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The detrimental effect of serum albumin on the re-spreading of a dipalmitoyl phosphatidylcholine Langmuir monolayer is counteracted by a fluorocarbon gas

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Abstract

We have recently reported that fluorocarbon gases exhibit an effective fluidizing effect on Langmuir monolayers of dipalmitoyl phosphatidylcholine (DPPC), preventing them from crystallizing up to surface pressures of ~40 mN m^{-1}, i.e. well above the DPPC's equilibrium surface pressure. We now report that gaseous perfluorooctyl bromide (gPFOB) promotes the re-spreading of DPPC Langmuir monolayers compressed on a bovine serum albumin (BSA)-containing sub-phase. The latter protein is known to maintain a concentration-dependent surface pressure that can exceed the re-spreading pressure of collapsed monolayers. This phenomenon was proposed to be responsible for lung surfactant inactivation. Compression/expansion isotherms and fluorescence microscopy experiments were carried out to assess the monolayers' physical state. We have found that, during expansion under gPFOB-containing air, the surface pressure of a DPPC monolayer on a BSA-containing sub-phase decreased to much lower values than when the DPPC monolayer was expanded in the presence of BSA under air (~0 mN m^{-1} vs. ~7.5 mN m^{-1} at 120 $Å^2$, respectively). Moreover, fluorescence images showed that, during expansion, the BSA-coupled DPPC monolayers, in contact with gPFOB, remained in the liquid-expanded state for surface pressures lower than 10 mN m^{-1}, whereas they were in a liquid-condensed semi-crystalline state, even at large molecular areas (120 $Å^2$), when expanded under air. The re-incorporation of the PFOB molecules in the DPPC monolayer during expansion thus competes with the re-incorporation of BSA, thus preventing the latter from penetrating into the DPPC monolayer. We suggest that combinations of DPPC and a fluorocarbon gas may be useful in the treatment of lung conditions resulting from a deterioration of the native lung surfactant function due to plasma proteins, such as in the acute respiratory distress syndrome.

Keywords: Dipalmitoyl phosphatidylcholine; Fluorocarbon; Perfluorooctyl bromide; Serum albumin; Langmuir monolayer; Fluorescence microscopy; Lung surfactant; Acute respiratory distress syndrome

1. Introduction

Protein adsorption at bio-interfaces plays a pivotal role in many biotechnological and medical applications. One particular example of bio-interface is the lung surfactant (LS). The endogenous LS is a complex mixture of phospholipids, mainly dipalmitoyl phosphatidylcholine (DPPC), neutral lipids, and four proteins designated SP-A, SP-B, SP-C and SP-D [1]. The main function of LS is to lower the work of breathing by reducing the surface tension at the air/water interface of the terminal airways to values as low as ~1 mN m^{-1} during expiration [2]. DPPC is generally accepted as being the lipid responsible for generating the near-zero surface tension needed at the interface [3]. It is also essential that LS be able to re-spread rapidly at the alveolar surface during inspiration.

Lack of effective surfactant in premature babies most often results in neonatal respiratory distress syndrome

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(NRDS). Administration of replacement lung surfactants (RLS) from animal origin has been reported beneficial and significantly reduces mortality rates [4]. Examples of such RLS include commercial preparations extracted from bovine (Survanta®, Ross Laboratories, Columbus, Ohio, USA) or porcine (Curosurf®, Chiesi Pharmaceutici, Parma, Italy) lungs [5].

Another severe lung condition, the acute respiratory distress syndrome (ARDS), shares many of the NRDS symptoms. However, ARDS does not respond positively to RLS therapy because ARDS is a more complicated pathology than the simple absence of surfactant [4]. In ARDS, substantial leakage of plasma proteins and inflammatory mediators, such as albumin, C-reactive protein, hemoglobin and fibrinogen, occurs in the alveolar spaces [6,7]. The lack of efficacy of RLS therapy in ARDS results from the fact that plasma proteins and mediators exasperate LS function deterioration [8,9]. Numerous in vitro studies have demonstrated the surfactant inhibition properties of plasma proteins, in particular albumin [10,11].

