Phospholipid Monolayers

Preventing Crystallization of Phospholipids in Monolayers: A New Approach to Lung-Surfactant Therapy**

Frédéric Gerber, Marie Pierre Krafft, Thierry F. Vandamme, Michel Goldmann, and Philippe Fontaine*

We report that a fluorocarbon gas can have a highly effective fluidizing effect on a semicrystalline dipalmitoylphosphatidylcholine (DPPC) monolayer and can prevent it from crystallizing. DPPC monolayers are commonly being used as simplified models of lung surfactant.

The native lung surfactant (LS) is a complex mixture of lipids and proteins that forms a monolayer at the alveolus/air interface of mammalian species. This layer lowers the air/alveoli surface tension upon compression (that is, during expiration), reduces the work of breathing, and respreads easily on expansion (that is, during inspiration).[1–3] The main component of native LS is DPPC. It is accompanied by small amounts of other lipids and of four specific proteins, SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are amphiphilic proteins that were recently shown to play an important role in the surface activity of LS.[1,3] Although DPPC can generate near-zero surface tension at the air/water interface during compression, it is a poor LS when used alone. This is because it tends to form rigid, multilamellar structures in solution and, consequently, does not adsorb efficiently and rapidly at the air/water interface.

When compressed at an air/water interface at 25 °C, DPPC forms a monolayer that is in a liquid-condensed state at high surface density. It forms essentially two-dimensional crystalline domains at the interface.[1–3] Such crystallization opposes effective respreading of the phospholipid on the alveolar surface upon inspiration. It is believed that the unsaturated and anionic phospholipids (especially phosphatidylglycerol) and hydrophobic proteins present in the native surfactant compensate for these limitations (from the respiratory-function standpoint) of DPPC monolayers.[4] In particular, one of the proteins of LS, SP-B, was shown to induce a reversible folding transition at monolayer collapse, thereby allowing all the components of LS to remain at the interface during respreading.[3]

Over the past few years, several LS substitutes (for example, Curosurf (Chiesi Pharmaceutici, Parma, Italy) and Survanta (Ross Laboratories, Columbus, OH, USA)) have been developed that are now clinically used in the treatment of neonatal respiratory distress syndrome. These replacement LSs consist of phospholipidic fractions of bovine (Survanta) and porcine (Curosurf) lung surfactant. However, these natural extracts are not entirely devoid of inherent immunological risks and potential viral contamination. Other drawbacks include the limited supply of animal surfactant, the cost of the purification procedure, and the difficulty of achieving batch-to-batch consistency. Therefore, there is a clear need for alternative synthetic LS substitutes.[1–4]

We found that a fluorocarbon gas very effectively inhibits the crystallization of DPPC on compression and facilitates its respreading on decompression. Because of their high biological inertness, remarkable ability to solubilize oxygen, and extremely low solubility in water, fluorocarbons (FCs) have been investigated for various biological applications, especially intravascular oxygen transport[5,6] and the stabilization of gaseous microbubbles that are used as contrast agents in ultrasound imaging.[5,6] Partial liquid ventilation with FCs has been explored as a treatment for acute respiratory distress syndrome (ARDS).[7–10] Improved oxygenation and lung compliance were achieved.[7] Recently, delivery of vaporized FCs to oleic acid injured ARDS sheep has resulted in significant and sustained improvement in gas exchange and lung compliance.[11,12] Although these results suggest that FCs may be useful in pulmonary-disease therapy, no study aimed at determining the influence of FCs on lung surfactant or lung-surfactant models appears to have been reported.

We have investigated the effects of an FC gas (gFC) on the physical state of DPPC Langmuir monolayers upon compression. Langmuir monolayers provide useful model systems for studying the lung surfactant. Although great care is necessary when extrapolating Langmuir monolayer behavior to lung-surfactant behavior in vivo, general correlations between in vitro and in vivo behavior have started to emerge.[13] The behavior of surfactants in monolayers is characterized by surface pressure–molecular area (π–A) compression isotherms. Compression isotherms of DPPC monolayers were measured under an atmosphere saturated with various gFCs. Fluorescence microscopy (FM) and grazing incidence X-ray diffraction (GIXD) were used to determine the morphology and degree of order of the organized domains within the monolayers. Perfluorooctyl bromide (PFOB) was selected as the gFC because it is one of the most thoroughly investigated FCs for biomedical applications,[14] particularly for pulmonary applications. Both the ability of gPFOB to prevent the formation of crystalline DPPC domains and its ability to dissolve such domains once formed were determined.

