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Phospholipid packing and hydration in pulmonary surfactant membranes and films as sensed by LAURDAN

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ABSTRACT

The efficiency of pulmonary surfactant to stabilize the respiratory surface depends critically on the ability of surfactant to form highly packed films at the air–liquid interface. In the present study we have compared the packing and hydration properties of lipids in native pulmonary surfactant and in several surfactant models by analyzing the pressure and temperature dependence of the fluorescence emission of the LAURDAN (1-[6-(dimethylamino)-2-naphthyl]dodecan-1-one) probe incorporated into surfactant interfacial films or free-standing membranes. In interfacial films, compression-driven changes in the fluorescence of LAURDAN, evaluated from the generalized polarization function (GPF), correlated with changes in packing monitored by surface pressure. Compression isotherms and GPF profiles of films formed by native surfactant or its organic extract were compared at 25 or 37 °C to those of films made of dipalmitoylphosphatidylcholine (DPPC), palmitoyloleoylphosphatidylcholine (POPC), DPPC/phosphatidylglycerol (PG) (7:3, w/w), or the mixture DPPC/POPC/palmitoyloleoylphosphatidylglycerol (POPG)/cholesterol (Chol) (50:25:15.10), which simulates the lipid composition of surfactant. In general terms, compression of surfactant films at 25 °C leads to LAURDAN GPF values close to those obtained from pure DPPC monolayers, suggesting that compressed surfactant films reach a dehydrated state of the lipid surface, which is similar to that achieved in DPPC monolayers. However, at 37 °C, the highest GPF values were achieved in films made of full surfactant organic extract or the mixture DPPC/POPC/POPG/Chol, suggesting a potentially important role of cholesterol to ensure maximal packing/dehydration under physiological constraints. Native surfactant films reached high pressures at 37 °C while maintaining relatively low GPF, suggesting that the complex three-dimensional structures formed by whole surfactant might withstand the highest pressures without necessarily achieving full dehydration of the lipid environments sensed by LAURDAN. Finally, comparison of the thermotropic profiles of LAURDAN GPF in surfactant model bilayers and monolayers of analogous composition shows that the fluorophore probes an environment that is in average intrinsically more hydrated at the interface than inserted into free-standing bilayers, particularly at 37 °C. This effect suggests that the dependence of membrane and surfactant events on the balance of polar/non-polar interactions could differ in bilayer and monolayer models, and might be affected differently by the access of water molecules to confined or free-standing lipid structures.

1. Introduction

Pulmonary surfactant is a lipid–protein complex synthesized and secreted by the type II pneumocytes into the thin water layer covering the alveolar spaces of mammalian lungs. The surfactant main function is to reduce the surface tension at the air–liquid interface, preventing lung collapse at the end of expiration [1]. The activity of surfactant at the alveoli involves three main processes: (i) transfer of surface active material from the aqueous hypophase where it is secreted, into the interface, (ii) reduction of surface tension to values close to 0 mN/m during compression at expiration and (iii) re-extension of the surface active film upon expansion at inspiration [2].

Representative values for the composition of lung surfactant as obtained from bronchoalveolar lavage are approximately 85–90% phospholipid, 6–8% specific surfactant-associated proteins and 5–10% neutral lipids by weight [3,4]. Phosphatidylcholine (PC) is by far the most prevalent class, with other phospholipid classes being present in much smaller amounts including phosphatidylglycerol (PG) and phosphatidylinositol (PI), together with minor traces of phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin (SM). The most abundant single component of lung surfactant is the disaturated phospholipid dipalmitoyl phosphatidylcholine (DPPC), which represents approximately 40% of the total weight and is the

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main responsible for the surface active properties of lung surfactant [4,5]. It is widely accepted that densely packed films such as those formed by DPPC-enriched lipid mixtures are competent to reduce to a minimum the surface tension at the air–liquid interface as required to avoid alveolar collapse at the end of expiration [4,6]. However, recent results have shown that stably low surface tensions can be also achieved by purely unsaturated phospholipid films, if they are compressed at a fast enough rate [7]. Rapid compression in this later case has been proposed to produce highly viscous amorphously packed films with the solid-like properties and stability of a vitrified two-dimensional glass. Molecular lipid packing at the interface is therefore a major feature to define stability of pulmonary surfactant films in highly compressed states.

Moreover, the composition of the lipid fraction of surfactant plays also a major role in determining the lateral structure of surfactant membranes and films. Pulmonary surfactant films have been shown to segregate under compression micron-size ordered regions enriched in saturated phospholipids, sorted from other more disordered regions where unsaturated phospholipids and hydrophobic proteins would preferentially partition [8,9]. The occurrence of such a phase segregation under physiological conditions of temperature is a matter of discussion [10], but it seems to be an intrinsic property of the particular composition of surfactant over a wide range of environmental conditions, at both micro- and nanoscopic levels [11,12]. Structural studies with different surfactant models suggest that the presence of a proper combination of low- and high-melting temperature phospholipid species, competent to sustain phase segregation, may be important for the surfactant function. These composite films would simultaneously provide structural stability at high compression and a dynamic environment to facilitate structural transformations thought to occur during breathing cycling [2,5,6]. Segregation of ordered and disordered phases has been demonstrated also in pulmonary surfactant bilayers [13,14], confirming the correlation of the properties of lipids and proteins to self-organize in both bilayer and interfacial monolayer models. However, the correspondence between lateral organization and molecular packing in surfactant bilayers and monolayers cannot be directly established, as both types of models are subjected to very different constraints. As a matter of fact, phospholipid monolayers are widely used as models of biological membranes [15], yet the exact correspondence of lateral packing and molecular organization is a matter of controversy.

In the present study we have approached a parallel characterization of lateral organization and packing properties of lipids in both pulmonary surfactant free-standing membranes and interfacial films, taking advantage of the unique spectroscopic properties of LAURDAN (1-[6-(dimethylamino)-2-naphthyl]dodecan-1-one). The fluorescence emission of this probe is highly sensitive to the level of hydration of the head group phospholipid region of membranes, which is affected by both packing and lateral organization [16–18]. LAURDAN fluorescence has been in fact recently used to obtain information on the lateral organization of simple interfacial phospholipids monolayers, as it can reveal compression-driven segregation of condensed domains and three-dimensional transitions with microscopic resolution [19]. In our study, we have characterized lipid packing/hydration as assessed by LAURDAN in more complex membranes and films such as those mimicking pulmonary surfactant and some of its models.

2. Materials and methods

2.1. Materials

1-[6-(Dimethylamino)-2-naphthyl]dodecan-1-one (LAURDAN) was from Molecular Probes (Eugene, OR). 1,2-Dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), egg yolk 1,2-diacylphosphatidylglycerol (PG) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Chloroform and methanol HPLC grade solvents were from Scharlab (Barcelona, Spain).

2.2. Pulmonary surfactant purification

Pulmonary surfactant was purified from bronchoalveolar lavage of porcine lungs as described in detail elsewhere [20]. Stock suspensions of native pulmonary surfactant were made in 5 mM pH 7 Tris buffer containing 150 mM NaCl. The total concentration of phospholipid in surfactant samples was estimated by phosphorus quantitation upon phospholipid mineralization [21]. LAURDAN-labelled native surfactant was prepared by adding a small aliquot of LAURDAN (typically 1% with respect to phospholipid, mol/mol) in dimethylsulfoxide (DMSO) to a surfactant suspension under vigorous vortexing, and the mixture was incubated in the dark for 30 min at room temperature.

The hydrophobic components of surfactant (organic extract, OE), including the full lipid fraction and the hydrophobic proteins SP-B and SP-C, were obtained by extraction with chloroform/methanol [22].