More precisely, it has been demonstrated that serum albumin exercises a concentration-dependent surface pressure that can exceed the re-spreading pressure of collapsed monolayers in vitro [4]. As a result, the collapsed surfactant monolayer cannot re-spread effectively during expansion, leading to higher minimum surface pressure and to alteration of the compression/expansion isotherms. It was concluded that the re-spreading pressure may be as important as the minimum surface tension in the design of new replacement surfactants that could be beneficial in ARDS therapy [4].

We have recently reported that a fluorocarbon gas (gFC) provides a highly effective fluidizing effect on a DPPC monolayer, preventing it from crystallizing during compression, and hence, from loosing its LS properties [12,13]. It was also found that gFC facilitated the re-spreading of DPPC molecules during expansion. The specific properties of fluorocarbons, including exceptional chemical and biological inertness, high gas-dissolving capacity, low surface tension, excellent spreading characteristic and high fluidity, have triggered numerous applications of these compounds in diagnosis and therapy [14–17]. We have suggested that certain fluorocarbons, in combination with DPPC, may prove useful in RLS compositions [18].

The primary goal of the present study was to evaluate the effect of gaseous perfluorooctyl bromide (gPFOB) on a DPPC monolayer compressed over a sub-phase containing bovine serum albumin (BSA). PFOB has been chosen because it has been selected for intravascular oxygen transport [15,19] and liquid ventilation [20]. The isotherms of DPPC monolayers compressed and expanded on pure water, or on a sub-phase containing BSA, have been determined. In each experiment, the atmosphere above the monolayer was air, or air saturated with gPFOB. The physical state of the monolayers, fluid liquid-expanded (LE) or semi-crystalline liquid-condensed (LC), was investigated using fluorescence microscopy (FM).

2. Materials and methods

2.1. Materials

Perfluorooctyl bromide (PFOB, perflubron) was kindly provided by Alliance Pharmaceutical Corp. (San Diego, CA, USA). L-α-l,2-dipalmitoyl-*sn*-3-glycerophosphatidylcholine (DPPC, purity >99%) and bovine serum albumin (BSA, essentially fatty acid free, purity >96%) came from Sigma. Spreading solutions of DPPC (1.0 mmol L^{-1}) were prepared in analytical grade chloroform. Water was purified 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-l,3-diazol-4-yl)amino]hexanoyl-l-hexadecanoyl-*sn*-glycero-3-phosphocholine, $NBDC_6$-HPC) was purchased from Molecular Probes (Eugene, OR, USA). It was used at a lipid mole ratio of 1%. Saturation of air with PFOB at 25 °C leads to a PFOB concentration of 0.5 mL L^{-1}.

2.2. Compression isotherms

Surface pressure (π) versus molecular area (A) isotherms were recorded on a Langmuir minitrough (Riegler and Kirstein, Potsdam, Germany) with a working surface area of ~120 cm^2 and a sub-phase volume of ~100 mL. The trough was equipped with two movable barriers (compression speed: 4 $Å^2$ $molecule^{-1}$ min^{-1}). π was measured using the Wilhelmy plate method. The trough was enclosed in a gas-tight box that was flushed with air or with air saturated with gPFOB, depending on the experiments. In the latter case, air was allowed to bubble at room temperature through liquid PFOB before being flushed into the gas-tight box. The flow rate of the gas phase (air or gPFOB-saturated air) was set to 1.2 L min^{-1}. Under these conditions, the evaporation rate of PFOB was measured to be ~6 mL h^{-1}. Temperature was regulated at 25±0.5 °C. The errors on π and A were estimated to ±1 mN m^{-1} and 1 $Å^2$, respectively. In the experiments where DPPC monolayers were compressed on water, 15 μL of the DPPC spreading solution were spread on water and the spreading solvent was allowed to evaporate for 15 min before compression. In experiments involving BSA, 15 μL of the DPPC spreading solution were spread on a cleaned sub-phase containing BSA (0.25 mg L^{-1}) in order to avoid pre-adsorption of the protein. The isotherms were recorded 1 h30 after spreading of the DPPC solution. The pH of the sub-phase was ~6.8. The isoelectric point of BSA is 5.4.