[*] F. Gerber, Dr. M. P. Krafft
Institut Charles Sadron (CNRS UPR 22)
6 rue Boussingault, 67083 Strasbourg Cedex (France)
Fax: (+33) 388-414-099
E-mail: krafft@ics.u-strasbg.fr
F. Gerber, Dr. T. F. Vandamme
Laboratoire de Chimie Bioorganique (UMR 7514)
Université Louis Pasteur
74 Route du Rhin, 67401 Illkirch (France)
M. Goldmann, Dr. P. Fontaine
Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE, UMR 130)
Centre Universitaire Paris Sud, Bât 209D
91898 Orsay Cedex (France)
and
Institut des Nano-Sciences de Paris (INSP-UMR CNRS 7588)
Campus Boucicaut
140 rue de Lourmel, 75015 Paris (France)

[**] We thank Alliance Pharmaceutical Corporation (San Diego, CA, USA) for the gift of perfluorooctyl bromide.

 DOI: 10.1002/ange.200462978

Isotherms of DPPC monolayers compressed at 25 °C on a pure water subphase under an atmosphere of N_2 and under an atmosphere of N_2 saturated with PFOB (vapor pressure: 10.5 torr at 25 °C) are shown in Figure 1. When compressed under pure N_2, DPPC undergoes a first-order phase transition from a liquid-expanded (LE) phase to a liquid-condensed (LC) phase at a surface pressure of $\pi \approx 13\ mN\,m^{-1}$. The LE/LC coexistence region is evidenced by the presence of a plateau on the isotherm and by the observation of discrete domains of LC phase within a continuous LE phase, as visualized with FM (inset (a) in Figure 1). This is in agreement with previous work.[15] When surface pressure increases, the LC domains increase in size, become more numerous, and progressively merge into a continuous LC phase.

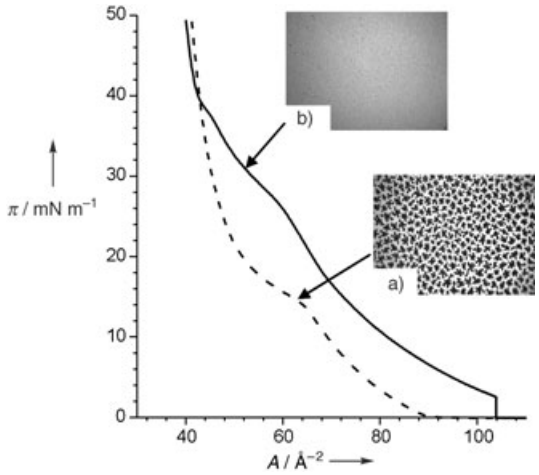

Figure 1. Compression isotherm of DPPC measured at 25 °C in an atmosphere of N_2 (dashed line) or N_2 saturated with PFOB (solid line). Insets: fluorescence images of a) the DPPC monolayer at $\pi = 15\ mN\,m^{-1}$ showing the crystalline domains and b) the DPPC monolayer in contact with PFOB ($\pi = 30\ mN\,m^{-1}$).

Compressing the DPPC monolayer under an N_2 atmosphere saturated with gPFOB changes the phase behavior drastically. The transition at $\pi \approx 13\ mN\,m^{-1}$ (Figure 1) has disappeared. The new transition seen at $\pi \approx 28\ mN\,m^{-1}$ is no longer of the LE/LC type, as assessed from the fluorescence images, which are bright and featureless not only at $30\ mN\,m^{-1}$ (inset (b) in Figure 1) but throughout the whole range of π values. The fact that the dye remains soluble for all π values suggests the formation of two LE phases, with a transition through a collective tilt. This indicates that the DPPC monolayer in contact with gPFOB is in a homogeneous fluid LE phase throughout the range of surface pressures investigated. Below $\pi = 40\ mN\,m^{-1}$, the isotherm is shifted towards larger molecular areas, a fact indicating that some PFOB is incorporated into the DPPC monolayer. These experiments demonstrate that gPFOB molecules interact with DPPC molecules, prevent the formation of the LC phase, and hence induce a fluidizing effect in the monolayer.