2.3. Pressure-fluorescence measurements in multi-well plates

To analyze the fluorescent properties of LAURDAN in interfacial films sustaining different lipid packing conditions, experiments were carried out by measuring simultaneously the LAURDAN emission and the surface pressure of films formed in 12- or 6-well microplates. Each well was filled with 2 mL (in 12-well plates) or 3 mL (in 6-well plates) of 5 mM pH 7.0 Tris buffer containing 150 mM NaCl. Then, small aliquots of a 0.1 mg/mL DPPC solution in chloroform–methanol (2:1 v/v), mixed or not with 5 μL of a 0.1 mg/mL LAURDAN solution in the same solvent, were deposited on top of the buffer solution in each well and the surface pressure was measured with a platinum wire connected to the surface pressure sensor of a surface balance (Nima Technology, Inc., Coventry, UK). The LAURDAN fluorescence spectra were obtained from each well in a Horiba Jobin Yvon (HJY) Fluoromax II photon counting spectrofluorometer (Longjumeau, France) fitted with a 150-W xenon arc lamp (Osram) and the HJY F-3000 fiber-optic adaptor in the sample holder. A 1-m fused-silica randomized bifurcated fiber-optic bundle (HJY) allowed remote sensing of the fluorescence by carrying the light to and from the samples placed in the HJY Micromax 384 microwell-plate reader (http://www.jobinyvon.com/MicroMax/). The excitation wavelength used was 370 nm and the emission spectra were recorded in the 430- to 540-nm range. A combination of 290- to 420-nm bandpass (Schott UG-1) and 420-nm long pass (Schott GG-420) glass filters were used in the excitation and emission paths of the F-3000 mount, respectively, to eliminate stray light in the fluorescence measurements. These measurements were made at 25 ± 1 °C.

2.4. Fluorescence and compression isotherms

Stock solutions of the different lipids and lipid mixtures with LAURDAN (1% with respect to phospholipid, mol/mol) were prepared in chloroform–methanol (2:1 v/v) and used to form films by spreading directly at the interface of a specially designed LB trough (190 cm^2, Nima Technology, Inc., Coventry, UK) equipped with a continuous Teflon-ribbon barrier able to sustain maximal surface pressures with no leakage, and thermostated at the desired temperature. The trough was set inside a custom-made closed chamber to ensure isolation from external light and controlled humidity. Before any given lipid mixture was spread, the trough was filled with 5 mM pH 7.0 Tris buffer containing 150 mM NaCl prepared in ultra-pure water (first deionized to Milli Q quality in a Millipore system, then distilled in the presence of potassium permanganate) [23], thermostated at the desired temperature and

equilibrated for at least 15 min to ensure water saturation of the air phase. Then the proper volume of organic solution mixtures, or of LAURDAN-labelled native surfactant, was spread at the interface. After waiting another 15 min to allow for solvent evaporation and film equilibration, the different films were compressed stepwise at a rate of 60 cm^2/min area change. At the desired pressures, compression was stopped and the LAURDAN emission spectrum was recorded with the setup described above, but introducing the common leg of the bifurcated fiber-optic bundle inside the (removable) focussing tube accessory of the Micromax 384 and placing the sampled surface, orientated at an angle of 90º, at the focal distance.

2.5. Fluorescence of LAURDAN in multilamellar suspensions

Appropriate volumes of chloroform–methanol solutions of DPPC, POPC, DPPC/PG (7:3 w/w), the mixture DPPC/POPC/POPG/Chol (50:25:15:10 w/w) or surfactant OE and LAURDAN (1% with respect to phospholipid, mol/mol) were introduced in round-bottomed glass tubes before carrying every solution to dryness under a mild nitrogen flow. To remove potential remaining traces of the organic solvent, all samples were evaporated under high vacuum for 1.5 h. The resulting lipid film was then hydrated with an appropriate volume of 5 mM pH 7.0 Tris buffer containing 150 mM NaCl in a thermomixer for 1 h at 50 °C, with periodical vigorous stirring. Final phospholipid concentration in the suspensions was 10 μg/mL. The fluorescence spectra of all samples were recorded in an Aminco-Bowman Series 2 luminescence spectrometer equipped with thermostated cells, using an excitation wavelength of 370 nm and recording the emission between 400 and 550 nm.

2.6. Data analysis: LAURDAN generalized polarization function

The LAURDAN emission is typically blue in dehydrated environments such as those provided by the headgroup region in the lipid gel phase, but it shifts from blue to green in the more hydrated liquid-crystalline phase [24,25]. To quantify the emission spectral changes, the generalized polarization function (GPF) was defined analogously to the fluorescence polarization function as:

$$GPF = (I_B - I_R) / (I_B + I_R)$$

where the relative parallel and perpendicular orientations of the polarizer are replaced by the intensities at the blue and red edges of the emission spectrum (I_B and I_R, respectively), at a given excitation wavelength [24,25]. It is important to underline that the GPF measurements of our experiments do not involve the use of polarizers. This well-characterized function is sensitive to the extent of water dipolar relaxation processes in the lipid bilayers [26].

3. Results

3.1. Fluorescence of LAURDAN in DPPC monolayers

The emission spectrum of the LAURDAN probe is very sensitive to its environment in membranes, particularly to the hydration level of the lipid headgroup region. The latter is strongly influenced by changes in the lipid packing as a result, for example, of changes in pressure or temperature. The LAURDAN fluorescence is blue in densely packed lipid gel phases (where no water is allowed) but changes to green in liquid-crystalline phases with higher hydration levels [24,25]. When studying interfacial DPPC monolayers formed by spreading lipid solutions in multi-well plates, the surface area remains constant so that changes in surface pressure are achievable by increasing the lipid density at the air–liquid interface.

Fig. 1 illustrates the capability of LAURDAN to sense progressively tighter lipid packing in DPPC monolayers as a result of an increasing density of lipid molecules at the air–liquid interface. Fig. 1A shows the blue shift of the LAURDAN emission observed upon spreading of increasing amounts of DPPC on the interface. By adding more material, the lipid density and packing increases, leading to a rise of the surface pressure and therefore to a blue shift in the LAURDAN emission, presumably due to progressive dehydration of the probe at the interface. This behaviour arises from the intramolecular charge transfer character of the lowest-lying electronic excited state of LAURDAN formed in polar media vs. the higher energy of the locally excited state which occurs in non-polar environments. Fig. 1B depicts both the surface pressure and the generalized polarization function (GPF) calculated from the LAURDAN emission spectra of Fig. 1A, obtained from films formed by spreading the different amounts of DPPC at the interface. At low lipid loads, the DPPC film is in a liquid-expanded phase (LE) and the LAURDAN emission peaks near 490 nm. When the lipid density increases the molecules are forced to pack more tightly, leading to a liquid-condensed phase (LC) where the LAURDAN emission peaks at ca. 440 nm. At intermediate lipid densities, presumably producing a coexistence of LE and LC phases, the LAURDAN fluorescence exhibits both blue and green contributions leading to broader emission spectra. Fig. 1B shows how the GPF values calculated from the LAURDAN emission spectra strongly correlate with the surface pressure, illustrating the sensitivity of the probe fluorescence to the lipid packing.

3.2. LAURDAN fluorescence in compressed Langmuir films

Fig. 2 shows compression isotherms obtained from DPPC or POPC films containing either 1% or 3% LAURDAN (probe-to-lipid, mol/mol). Isotherms of disaturated phospholipids like DPPC reach surface pressures above 60 mN/m at temperatures below its gel-to-fluid phase transition temperature (41 °C for DPPC), reaching a solid-like metastable state. The DPPC isotherm also shows a conspicuous plateau at around 10 mN/m, pressures at which a well-characterized LE-LC two-dimensional transition occurs. The insets in Fig. 2 confirm how the fluorescence of LAURDAN is sensitive to the compression state of DPPC films at a wide range of surface pressures. The figure allows also comparison of the LAURDAN spectra in progressively compressed DPPC films carrying different amounts of probe. Films containing either 1% or 3% probe show a similar blue shift of the LAURDAN emission—and a similar change in GPF—upon compression although the spectra of films containing a higher proportion of probe exhibit a much better signal-to-noise ratio. Still, monomolecular films carrying as little as 1% probe-to-lipid ratio can produce emission spectra amenable to be analyzed in terms of pressure/GPF dependence. We therefore maintained the lowest probe loading to carry out all the experiments of this study, in order to minimize potential probe-promoted perturbations of the structure of the films [27]. The LAURDAN GPF increases moderately, following a progressive lipid packing in the LE phase, at pressures below 10 mN/m in the DPPC films. The compression-promoted increase in GPF undergoes an inflection point (marked by the lower arrow in Fig. 2A) at the beginning of the LE-LC plateau, indicating that this lateral transition is a major factor promoting the dehydration of LAURDAN that causes its emission blue shift. The dependence of GPF with the surface pressure displays a second inflection point (marked by the upper arrow in Fig. 2A) at around 15–17 mN/m, presumably once the LE-LC transition has been completed.