2.3. Fluorescence microscopy

Fluorescence microscopy (FM) was achieved with the above balance equipped with an Olympus fluorescence microscope (20× power objective) mounted on a *xy* translation stage, so as to allow scanning of the trough over different regions. 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 via a Hamamatsu intensified camera. The microscope was linked to the gas-tight box of the trough through an extensible gusset, allowing control of the partial pressure of PFOB. The surface pressure was kept constant during the FM experiments. The fluorescent probe $NBDC_6$-HPC is soluble into liquid-expanded (disordered) phases, which appear bright under the microscope, and is excluded from the liquid-condensed (semi-crystalline) phases, which then appear as dark domains in the images [21].

3. Results and discussion

3.1. Isotherms of Langmuir monolayers of DPPC

3.1.1. On pure water

When compressed under air, DPPC underwent a first order phase transition from a liquid-expanded (LE) state to a liquid-condensed (LC) state at a surface pressure of π ~13 mN m^{-1}, as shown by the presence of a plateau on the isotherm (Fig. 1) [22]. At the onset of the LE/LC coexistence region, as

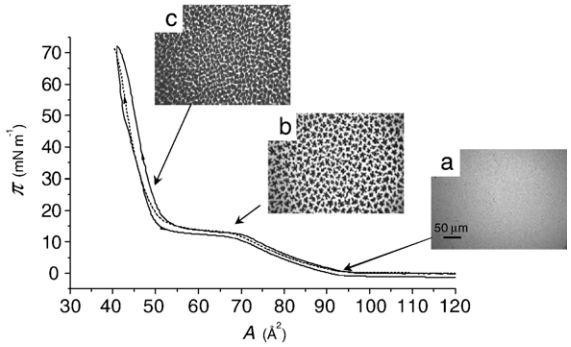

Fig. 1. Surface pressure (π) versus molecular area (A) isotherms of a Langmuir monolayer of DPPC compressed on pure water (25±0.5 °C) under air. The first compression—expansion cycle is represented by a solid line and the second compression by a dashed line. Fluorescence micrographs of the DPPC monolayer at (a) 2 mN m^{-1}, (b) 13 mN m^{-1} and (c) 20 mN m^{-1}, show the formation of semi-crystalline LC domains in the last two cases. The fluorescent dye is preferentially soluble in the disordered liquid-expanded regions of the monolayer, which appear bright, while the liquid-condensed domains, from which the dye is excluded, appear dark [21].

visualized by FM, LC domains appeared, became more numerous upon compression, and progressively merged into a continuous LC phase.

Monitoring the expansion of the DPPC monolayer by FM showed the progressive fragmentation of the continuous LC phase into discrete LC domains. The size of the LC domains progressively decreased, until they disappeared at $\pi < \sim 5$ mN m^{-1} ($A \sim 80$ Å^2). When the DPPC monolayer was subsequently re-compressed after expansion, no significant variation was seen in the isotherms, which means that no significant amount of DPPC molecules was desorbed to the sub-phase during the compression/expansion cycle. The limiting molecular area (A_∞) was ~50 Å^2. The surface pressure of collapse (π_{coll}) was ~71 mN m^{-1}, i.e. the minimum surface tension was close to 1 mN m^{-1}.

3.1.2. On a sub-phase containing bovine serum albumin

The influence of human serum albumin (HSA) on Langmuir monolayers of various zwitterionic and anionic phospholipids has been investigated using Brewster angle microscopy (BAM) and grazing-incidence X-ray diffraction (GIXD) [23,26]. It was shown that a mixed HSA/phospholipid monolayer was formed that results from adsorption and penetration of the albumin into the phospholipid monolayer. Upon compression, at 30 mN m^{-1}, the protein was squeezed out from the monolayer into the sub-phase. Depending on the pH of the sub-phase, i.e. on the electrostatic interactions between the protein and the phospholipids, a variety of inhomogenous phases with various morphologies (e.g. honeycomb, block, or stripe phases) were observed by BAM. The phase behavior and structural changes of monolayers of zwitterionic and anionic phospholipids coupled with HAS have been investigated by GIXD.