To assess the effect of gPFOB on already formed crystalline DPPC domains, we have compressed DPPC monolayers at $\pi = 10\ mN\,m^{-1}$, thus causing the formation of LC domains. gPFOB-saturated N_2 was then allowed to flow into the gas-tight box enclosing the trough. The fluorescence images of Figure 2 show that five minutes after the introduction of gPFOB the LC domains have become significantly smaller. After three minutes, the domains have totally disappeared, a result indicating that the DPPC monolayer has become totally fluid.

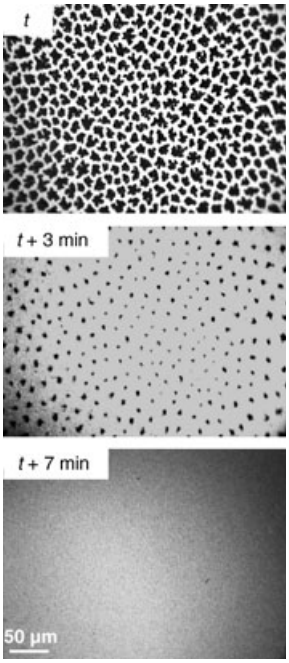

Figure 2. Fluorescence images of a DPPC monolayer compressed at $\pi = 10\ mN\,m^{-1}$ under N_2 (upper image). At time *t*, the atmosphere of N_2 above the monolayer started to be saturated with gPFOB. One can see that the LC domains progressively disappear with time. After 7 min, the monolayer is totally homogeneous and fluid.

To secure another unambiguous, independent assessment of any possible crystalline regions of the monolayer, we performed GIXD experiments. The X-ray beam hits the surface of water at an incidence lower than the critical angle of total external reflection for water (≈ 2.5 mrad at 7.7 keV). Under these conditions, an evanescent wave propagates along the air/water interface. If an ordered monolayer is present at the interface, the wave is diffracted and Bragg peaks are obtained. GIXD experiments thus provide information on the existence and structure of ordered zones within the monolayer.[16] N_2 is replaced by He gas to reduce the X-ray scattering by the gas phase, which can mask the surface-diffraction peaks.

Figure 3a shows the integrated diffracted X-ray intensity (I/I_0) as a function of the in-plane scattering wave vector q_{xy} of a DPPC monolayer compressed at $\pi = 20\ mN\,m^{-1}$. At this surface pressure, two Bragg peaks are obtained that indicate a rectangular unit cell (tilted L_{2d} phase). The calculated area per chain ($\approx 23\ Å^2$) and the tilt angle obtained from Bragg rod analysis are in agreement with published data.[17] When the He atmosphere is saturated with gPFOB, the diffraction

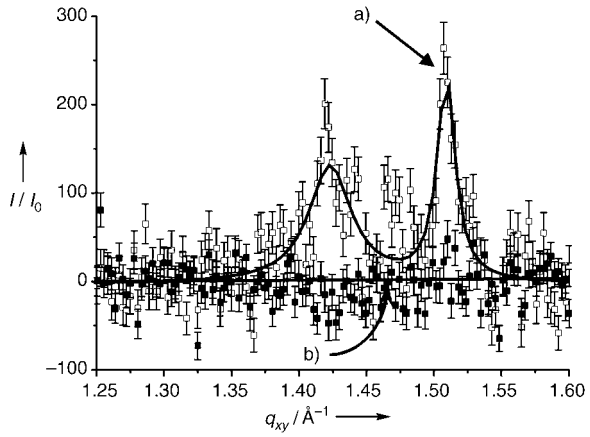

Figure 3. Diffracted intensity of the grazing X-rays I/I_0 as a function of the in-plane scattering wave vector q_{xy}. a) When compressed at $\pi = 20\ \mathrm{mN\,m^{-1}}$ under He, the DPPC monolayer shows the two Bragg peaks characteristic of the tilted LC phase at 1.42 and 1.51 Å^{-1}. b) When the helium is saturated with gPFOB, the peaks disappear rapidly.

peaks disappear within a few minutes, thereby indicating the dissolution of the LC domains, which allows the rapid respreading of the DPPC molecules. Furthermore, when pure He was subsequently allowed to sweep the gas-tight box while the compression of the DPPC monolayer was maintained at $\pi = 20\ \mathrm{mN\,m^{-1}}$, the two diffraction peaks were seen to slowly reappear, thereby showing reformation of the LC domains. This establishes the facts that PFOB molecules are adsorbed into the DPPC monolayer and that they inhibit the organization of the DPPC molecules into LC domains. When the inflow of gPFOB is stopped, while the flow of He gas is maintained, the PFOB molecules adsorbed at the interface are removed and the DPPC monolayer recovers its normal behavior, that is, crystallizes.