Fig. 2 further shows that compression of POPC films also produces a moderate but progressive shift of the LAURDAN emission to shorter wavelengths, in spite of the POPC films maintain a LE phase at all the compression states achieved at temperatures well above its melting transition. The increase of LAURDAN GPF observed in these films upon increasing of surface pressure is therefore strictly associated with compression-induced partial dehydration of POPC molecules due to progressive packing. The absolute values of the maximum GPF

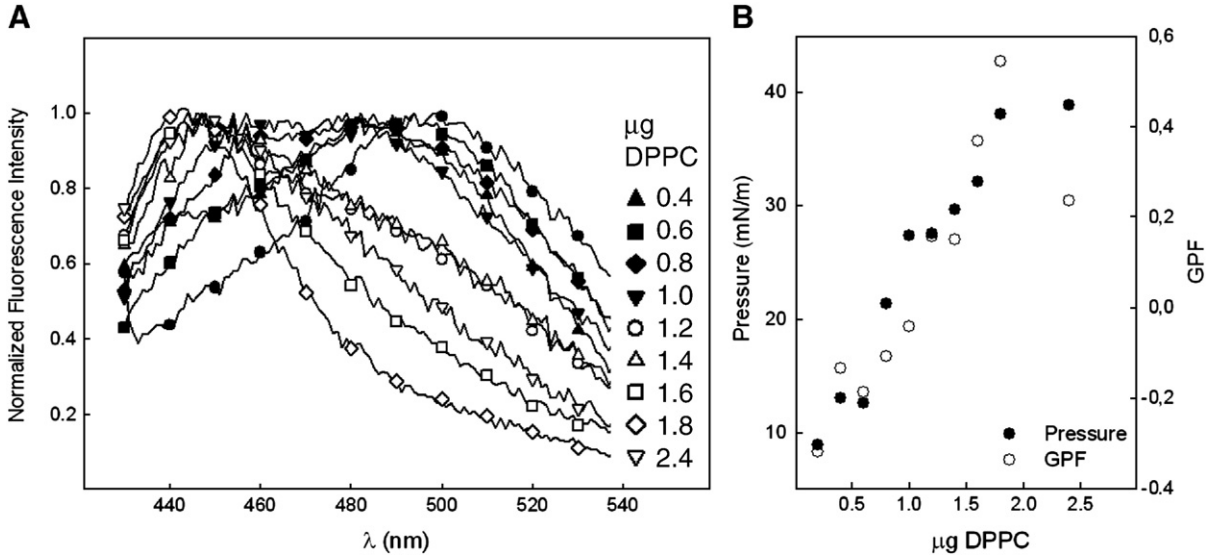

Fig. 1. LAURDAN fluorescence in DPPC monolayers. A: Fluorescence spectra of LAURDAN in monolayers prepared by spreading the indicated amounts of DPPC (from a solution 0.1 mg/mL) and LAURDAN in organic solution on top of a subphase Tris 5 mM pH 7 containing NaCl 150 mM. B: Plot of the surface pressure (closed circles), as measured with a surface balance, and the fluorescence of LAURDAN, evaluated from the spectral Generalized Polarization Function (GPF, open circles), versus the density of DPPC at the interface.

reached upon compression of POPC films (ca. −0.2 at ~70 Å^2/molecule) are markedly lower than those calculated from the LAURDAN spectra in compressed DPPC films (~0.43 at ~40 Å^2/molecule), indicating that POPC molecules are less packed and better hydrated at maximal compression than DPPC.

Given the sensitivity of the LAURDAN fluorescence to the lateral organization and compression state of interfacial phospholipid films, we have analyzed its behaviour in more complex films such as those spontaneously formed by interfacial adsorption of pulmonary surfactant lipid–protein complexes. Fig. 3A depicts the pressure–area isotherm of a film formed by direct spreading of an aliquot of LAURDAN-labelled porcine pulmonary surfactant at the air–liquid interface. The compression isotherm of native surfactant has the expected morphology, including a marked plateau at 45–50 mN/m

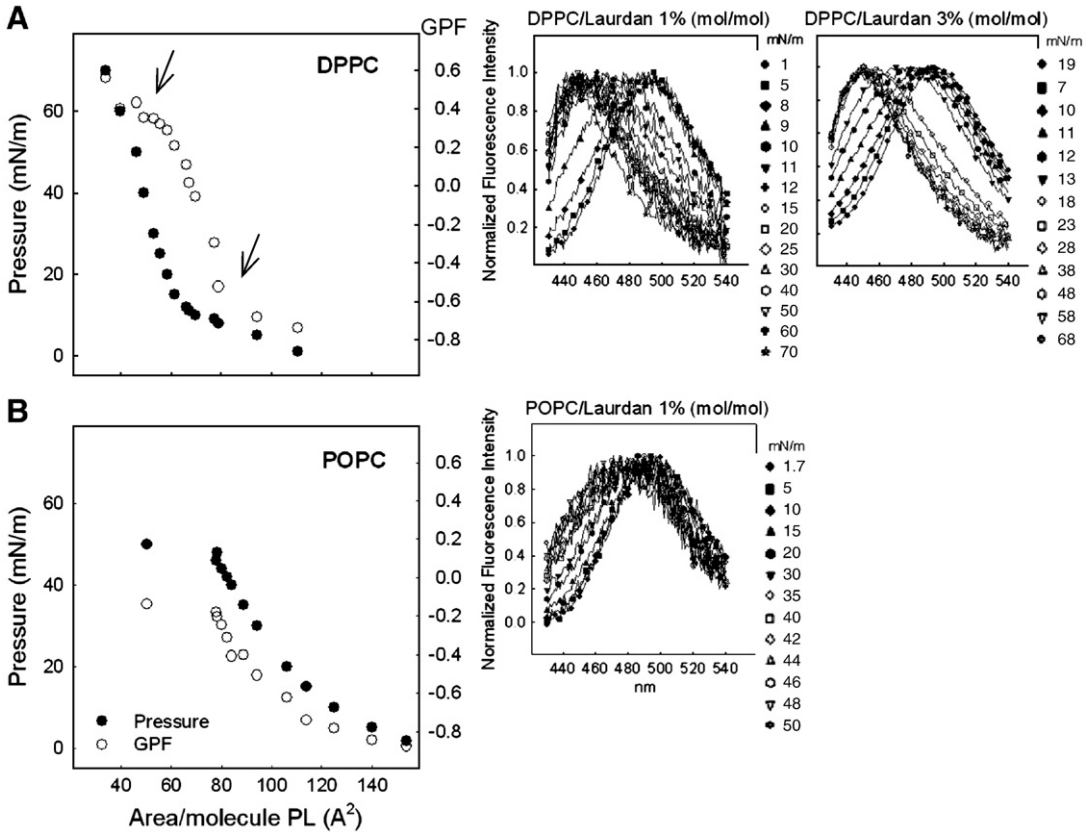

Fig. 2. LAURDAN fluorescence in compressed Langmuir films. Surface pressure (closed circles) and LAURDAN GPF (open circles) compression isotherms plotted versus the area/molecule obtained from Langmuir films made of pure DPPC (A) or POPC (B), containing 1% (mol/mol) of the fluorescent probe. The arrows remark conspicuous inflection points in the GPF profile. Insets: Fluorescence spectra of LAURDAN obtained from the indicated monolayers prepared by spreading a phospholipid/LAURDAN mixture in organic solution on top of a Tris 5 mM pH 7 subphase, containing NaCl 150 mM, and compressed to the corresponding pressures.

