Segregation of Saturated Chain Lipids in Pulmonary Surfactant Films and Bilayers

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ABSTRACT The physical properties of organized system (bilayers and monolayers at the air water interface) composed of bovine lipid extract surfactant (BLES) were studied using correlated experimental techniques. 6-Dodecanoyl-2-dimethylamino-naphthalene (LAURDAN)-labeled giant unilamelar vesicles (mean diameter ~30 μm) composed of BLES were observed at different temperatures using two-photon fluorescence microscopy. As the temperature was decreased, dark domains (gel-like) appeared at physiological temperature (37°C) on the surface of BLES giant unilamelar vesicles. The LAURDAN two-photon fluorescent images show that the gel-like domains span the lipid bilayer. Quantitative analysis of the LAURDAN generalized polarization function suggests the presence of a gel/fluid phase coexistence between 37°C to 20°C with low compositional and energetic differences between the coexisting phases. Interestingly, the microscopic scenario of the phase coexistence observed below 20°C shows different domain's shape compared with that observed between 37°C to 20°C, suggesting the coexistence of two ordered but differently organized lipid phases on the bilayer. Epifluorescence microscopy studies of BLES monomolecular films doped with small amounts of fluorescent lipids showed the appearance and growth of dark domains (liquid condensed) dispersed in a fluorescent phase (liquid expanded) with shapes and sizes similar to those observed in BLES giant unilamelar vesicles. Our study suggests that bovine surfactant lipids can organize into discrete phases in monolayers or bilayers with equivalent temperature dependencies and may occur at physiological temperatures and surface pressures equivalent to those at the lung interface.

INTRODUCTION

Pulmonary surfactant is a lipid-protein mixture secreted by the type-II cells of the terminal air-space and provides lung stability during normal respiration. Surfactant form multilayers or films at the lung air-aquas interface, and these layers reduce the surface tension of the interface to near 1 mN/m values (Goerke, 1998; Schürch et al., 1998). These layers have been demonstrated to be multiples of bilayers at the lung interface, and the exact organization of the lipids and proteins in such layers is unclear. In vitro studies of adsorption of bovine surfactant on to the surface of a captive air-bubble (simulating the alveoli) suggest the existence of bilayers of surfactant at the air-water interface (Amrein et al., 2000; Schürch et al., 1998). A recent study shows that a particular organization of lipids in surfactant may facilitate the specific interactions surfactant proteins with gel-like regions of surfactant films (Worthman et al., 2000). Such specific interactions may lead to formation of highly surface-active lipid-protein complexes as well as sorting of lipids at the lung air-water interface (Greise and Beck, 1999). Recent studies have demonstrated that in solvent-spread films of calf and porcine surfactant condensed domains appear as the packing density of the films is increased (Discher et al., 1996; Grunder et al., 1999; Nag et al., 1998; Piknova et al., 2001). A number of studies also suggest that surfactant films are enriched in dipalmitoylphosphatidylcholine (DPPC), and because of the high melting phase transition (41°C), it is the only surfactant component, films of which can stand very high packing density at 37°C (Goerke, 1998; Hawco et al., 1981; Keough et al., 1985; Nag et al., 1996, 2000). Surfactant also exhibit broad, first order, fluid to gel transitions upon cooling below 41°C (Dluhy et al., 1989, Keough et al., 1985), however the organization of surfactant components in such systems are not known.

Among other lipids, surfactant contains significant amounts dipalmitoylphosphatidylcholine (DPPC), fluid-PC, and phosphatidylglycerol, and thus is ubiquitous in its composition compared with most eukaryotic membranes (for recent reviews, see Batenburg and Haagsman, 1998; Nag et al., 2000; Veldhuizen et al., 1998). Surfactant also contains small amounts of specific lipid associated proteins called SP-A, SP-B, SP-C, and SP-D (~10% by weight of lipids) (Johansson and Curstedt, 1997). Bovine lipid extract surfactant (BLES) is a clinical surfactant preparation and contains all lipid components of surfactant, except the neutral lipids (i.e., cholesterol, triglycerides), however the hydrophobic SP-B and SP-C at ~2% weight of the lipids are present (Nag et al., 2000; Yu et al., 1983). The molecular composition of BLES is close to surfactant from humans as recently determined by mass spectrometry (Postle et al., 1999; Nag et al., 2000), and BLES has mainly been used for

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clinical therapy and trials in lung disease such as acute-respiratory distress syndrome (Yu et al., 1983; Veldhuizen et al., 1998).

In this work we have studied the lipid organization in BLES in mainly bilayers in comparison to its organization in monomolecular films. To understand the properties of lipid packing, we have examined the lipid phase transition in BLES in different model systems such as multilamellar vesicles using differential scanning calorimetry and monolayers and giant unilamellar vesicles (GUVs) using fluorescence microscopy. In particular, we exploited the advantage inherent in two-photon excitation microscopy, which allowed us to directly observe the temperature-induced changes in lipid phase equilibria using LAURDAN-labeled GUVs. These "cell size" vesicles are becoming convenient models for the study of membrane physical properties (Menger and Keiper, 1998) and allows for the direct visualization of lipid domain formation induced by temperature in free-standing bilayers (Bagatolli and Gratton 1999, 2000a,b; Bagatolli et al., 2000a,b; Dietrich et al., 2001) and in this study of a natural hydrophobic extracts from bovine lungs. This study points to a novel microscopic view of lipid lateral organization in lung surfactant, which can possibly facilitate its biophysical function in the alveoli.

MATERIALS AND METHODS

BLES, a clinical preparation in saline (27 mg/ml), was a generous gift from BLES Biochemicals (London, Ontario, Canada). Hydrophobic extracts of BLES were prepared in organic solvents using methods of Bligh and Dyer (1959), as modified by Yu et al. (1983). Monolayer and bilayer formations were made with this BLES extract and contained all components as in its supplied emulsions in saline determined by mass spectrometry (Veldhuizen et al., 1998). The extracts in solvents were dried under a stream of nitrogen and redissolved in chloroform:methanol (3:1, vol/vol) to a desired phospholipid concentrations of 1 mg/ml. The phospholipid concentrations were measured by standard phosphorous assay method (Rouser et al., 1970).

The fluorescent probe, 1-palmitoyl-2- (nitro-benoxa-diazole) dodecanoyl phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). This probe has been shown to partition in the liquid expanded (or fluid) phase of DPPC and porcine surfactant monolayer films (Nag et al., 1998; Worthman et al., 1997). The fluorescent probe, 6-dodecanoyl-2-dimethylamino-naphthalene (LAURDAN), was obtained from Molecular Probes (Eugene, OR). LAURDAN displays homogeneous distribution on phospholipid membranes displaying phase coexistence and shows a lipid phase-dependent emission spectral shift, i.e., the emission of LAURDAN is blue in the gel lipid phase and green in the fluid lipid phase (see below; Bagatolli and Gratton, 1999, 2000a,b). All experiments were performed using doubly distilled deionized water, with resistivity above 18 MΩ.