In conclusion, gaseous PFOB was found to suppress the LC phase of DPPC Langmuir monolayers and, hence, the formation of crystalline domains. When contacted with the gFC, the DPPC monolayer remains in the LE phase throughout the range of surface pressures investigated (up to $\pi = 40\ \mathrm{mN\,m^{-1}}$), as assessed by compression isotherms, FM, and GIXD experiments. It was also found that gFCs can induce the dissolution of pre-existing LC-phase domains, and hence facilitate the respreading of the DPPC molecules on the water surface, as shown by FM and GIXD; gPFOB, thus, demonstrated a highly effective fluidizing effect on the DPPC monolayer. Other fluorocarbons, including perfluorooctylethane, bis(perfluoroalkyl)ethanes, and perfluorodecalin, were also effective. The insights provided by this study suggest that some FCs, whose biocompatibility is well documented, in combination with DPPC, may prove useful in lung-surfactant-substitute compositions.

Experimental Section

L-α-1,2-Dipalmitoyl-*sn*-3-glycerophosphatidylcholine (DPPC) was obtained from Sigma in >99% purity. Spreading solutions of DPPC (1.0 mmol L^{-1} for minitrough experiments and 2.0 mmol L^{-1} for GIXD experiments) were prepared in chloroform (analytical grade). Water was purified by using a Millipore system (pH 5.5, surface tension = 72.1 mN m^{-1} at 20 °C, resistivity = 18 MΩ cm). The fluorescent dye (2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, NBDC$_6$-HPC) was purchased from Molecular Probes (Eugene, OR, USA), and used at a lipid mole ratio of 1%.

Surface pressure versus molecular area isotherms were recorded on a Langmuir minitrough (Riegler&Kirstein, Potsdam, Germany) equipped with two movable barriers (speed = 2.0 mm min^{-1}). The surface pressure was measured by using the Wilhelmy plate method. The trough was enclosed in a gas-tight box that was flushed either with pure N_2 or with N_2 saturated with gPFOB. In the latter case, N_2 was allowed to bubble at room temperature through the liquid fluorocarbon before being flushed into the gas-tight box. The flow rate of the gas phase (N_2 or PFOB-saturated N_2) was set to 1.2 L min^{-1}. The temperature was regulated to 25 ± 0.5 °C. DPPC solution (15 μL) was spread on the water surface. The errors in π and A were estimated as ±1 mN m^{-1} and ±1 Å^2, respectively.

Fluorescence microscopy was achieved with the above Langmuir balance equipped with an Olympus fluorescence microscope fitted with a 20×-power objective. An Olympus 100-W high-pressure mercury lamp was used for excitation. A dichroic mirror/barrier filter assembly was used to filter and direct the excitation light onto the monolayer (450–490 nm) and to filter out the emitted fluorescence (520 nm). The emitted fluorescence was collected by the objective and detected with a Hamamatsu intensified camera. The microscope was linked to the gas-tight box of the trough through an extensible gusset to allow easy control of the partial pressure of PFOB. The surface pressure was kept constant throughout the fluorescence microscopy experiments.

GIXD experiments were perfomed at the D41B beamline of the LURE-DCI synchrotron source (Orsay, France). The teflon Langmuir trough mounted on the diffractometer was equipped with a movable single barrier. The surface pressure π (as measured by the Wilhelmy plate method) was kept constant throughout a given scan. The trough was enclosed in a gas-tight box with kapton windows filled with water-saturated helium. The temperature was regulated to 20 ± 0.5 °C. Approximately 50 μL of DPPC solution were spread on the water surface. The film was compressed stepwise and Bragg peaks were recorded at each set pressure step. The X-ray wavelength, α = 1.646 Å, of the incoming X-ray beam was selected by using a focusing Ge(111) monochromator. The grazing angle of incidence (θ_i = 2.08 mrad) was set slightly below the critical angle for total external reflection of the X-rays at the air/water interface (about 2.8 mrad at 1.646 Å). For the acquisition of the diffracted intensity, we used a new set up composed of a two-dimensional detector and a single vertical slit positioned between the sample and the detector.[18] The q_{xy} resolution was 0.007 Å^{-1} for the q range explored. The total duration of a scan was typically 10 min. The shape of the Bragg rods gave information about the tilt angle τ and tilt azimuth φ.[19] In this study, the nearest-neighbor tilted phase (L_{2d}, $\tau \neq 0$, $\varphi = 0$) was observed, according to the nomenclature introduced in Ref. [17]. For this L_{2d} phase, the diffraction pattern exhibited two peaks. The peak located at low q_{xy} corresponds to the degenerate [11] and [$\bar{1}$1] out-of-plane reflections, and the other peak corresponds to the [02] in-plane reflection.

