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Surface Characterization of Bovine Milk Phospholipid Monolayers by Langmuir Isotherms and Microscopic Techniques

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Monolayers were prepared from phospholipids extracted from bovine milk and used as a model system to mimic the native milk fat globule membrane (MFGM) surface structure in various microscopic experiments. The natural complex mixtures of phospholipids were isolated from bovine raw milk, raw cream, processed whole milk, and buttermilk powder by total lipid extraction and solid-phase extraction. A Langmuir film balance mounted on an epifluorescence microscope was used to analyze the physical behavior of the monolayer films and the phase coexistence resulting from the formation of phospholipid microdomains within these films. Atomic force microscopy was used for nanometer-scale topographic resolution of the microdomains. This study allowed comparison of the behavior of phospholipid monolayers from dairy products at different stages of processing, analysis of the formation of microdomains, and the study of the effect of milk processing on lipid−lipid interactions and phase coexistence. It was observed that milk processing changes the physical behavior of phospholipid monolayers by altering the phospholipid profile and the fatty acid distribution.

KEYWORDS: Bovine milk; Langmuir isotherms; phospholipid monolayers; milk fat globule membrane

INTRODUCTION

The backbone of the milk fat globule membrane (MFGM) is composed of a phospholipid matrix, a mixture of hundreds of phospholipids (including different combinations of bulky head groups and fatty acyl chains), in which proteins and cholesterol are embedded (*1*). Milk processing (centrifugation, homogenization, pasteurization, and churning) alters the composition and structure of the MFGM (*2*−*4*). The effect of milk processing on the phospholipid profile of dairy products upon processing has recently been investigated in our laboratory (*5*). In the work presented here we extend our exploration into the effects milk processing has on the composition and structure of the MFGM by studying model monolayer systems composed of phospholipids extracted from milk. These model monolayer systems, designed to mimic the structure of the native MFGM, allow us to further understand phospholipid microdomain formation within the MFGM (*5*).

Confocal laser scanning microscopic (CLSM) images of native milk fat globules stained with a fluorescent phospholipid analogue reveal the presence of lateral heterogeneities or domains in the organization of the MFGM components at the surface of the globules (*5*). However, the physicochemical properties of this native system cannot readily be studied. Therefore, monolayer films composed of phospholipids extracted from four different dairy products were used to mimic the milk fat globule membrane. Our investigation of these systems gives us insight into lipid−lipid interactions, the formation of domains on the native MFGM surface, and the overall structure of the MFGM.

Phospholipid monolayers provide a convenient model system for the exploration of membrane structure and help to overcome the complexity of examining native biomembranes. Veatch and Keller (*6*) mapped phase diagrams of binary and ternary lipid systems by varying composition and temperature. Their work, as well as that of others, has shown there are conditions that result in partitioning of the phospholipids into different phases, thus leading to phase coexistence and formation of microdomains in membranes. Immiscibility behavior of these phases usually depends on the lipid mixtures. This phase separation leads to lateral heterogeneity in the membrane, which is a direct result of liquid−liquid immiscibility (*7*). Domain formation has been observed in different native and model membranes (*6*−*8*). These domains, often called lipid rafts, are thought to be enriched in sphingomyelin and cholesterol (*7*) and to be a liquid-ordered, l_o, phase coexisting with a liquid-disordered, l_d, phase. The liquid-ordered phase generally contains a larger fraction of the more saturated and longer chain phospholipids, and, as a result, the fatty acid chains are more ordered, typically showing more *trans* conformations along the carbon backbone. In contrast, the liquid-disordered phase contains a larger fraction of the more unsaturated and shorter chain phospholipids, and thus the fatty acid chains are in a less ordered conformation with a larger degree of *gauche* defects along the carbon backbone. The coexistence of a liquid-ordered phase with a liquid-disordered phase depends on

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the presence of cholesterol (*9*), but most importantly it depends on the presence of sphingolipids, such as sphingomyelin (*10*). The sizes and shapes of the liquid-ordered domains vary according to temperature, pressure, and chemical composition (*11*).

Model lipid systems give insight into the physical and chemical properties of biomembranes. An easy and efficient model system to study biomembranes and intermolecular interactions, such as lipid−lipid and lipid−protein interactions, is a monolayer spread on a Langmuir trough (*12*). Using a Langmuir trough, the pressure and temperature can be controlled, and their effects on the phospholipid monolayer films can be carefully monitored using various microscopic techniques. Epifluorescence microscopy is a convenient technique used to observe phospholipid monolayer films at the air−water interface. Using this technique involves adding a fluorescent phospholipid analogue, which is composed of a phospholipid molecule labeled with a fluorescent moiety at either the head or tail of the phospholipid, to the phospholipid mixtures. The labeled phospholipid is considered to be an impurity, but at low concentration (2% by mass), it does not introduce significant perturbations to the membrane system (*13*). Contrast in the fluorescence microscopy image results from partitioning of the labeled phospholipids exclusively into the liquid-disordered phase. The phase separation has been confirmed by techniques that do not require dyes (*13*). Common model systems use a binary or ternary phospholipid mixture. In this study, a system that was closer to the native MFGM was investigated by using the natural complex mixture of phospholipids found in four milk products.

Atomic force microscopy (AFM) has been used to acquire topographic images with nanometer resolution of the organizational structure in supported lipid monolayers and bilayers (*14*, *15*). In liquid-ordered domains, the saturated acyl chains are more tightly packed and stretched out than in the liquid-disordered phase, which is composed of more unsaturated lipids (*16*). The membrane is usually more laterally compressed, with less permeability and greater thickness in the liquid-ordered domains (*16*); therefore, the liquid-ordered domains are "taller" than the liquid-disordered phase. AFM, allowing resolution in the nanometer range, appears to be a useful tool to measure this topographic height difference between the two phases and to correlate this with the fluorescence images (*14*).

In this study, a Langmuir film balance mounted on an epifluorescence microscope and AFM were used to analyze the physical behavior of the monolayer films of phospholipids extracted from raw milk (RM), raw cream (RC), processed milk (PM), and buttermilk powder (BP). A comparison of the physical behavior of phospholipid monolayers from dairy products at different stages of processing was made. These two powerful microscopic techniques provide us with the ability to analyze lipid−lipid interactions and the formation of microdomains, analogous to lipid rafts, upon increasing temperature and pressure. Ultimately, we have been able to relate the effect milk processing has on milk phospholipid composition to lipid−lipid interactions and lipid phase separation.