Giant unilamellar vesicle preparation

Stock solutions of BLES were made in chloroform at a final concentration of 0.2 mg/ml for the vesicle formation. GUVs were prepared by the electroformation method (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1987; Angelova et al., 1992) in a special temperature-controlled chamber (Bagatolli and Gratton, 1999, 2000a,b). Briefly, ~3 μl of the BLES lipid stocks solution were spread on each of two platinum (Pt) wires under a stream of N_2. To remove the residual of organic solvent the samples were lyophilized for ~2 h. The wires were covered with water previously heated to temperatures corresponding to the fluid phase (58°C for GUVs composed of BLES). The Pt wires were connected to a function generator (Hewlett-Packard, Santa Clara, CA) and a low frequency AC field (sinusoidal wave function with a frequency of 10 Hz and peak to peak amplitude of 2 V) was applied for 90 min. After the vesicles formed, the AC field was turned off, and the temperature scan (cooling) was initiated at a similar rate that was used in the differential scanning calorimetry (DSC) experiments. A CCD color video camera (CCD-Iris, Sony, Tokyo) attached to the inverted microscope was used to follow vesicle formation and to select the target vesicle (Bagatolli and Gratton, 1999). The temperature was measured inside the sample chamber using a digital thermocouple (model 400B, Omega Inc., Stamford, CT) with a precision of ±0.1°C. The LAURDAN labeling procedure was done in one of two ways. Either the fluorescent probe was premixed with BLES preparation in chloroform or a small amount (less than 1 μl) of LAURDAN in dimethylsulphoxide was added after the vesicle formation (final LAURDAN/lipid ratio 1:500 mol/mol in both cases). The sample behavior during the cooling cycle was independent of the labeling procedure. The mean diameter of the GUVs was ~30 μm. To check the lamellarity of the giant vesicles we imaged several vesicles (up to 20 vesicles in different regions of the Pt wires) labeled with LAURDAN at the equatorial region of the GUV using the two-photon excitation microscope. We found similar fluorescence intensities among the different vesicles. Because the existence of multilamellar vesicles would give rise to different intensity images due to the presence of different numbers of LAURDAN labeled lipid bilayers, we concluded that the vesicles were unilamellar in agreement with previous observations done on GUVs using the electroformation method (Mathivet et al., 1996; Bagatolli and Gratton, 1999, 2000a,b; Bagatolli et al., 2000a,b).

Experimental apparatus for two-photon excitation microscopy measurements

Two-photon excitation is a nonlinear process in which a fluorophore absorbs two photons simultaneously. Each photon provides half the energy required for excitation. The high photon densities required for two-photon absorption are achieved by focusing a high peak power laser light source on a diffraction-limited spot through a high numerical aperture objective. Therefore, in the areas above and below the focal plane, two-photon absorption does not occur because of insufficient photon flux. This phenomenon allows for a sectioning effect without using emission pinholes as in confocal microscopy. Another advantage of two-photon excitation is the decreased extent of photobleaching and photodamage above and below the focal plane. For our experiments we used a scanning two-photon fluorescence microscope whose design and performance was discussed elsewhere (So et al., 1995, 1996). The two-photon excitation images were collected on an Axiovert 35 inverted microscope (Zeiss, Thornwood, NY) with a Zeiss 20X LD-Achroplan (0.4 N.A., air) using a titanium-sapphire laser excitation source (Coherent, Palo Alto, CA) tuned to 780 nm, pumped by a frequency-doubled Nd:Vanadate laser (Coherent, Palo Alto, CA). The laser was guided by a galvanometer-driven *x-y* scanner (Cambridge Technology, Watertown, MA) to achieve beam scanning in both *x* and *y* directions. A frequency synthesizer (Hewlett-Packard) controlled the scanning rate of 9 s to acquire a 256 × 256 pixel frame. Experiments were conducted by exciting LAURDAN with circular polarized light, a necessary condition to obtain the "generalized polarization" (GP) images in the center cross-section of the vesicle (Bagatolli and Gratton, 2000b). To change the polarization of the laser light from linear to circular, a quarter wave-plate (CVI Laser Corporation, Albuquerque, NM) was placed before the light entered the microscope. The fluorescence emitted from the sample was passed first through a broad band-pass filter from 350 to 600 nm (BG39 filter; Chroma Technology, Inc., Brattleboro, VT) to remove light scattered from the excitation light. A two-channel detection system is used to simultaneously collect the "red" and "blue" images necessary to calcu-

late the LAURDAN GP function (see below for a description of the GP calculation). A custom-built digitizer card (ISS, Urbana, IL) in a personal computer was used for acquisition in the photon counting mode. The diameters of the vesicles were measured by using size-calibrated fluorescent spheres (latex FluoSpheres, polystyrene, blue fluorescent 360/415, diameter 15.5 μm, Molecular Probes Inc., Eugene, OR). We determined that the pixel size in our experiments correspond to 0.52 μm.

LAURDAN GP measurements

The membrane probe LAURDAN was used due to its ability to detect changes in the water penetration into the bilayer surface that correlates strongly with the membrane phase state (Parasassi et al., 1990, 1991, 1998; Parasassi and Gratton, 1995). The emission spectrum of LAURDAN in a pure phospholipid bilayer is centered at 440 nm when the membrane is in the gel phase and at 490 nm when in the liquid crystalline phase. The GP or the "generalized polarization," gives a mathematically convenient and quantitative way to measure the LAURDAN emission spectral shift. The GP calculation is given as:

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

in which I_B and I_R are the blue (440 nm) and red (490 nm) emission intensity readings, respectively. This well-characterized function is sensitive to the extent of water dipolar relaxation processes in the lipid bilayer allowing the determination of the phase state of an unknown sample with the characteristic values obtained in pure gel (high GP, low extent of water dipolar relaxation) or fluid (low GP, high extent of water dipolar relaxation) phases. Full discussions of the use and mathematical significance of the GP has been published elsewhere (Parasassi et al., 1990, 1991, 1998; Parasassi and Gratton, 1995; Bagatolli and Gratton, 1999, 2000a,b). In our two-photon, dual-channel instrument, the fluorescence emission was split into "red" and "blue" channels by using a Chroma Technology 470DCXR-BS dichroic beam splitter in the emission path. Two optical bandpass filters centered at 446 $\pm$ 23 nm and at 499 $\pm$ 23 nm (Ealing electro-optics, New Englander Industrial Park, Holliston, MA) were placed in the appropriate path to further isolate the red and blue parts of the emission spectrum. Two separate photo multipliers (R5600-P, Hamamatsu, Brigdewater, NJ) were used in the photon counting mode. Corrections for the wavelength dependence of the emission was accomplished through the comparison of known solutions (LAURDAN in dimethylsulphoxide, GP = -0.46; LAURDAN in DMPC vesicles T = 20°C, GP = 0.58) measured with a Model PC1 (ISS Inc, Champaign, Urbana) steady-state fluorometer. To obtain the mean GP values at the three different temperature regimes, the LAURDAN GP histograms obtained from the images were analyzed using Gaussian functions (Bagatolli and Gratton, 2000 a,b). Although there are no standard theories available for the GP distribution function, the best fit of GP histograms obtained from GP images in the pure gel and fluid phases was Gaussian (Bagatolli and Gratton, unpublished observations). Therefore, this function is used to determine the mean GP values at the phase coexistence temperature region. The assignment of two populations to get the mean GP value in each visible phase at the phase coexisting temperature regime is based on the lateral phase separation picture. In our experiments the physical characteristics of the observed lipid domains (phase state and shape) were well reproducible from one GUV to another in the same preparation or among GUVs from different preparations.

Differential scaning calorimetry of BLES

The BLES extract in chloroform:methanol (3:1, vol/vol) was dried under a stream of nitrogen and resuspended in doubly deionized water at a desired concentration of 5 mg/ml. The resuspension was performed in a water bath at 50°C, and the suspension was vortexed at a rate of 20 cycles/s, to form bilayer suspensions of mostly multilamellate structures (as determined by transmission electron microscopy, data not shown). DSC (Microcal-II, Micorcal Inc., Northamton, MA) measurements were performed on these suspensions over a temperature range of 50°C to 10°C, using at least four cycles of heating and cooling at a rate of 10°C/h. The details of the DSC methods and emulsion preparations as applied to studies of various phospholipid preparations are discussed in details elsewhere (Keough et al., 1985; Nag et al., 1996). We also performed DSC measurements of BLES in physiological saline (27 mg/ml) as obtained from the supplier. There was no appreciable difference in the calorimetrically detected enthalpy change between the two BLES preparation (although some differences in multilamellate bilayer arrangements were noted in TEM) or between cycles of heating and cooling, suggesting that the gel-liquid crystalline phase transitions were reversible in both conditions and emulsions.

Film preparation and imaging

The BLES in chloroform:methanol (3:1) containing 1 mol % (total BLES phospholipids by weight) of NBD-PC was spread on the air-water interface of a epifluorescence microscopic surface balance (Kibron Scientific, Helsinki, Finland). Details of design and performance of a similar surface balance has been discussed elsewhere (Nag et al., 1990). All studies were performed on the surface balance at an ambient room temperature (23°C $\pm$ 1°C). The films were compressed to the desired surface pressure and epifluorescence images obtained directly from the air-water interface were video recorded at defined surface pressures by methods discussed in details elsewhere (Nag et al., 1990, 1998).

RESULTS

To explore the characteristic of the lipid lateral organization in BLES bilayers at the level of single vesicles, two-photon excitation fluorescent images of LAURDAN-labeled GUVs were taken at the polar region of the vesicle during cooling from a temperature range of 55°C to 10°C. Fig. 1 (top panel) shows that above 37°C the two-photon images display a homogeneous LAURDAN fluorescence intensity. At 37°C, small nonfluorescent areas are observed on the GUV surface showing the presence of lipid phase separation. At lower temperatures the dark domains increase in size (Fig. 1, bottom panel). As the temperature is kept constant the domains move freely and rapidly on the GUV surface (Fig. 1, center panel). LAURDAN intensity images taken at the equatorial region of the GUVs at temperatures corresponding to the phase coexistence region displayed a continuous fluorescent ring (insert as A in Fig. 1, center panel), showing that LAURDAN homogeneously partitions between the different lipid phases as also observed previous in various lipid systems (Bagatolli and Gratton, 2000a,b, 2001). This last observation removes the possibility of probe segregation from one of the coexisting phases. The lack of fluorescence from the dark lipid domains observed at the the polar region of the vesicle is due to the photoselection effect (Parasassi et al., 1997; Bagatolli and Gratton, 1999, 2000a,b). At the polar region of the GUV, the LAURDAN dipole in gel domains remains perpendicular to plane of polarization of the light, minimizing absorption and hence emission (Parasassi et al., 1997; Bagatolli and Gratton,