The isotherms of the DPPC monolayer spread on a BSA-containing sub-phase compressed under air are shown in Fig. 2. Upon compression, the isotherm is significantly shifted toward larger molecular area (A) values, as compared to the isotherm obtained on pure water. At the beginning of the compression (A ~120 Å^2), π is ~20 mN m^{-1} in the presence of BSA, while it is close to zero on pure water. This means that BSA is incorporated into the DPPC monolayer upon compression and forms a mixed DPPC/BSA monolayer, in agreement with previous reports [23–26].

The physical state of the mixed DPPC/BSA monolayer has been investigated by FM. For $\pi < 30$ mN m^{-1}, FM images showed a dark grey, uniform phase. This phase is very different from the bright and featureless images typical of fluid liquid-expanded phases, as seen in Fig. 1a. The dark appearance of the FM images indicates that the fluorescent dye is not soluble in the mixed DPPC/BSA monolayer. This phase did not show any presence of LC domains, which supports the fact that BSA had penetrated into the monolayer and had interacted with the DPPC molecules, preventing the formation of LC domains. However, no specific features, such as inhomogeneous phases, as suggested in [24], have been observed, probably due to the difference of resolution between FM and BAM techniques.

For $\pi > 30$ mN m^{-1}, the shape of the compression isotherm became more similar to that of DPPC spread on pure water. The presence of numerous small LC domains forming a practically continuous LC phase was observed (Fig. 2a), which shows that BSA had been squeezed out from the DPPC monolayer, allowing the formation of LC domains. The number and size of these domains increased with π, until they formed a continuous LC phase at ~40 mN m^{-1} (Fig. 2b). The value of π_{coll} (~71 mN m^{-1}) of the DPPC monolayer in the presence of BSA and of A_∞ (~50 Å^2), are similar to those obtained on pure water. The fluorescence micrograph (Fig. 2c) illustrates a fracture of the monolayer that occurred during collapse. Moreover, the value of A_∞ determined on the second compression was similar to that measured after the first compression, which suggests that the stability of the DPPC monolayer is not affected by the presence of the BSA in the sub-phase.

Upon expansion of the DPPC monolayer, BSA was re-adsorbed at the interface, maintaining a surface pressure of 20 to 10 mN m^{-1} for A ranging from 50 to 120 Å^2. Monitoring monolayer expansion clearly showed the role of BSA in

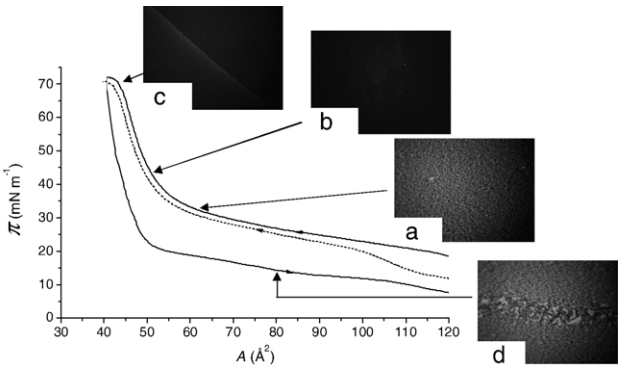

Fig. 2. π/A isotherms of a DPPC Langmuir monolayer spread on a sub-phase containing BSA compressed under air. The first compression—expansion cycle is represented by a solid line and the second compression by a dashed line. FM images of the DPPC monolayer upon compression at (a) 30 mN m^{-1}, (b) 40 mN m^{-1} and (c) 71 mN m^{-1}, and, upon expansion, at (d) 80 Å^2 (25 °C).