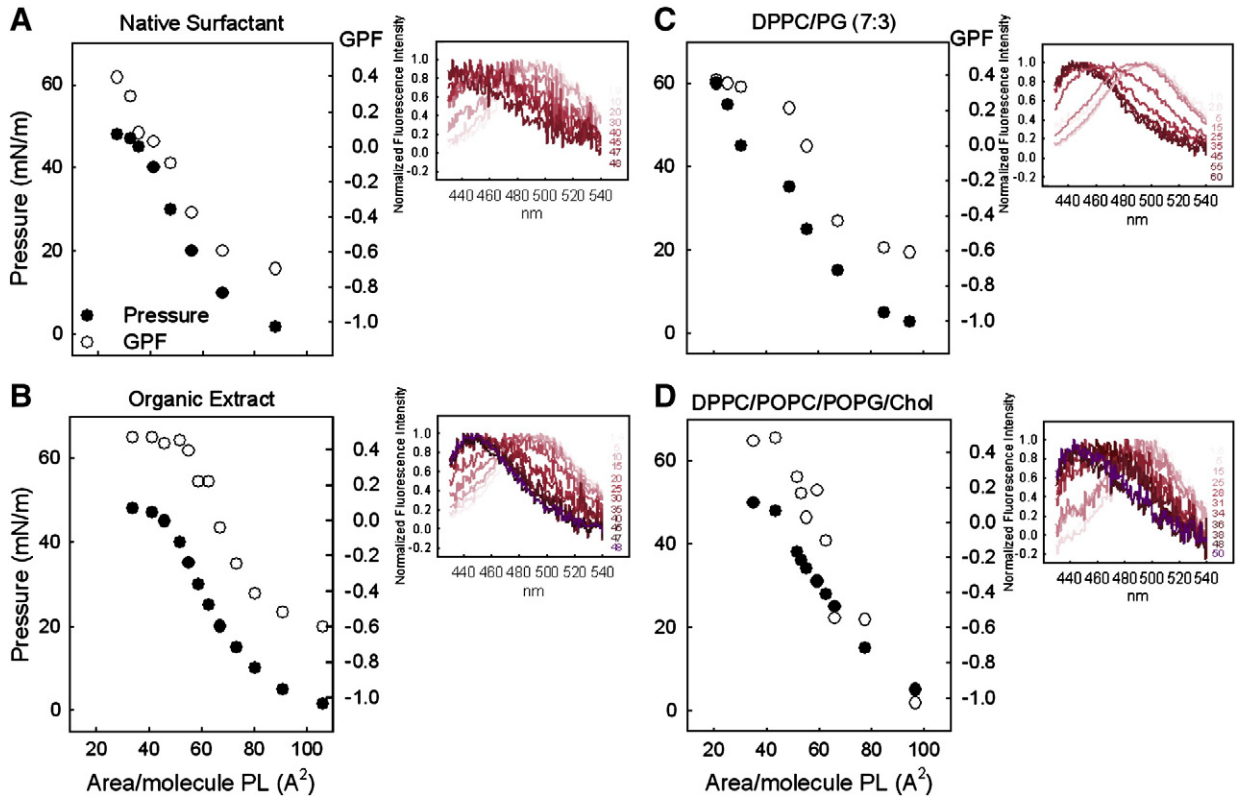

Fig. 3. LAURDAN fluorescence in compressed monolayers made of complex lipid mixtures. Surface pressure (closed circles) and LAURDAN GPF (open circles) compression isotherms plotted versus the area/molecule obtained from Langmuir films made of native surfactant (A), its organic extract containing all the lipids plus the hydrophobic proteins SP-B and SP-C (B), the mixture DPPC/PG (7:3, w/w) (C) or the quaternary system DPPC/POPC/POPG/Chol (50:25:15:10, w/w/w/w) (D), containing in all cases 1% (mol/mol) of LAURDAN. Insets: Fluorescence spectra of LAURDAN recorded from the corresponding interfacial monolayers compressed to the indicated pressures (low to high pressures represented by light to darker tones).

that has been associated with compression-driven three-dimensional transitions promoting formation of multilayer structures [8,9,28]. Raw fluorescence spectra of LAURDAN in these surfactant films, obtained at different compression states, are noisy but still show a marked blue shift associated with progressive compression-driven packing at the interface. A plot of the GPF values calculated from the LAURDAN spectra against the area/molecule ratio follows the increase in pressure up to values around 0.4, just slightly lower than GPF from DPPC films. Therefore, the hydration level of LAURDAN at the interfacial surfactant films approaches more the environment sensed by the probe in DPPC films than that in POPC layers, confirming that compression to maximal pressures of the native pulmonary surfactant films probably ends in formation of DPPC-enriched highly packed structures. Interestingly, the increase of GPF with compression seems to reach a plateau at a GPF of around 0.1, which only increases further up to 0.4 once compression has taken the isotherm well into the surface pressure plateau associated with three-dimensional folding of the surface film. A parallel increase of LAURDAN GPF and surface pressure is even clearer in interfacial films formed by spreading of the pulmonary surfactant organic extract containing the full lipid complement of surfactant and the hydrophobic proteins SP-B and SP-C (Fig. 3B). Isotherms from surfactant organic extract films also exhibit a plateau at around 45 mN/m associated with a maximal LAURDAN GPF above 0.4. Films from organic extract, however, always reach higher pressures and higher levels of dehydration, as monitored by LAURDAN, than films of the whole native surfactant compressed to a similar extent.

However, the isotherms of films formed from complex systems, such as the whole native surfactant or its organic extract, cannot be easily interpreted in terms of simple factors. The changes in the fluorescence properties of LAURDAN probe molecules inserted into these films have probably multiple contributions including segregation of phases and domains, compression-driven 2D and 3D structural transitions, and changes in lateral packing. To facilitate interpretation of the complex behaviour of surfactant films, we have compared their pressure/GPF–area isotherms with those obtained by compressing films formed from simplified lipid mixtures mimicking part of the compositional complexity of pulmonary surfactant. Fig. 3C shows the compression isotherm and LAURDAN spectra from a film made of a mixture DPPC/PG (7:3, w/w), widely used to reconstitute pulmonary surfactant-mimetic systems [3]. This mixture, containing 30% unsaturated phospholipid, is more fluid and dynamic than pure DPPC and contains also the anionic PG required to optimize lipid–protein interactions with the hydrophobic fraction of surfactant proteins [29,30]. The DPPC/PG isotherm exhibits a plateau at pressures below 40 mN/m, while the other films level off at pressures higher or around 40–45 mN/m or higher. Fig. 3D allows also comparison of isotherms from films made of the mixture DPPC/POPC/POPG/Chol (50:25:15:10, w/w/w/w), which possess roughly similar saturated/unsaturated and zwitterionic/anionic phospholipid compositional balances than the whole surfactant, and cholesterol levels in the range of the physiological values. Films made from the two types of mixtures showed also a good correlation between the increase in surface pressure and the progressive increase of LAURDAN GPF once subjected to compression, and reached maximal GPF values comparable to those calculated from the spectra of LAURDAN in compressed surfactant films.