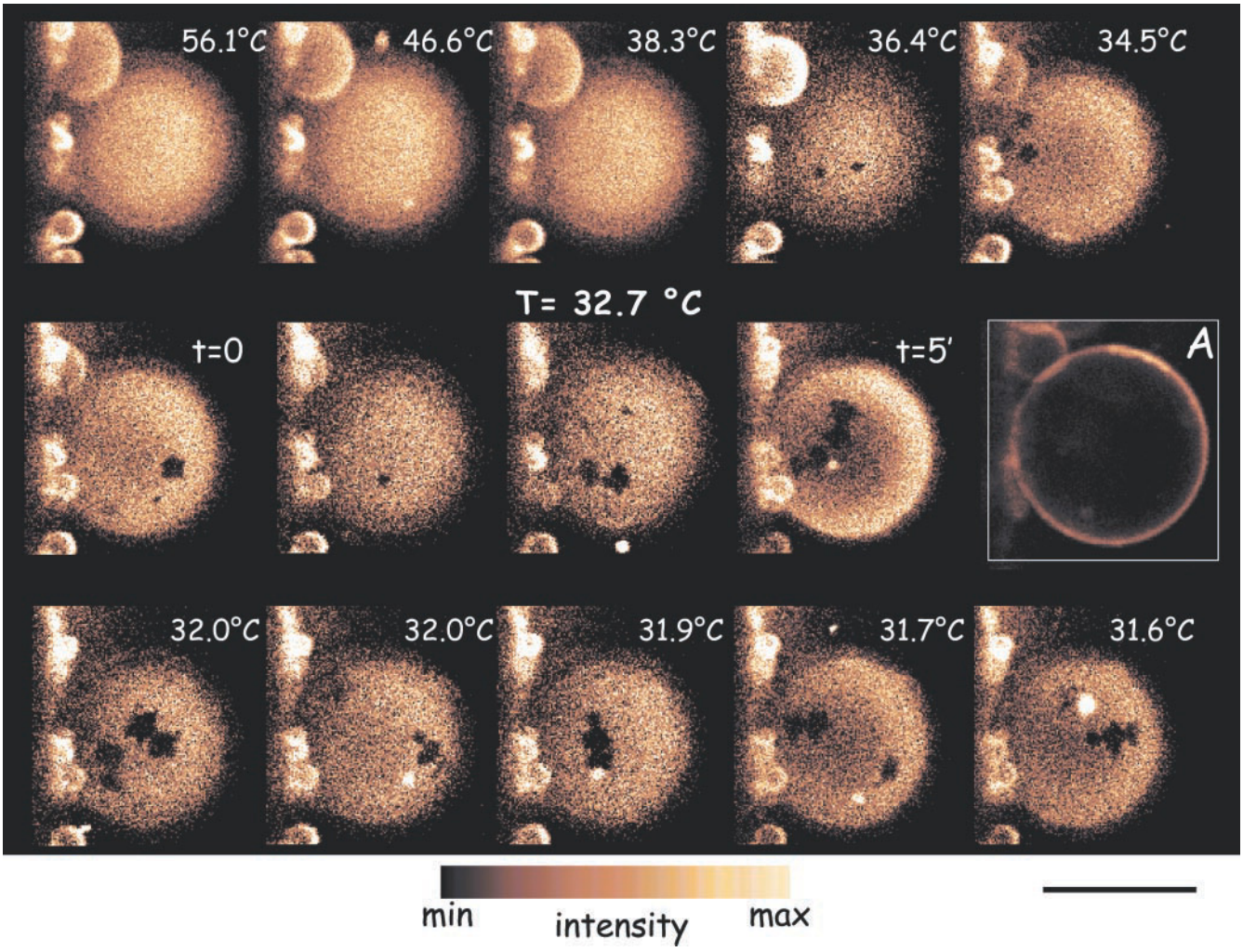

FIGURE 1 Two-photon excitation fluorescence intensity images of LAURDAN-labeled GUVs composed of BLES (false color representation) as a function of a temperature during cooling from 56°C to 31°C. The images were taken at the top part of the GUV. The center panel shows the time evolution of the gel (*black*) domains (5 min) in a single GUV at constant temperature (32.7°C). The starting point of the optically resolvable nucleation of gel domains (*dark areas*) was found around 37°C. The scale bar corresponds to 35 μm. The insert *A* in the center panel shows a fluorescent image taken at the equatorial region of the BLES GUV showing the presence of LAURDAN in the entire bilayer at the phase coexistence temperature region. This last image was obtained with a blue band pass filter and display higher fluorescent intensity on the region corresponding to the gel domain (see text).

1999, 2000a,b). These last observations agree with previous observations in others phospholipid binary mixtures at the gel/fluid phase coexistence temperature regime (Bagatolli and Gratton, 2000a,b).

At temperatures below 20°C the structure of the domain in BLES GUVs displays shape changes comparing with that observed above 20°C (Fig. 2). At this low temperature regime, from images taken at the equatorial region of the vesicle, we observed that LAURDAN is homogeneously distributed (Fig. 3 *A*, right), demonstrating that probe segregation from one of the coexisting phases does not occur. This phase coexistence appears different from that observed at the fluid/gel phase-coexistence temperature regime (between 20°C and 37°C, Fig. 2).

To explore the phase state of the lipid membrane the LAURDAN images at the center cross-section of the BLES vesicles were analyzed using the GP function as shown in Fig. 3 *A*. The GP histogram obtained at the fluid phase is broad, and the center GP value is ~ -0.3 showing a high extent of solvent dipolar relaxation process in the membrane. This last observation is in agreement with that observed for single phospholipid GUVs at the fluid phase temperature regime (Bagatolli and Gratton, 1999, 2000a). At temperatures corresponding to the phase coexistence temperature regime (37°C–20°C), two distinct regions of GP values are seen around the circumference with the more condensed domains exhibiting the largest GP values (Fig. 3 *A*, center). Correspondingly, the GP histogram must be fit with two GP components rather than one (Fig. 3 *B*). It is important to remark that the gel lipid domains span the lipid bilayer as clearly seen in Fig. 3 *A* (center image, white arrow). The same picture is corroborated in Fig. 1 (center

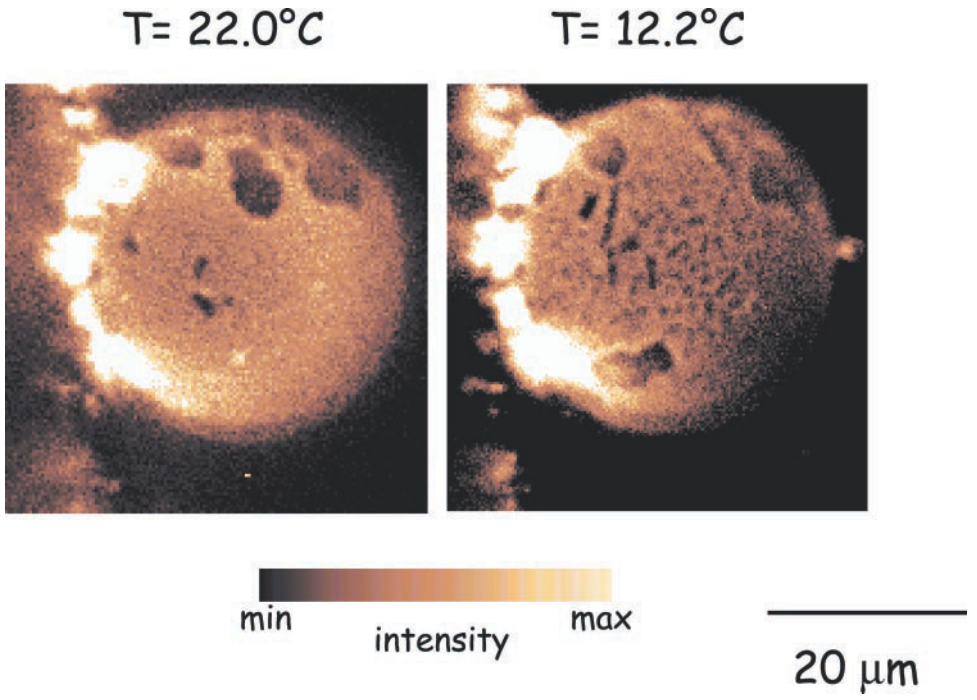

FIGURE 2 Two-photon excitation fluorescence intensity images of LAURDAN-labeled BLES GUVs at a low temperature regime. The images have been taken at the top part of the GUV. The ordered phase or black regions at 12.2°C are elongated and irregular shaped compared with those observed at 22°C, showing the coexistence of two ordered phases below 20°C (see text).

panel) in which the lipid domains at constant temperature do not change the shape and are always nonfluorescent in the images taken at the polar region of the GUV. An independent behavior between both leaflets of the bilayer is not consistent with the last mentioned picture, considering that LAURDAN is present in both sides of the bilayer (Parasassi et al., 1997). These findings are in agreement with those observed in phospholipid binary mixtures at the gel/fluid phase coexistence (Bagatolli and Gratton, 2000a,b, 2001) and in quaternary (phospholipid/cholesterol/sphyngomyelin/ganglioside) and natural (Brush Border membrane lipid extracts) mixtures displaying fluid ordered/fluid disordered phase coexistence (Dietrich et al., 2001).