MATERIALS AND METHODS

Samples and Reagents. Bovine raw milk was collected from the bulk tank of milk from the dairy herd at the Dairy Products Technology Center (California Polytechnic State University, San Luis Obispo, CA). Processed whole milk was bought from Producers Dairy Foods, Inc. (Fresno, CA) (pasteurized at 76 °C for 15 s and homogenized at 140 bar in the first stage and at 100 bar in the second). Buttermilk powder was provided by Land O'Lakes, Inc. (Arden Hills, MN). Milli-Q water (18 MΩ·cm), used as trough subphase, was purified by means of a Millipore filtration device (Millipore, Milli Q). All solvents (chloroform, methanol, hexane, and diethyl ether) were from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. Strata silica SI-1 normal phase solid-phase extraction cartridges (bed weight = 2 g, volume = 12 mL) were purchased from Phenomenex (Torrance, CA). The headgroup-labeled fluorescent phospholipid analogue Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red-DHPE, 0.1 mL/mL in chloroform, Invitrogen, Molecular Probes (Carlsbad, CA)), was used to provide contrast between lipid phases.

Raw milk and processed milk were freeze-dried before Folch (*17*) extraction. Raw cream was obtained by centrifugation of raw milk at 3000*g* for 5 min at 20 °C (Eppendorf centrifuge 5810 R). The fat layer at the top of the centrifuge tube was recovered. The phospholipid mixtures from raw milk, raw cream, processed milk, and buttermilk powder were obtained by total lipid extraction using the Folch (*17*) method followed by a solid-phase extraction using the method developed by Bitman et al. (*18*).

Lipid Profiling. The phospholipid mixtures were analyzed by electrospray ionization−tandem mass spectrometry (ESI-MS/MS) as described previously by Gallier et al. (*19*). The ratio saturated/unsaturated was calculated as follows: saturated/(monounsaturated + polyunsaturated).

Presence of Residual Proteins. Protein contamination on the phospholipid extracts was determined using the Pierce bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Pittsburgh, PA). The procedure followed is described by the manufacturers. Briefly, 100 mL of the emulsified phospholipid fraction was dispersed in a mixture of BCA/copper complex. The mixture was allowed to react at 37 °C for 30 min, color development was measured at 560 nm, and the results were compared to a standard curve made with bovine serum albumin. The method is sensitive to 25 mg/mL (25 ppm).

Langmuir Film Balance. Fluorescence microscopy was used to examine phase separation of phospholipid monolayer films deposited on an air−water interface. A KSV Minitrough Langmuir film balance (KSV Instruments, Helsinki, Finland) with a Wilhelmy plate was mounted on an Olympus BX60 microscope. An Olympus QColorA mercury lamp served as a radiation source. Incident light was passed through an excitation filter to select wavelengths ranging from 510 to 590 nm. Texas Red-DHPE was used as the fluorescent dye at a concentration of 2% of the total phospholipid content by mass. The phospholipid mixtures were dissolved in chloroform to a concentration of 1 mg/mL.

The surface pressure was measured continuously as a function of average total molecular area. The Wilhelmy plate was cleaned by heating it in a Bunsen burner flame until it was glowing orange. The water surface was cleaned before and between each isotherm until a surface pressure value of < 0.1 mN/m was achieved after a full compression of the trough barriers. The phospholipids were carefully spread onto the surface with a gastight microsyringe, and the solvent was allowed to evaporate over 5 min. The films were subsequently compressed at a constant rate of 10 mm/min. Langmuir compression isotherms (surface pressure versus surface area, $\pi-A$) and fluorescence images were collected at 16, 20, 24, and 27 °C using the proprietary KSV software and QCapture software (QImaging, Surrey, BC, Canada), respectively. All isotherms were collected at least twice at each temperature to confirm the reproducibility of the measurement. Images were acquired at target surface pressures (in increments of 5 mN/m) and analyzed with ImageJ software (U.S. National Institutes of Health, Bethesda, MD). The total fraction of nonfluorescent domains in epifluorescence images of the monolayers was analyzed using at least four thresholded images recorded from different regions of the monolayer at each target surface pressure and each experimental temperature.

Langmuir−Blodgett (LB) Films. Each phospholipid mixture (40 μL) was deposited onto the air−water interface on the trough. The film was allowed to equilibrate for 5 min and was subsequently compressed up to 20 mN/m at 20 °C (for RM, RC, and PM phospholipid films) or up to 30 mN/m at 25 °C (for BP phospholipid films) and then transferred to a clean glass microscopic slide previously immersed into the subphase by lifting the support at a constant speed of 5 mm/min.

Atomic Force Microscopy. An MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA) was used in both contact and AC modes to image the surface of the phospholipid monolayers. The images were processed using Igor Pro 6.05 (Wavematrics Inc., Portland, OR) and the proprietary MFP-3D software (Asylum Research) with a second-order planefit and first-order flatten. Contact silicon cantilevers (NSC35/AlBS, n-type silicon (phosphorus doped)/aluminum backside coated)

Table 1. Phospholipid Composition of Raw Milk, Raw Cream, Processed Milk, and Buttermilk Powder[a]

phospholipid	raw milk (mol %)	raw cream (mol %)	processed milk (mol %)	buttermilk powder (mol %)
total LysoPC	1.0 ± 0.1	0.7 ± 0.1	1.08 ± 0.02	3.6 ± 0.2
total PC	36.6 b	31.1 a	40.3 c	44.3 d
total SM	21.8 b	17.7 a	24.1 b	23.9 b
total ePC	5.4 b	4.4 a	5.2 b	6.8 c
total LysoPE	0.5 b	0.4 a	0.5 b	0.4 a
total PE	22.6 c	24.8 d	20.8 b	7.3 a
total PE-cer	0.0087 b	0.0074 ab	0.0055 ab	0.0019 a
total ePE	1.0 c	0.9b c	0.9 b	0.3 a
total PI	2.9 b	8.8 d	2.3 a	7.1 c
total PS	1.7 a	6.7 c	1.3 a	4.0 b
total PG	4.6 ± 0.8	3.6 ± 0.5	2.3 ± 0.1	0.51 ± 0.03
total PA	1.8 c	0.8 a	1.2 b	1.8 c

[a] The phospholipids were extracted following the Folch method for total lipid extraction and the Bitman method for solid-phase extraction. Means within a row with different letters differ ($P < 0.05$). Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; ePC, ether phosphatidylcholine; ePE, ether phosphatidylethanolamine; PE-cer, phosphoethanolamine-ceramide.