Fig. 4 plots the surface pressure dependence of the calculated GPF values of LAURDAN fluorescence for the different films studied. These curves provide a calibration standard to correlate the GPF values provided by LAURDAN with any given packing state in the particular lipid-based layers analyzed herein. In general terms, films containing

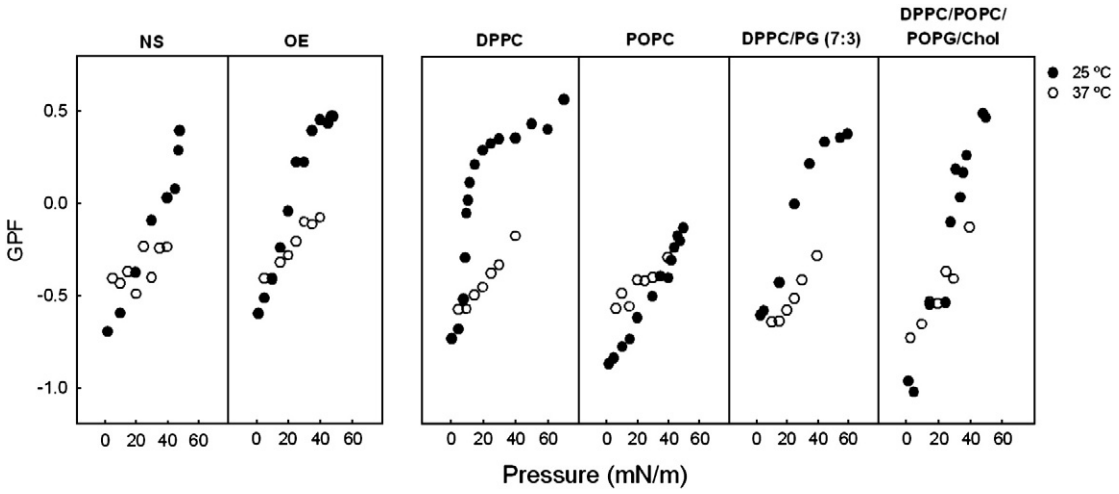

Fig. 4. LAURDAN monitoring of lateral packing in interfacial films. Plots of LAURDAN fluorescence, evaluated by the Generalized Polarization Function, versus surface pressure, as measured with a surface balance, in progressively compressed films of the indicated composition containing 1% (mol/mol) LAURDAN, formed on top of a subphase Tris 5 mM pH 7 in the presence of NaCl 150 mM. Data have been obtained by preparing and compressing the films at 25 °C (closed circles) or 37 °C (open circles).

saturated phospholipid species, including those formed by the whole native surfactant or its organic extract, give rise upon compression to higher values of LAURDAN GPF than those obtained from films made exclusively from unsaturated phospholipids. Compression of surfactant films to pressures at the plateau produces GPF values in the order of 0.4–0.5, quantitatively comparable to those reached by DPPC-containing mixtures. This fact indicates that, in all these films, maximal dehydration of LAURDAN probe is probably achieved in the highly packed state of DPPC-enriched environments. Interestingly, compression of pure DPPC films also leads to a plateau at similar values of GPF, which can exceed 0.5 upon compression beyond 60 mN/m, presumably once DPPC films transit from a tilted condensed (TC) into a solid-like two-dimensional phase. The films were also analyzed at 37 ± 2 °C, and the data have also been included in Fig. 4. In general, the maximal pressure reached by the films at 37 °C was substantially lower than the pressure reached by the films at 25 °C before collapse. This effect is probably due to the fact that kinetically defined collapse is faster at 37 °C than the fastest compression speed achievable in our Langmuir balance [28,31].

Fluorescence measurements along the compression isotherms in Langmuir balances thermostated at 37 °C are a technical challenge and produce worse LAURDAN spectra in terms of signal-to-noise ratio than those obtained at 25 °C. Fluorescence spectra from the most complex mixtures such as native surfactant or its organic extract are the noisiest ones but still display a good correlation between GPF and the surface pressure. At 37 °C, the LAURDAN GPF values associated with the compressed states of all the DPPC-containing films were markedly lower than those obtained at 25 °C, and not so much different than the values obtained from the POPC film, indicating that LAURDAN could be in a relatively hydrated environment at 37 °C, close to that offered by a liquid-expanded phase where the GPF would practically depend only on the lateral pressure. It is of interest that only the films containing cholesterol can produce at 37 °C slightly higher GPF values, perhaps indicating some sensitivity of the LAURDAN fluorescence to the presence of cholesterol-containing liquid-ordered type interfacial phases where the probe would be slightly more dehydrated than in liquid-expanded regions.

3.3. LAURDAN fluorescence in surfactant bilayers

Fig. 5 summarizes the fluorescence spectra of LAURDAN incorporated into natural surfactant, aqueous suspensions of surfactant organic extract or in multilamellar suspensions of DPPC, POPC, DPPC/PG (7/3, w/w) or DPPC/POPC/POPG/Chol (50:25:15:10 w/w/w) mixtures at different temperatures. As it occurs in interfacial monolayers and has been reported previously, the LAURDAN fluorescence is highly sensitive to the hydration state of the phospholipid head group region of bilayers, which also depends on the phase, lateral organization and lipid packing, and is strongly influenced by temperature [16,18,25]. Fig. 6 depicts the GPF thermotropic profiles of the different systems tested, calculated from the spectra in Fig. 5. Natural surfactant membranes, as well as suspensions prepared from the surfactant organic extract, show a broad thermotropic transition of LAURDAN GPF, corresponding probably to progressive melting from an ordered into a disordered phase as it has been well characterized previously by DSC or IR spectroscopy [13,32,33]. Such a transition occurs with an apparent inflection point at around 32 °C. DPPC bilayers show a much more abrupt transition, at ca. 40 °C. Bilayers reconstituted from DPPC/PG or DPPC/POPC/POPG/Chol mixtures also display broadened GPF profiles, centered at around 30–32 °C. In all these membrane systems, the maximal GPF of LAURDAN reaches similar values around 0.5. In contrast, in membranes made of pure POPC, LAURDAN GPF experiences a much more limited variation with temperature, reaching a maximum value slightly above 0.0 at the lowest temperature tested.

Table 1 summarizes and compares the maximal values obtained at 25 and 37 °C for LAURDAN GPF in monolayers and bilayers of the different systems investigated. Table 1 also provides the surface pressure and area/molecule values at which the interfacial films exhibit those maximal GPF values. In general, the maximum GPF values we measured in monolayers were always lower than the GPF values obtained from bilayers of comparable composition at equivalent environmental conditions. In both monolayers and bilayers of any of the tested compositions, LAURDAN shows lower GPF at 37 than at 25 °C, consistent with a lower packing and higher hydration of the headgroup region of the lipid layers at the higher temperatures. The differences between bilayers and monolayers were substantially larger at 37º than at 25 °C. If positive and negative values of the GPF are associated with dominant blue (dehydrated, condensed packing) or green (hydrated, expanded packing) components, respectively, values of Table 1 indicate that pure DPPC layers have a condensed character in both bilayers and monolayers, either at 25 or 37 °C, while POPC bilayer and monolayer structures would be expanded, fully hydrated, at the two temperatures tested. The mixture DPPC/PG seems to show in average a condensed character at 25 °C, in both monolayers and bilayers, while it is fairly expanded and hydrated in all the structures at 37 °C. Interestingly enough, all the mixtures containing cholesterol, including natural surfactant, its organic extract