At temperatures below 20°C the GP images and histograms are consistent with a very low extent of water dipolar relaxation in the whole BLES GUV (GP ~0.55 and a narrow GP histogram) in agreement with observations on single phospholipid component GUVs in the gel phase (Bagatolli and Gratton, 1999, 2000a,b). This last finding shows that the two different regions observed at the polar region of the GUV in Fig. 2 correspond to the coexistence of two different highly ordered phases. This last fact supports the idea that another phase transition may occur in the lower temperature range.

Fig. 4 shows the change in heat released upon cooling BLES emulsion as determined using DSC. The DSC data show that BLES emulsions undergo broad and complex thermotropic phase transition with a peak around 28°C and a main enthalpy change between 35°C to 10°C and is consistent with the microscopic scenario observed in GUVs.

Fig. 5 *A* shows a surface pressure-area isotherm (left) of solvent-spread films of BLES, along with images observed in such films at the surface pressures indicated by the arrows. The condensed domains in the BLES films appear at a surface pressure ~12 mN/m and then grow in size with increasing film compression. The fluorescent probe NBD-PC is dispersed homogeneously in the expanded or fluid phase and is excluded from the condensed phase. The isotherm and pattern of condensed domain growth in BLES up to 20 mN/m are similar to those previously observed in porcine and calf lung surfactant extract films (Discher et al., 1996, 1999; Nag et al., 1998). However, above 20 mN/m the disappearance of condensed domains in porcine and calf lung surfactant extract films did not occur in BLES films, possibly due to the absence of neutral lipids in this system (Yu et al., 1998) and was similar to those observed in films of calf surfactant phospholipid fraction (Piknova et al., 2001).

Similarities between the shape and size of the ordered lipid domains obtained in monolayers and bilayers are shown in Fig. 5 *B*. The shape of the lipid domains observed in both cases are not circular and match very well with those observed in binary phospholipid mixtures displaying gel/fluid phase coexistence (Bagatolli and Gratton, 2000b, 2001).

DISCUSSION

Despite numerous efforts to examine surfactant at the lung air-water interface, it is not clear to date how the surface

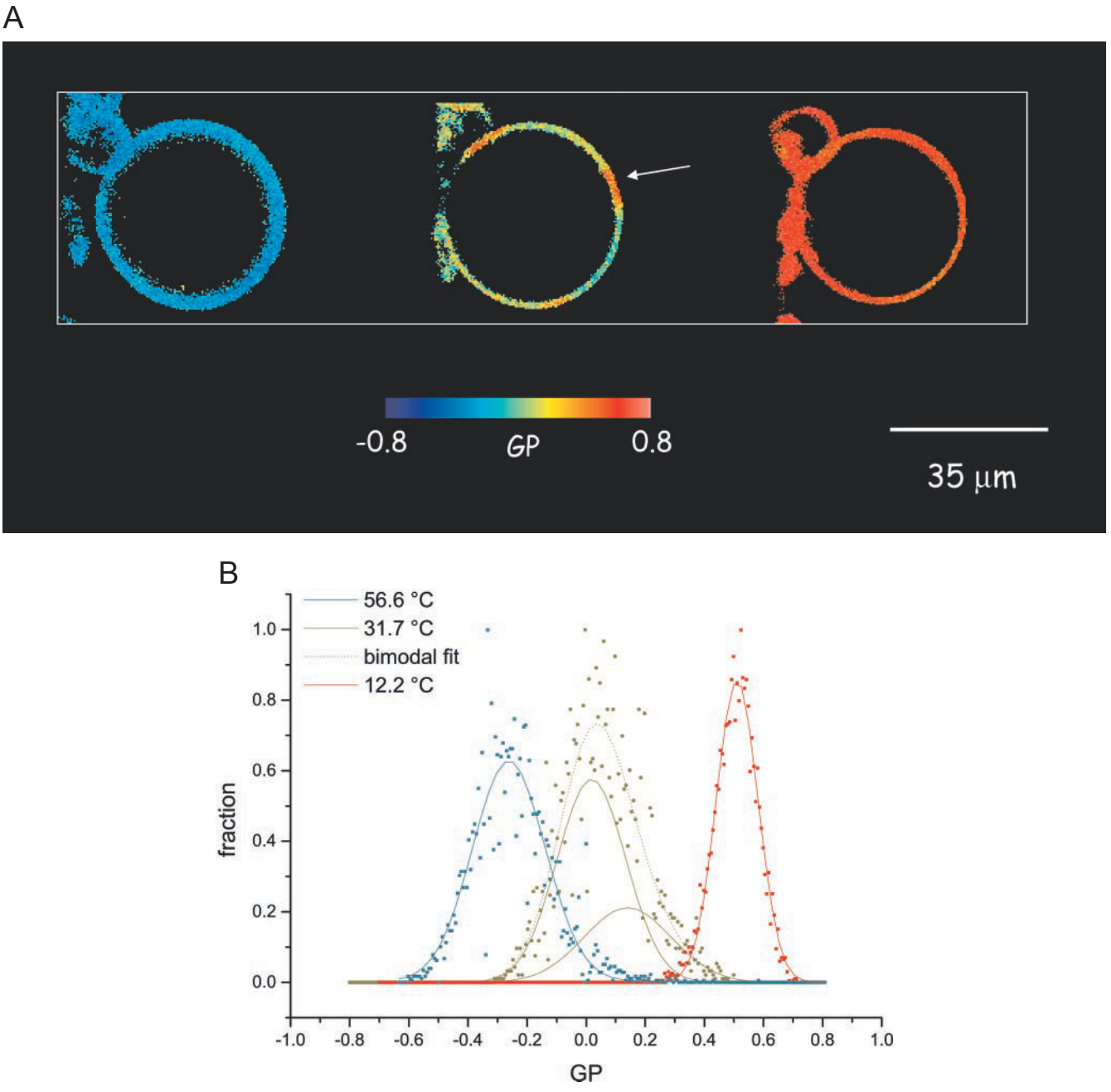

FIGURE 3 Two-photon excitation LAURDAN GP images of a single GUV composed of BLES obtained with circular polarized light at 56.1°C (*left*), 37.1°C (*center*), and 12.2°C (*right*) are shown in *A*. The white arrow in the center image indicates an ordered or gel-domain region (*orange*) that spans the lipid bilayer. Experimental and fitted GP histograms corresponding to the images in *A* are shown in *B*.

tension of this interface reaches low values close to 1 mN/m, considering surfactant contains 30% to 40% by weight fluid lipids (Daniels et al., 1990; Keough, 1985; Keough et al., 1985; Postle et al., 1999; Veldhuizen et al., 1998). Our experiments in different model system were designed to expand the knowledge about the topography in BLES-containing interfaces, in particular comparing the microscopic scenario at the level of single vesicles (bilayers) with monolayers.