Table 2. Degree of Unsaturation of the Phospholipids Present in Raw Milk, Raw Cream, Processed Milk, and Buttermilk Powder Obtained Following the Folch Method for Total Lipid Extraction and the Bitman Method for Solid-Phase Extraction[a]

		raw milk (mol %)	raw cream (mol %)	processed milk (mol %)	buttermilk powder (mol %)
total PL	saturated	36.6 b	27.9 a	35.2 b	35.7 b
	monounsaturated	24.9 ± 3.3	25.3 ± 3.0	24.9 ± 2.1	23.6 ± 3.1
	polyunsaturated	38.5 a	46.7 c	39.7 ab	40.5 b
	saturated/unsaturated	0.6 b	0.4 a	0.5 b	0.6 b

[a] Means within a row with different letters differ ($P < 0.05$). Abbreviation: PL, phospholipids.

from MikroMasch (San Jose, CA) were used for contact mode using the longest of the three tips of the cantilever. Olympus OMCL-AC cantilevers (aluminum coated, Olympus Corp., USA) were used for AC mode imaging.

RESULTS AND DISCUSSION

The acyl chain composition of the phospholipids (length and degree of unsaturation) and the type of headgroup play key roles in phase separation (*16*). Cholesterol and saturated phospholipids, such as sphingomyelin, tend to pack in liquid-ordered domains, whereas the liquid-disordered domains are composed mainly of unsaturated phospholipids, such as PC, PE, PI, and PS. Biomembranes are expected to be a fluid, liquid-crystalline phase as they contain low acyl chain melting temperature phospholipids (*10*). However, the amount of saturated acyl chains and high melting temperature sphingomyelin and cholesterol present in the biomembranes favors a liquid-ordered phase coexisting with a liquid-disordered phase. Cholesterol has a condensing effect on the permeability, molecular ordering, and lateral phase separations in biomembranes (*20*).

Physical Behavior of Milk Phospholipids Is Affected by Surface Pressure, Temperature, and Composition. The phospholipid mixtures are composed of hundreds of species, charged and uncharged, saturated and unsaturated, all with different headgroups and acyl chains. The phospholipid profile (**Table 1**) and degree of saturation (**Table 2**) of the four mixtures showed significant differences between the four phospholipid extracts, mainly a lower amount of phosphatidylethanolamine in the BP phospholipid extracts and a lower degree of saturation of the RC phospholipids. Structural data on complex lipid systems are very scarce; however, there are a few published studies examining pulmonary surfactant, a lipid−protein mixture (*21*, *22*), pulmonary surfactant phospholipids (*23*, *24*), or brush border membrane lipids (*25*). To draw conclusions, references must then be made to ternary systems. It is not possible to pinpoint which phospholipid is responsible for particular results observed in such a complex and diverse system; however, the four films under study can be compared with each other.

Effect of Surface Pressure and Temperature on Phase Coexistence Deduced from the Compression Isotherms. The monolayer films of phospholipids from buttermilk powder, processed milk, raw milk, and raw cream were compressed up to the collapse pressure at 16, 24, and 27 °C (results not shown) and 20 °C (**Figure 1**) to probe the effect of surface pressure on lipid−lipid interactions. The four dairy phospholipid mixtures had similar compression isotherms at all temperatures. A plateau, typical of a gas phase, at very low pressures (< 1 mN/m) was followed by a gradual increase reaching a second plateau near the collapse pressure (> 50 mN/m) (**Figure 1**). Unfortunately, the physiological temperature of a cow's udder, 37 °C, could not be achieved with the present equipment. The physical behavior of the mixtures did not differ significantly, according to the compression isotherms.

The temperature did not affect the $\pi-A$ isotherms (results not shown for BP, RC, and PM films; **Figure 2**). On the $\pi-A$ isotherms (**Figures 1** and **2**), at all temperatures, a kink was observed at 55 mN/m preceded by a plateau and a collapse of the phospholipid monolayer films at about 60 mN/m. At this point, some material can be absorbed at the surface of the Wilhelmy plate, hindering the measurement of the surface pressure. Very few investigations (*21*, *24*, *25*) have been carried out on complex systems; therefore, it is difficult to make comparisons with the current results to explain the phenomenon happening at surface pressures of > 50 mN/m.

Effect of Surface Pressure on Phase Separation Studied by Fluorescence Microscopy. Because phospholipids do not possess an intrinsic fluorescence, the observation of phospholipid monolayers under a fluorescence microscope requires the use of a dye. Texas Red-DHPE was used as a headgroup-labeled fluorescent analogue. Contrast in the images results from different dye solubilities in the coexisting phases. The lateral distribution of the dye revealed the coexistence of two phases at different pressures and temperatures and different physical behaviors among the four mixtures under study. The dark areas correspond to a liquid-ordered phase with low dye solubility (*13*), and the fraction

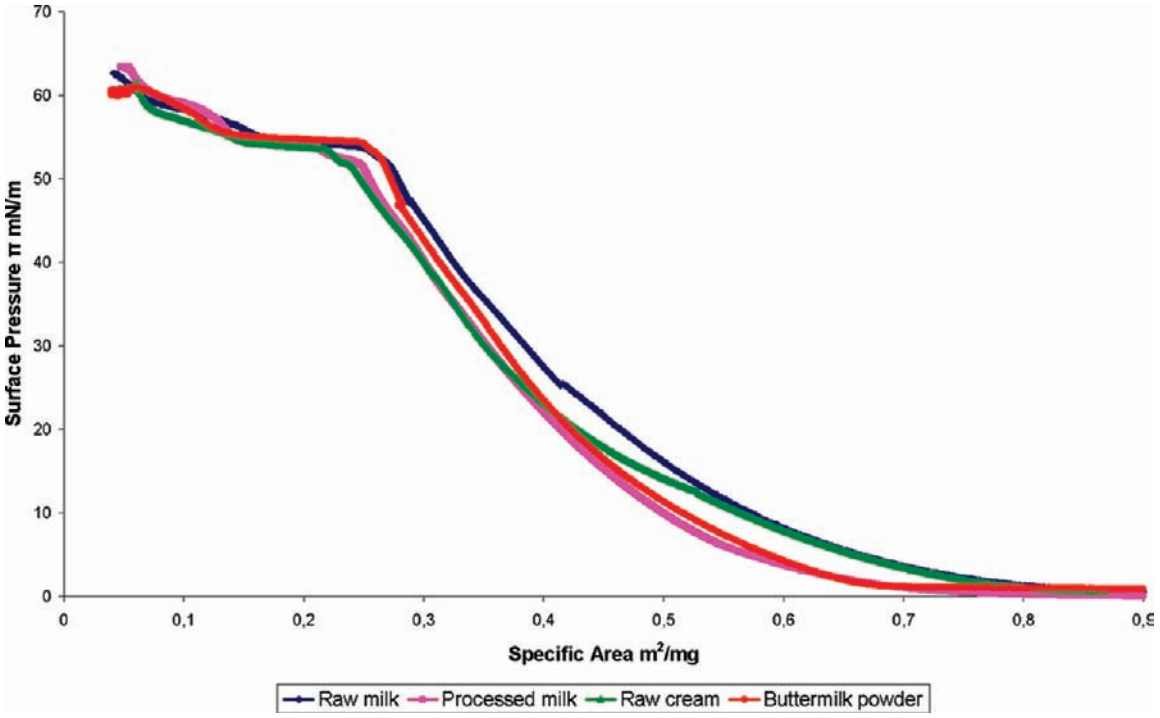

Figure 1. Compression isotherms of monolayer films of phospholipids extraction from RM, PM, RC, and BP. The isotherms were recorded at 20 °C.