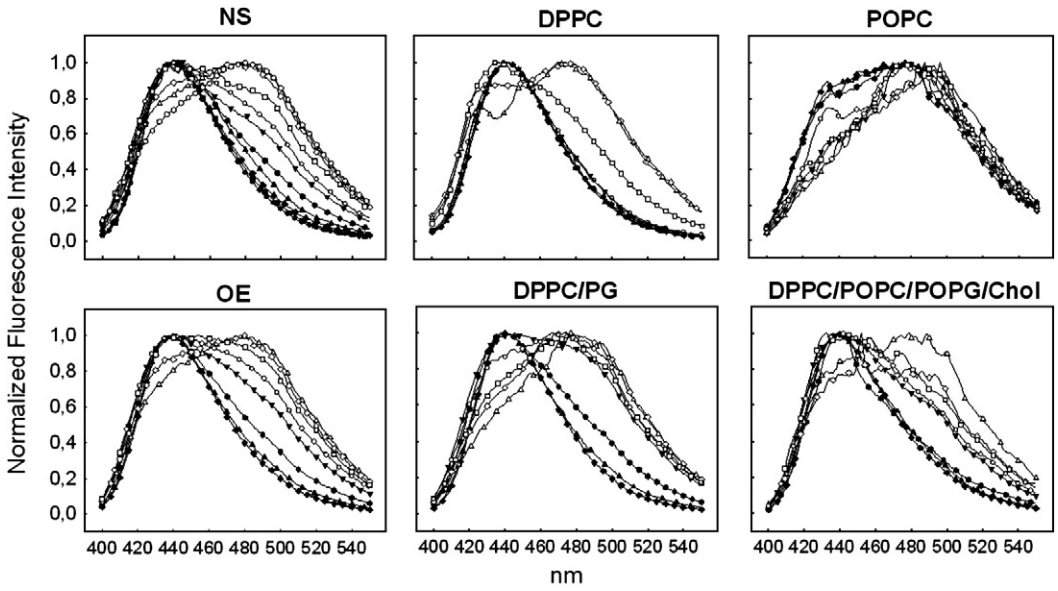

Fig. 5. LAURDAN fluorescence in bilayers. Fluorescence spectra of LAURDAN incorporated into native pulmonary surfactant membranes, into suspensions of its reconstituted organic extract, or in bilayers made of DPPC, POPC, DPPC/PG (7:3, w/w) or DPPC/POPC/POPG/Chol (50:25:15:10, w/w/w/w), recorded at 10 °C (—◆—), 15 °C (—■—), 20 °C (—♦—), 25 °C (—▲—), 30 °C (—●—), 35 °C (—▼—), 37 °C (—○—), 40 °C (—□—), 45 °C (—◇—), 50 °C (—△—) and 55 °C (—○—).

or the quaternary surfactant model system, exhibit a condensed-like dehydrated character at 25 °C in both bilayers and films. However, they perform differently at 37 °C, behaving at this temperature as rather condensed in bilayers but relatively hydrated, presumably expanded, in interfacial films.

4. Discussion

The composition of the lipid fraction of surfactant plays a major role in determining the lateral structure of surfactant membranes and films, as well as the susceptibility of the interfacial films to sustain a high packing during the reduction of the respiratory surface that occurs at expiration. A highly packed state is strictly required to prevent exposure of water molecules to air so as to reduce the surface tension to the values required to stabilize the alveolar spaces [34].

In the present work we have studied and compared the packing properties and the extent of hydration of pulmonary surfactant lipids in both mono- and bilayers, taking advantage of the unique spectroscopic properties of LAURDAN. In interfacial films, the probe emission shows a blue shift when the monolayers are compressed to high pressures and LAURDAN displays a low hydration level. The LAURDAN GPF values obtained from surfactant films compressed at 25 °C approach those from pure DPPC monolayers taken to high compression rates, suggesting that the environment sensed by the fluorescent probe in compressed surfactant films is similar to that sensed in DPPC films in terms of packing and dehydration. This feature would be consistent with a classical hypothesis in the pulmonary

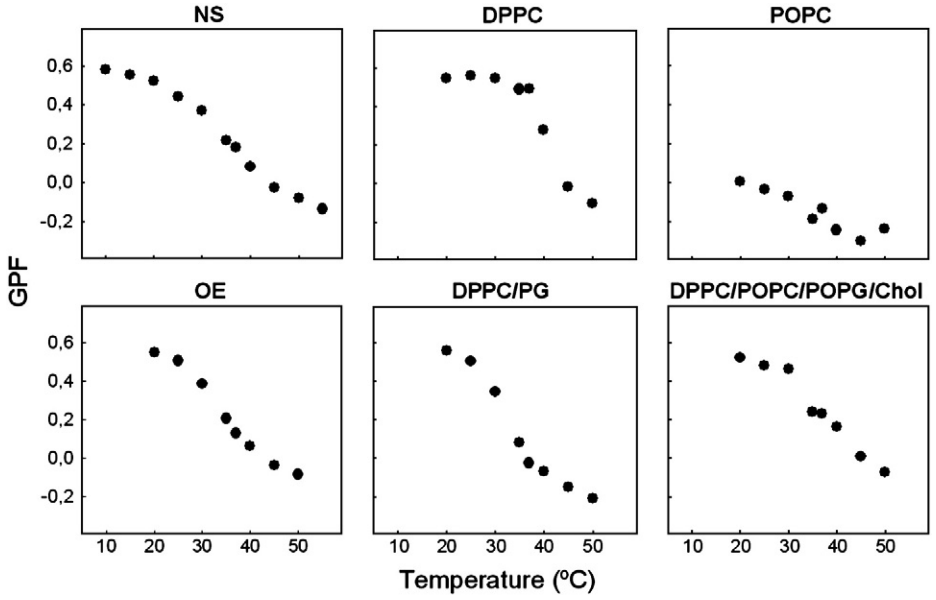

Fig. 6. Thermotropic behaviour of LAURDAN fluorescence in bilayers. Temperature dependence of LAURDAN fluorescence in native pulmonary surfactant membranes or in membranes reconstituted from surfactant organic extract, DPPC, POPC, DPPC/PG (7:3, w/w) or DPPC/POPC/POPG/Chol (50:25:15:10, w/w/w/w), as assessed by the Generalized Polarization Function.

Table 1
Comparison of LAURDAN fluorescence in surfactant-related films taken to compression-driven maximal packing and in free-standing bilayers.

Material	T °C	Monolayers			Bilayers
		GPF max	π (mN/m)*	A/mol (Å²)*	GPF
NS	25	0.393	48	32.5	0.442
	37	−0.244	40.2	10.5	0.181
OE	25	0.435	45	47.3	0.507
	37	−0.079	39.6	49.5	0.130
DPPC	25	0.431	70.0	38.7	0.560
	37	0.065	44.4	51.7	0.493
POPC	25	−0.202	47.3	70.1	−0.034
	37	−0.295	40.0	69.3	−0.134
DPPC/PG	25	0.342	50.0	23.7	0.505
	37	−0.286	40.0	33.1	−0.025
DPPC/POPC/POPG/Chol	25	0.224	45.0	49.6	0.482
	37	−0.132	40.0	54.9	0.231

* Surface pressure and area/molecule at which maximal GPF values are reached upon compression.

surfactant field, which proposes that compression of surfactant films to high enough pressures probably ends in formation of DPPC-enriched highly packed structures responsible for the maximal stability of the lungs under physiological conditions [6,34]. However, our experiments suggest that at 37 °C surfactant films are less condensed and dehydrated than pure DPPC monolayers, indicating that achieving very high surface pressures must rely in something else than condensing the films to solid-like states. In interfacial films formed by adsorption of aqueous suspensions of native surfactant, presumably composed of a monolayer and associated bilayers, the highest GPF values are reached only at the end of the large characteristic plateau of its pressure–area isotherm, occurring at around 45 mN/m. Such plateau has been attributed to the formation of folds and three-dimensional structures associated with the interface, promoted by the hydrophobic surfactant proteins SP-B and SP-C and dependant on the presence of unsaturated lipid species and/or cholesterol [2,28,31,35]. The end of the plateau seems to correlate with the maximum dehydration, as sensed by LAURDAN, and precedes the final increase in surface pressure taking surfactant films to the minimal surface tension. Interestingly enough, films containing cholesterol yield upon compression the highest values of GPF at 37 °C. This suggests that certain proportions of cholesterol may help to achieve highly packed dehydrated states in complex films such as those formed by the whole surfactant, particularly under physiological temperature conditions. Recent studies have shown in fact that addition of cholesterol to clinical surfactants—typically depleted of cholesterol—enhance significantly the stability of their interfacial films subjected to compression, under physiological conditions of temperature and humidity [36]. The presence of ca. 5–10% cholesterol by weight in the composition of the natural surfactant may then have an important structural contribution, beyond modulating the lateral organization of lipids and proteins in surfactant membranes [13,14], and suggests a possible strategy to optimize better therapeutic surfactant preparations. The proportion of cholesterol should not probably rise above a certain level, considering that an exacerbated content of neutral lipids is deleterious for the surfactant function [37,38].