Phase coexistence in BLES bilayers and monolayers

In agreement with previous observations done in phospholipid binary mixtures (Bagatolli and Gratton, 2000a,b), LAURDAN did not show a preferential partition between the fluid and gel lipid domains in BLES GUVs (Fig. 1, center panel). This last phenomenon allowed for visualiza-

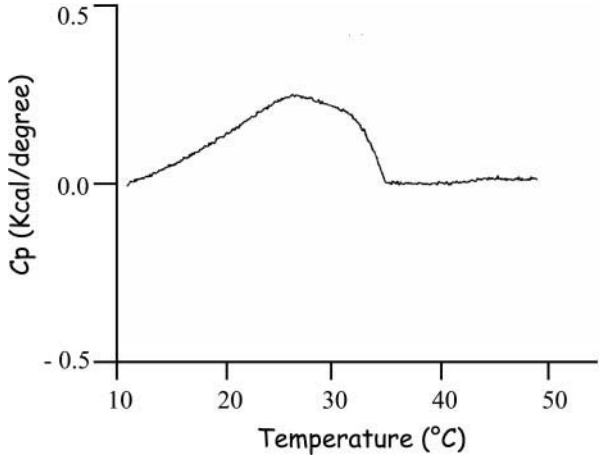

FIGURE 4 Typical DSC cooling thermogram of BLES emulsions obtained between 50°C to 10°C. Heating and cooling cycles did not show any significant differences, suggesting the liquid crystalline to gel transition was reversible.

tion of domain morphology through the photoselection effect in the polar region of the GUV and determination of the domain phase state through calculation of LAURDAN′s GP function in the equatorial region of the vesicle (Bagatolli and Gratton, 2000a,b).

It is important to remark that the LAURDAN fluorescence properties and the shape of the laterally ordered lipid domains observed in our BLES experiments are different from the picture obtained by Dietrich et al. (2001) in LAURDAN-labeled GUVs composed of samples that display fluid ordered/fluid disordered phase coexistence ("raft"-like mixtures composed of artificial and natural lipid mixtures containing mainly phospholipids, sphingomyelin, and cholesterol). In "raft" mixtures the lipid phase coexistence is characterized by the coexistence of circular domains and both coexisting phases show LAURDAN fluorescence intensity in images taken at the GUV polar region because both phases are fluid (Dietrich et al., 2001). Similar circular liquid ordered domains have also been observed in POPC/

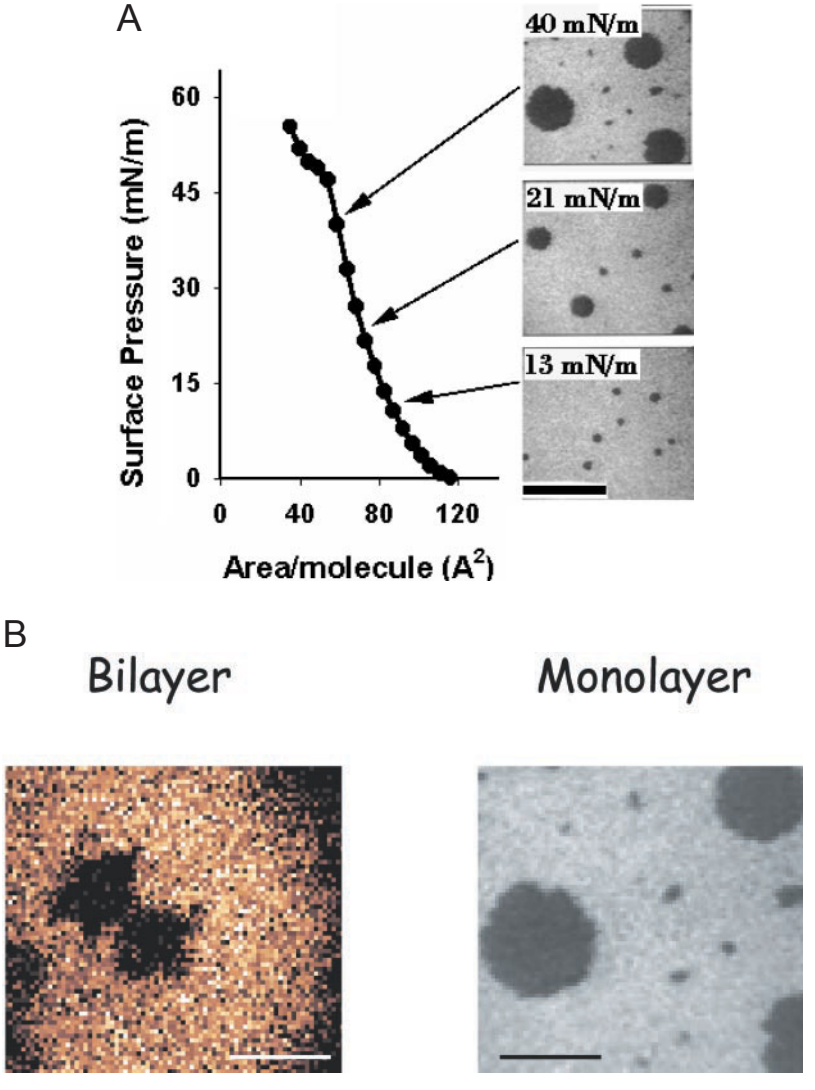

FIGURE 5 (*A*) Surface pressure-area isotherms of BLES solvent spread films and typical images observed in such films using epifluorescence microscopy. The black regions in the fluorescent images are condensed domains, which excluded the fluorescent probe NBD-PC that remains in homogeneously fluorescent phase. Scale bar is 20 μm. (*B*) Comparison between condensed and gel domains obtained in monolayers and bilayers, respectively; scale bar corresponds to 12 μm.

cholesterol monolayer films by fluorescence microscopy (Worthman et al., 1997). On the other hand, the dark domains in BLES GUVs between 37°C to 20°C have very similar physical characteristics to those observed in LAURDAN labeled-GUVs composed of binary phospholipid mixtures displaying gel/fluid phase coexistence (Bagatolli and Gratton, 2000a,b) and in LAURDAN labeled-GUVs composed of cholesterol-depleted Brush Border membranes lipid extracts (Dietrich et al., 2001). In this last natural lipid extract the ordered domains were suggested to be sphingomyelin-enriched gel-like phase (Dietrich et al., 2001). These observations strongly suggest that BLES bilayer display a gel/fluid phase coexistence between 37°C to 20°C. This last picture is novel for a natural multicomponent lipid mixture having a gel/fluid phase transition at physiological temperature (37°C).