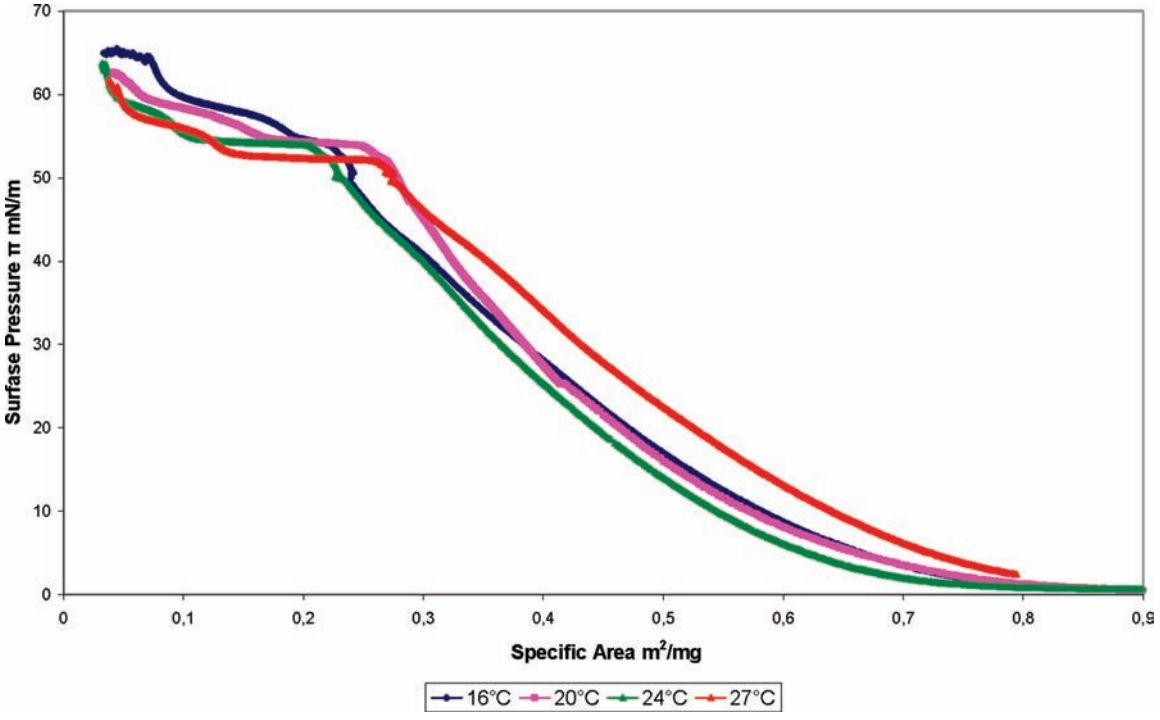

Figure 2. Compression isotherms of monolayer films of phospholipids extracted from raw milk. The isotherms were recorded at 16, 20, 24, and 27 °C.

of these domains increases upon lateral compression of the phospholipid monolayer film. Fluorescence microscopic images (**Figures 3**−**6**) demonstrate the phase separation within phospholipid films at 20 °C (images at 16, 24, and 27 °C not shown), not revealed by the compression isotherms.

No phase transition, or "kink", was observed on the compression isotherms between 10 and 40 mN/m for each film (**Figure 1**); however, the fluorescence microscopic images (**Figures 3**−**6**) revealed the presence of a phase separation. The compression isotherm is the resulting effect of each phospholipid present in the complex mixture and the interactions with each other. Fluorescence images (**Figures 3**−**6**) showed differences among the phospholipid mixtures, indicating a different physical behavior according to the type of processing the dairy product had undergone. Although the shape and size of the domains of RM, RC, and PM phospholipid films were quite similar at all pressures (**Figures 4**−**6**), the shape and size of the domains of the BP mixture diverged (**Figure 3**).

At 16, 24, and 27 °C (results not shown) and 20 °C (**Figures 4**−**6**) and at very low surface pressure (< 1 mN/m), a gas phase was observed in RC, PM, and RM monolayer films followed by a two-dimensional (2D) foam-like structure at surface

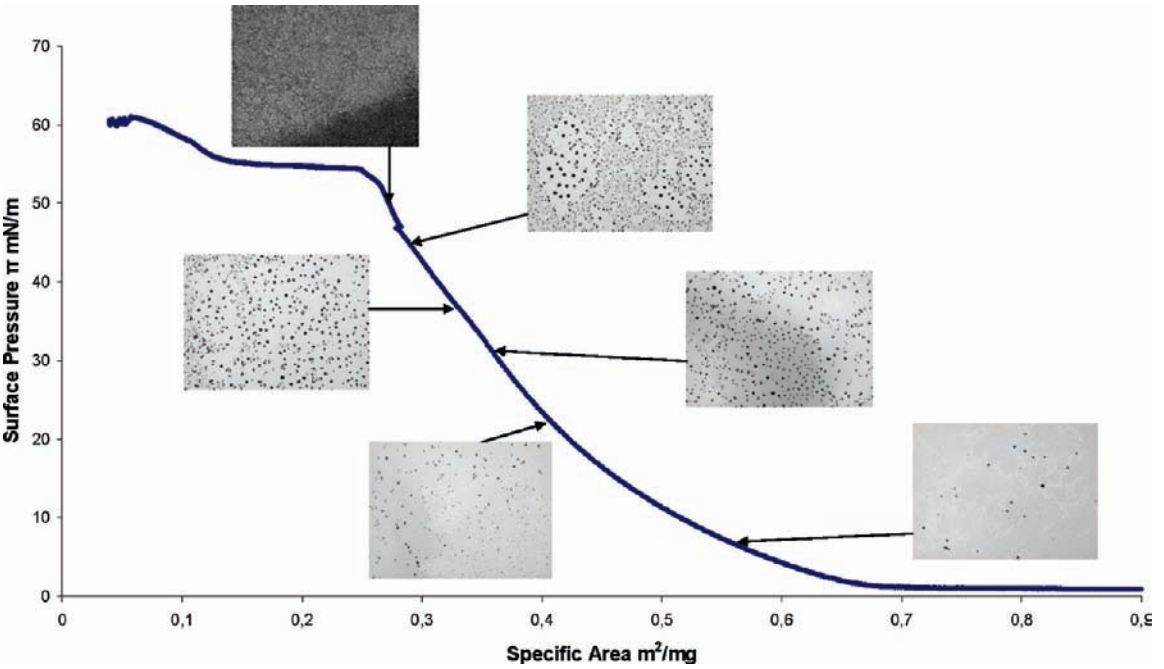

Figure 3. Compression isotherm and fluorescence images of BP monolayers at 20 °C.