hindering the re-spreading of the DPPC: at the end of the expansion ($A \sim 120$ Å^2), π was still ~ 7.5 mN m^{-1}, instead of ~ 0 mN m^{-1} in the absence of BSA. In parallel, FM showed that, even at large A values (~ 80 Å^2), the phase was clearly not fluid. Large regions of LC phase, hardly fragmented, were visible (Fig. 2d). In other regions, numerous small LC domains were present. By contrast, the DPPC monolayer expanded on pure water under the same conditions was totally fluid. This shows that BSA re-adsorption during expansion of the DPPC monolayer prevented the re-spreading of the DPPC molecules, which, in the case of lung surfactant, would hinder lung function [4].

3.2. Isotherms of Langmuir monolayers of DPPC in contact with a fluorocarbon gas

3.2.1. On pure water

The behavior of a DPPC monolayer compressed on pure water under an atmosphere of air saturated with gPFOB has recently been investigated by FM and GIXD [12,13]. The gPFOB molecules adsorb and penetrate into the DPPC monolayer (as assessed by the surface pressure increase on the isotherm), drastically changing its phase behavior (Fig. 3). The LE/LC phase transition pressure of DPPC, which was typically found at ~ 13 mN m^{-1} in the absence of gPFOB, was increased to ~ 40 mN m^{-1}. The transition observed at ~ 28 mN m^{-1} in the presence of gPFOB was no longer of the LE/LC type, but likely corresponds to a re-orientation of the PFOB molecules inserted in the DPPC monolayer. The DPPC monolayer in contact with gPFOB is in a homogenous, fluid LE phase up to ~ 40 mN m^{-1}, above which the PFOB molecules are excluded from the monolayer and likely form a thin liquid film on top of the DPPC monolayer.

Upon expansion, below 40 mN m^{-1}, the isotherm was shifted towards larger molecular areas, indicating that the PFOB molecules were reincorporated into the DPPC monolayer, thus increasing the re-spreading properties of the DPPC molecules, as assessed by FM. The DPPC monolayer contacted with gPFOB was stable until ~ 71 mN m^{-1} and no significant loss of DPPC molecules to the sub-phase occurred during the compression/expansion cycle. These experiments demonstrate

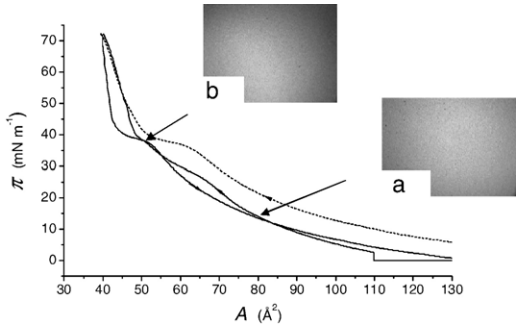

Fig. 3. π/A isotherms of a DPPC monolayer spread onto pure water under an atmosphere of gPFOB-saturated air (25 °C). The first compression—expansion cycle is represented by a solid line and the second compression by a dashed line. FM images at (a) 13 mN m^{-1} and (b) 35 mN m^{-1} show that the DPPC monolayer in contact with gPFOB remains fluid up to 40 mN m^{-1}.

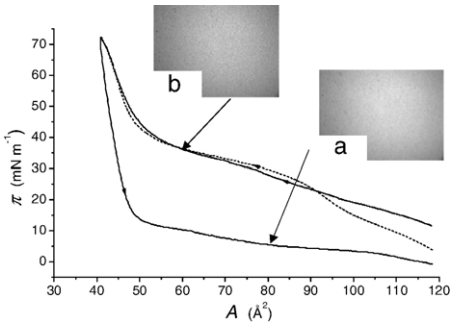

Fig. 4. π/A isotherms of a DPPC Langmuir monolayer spread on a sub-phase containing BSA and compressed under gPFOB-saturated air (25 °C). The first compression—expansion cycle is represented by a solid line and the second compression by a dashed line. Fluorescence micrographs of the DPPC monolayer (a) upon compression at 35 mN m^{-1} and (b) upon expansion at 80 Å^2.

that the gPFOB molecules interact with DPPC molecules and prevent the formation of the LC phase, and hence, induce a fluidizing effect in the monolayer.