Our study illustrates that the LAURDAN fluorescence can be used as a tool to evaluate the formation of interfacial surfactant films using spectroscopic measurements. The classic functional assessment of the surfactant function in surface balances hinders a massive analysis of samples and the search for new drugs and additives bound to produce better therapeutic surfactant preparations. Other spectroscopic methods have been designed to measure the surfactant surface activity [39], but none of them measure properties directly correlated with surface pressure in quantitative terms. We propose that surfactant film formation can be easily followed and quantitated in multiple samples by monitoring how fast high enough LAURDAN GPF is reached at the interface.

Our study also sought the comparison of lipid packing and hydration in bilayer and monolayer surfactant models. We expected that comparison of the LAURDAN fluorescence from bilayers and monolayers of similar composition, under similar conditions, could allow a direct quantitative evaluation of the phospholipid packing in surfactant bilayers taking as a reference the fluorescent properties of monolayers with a defined (compression-controlled) packing state. A similar approach has been recently performed by the group of Bagatolli [19]. Their study compared the spectroscopic properties of interfacial monolayers and supported planar bilayers with similar geometry under exactly the same experimental setup, finding that a correspondence between GPF values from bilayers and monolayers of DOPC or DPPC occurred when the corresponding interfacial films were compressed to 26 ± 2 and 28 ± 3 mN/m, respectively. We also expected that the good correlation observed in our experiments between surface pressure and LAURDAN GPF values in different interfacial films could permit in principle their use as a potential calibration to infer packing parameters in membranes, once the LAURDAN spectra is obtained from bilayers of equivalent composition. However, to our surprise, the maximum values of LAURDAN GPF we obtained in compressed monolayers were always lower than those calculated from free-standing bilayers at the same temperature, the difference being more substantial at higher temperature values. We are aware that our experiments compare flat monolayers, oriented perpendicularly to the incoming excitation light, with randomly orientated bilayers in free-standing liposomes. Although the lack of preferential polarization in the excitation beam we have used in our experiments should in principle prevent appearance of important differences due to orientational photoselection effects, we cannot rule out slightly reduced excitation of the probe molecules that are rigidly oriented in planes parallel to the incident light. That could be the case for LAURDAN in solid-like monolayer phases, where the probe should emit in the blue region of the spectrum producing relatively underestimated GPF values. However, we would expect that such photoselection-promoted underestimation of GPF would be more important at lower temperatures, when orientational differences of the probe in monolayers would be maximal but the observed effect is just the opposite. Differences in GPF when comparing equivalent bilayers and monolayers were higher at 37 than at 25 °C. Therefore, we can rationalize that the large differences in GPF are at least in part also due to real differences in the average hydration of the environments sensed by the probe.

In the experiments carried out by Brewer et al., the fluorescence properties of LAURDAN were determined from specific regions of the interfacial films, so that a correlation could be directly established between the probe hydration in bilayers and that in certain regions of the monolayers compressed to around 28–30 mN/m. In contrast, our GPF spectroscopic data evaluate the "average" packing/hydration state of the films, in a way that in our opinion could be comparable to the way GPF is determined from the bulk spectroscopic examination of membrane suspensions. The conclusion must be that packing/hydration of interfacial films as sensed by LAURDAN is likely heterogeneous, with some regions exhibiting packings that are similar to the average packing of bilayers of analogous composition, but that contribute only partially to an average state that in general terms seems to be more hydrated than the average state of the corresponding bilayers. It is possible that some regions of the interfacial films could include packing defects, or would be associated with excluded, partially collapsed, structures in which the probe would be exposed to a more hydrated/less packed environment. It could be also possible that structural fluctuations in interfacially confined films could be associated with higher exposure of phospholipid molecules to water than that occurring in free-standing bilayers,

where fluctuations would be less restricted and could permit more efficient shielding of the lipid headgroups from water. This would explain why differences between hydration in bilayers and monolayers are larger for higher temperature values. A problem of the fluorescent measurements as obtained in bulk, either from liposome suspensions or from the monolayers, is that potential inhomogeneities in the distribution of the probe cannot be discarded. LAURDAN has been described as a probe with similar partition into ordered and disordered phases, but differences in incorporation and distribution of the probe in interfacial films spread at low initial surface pressures and in self-organized free-standing bilayers, cannot be easily discarded. This could enhance the differences observed when comparing bilayers and monolayers.

The effect of hydration or packing defects on the stability of compressed states in interfacial phospholipid films has a particular physiological relevance in the case of the pulmonary surfactant. It is widely assumed that humidity in the distal airspaces is on the order of 100%, and this could be a source of instability for the compressed states of surfactant films, achieved at the end of expiration, when surface tension has to be reduced below 5 mN/m. As a matter of fact, it has recently been demonstrated that the ability of surfactant preparations to reach and sustain very low surface pressures is very different in dry– and humid air–water interfaces [36,40,41]. Compressed films made of clinical surfactants with different compositions are very unstable in moist air interfaces while they are competent to maintain very low surface tensions during repetitive compression-expansion cycling if exposed to dry air [36,40]. Films formed by the whole natural surfactant, however, were equally stable and competent to achieve low enough surface tensions under humid or dry air [36], a quality that might be implemented by addition of cholesterol to the clinical surfactants, the composition of which lacks the sterol. Our experiments show that the presence of cholesterol helps to achieve lower levels of hydration during compression of the films, as monitored by LAURDAN, presumably because cholesterol contributes to seal packing defects in the lipid layers, a property well documented in bilayers [42–44]. Further experiments should confirm the importance of cholesterol, a relatively underestimated component of pulmonary surfactant, to optimize phospholipid packing in surfactant layers and therefore to enhance stability and surface performance of surfactant films under the very demanding physiological conditions.