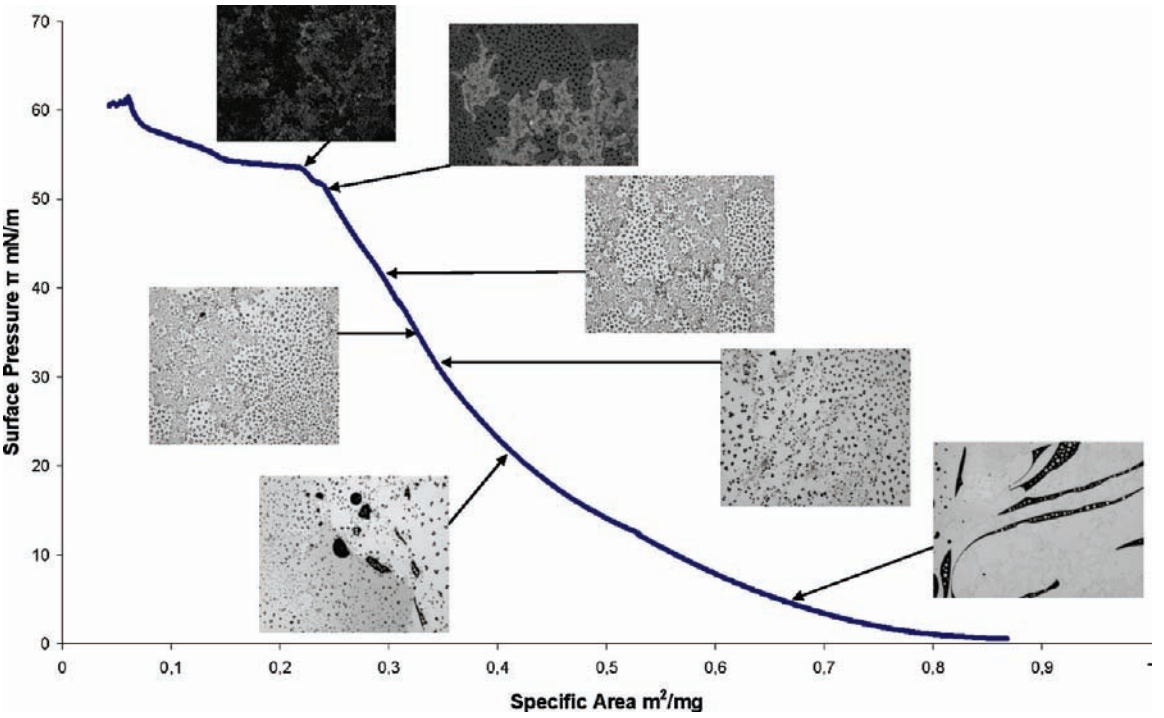

Figure 4. Compression isotherm and fluorescence images of RC monolayers at 20 °C.

pressures up to 20 mN/m, where the stripes, resulting from the conversion of circular bubbles from the gas phase into polygonal networks (*26*), ruptured, leading to a liquid-disordered phase coexisting with a liquid-ordered phase. Upon further compression, the domains grew in size. At the kink at about 55 mN/m, it can be hypothesized that more than two phases coexist, as shades of gray were observed in the liquid-disordered phase from 40 to 45 mN/m, or that the collapse of the film produced artifacts arising from the overlapping of collapsed regions of the film. The gas phase and the 2D foam were nonexistent in the BP monolayer films (**Figure 3**). This could be due to the lower amount of PE of the BP phospholipids (*19*) as this was the major difference in the composition of BP phospholipids compared to the other mixtures (**Table 1**).

The nonhomogeneous repartition of the dark domains (**Figures 3**−**6**) within the bright phase indicates a heterogeneous distribution of the phospholipids within the mixtures. This indicates that the films might be richer in some phospholipids than others in some areas, possibly a higher concentration of anionic phospholipids or saturated phospholipids. Each phospholipid monolayer film behaved differently, giving different shapes and packing arrangement (**Figures 3**−**6**).

Gas Phase, 2D Foam, and Domain Growth. Upon compression, phase separation occurred and the liquid-ordered domain

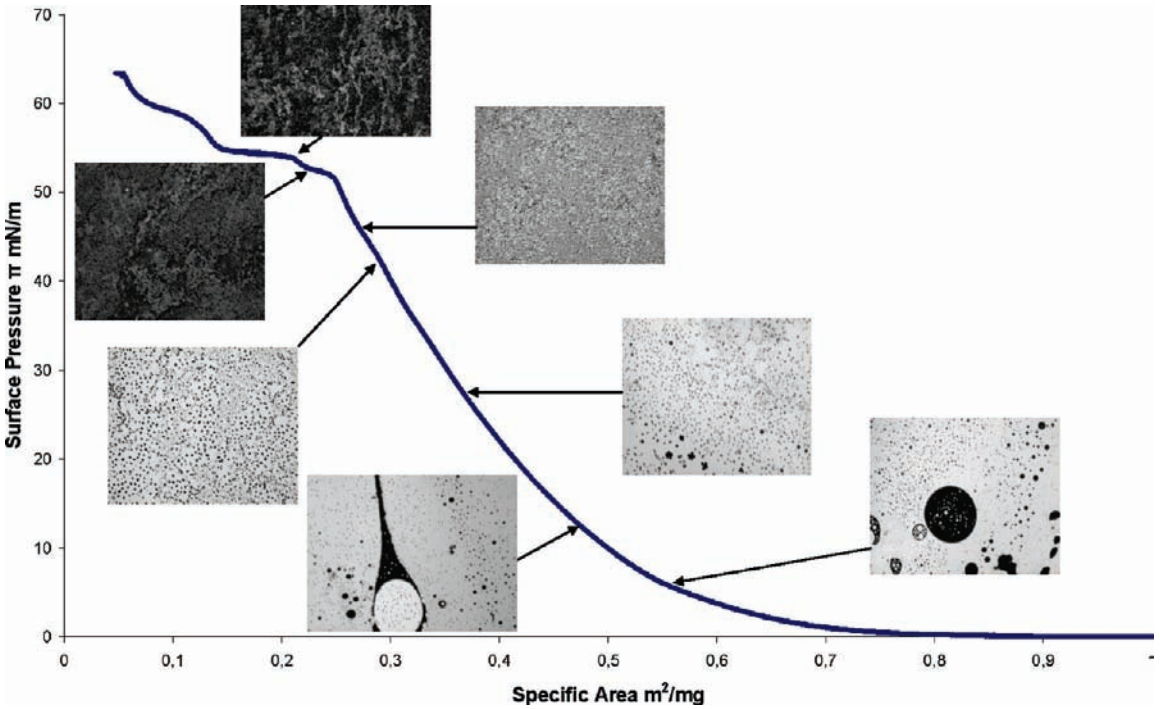

Figure 5. Compression isotherm and fluorescence images of PM monolayers at 20 °C.