3.2.2. On a sub-phase containing bovine serum albumin

Fig. 4 shows the isotherms of a DPPC monolayer spread on a BSA-containing sub-phase and compressed under a gPFOB-saturated air atmosphere. During the first compression, the isotherm was significantly less shifted towards larger A values than the isotherm of DPPC compressed on a BSA-containing sub-phase under air. This indicates that, in the presence of gPFOB molecules, the incorporation of BSA in the DPPC monolayer is less pronounced. It is also noteworthy that the initial surface pressure recorded with BSA in the sub-phase when the DPPC monolayer was contacted with gPFOB was lower than the initial surface pressure recorded in the absence of gPFOB (i.e. 12 mN m^{-1} vs. 20 mN m^{-1}, respectively). This also indicates that the presence of gPFOB counteracts the adsorption of BSA in the DPPC monolayer. The fact that, for $\pi < 30$ mN m^{-1}, the FM images showed the dark grey uniform phase already seen in Section 3.1.2, indicates that the presence of gPFOB limits but does not totally inhibit the adsorption of BSA. For 30 mN m$^{-1} < \pi < 40$ mN m^{-1}, a bright and featureless image, typical of a LE phase, was seen. Within this pressure range, BSA had been squeezed out, while PFOB molecules were still present in the DPPC monolayer, resulting in a definite fluidizing effect. For $\pi > 40$ mN m^{-1}, very small LC domains were present on the FM images, meaning that the PFOB molecules were being squeezed out and went on top of the DPPC monolayer.

The striking point is that, contrarily to what happened in the absence of gPFOB (Fig. 2), π decreased strongly upon expansion, reaching even ~ 0 mN m^{-1} for the largest A values (~ 120 Å^2), as compared to 7.5 mN m^{-1} when BSA was present and gPFOB absent. This means that the presence of the fluorocarbon molecules hindered the re-adsorption of the protein upon expansion.

This view is confirmed by the FM images obtained at $A \sim 80$ Å^2, which were uniformly bright, meaning that the monolayer was in the LE state (Fig. 4), as compared to the FM

images obtained in the presence of BSA, but without gPFOB, which clearly showed the presence of LC domains (Fig. 2d). The values of π_{coll} and A_∞ obtained after the first compression, i.e. $\sim$71 mN m^{-1} and $\sim$50 Å^2, respectively, show that the DPPC monolayer was not destabilized when BSA and gPFOB were present simultaneously. The second compression isotherm did not show any significant difference with the first compression isotherm.

4. Conclusions and perspectives

The presence of a fluorocarbon gas in the atmosphere above a Langmuir monolayer of DPPC profoundly modifies the effect of serum albumin on the compression/expansion behavior and morphology of this monolayer. Most importantly, the isotherms and fluorescence micrographs provide evidence that the fluorocarbon gas facilitates the re-spreading of a collapsed DPPC Langmuir monolayer during expansion on a sub-phase containing bovine serum albumin. The substantial residual surface pressure (π=7.5 mN m^{-1}) still observed at the end of expansion (A=120 Å^2) when BSA is present in the sub-phase, returns to essentially 0 mN m^{-1} when the fluorocarbon gas is introduced in the atmosphere above the monolayer. In parallel, the numerous LC domains seen on expansion of the DPPC monolayer, even at large A values, when BSA is present, are no longer seen when the fluorocarbon is added. The observed fluidizing effect induced by the fluorocarbon thus counteracts the deleterious effect of albumin, which adsorbs at the interface during expansion, and develops a surface pressure that maintains the DPPC monolayer in a liquid condensed state, thus hindering lung function. These results suggest that combinations of a fluorocarbon gas and DPPC could be useful in the design of new replacement lung surfactants effective against the acute respiratory syndrome, in which the inactivation of the lung surfactant is largely driven by serum proteins.

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