Acknowledgments

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References

[1] C.B. Daniels, S. Orgeig, Pulmonary surfactant: the key to the evolution of air breathing, News Physiol. Sci. 18 (2003) 151–157.
[2] A.G. Serrano, J. Perez-Gil, Protein–lipid interactions and surface activity in the pulmonary surfactant system, Chem. Phys. Lipids 141 (2006) 105–118.
[3] O. Blanco, J. Perez-Gil, Biochemical and pharmacological differences between preparations of exogenous natural surfactant used to treat respiratory distress syndrome: role of the different components in an efficient pulmonary surfactant, Eur. J. Pharmacol. 568 (2007) 1–15.
[4] R. Veldhuizen, K. Nag, S. Orgeig, F. Possmayer, The role of lipids in pulmonary surfactant, Biochim. Biophys. Acta 1408 (1998) 90–108.
[5] J. Perez-Gil, Structure of pulmonary surfactant membranes and films: the role of proteins and lipid–protein interactions, Biochim. Biophys. Acta 1778 (2008) 1676–1695.
[6] R. Wustneck, J. Perez-Gil, N. Wustneck, A. Cruz, V.B. Fainerman, U. Pison, Interfacial properties of pulmonary surfactant layers, Adv. Colloid Interface Sci. 117 (2005) 33–58.
[7] E.C. Smith, J.M. Crane, T.G. Laderas, S.B. Hall, Metastability of a supercompressed fluid monolayer, Biophys. J. 85 (2003) 3048–3057.
[8] B.M. Discher, K.M. Maloney, W.R. Schief Jr., D.W. Grainger, V. Vogel, S.B. Hall, Lateral phase separation in interfacial films of pulmonary surfactant, Biophys. J. 71 (1996) 2583–2590.
[9] K. Nag, J. Perez-Gil, M.L. Ruano, L.A. Worthman, J. Stewart, C. Casals, K.M. Keough, Phase transitions in films of lung surfactant at the air–water interface, Biophys. J. 74 (1998) 2983–2995.
[10] W. Yan, S.C. Biswas, T.G. Laderas, S.B. Hall, The melting of pulmonary surfactant monolayers, J. Appl. Physiol. 102 (2007) 1739–1745.
[11] A. Cruz, D. Schurch, V. Picardi, L. Vazquez, J. Pérez-Gil, Compression-driven segregation of micro- and nano-domains in pulmonary surfactant layers, Biophys J Suppl. S (2007) 423A.
[12] Y.Y. Zuo, E. Keating, L. Zhao, S.M. Tadayyon, R.A. Veldhuizen, N.O. Petersen, F. Possmayer, Atomic force microscopy study of functional and dysfunctional pulmonary surfactant films. I. Micro- and nano-structures of functional pulmonary surfactant films and the effect of SP-A, Biophys. J. 95 (2008) 2779–2791.
[13] J. Bernardino de la Serna, J. Perez-Gil, A.C. Simonsen, L.A. Bagatolli, Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures, J. Biol. Chem. 279 (2004) 40715–40722.
[14] J.B. de la Serna, G. Oradd, L.A. Bagatolli, A.C. Simonsen, D. Marsh, G. Lindblom, J. Perez-Gil, Segregated phases in pulmonary surfactant membranes do not show coexistence of lipid populations with differentiated dynamic properties, Biophys. J. 97 (2009) 1381–1389.
[15] H. Brockman, Lipid monolayers: why use half a membrane to characterize protein-membrane interactions? Curr. Opin. Struct. Biol. 9 (1999) 438–443.
[16] L.A. Bagatolli, Direct observation of lipid domains in free standing bilayers: from simple to complex lipid mixtures, Chem. Phys. Lipids 122 (2003) 137–145.
[17] L.A. Bagatolli, T. Parasassi, G.D. Fidelio, E. Gratton, A model for the interaction of 6-lauroyl-2-(N, N-dimethylamino)naphthalene with lipid environments: implications for spectral properties, Photochem. Photobiol. 70 (1999) 557–564.
[18] C.C. De Vequi-Suplicy, C.R. Benatti, M.T. Lamy, Laurdan in fluid bilayers: position and structural sensitivity, J. Fluoresc. 16 (2006) 431–439.
[19] J. Brewer, J.B. de la Serna, K. Wagner, L.A. Bagatolli, Multiphoton excitation fluorescence microscopy in planar membrane systems, Biochim. Biophys. Acta 1798 (2010) 1791–1798.
[20] H.W. Taeusch, J.B. de la Serna, J. Perez-Gil, C. Alonso, J.A. Zasadzinski, Inactivation of pulmonary surfactant due to serum-inhibited adsorption and reversal by hydrophilic polymers: experimental, Biophys. J. 89 (2005) 1769–1779.
[21] G. Rouser, S. Fkeischer, A. Yamamoto, Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[22] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[23] A. Cruz, J. Perez-Gil, Langmuir films to determine lateral surface pressure on lipid segregation, Meth. Mol. Biol. 400 (2007) 439–457.
[24] T. Parasassi, G. De Stasio, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186.
[25] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 60 (1991) 179–189.
[26] L.A. Bagatolli, E. Gratton, Direct observation of lipid domains in free-standing bilayers using two-photon excitation fluorescence microscopy, J. Fluoresc. 11 (2001) 141–160.
[27] A. Cruz, L. Vazquez, M. Velez, J. Perez-Gil, Influence of a fluorescent probe on the nanostructure of phospholipid membranes: dipalmitoylphosphatidylcholine interfacial monolayers, Langmuir 21 (2005) 5349–5355.
[28] W.R. Schief, M. Antia, B.M. Discher, S.B. Hall, V. Vogel, Liquid-crystalline collapse of pulmonary surfactant monolayers, Biophys. J. 84 (2003) 3792–3806.
[29] E.P. Ingenito, R. Mora, L. Mark, Pivotal role of anionic phospholipids in determining dynamic behavior of lung surfactant, Am. J. Respir. Crit. Care Med. 161 (2000) 831–838.
[30] J. Perez-Gil, C. Casals, D. Marsh, Interactions of hydrophobic lung surfactant proteins SP-B and SP-C with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol bilayers studied by electron spin resonance spectroscopy, Biochemistry 34 (1995) 3964–3971.
[31] W. Yan, B. Piknova, S.B. Hall, The collapse of monolayers containing pulmonary surfactant phospholipids is kinetically determined, Biophys. J. 89 (2005) 306–314.
[32] R.A. Dluhy, K.E. Reilly, R.D. Hunt, M.L. Mitchell, A.J. Mautone, R. Mendelsohn, Infrared spectroscopic investigations of pulmonary surfactant. Surface film transitions at the air–water interface and bulk phase thermotropism, Biophys. J. 56 (1989) 1173–1181.
[33] H. Trauble, H. Eibl, H. Sawada, Respiration–a critical phenomenon? Lipid phase transitions in the lung alveolar surfactant,, Naturwissenschaften 61 (1974) 344–354.
[34] J. Goerke, Pulmonary surfactant: functions and molecular composition, Biochim. Biophys. Acta 1408 (1998) 79–89.
[35] F. Lhert, W. Yan, S.C. Biswas, S.B. Hall, Effects of hydrophobic surfactant proteins on collapse of pulmonary surfactant monolayers, Biophys. J. 93 (2007) 4237–4243.
[36] Y.Y. Zuo, E. Acosta, Z. Policova, P.N. Cox, M.L. Hair, A.W. Neumann, Effect of humidity on the stability of lung surfactant films adsorbed at air–water interfaces, Biochim. Biophys. Acta 1758 (2006) 1609–1620.
[37] L. Gunasekara, S. Schurch, W.M. Schoel, K. Nag, Z. Leonenko, M. Haufs, M. Amrein, Pulmonary surfactant function is abolished by an elevated proportion of cholesterol, Biochim. Biophys. Acta 1737 (2005) 27–35.

[38] Z. Leonenko, S. Gill, S. Baoukina, L. Monticelli, J. Doehner, L. Gunasekara, F. Felderer, M. Rodenstein, L.M. Eng, M. Amrein, An elevated level of cholesterol impairs self-assembly of pulmonary surfactant into a functional film, Biophys. J. 93 (2007) 674–683.
[39] A. Ravasio, A. Cruz, J. Perez-Gil, T. Haller, High-throughput evaluation of pulmonary surfactant adsorption and surface film formation, J. Lipid Res. 49 (2008) 2479–2488.
[40] E.J. Acosta, R. Gitiafroz, Y.Y. Zuo, Z. Policova, P.N. Cox, M.L. Hair, A.W. Neumann, Effect of humidity on lung surfactant films subjected to dynamic compression/expansion cycles, Respir. Physiol. Neurobiol. 155 (2007) 255–267.
[41] Y.Y. Zuo, R. Gitiafroz, E. Acosta, Z. Policova, P.N. Cox, M.L. Hair, A.W. Neumann, Effect of humidity on the adsorption kinetics of lung surfactant at air–water interfaces, Langmuir 21 (2005) 10593–10601.
[42] D. Arrais, J. Martins, Bilayer polarity and its thermal dependency in the l(o) and l (d) phases of binary phosphatidylcholine/cholesterol mixtures, Biochim. Biophys. Acta 1768 (2007) 2914–2922.
[43] L. Norlen, Skin barrier structure and function: the single gel phase model, J. Invest. Dermatol. 117 (2001) 830–836.
[44] J.J. Theunissen, R.L. Jackson, H.J. Kempen, R.A. Demel, Membrane properties of oxysterols. Interfacial orientation, influence on membrane permeability and redistribution between membranes, Biochim. Biophys. Acta 860 (1986) 66–74.