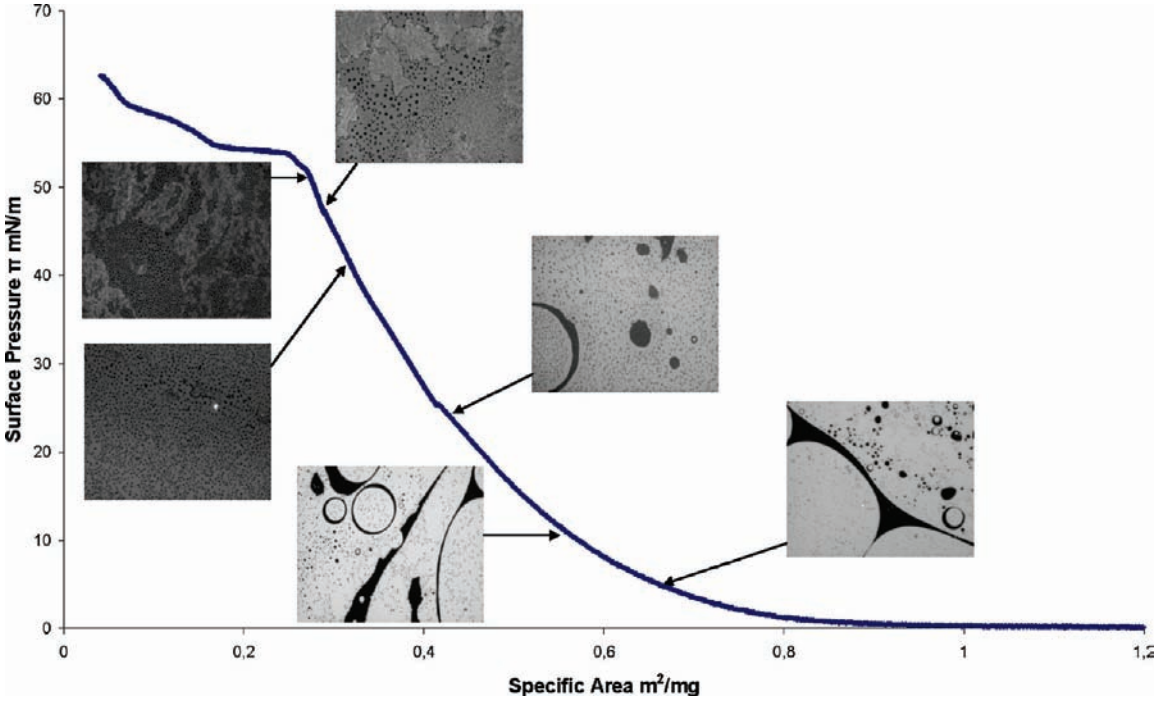

Figure 6. Compression isotherm and fluorescence images of RM monolayers at 20 °C.

size grew as the liquid-disordered phase was squeezed (**Figure 7**). At lower surface pressure (< 20 mN/m), some domains were larger than at higher surface pressure due to the presence of a gas phase (< 1 mN/m) and a 2D foam phase (> 1 mN/m) for RM, RC, and PM films at the four temperatures (**Figures 4**−**6**, images at 16, 24, and 27 °C not shown). At pressures of < 20 mN/m, the contrast is mainly due to a liquid-disordered/gas separation and, at higher pressures, to a liquid-ordered/liquid-disordered separation. The gas phase appears dark as a result of the low molecular density of phospholipids, both labeled and unlabeled. A gaseous phase is not completely a disordered-like phase, but the molecules exhibit a preferential orientation relative to the surface normal (*12*). Therefore, the quantitative measurement of domain growth (**Figure 7**) for the phospholipid mixtures showing a 2D foam structure is relevant only for pressures between 20 mN/m (when the 2D foam disappears) and 45 mN/m (before the collapse pressure). As the buttermilk powder phospholipid monolayer does not present any gas phase or 2D foam structure (**Figure 3**), a quantitative analysis of the domain growth (**Figure 7**) is relevant at any pressure below the collapse pressure. The buttermilk powder phospholipid monolayers had an overall smaller area fraction of liquid-ordered domains at 20 °C (**Figure 7**) compared

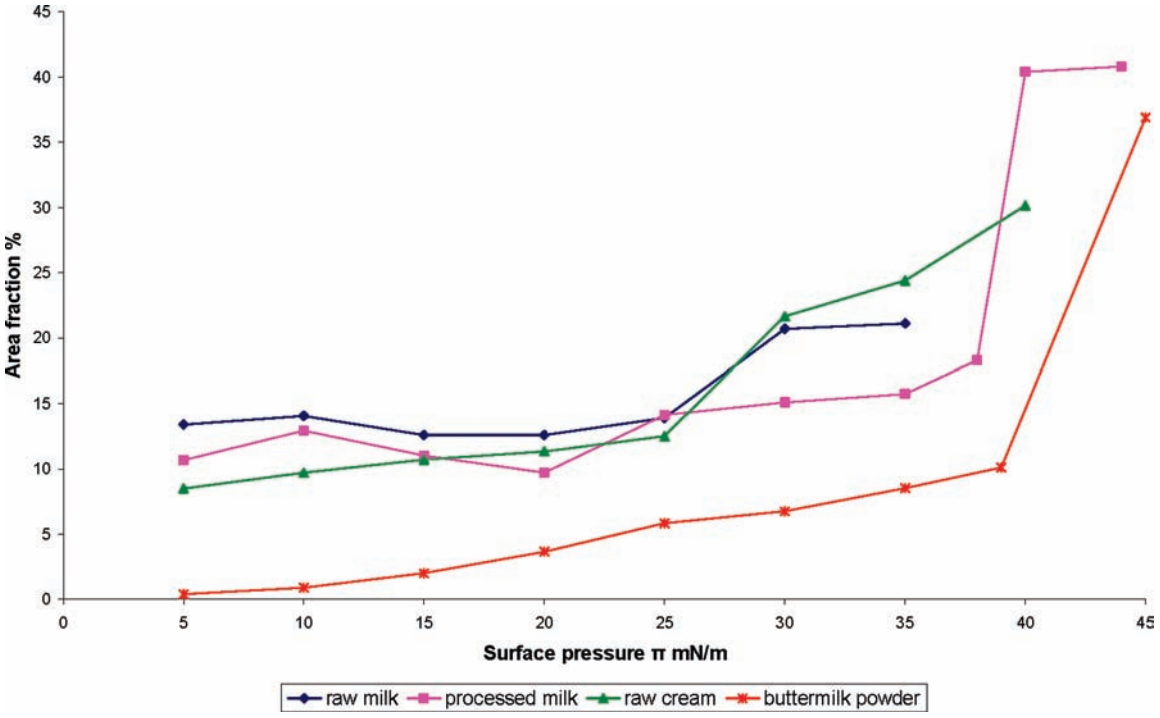

Figure 7. Domain growth upon varying surface pressure at 20 °C. RC, RM, and PM films show a gas phase at pressures of <20 mN/m.