For phospholipid binary mixtures at the phase coexistence temperature regime the difference in the GP center values (ΔGP), associated with the fluid and gel domains, is related to the miscibility of the binary lipid mixture components (Bagatolli and Gratton, 2000b). We found that an increase in the ΔGP values occurred as the miscibility of the lipid mixture decreases (Bagatolli and Gratton, 2000b). The explanation given for this behavior was related to the compositional and energetic differences between the fluid and gel domains, i.e., the higher the miscibility of the mixture the lower the compositional and energetic differences between the fluid and gel domains, hence the smaller the ΔGP (Bagatolli and Gratton, 2001). For BLES mixture we found a small ΔGP value similar to that observed for miscible phospholipid binary mixtures with a low GP value observed for the gel-like domains in respect to that obtained in a pure phospholipid gel phase (Bagatolli and Gratton, 2000b). Taking into account this last finding we conclude that BLES bilayers display a relative good miscibility among the lipid components showing low energetic and compositional differences between the gel and fluid domains. This last observation supports the idea that even though the gel domains in BLES bilayers has a relatively high percentage of saturated phospholipids, fluid-like lipid molecules may crystallize on the BLES gel domains introducing structural defects that favor water penetration. This last effect decreases the mean GP value of the gel phase in a similar manner to that observed in some particular binary phospholipid mixtures and in cholesterol-depleted brush border membrane lipid extracts (Bagatolli and Gratton, 2000a,b; Dietrich et al., 2001).

The growth of condensed domains in BLES films suggests that the gel-lipids in surfactant undergo a fluid to condensed phase transition with increasing packing density. These condensed domains are probably formed by segregation of mainly saturated chain phospholipids into organized structures in our BLES system. For example, condensed domains in films of DPPC were previously thought to be the "liquid condensed" regions of the films, because the molecular arrangements in such domains were not clear (Hollars and Dunn, 1998). Recent complementary techniques using synchrotron and neutron diffraction has conclusively shown that such condensed regions have a higher degree of chain tilt or are more perpendicular to the plane of the monolayer films and also compared with the fluid or expanded phase, and are thereby considered to be "tilt condensed" phases (Kaganer et al., 1999).

Similarities between the shape and size of the laterally ordered domains in monolayers and free-standing bilayers were observed from the experimental data. To the best of our knowledge, our study is the first one that films, and bilayers of the same complex system were visually compared. However, additional experiments are necessary to further evaluate the similarities between the phase coexistence's pictures in both model systems. For example, we do not rule out the possibilities of the effect of coupling between the hemilayers of the GUV to produce subtle differences in domain physical characteristics between planar films and free-standing bilayers. At present, we also do not know the lateral pressure of bilayers at the phase coexistence temperature regime to perform a complete correlation between both systems.

Fluid phase in BLES bilayers

Although we observed a homogeneous fluorescence distribution on the GUVs surface using LAURDAN, we found that the LAURDAN GP histogram in the fluid phase (above 37°C) is particularly broad (Fig. 3 *B*). This extensive GP heterogeneity found in the fluid phase of BLES does not correlate with images presenting large domains (micron size domains) as we observe in the fluid-gel temperature regime using the two-photon excitation fluorescence images. This heterogeneity of the GP histogram was previously observed at the fluid phase temperature regime in GUVs composed of single components and binary mixtures (Bagatolli and Gratton, 1999, 2000a,b) and in multilamellar vesicles composed of DOPC or DLPC (Parasassi et al., 1997). This last phenomenon was related to a nonrandom organization of lipids in the fluid phase (Bagatolli and Gratton, 1999, 2000a,b) as was suggested in some previous studies on phospholipid mixtures in the fluid phase (Jørgensen et al., 1993; Mouritsen, 1998; Worthman et al., 1997). The agreement between data on BLES and on the artificial PC mixtures can be associated with the high-saturated phospholipid content of BLES (~40% DPPC by weight). The nonrandom organization of lipids in BLES mixture at the fluid phase temperature regime is remarkable and to our knowledge is a novel observation for a natural lipid extract, again due to the peculiar composition of surfactant.