Received: December 17, 2004

Keywords: fluidizing effects · fluorocarbons · liquid crystals · monolayers · phospholipids

[1] J. A. Zasadzinski, J. Ding, H. E. Warriner, F. Bringezu, A. J. Waring, *Curr. Opin. Colloid Interface Sci.* **2001**, *6*, 506.

[2] R. H. Notter, *Lung Surfactants: Basic Science and Clinical Applications*, *Vol. 149*, Marcel Dekker, New York, **2000**.
[3] J. Ding, D. Y. Takamoto, A. von Nahmen, M. M. Lipp, K. Y. C. Lee, A. J. Waring, J. A. Zasadzinski, *Biophys. J.* **2001**, *80*, 2262.
[4] R. Veldhuizen, K. Nag, S. Orgeig, F. Possmayer, *Biochim. Biophys. Acta* **1998**, *1408*, 90.
[5] J. G. Riess, *Chem. Rev.* **2001**, *101*, 2797.
[6] E. G. Schutt, D. H. Klein, R. M. Mattrey, J. G. Riess, *Angew. Chem.* **2003**, *115*, 3336; *Angew. Chem. Int. Ed.* **2003**, *42*, 3218.
[7] M. R. Wolfson, N. E. Kechner, R. F. Roache, J. DeChadarevian, H. E. Friss, S. D. Rubenstein, T. H. Shaffer, *J. Appl. Physiol.* **1998**, *84*, 624.
[8] M. Sukumar, M. Bommaraju, J. E. Fisher, F. C. Morin, M. C. Papo, B. P. Fuhrman, L. J. Hernan, C. L. Leach, *J. Appl. Physiol.* **1998**, *84*, 327.
[9] C. L. Leach, J. S. Greenspan, D. Rubenstein, T. H. Shaffer, M. R. Wolfson, J. C. Jackson, R. DeLemos, B. P. Fuhrman, *N. Engl. J. Med.* **1996**, *335*, 761.
[10] A. T. Rotta, B. Gunnarsson, L. J. Hernan, B. P. Fuhrman, D. M. Steinhorn, *J. Crit. Care* **2000**, *28*, 202.
[11] J. U. Bleyl, M. Ragaller, U. Tscho, M. Regner, M. Hübler, M. Kanzow, O. Vincent, M. Albrecht, *Crit. Care Med.* **2002**, *30*, 1340.
[12] K. von der Hardt, E. Schoof, M. A. Kandler, J. Dötsch, W. Rascher, *Pediatr. Res.* **2002**, *51*, 177.
[13] M. M. Lipp, K. Y. C. Lee, D. Y. Takamoto, J. A. Zasadzinski, A. J. Waring, *Phys. Rev. Lett.* **1998**, *81*, 1650.
[14] J. G. Riess in *Handbook of Fluorous Chemistry* (Eds.: J. A. Gladysz, I. Horváth, D. P. Curran), Wiley-VCH, Weinheim, **2004**, p. 521.
[15] M. Lösche, E. Sackmann, H. Möhwald, *Ber. Bunsen-Ges.* **1983**, *87*, 848.
[16] P. Fontaine, M. Goldmann, P. Muller, M.-C. Fauré, O. Kolovanov, M. P. Krafft, *J. Am. Chem. Soc.* **2005**, *127*, 512.
[17] V. M. Kaganer, H. Möhwald, P. Dutta, *Rev. Mod. Phys.* **1999**, *71*, 779.
[18] P. Fontaine, M. Goldmann, M. Bordessoule, A. Jucha, *Rev. Sci. Instrum.* **2004**, *75*, 3097.
[19] J. Als-Nielsen, D. Jacquemain, K. Kjaer, F. Leveiller, M. Lahav, L. Leiserowitz, *Phys. Rep.* **1994**, *251*, 246.