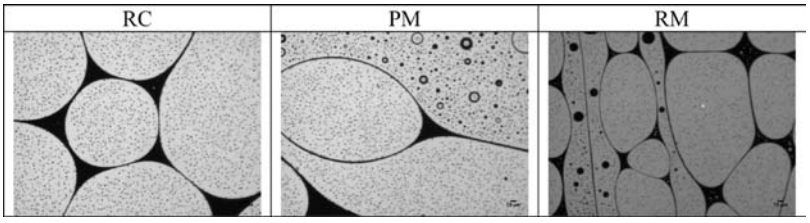

Figure 8. Fluorescence images of 2D foam structure observed on the Langmuir trough at 15 mN/m and 16 °C. Scale bar = 10 μm.

to the three other films; this may be related to the lower amount of PE (*5*). Waninge et al. (*27*) reported the effect of the concentration of PE on the equilibrium phase structures of MFGM lipid systems, shifting from a lamellar to a hexagonal phase at high PE concentration. Therefore, PE may play a role in the lateral organization of phospholipid systems, and the low amount of PE in the BP mixture (**Table 1**) (*19*) may explain why its physical behavior is different from the behavior of the other mixtures.

A gas phase results from significant contact of the hydrocarbon portions of the phospholipids with the water surface (*26*). When the available area for the monolayer is large, there is little effect of the phospholipids on the surface tension of water; the phospholipid intermolecular lateral interactions are weak, and the phospholipid monolayer is considered to be a two-dimensional gas. In the gas phase, torus domains are also observed (for example, the circular shapes in **Figure 8** at the top right of the PM image). These domains are common in systems of two immiscible fluid phases (*11*). The gas phase disappears by increasing the amount of phospholipids spread at the interface or by compressing the film; however, it is unlikely that a gas phase will occur at surface pressures of > 1 mN/m. Knobler (*26*) reported the presence of a 2D foam structure in monolayer films. The foam consists of interconnected straight stripes surrounded by the gas phase. The interconnected stripes form polygonal cells (**Figure 8**). A 2D foam structure was observed in RC, RM, and PM films at all temperatures at pressures between 1 and about 20 mN/m (**Figures 4**−**6**, images at 16, 24, and 27 °C not shown).

"Squeeze-Out" Phenomenon. Innocente et al. (*28*) extracted the water-soluble fraction of the MFGM containing a mixture of proteins, phospholipids, and a large amount of neutral lipids. They studied its surface properties with a Langmuir trough and reported a first phase transition (from a liquid-expanded film to a condensed film) at about 23 mN/m. This phase transition was not observed on the compression isotherms in the current study. The presence of this phase transition is related to either (i) the squeezing out of the proteins as observed by Bos and Nylander (*29*) studying β-lactoglobulin−phospholipid films or (ii) the presence of triglycerides as observed by compressing phospholipid films contaminated with triglycerides (results not shown). However, a similar kink (**Figures 1**−**6**) to the second phase transition at about 50 mN/m that Innocente et al. (*28*) observed was also present on the compression isotherms in this study at surface pressures around 55 mN/m (**Figures 1**−**6**). They associated this phase transition to a change from a condensed film to a solid film. However, this high surface pressure is not reached in the native milk system. The MFGM surface pressure is likely between 30 and 35 mN/m by reference to work from Demel et al. (*30*). To compare the present model systems to the native membrane, only the surface pressure under 40 mN/m will be considered.

It is very interesting to observe domains in the absence of cholesterol, or at least very low amounts of cholesterol, and also in the absence of proteins, two components usually found in biomembranes. In the current study, using the BCA protein assay, all phospholipid extracts showed no absorbance at 560 nm;

thus, they did not contain residual proteins. The amount of residual cholesterol in the phospholipid mixtures was not determined, but there was still some residual cholesterol left in the extracts, as reported in a previous study (*19*). All four dairy products originally contained a very low amount of cholesterol (*31*), and it is possible that some cholesterol was left after total lipid extraction followed by solid-phase extraction. Even though cholesterol is a nonpolar lipid, it does have one polar group, thus making it a surface-active molecule.

Addition of sphingomyelin or cholesterol led to a similar isotherm (results not shown) but an increase in domain size, supporting the hypothesis that the dark domains observed in the four phospholipid monolayer films are a liquid-ordered phase enriched in sphingomyelin and cholesterol as reported in the literature (*8*, *22*, *32*). This emphasizes the importance of the presence of sphingomyelin or cholesterol for phase coexistence and could be an important factor in choosing between different sources of phospholipids for liposome formation, for example, as soy phospholipids do not contain at all sphingomyelin (*33*) and egg phospholipids only a very small amount (*34*). A gas phase and a 2D foam (results not shown) were also observed after the addition of cholesterol to the buttermilk powder phospholipids. This suggests that residual cholesterol may be present in the raw milk, raw cream, and processed milk phospholipid mixtures and be responsible for the formation of a gas phase and 2D foam. When the cholesterol concentration in the phospholipid mixtures and the surface pressure were increased, a three-phase coexistence was observed (**Figure 9**); Veatch and Keller (*6*) speculated about the possibility of a three-phase coexistence appearing in giant unilamellar vesicles prepared with a mixture containing dioleoylphosphatidylcholine (DOPC), palmitoyl sphingomyelin, and cholesterol at 25 °C. Veatch (*35*) mapped the phase diagram of cholesterol, DOPC, and dipalmitoylphosphatidylcholine at 10 °C on the basis of observations by nuclear magnetic resonance and reported a three-phase coexistence at a certain lipid composition.

