of native human surfactant (Fig. 4). The response after dosing was immediate, with visible color changes (blue to pink) for rabbits that received our formulations. [The immediacy of response in infants treated with Curosurf is prognostic of decreased mortality and morbidity (*19*).] The two formulations and human surfactant were not significantly different from one another at any time point, but all three were significantly different from the saline group at 10 min ($P < 0.005$) and at 30 min ($P < 0.01$). These data suggest that lipid polymorphic phase behavior may play a role in the design of effective synthetic surfactants and may also be important in the functioning of native surfactant. Indeed, lipid-protein structures reminiscent of nonbilayer lipid (tubular myelin) have been shown to be essential in the rapid transfer of lipid to the air-water interface from lamellar bodies secreted by alveolar type II pneumocytes (*20*).

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11. Differential scanning calorimetry on the 7:3:7 DOPE-DPPC-cholesterol mixture (three successive heating and cooling scans between 10° and 70°C) produced no discernible transition. If either DOPE or DPPC had phase-separated from the mixture, the T_h of DOPE at 10°C (0.3 kcal/mol) (*21*) or the T_m (gel-to-liquid crystalline transition temperature) of DPPC at 41°C (8.7 kcal/mol) [B. D. Ladbrooke and D. Chapman, *Chem. Phys. Lipids* **3**, 304 (1969)] would have appeared. The absence of any endotherm-exotherm for this mixture was not surprising, given the broadness of the transition as measured by NMR (20° to 30°C) and the fact that enthalpies for lamellar-to-H_{II} transitions are typically low (<1 kcal/mol) [D. Marsh, *Handbook of Lipid Bilayers* (CRC Press, Boca Raton, FL, 1990), pp. 265–273].
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22. Surface pressure changes (expressed in millinewtons per meter) were recorded with a Wilhelmy balance (KSV Instruments, Trumbull, CT) with a roughened platinum plate suspended at the air-water interface. The subphase was contained within an acid-washed Teflon beaker. Upon injection of sample into the subphase, the solution was stirred briefly (~2 s) with a Teflon-coated stir bar. Liposomes were injected at $t = 0$. For cardiolipin studies, 100 μl of a MLV suspension (20 mg/ml) was injected at room temperature into a 25-ml subphase consisting of 10 mM Hepes and 150 mM NaCl (pH 7.5) with and without 50 mM $CaCl_2$. For DOPE studies, 100 μl of a MLV suspension [20 mg/ml, in 10 mM glycine and 150 mM NaCl (pH 9.6)] was injected at room temperature into a subphase (20 ml) consisting of either the same buffer solution or a solution of 10 mM acetate and 150 mM NaCl (pH 4.5).
23. Spectra were acquired on a Bruker AM360 NMR spectrometer operating at 145.8 MHz (8.5 T) on nonspinning samples with a 10-mm double-resonance probe. The resonance of small unilamellar vesicles of egg lecithin was used as an external chemical shift reference (0 ppm).
24. Data on x-ray diffraction intensity versus scattering angle were obtained with a two-dimensional (2D) image–intensified x-ray detector, as described [S. M. Gruner, R. P. Lenk, A. S. Janoff, M. J. Ostro, *Biochemistry* **24**, 2833 (1985)]. Samples were spun for 1 hour in a Beckman J2-21 centrifuge at 20,000*g* to settle the liposomes. The clear supernatant was decanted, and the resultant concentrated liposome suspension was sealed in thin-walled glass x-ray capillaries (diameter 1.5 mm). The capillaries were inserted into the thermostated (±0.5°C) copper jacket of the x-ray specimen stage. The resultant 2D powder diffraction patterns were azimuthally integrated and are displayed as x-ray intensity versus angle from the center of the diffraction pattern. The diffraction spacings were calibrated against silver stearate (long spacing, 48.68 Å).
25. We injected 100 μl of sample (20 mg/ml) into a 20-ml subphase consisting of 10 mM Hepes and 150 mM NaCl (pH 7.5). The Teflon beaker was placed inside a larger temperature-regulated bath. Liposomes were formed by hydrating a dried lipid film with a solution containing 10 mM glycine and 150 mM NaCl (pH ~9). The samples were extruded through a filter (pore size, 0.4 μm) and then dialyzed (cutoff molecular weight, 10,000) overnight at 5°C against an excess of 10 mM Hepes and 150 mM NaCl (pH 7.4). Although the samples were initially frozen and thawed 10 times between liquid N_2 and room temperature before dialysis, subsequent differential scanning calorimetry of samples not frozen and thawed indicated that the lipids were mixed ideally, and the freeze-thaw step was omitted. After dialysis, the liposomes were stored at 5°C until use.
26. We used the mini-Langmuir trough (KSV Instruments, 7.5 cm by 30 cm) equipped with a Wilhelmy balance and a dual-barrier mechanism. Barriers were composed of Delrin, a hydrophilic material that allowed solution contact when the liquid level was ~1 mm below the edge of the trough. Keeping the solution below the edge of the trough aided greatly our ability to compress DPPC-containing monolayers to very high values of Π (low values of γ) by avoiding overflow.
27. Rabbits (~30 g) were dosed with either 0.2 or 0.3 ml of MLVs passed through a 5-μm filter at a lipid concentration of 20 mg/ml (~140 to 200 mg per kilogram of body weight). Lung compliance was measured with constant-volume ventilation. Differences between mean dynamic lung compliance were tested with a one-way analysis of variance and Newmann-Keuls multiple-range testing.
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