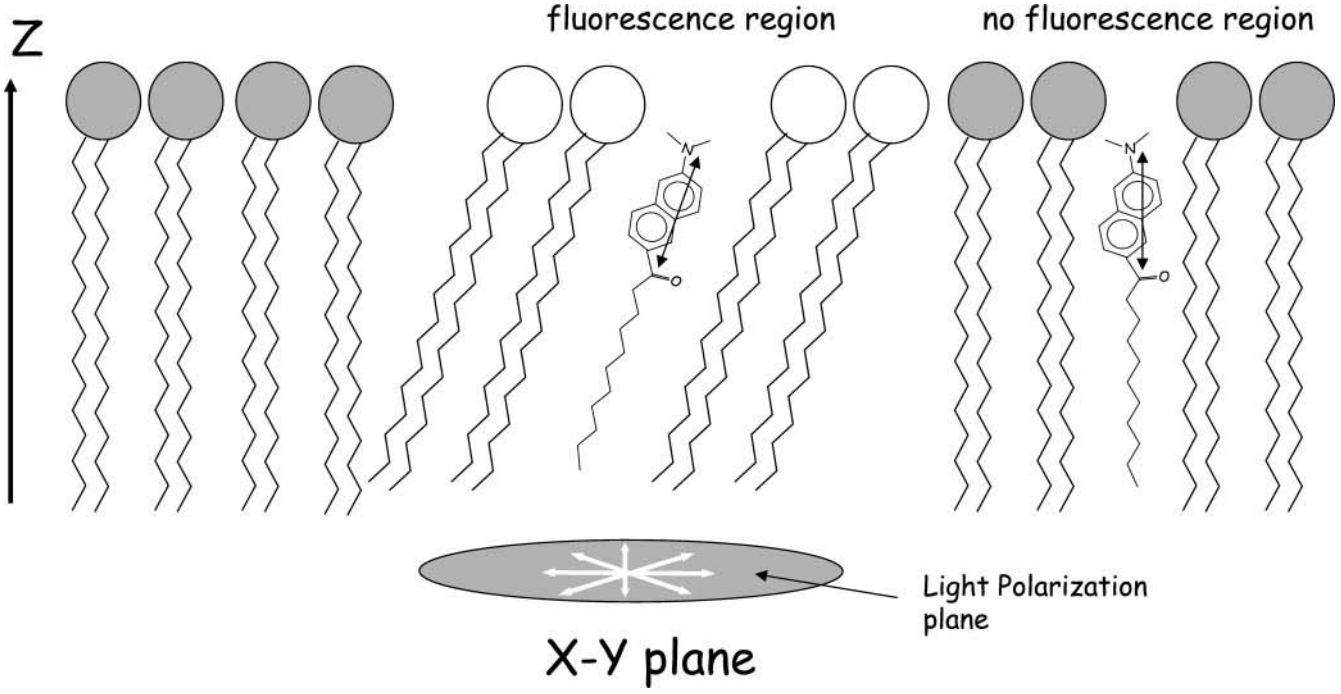

FIGURE 6 Schematic representation of the photoselection effect on LAURDAN-labeled BLES GUVs at the low temperature regime (below 20°C) using circular polarized excitation light. The different orientations of the coexisting highly ordered phases affect the LAURDAN position and hence the probe's excitation efficiency (see text).

Gel phase in BLES bilayers

The behavior of LAURDAN below 20°C in BLES bilayer is very peculiar, compared with previously studies synthetic lipid mixtures in GUV (Bagatolli and Gratton, 2001). The observation of domain coexistence in the polar region of BLES GUVs through the photoselection effect (Fig. 2) with the same low extent of dipolar relaxation process (high GP, Fig. 3) shows the coexistence of two distinct highly ordered phases at the low temperature regime. The observation of highly ordered domain coexistence at the polar region of the vesicles using LAURDAN is novel because it was not observed previously in any other GUVs of various phospholipid mixtures previously studied (Bagatolli and Gratton, 2000a,b). In general for GUVs composed of phospholipids (pure components or binary mixtures) at the gel phase temperature regime the low extent of solvent dipolar relaxation (high GP values) measured in the equatorial region of the GUVs is accompanied by a lack of fluorescence intensity at the vesicle's polar region. This last observation is explained by the fact that the photoselection effect operates at the polar region of the GUV, i.e., that the dipole of LAURDAN in the gel domains remains perpendicular to plane of polarization of the light, minimizing absorption, and hence emission (Parasassi et al., 1997; Bagatolli and Gratton, 1999, 2000a,b). This lack of fluorescent intensity at the polar region of the GUVs was observed even in samples that displayed the coexistence of two different gel phases (such as DPPE/DPPC or DMPE/DMPC; Bagatolli and Gratton, 2000a,b), suggesting similar orientation of the LAURDAN probe in the coexisting gel phases. The observations done in BLES GUVs at the low temperature regime can be explained by considering different LAURDAN orientations between the coexisting lipid domains (Fig. 6). As shown in Fig. 6, two different LAURDAN orientations will produce two different fluorescence intensity regions. Interestingly, the DSC result does not show a clear second peak between 20°C to10°C. However, we cannot discard the possible contribution of a second, low temperature-phase transition on the overall DSC curve, considering the broad and complex characteristic of the BLES thermogram (Fig. 4).

Previous studies on porcine surfactant monolayers have suggested that possibly a higher order phase transition may be occurring in the films at very high packing density or surface pressure (above 50 mN/m; Nag et al., 1998). This was observed as a disappearance of the condensed phase in the films at the high packing density and appearance of heterogeneous phase structures in such films, which was explained by a possible condensed to solid-like phase transition occurring in such systems (Nag et al., 1998; Veldhuizen et al., 1998). However, a recent study suggests that there is persistence of phase segregation in surfactant phopholipid (without SP-B/C and neutral lipids) extract films at very high surface pressure (69 mN/m) where a solid like phase is expected (Piknova et al., 2001). Neutron diffraction studies

from DPPC monolayers have suggested that a loss of the hydration shell of the lipid head group occurs at high surface pressures when the lipids enter a solid-like phase without further change of the chain orientations from the condensed phase (Brumm et al., 1994; Denicourt et al., 1994). However, this last phase transition in DPPC monolayers is not detectable in the phase transition isotherms (Brumm et al., 1994; Kaganer et al., 1999) in contrast to that found in porcine surfactant monolayers at high packing densities (Nag et al., 1998). The situation, however, in DPPC monolayers at high packing density is quite the opposite to those observed in BLES bilayers at the low temperature regime, in which there is no evidence of dehydration (both ordered phases display similar extents of water dipolar relaxation process, Fig. 3), and the photoselection effect suggests different lipid orientations between the ordered coexisting phases (Fig. 2). We believe that the low temperature phase transition found in BLES bilayers may be similar to that observed in BLES monolayers at high surface pressure. However, we prefer to be caution in invoking a coexistence of gel/solid phases in bilayers considering the complexity of BLES composition. Further studies are necessary to confirm this last hypothesis.

Gel domains span the lipid bilayer

An important feature of the two-photon excitation fluorescence images concerns the symmetry of the gel domains along the normal to the bilayer surface. Our BLES images show a coupling between the inner and outer leaflet of the bilayer. This last finding was observed in GUVs composed of lipid binary mixtures displaying gel/fluid phase coexistence (Korlach et al., 1999; Bagatolli and Gratton, 2000a,b) and synthetic and natural lipid mixtures displaying fluid ordered/fluid disordered phase coexistence (POPC/sphingomyelin/cholesterol 1:1:1 mixtures with and without the ganglioside G_{M1} and brush border membrane lipid extract from rat kidney; Dietrich et al., 2001). Our observation in BLES bilayers match with that observed by Dietrich et al. (2001) in cholesterol-depleted brush border membrane where a gel/fluid phase coexistence is suggested and support the picture that gel domains spanning the lipid bilayer are not confined to synthetic binary mixtures. This last fact opens exciting possibilities concerning the biological relevance of such phenomena.

CONCLUSIONS

In conclusion, this study suggests that gel and fluid phases may coexist at physiological temperatures in the membranous system of the lung interface. The saturated lipids in pulmonary surfactant can organize in phase-segregated domains in monomolecular films and bilayers. This transition from expanded to tilt-condensed phase in monolayers is equivalent and shows certain similarities and differences to the liquid crystalline to gel phase transition in bilayers, especially for a complex biological material. This process may allow for surfactant layers at the lung air-water interface to be enriched in the saturated phospholipid for the material to function properly due to the supra-molecular lipid arrangements.

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