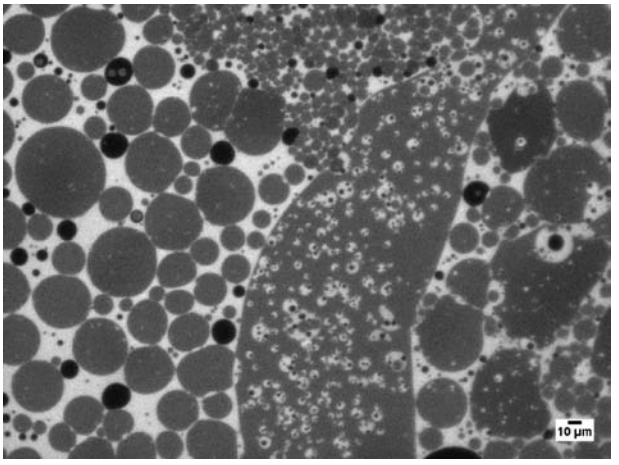

Figure 9. Fluorescence image of a three-phase coexistence. PM phospholipids (80 μL) were mixed with 2.5 μL of cholesterol (4.5 mg/mL in chloroform). The mixture was deposited on the air−water interface, and the film was compressed to 15 mN/m. Scale bar = 10 μm.

Domain Shapes Result from a Nonequilibrium State. The number of domains is a nonequilibrium property and depends on the quantitatively unpredictable nucleation process (*12*). The number of domains can be increased by increasing the temperature or by adding cholesterol (or other surface-active molecules), as both actions reduce the line tension, the energy per interface length between the liquid-ordered and liquid-disordered phases (*12*).

At 20 °C, the buttermilk phospholipids formed bean-like shapes which, with increasing surface pressure, tended to associate to give flower shapes (**Figure 10**). The raw cream phospholipids formed a shape similar to commas, which associated upon increasing surface pressure to give a "butterfly" shape (**Figure 10**). The raw milk phospholipids had a mixture of the shapes observed with the buttermilk phospholipids and the raw cream phospholipids (**Figure 10**). The processed milk phospholipid shapes were similar to the buttermilk phospholipid shapes but tended to be smaller and more compact (**Figure 10**).

The increase in temperature did not have a significant impact on the curve of the $\pi-A$ isotherms (**Figure 2**) but had an effect on the shape of the domains (**Figures 10** and **11**). The buttermilk phospholipid domains were a mix of round and bean-like shapes associating to give "florets" with increasing surface pressure at 27 °C (**Figure 11**). The flower shapes of the raw cream phospholipid domains were more stretched at 27 °C (**Figure 11**) than at 20 °C (**Figure 10**), leading to snowflake- and "Christmas tree"-like shapes, with some branching also observed in RC phospholipid films. The raw milk phospholipid domains were more condensed at 27 °C (**Figure 11**). The processed milk phospholipid domains had more stretched flower shapes at 27 °C (**Figure 11**). The shape and size of the liquid-ordered domains were rather similar between the monolayer films from the same phospholipid source at 16, 20, and 24 °C (**Figures 3**−**6**, images at 16 and 24 °C not shown). A change in the shape and size of the domains for all phospholipid films appeared at 27 °C (images not shown), with more stretched domains.

The shapes of the lipid domains of the different monolayer films observed under epifluorescence microscopy are the result of the lateral arrangement of all the different phospholipids present in the natural complex mixtures at the air−water interface. The shapes of the domains could be related to the degree of saturation (**Table 2**) and the diversity of the headgroups of the polar lipids (**Table 1**) present in each extract (*5*). Long-range electrostatic forces exist between the phospholipid molecules at the air−water interface (*11*), and domain shapes result from a competition between line tension and long-range electrostatic dipolar repulsions. Circular shapes result from the minimization of the edge energy of the domains (*16*) and are expected for an isotropic substance (*36*). The smoothing of the boundary by the line tension

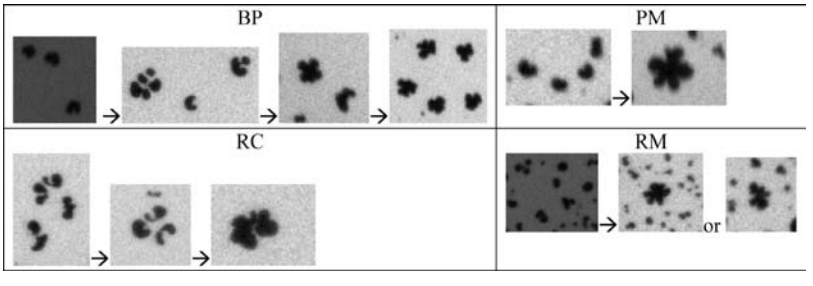

Figure 10. Fluorescence images of the different shapes observed on the Langmuir trough at surface pressures between 25 and 35 mN/m and at 20 °C. (The arrows indicate shapes upon increasing surface pressure.)

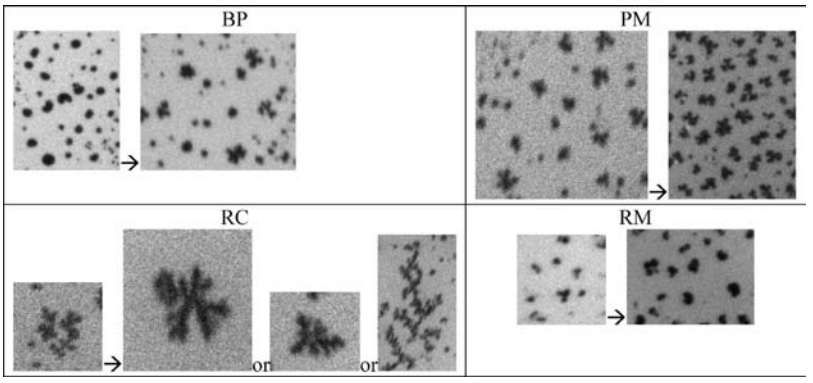

Figure 11. Fluorescence images of the different shapes observed on the Langmuir trough at surface pressures between 25 and 35 mN/m and at 27 °C. (The arrows indicate shapes upon increasing surface pressure.)

tends to lead to regular shapes. Coffee bean and clover leaf shapes appear when the smoothing is not terminated (*13*). The formation of domains is limited by the rate of nucleation. On compression, the domains become larger and electrostatically unstable and eventually form higher harmonic shapes such as flowers (*11*). Upon compression, domain size increases as do electrostatic forces (*37*); therefore, shape changes are likely to happen.

Keller et al. (*36*) mentioned the possibility of chiral crystal shapes upon compression resulting from long-range molecular order. The metastable snowflake shape (**Figure 11**) might be temporary and disappear within hours. The same kinds of flowers or snowflakes were observed by Discher et al. (*23*) using pulmonary surfactant phospholipids; they attributed these shapes to a nonequilibrium state, becoming more regular (circular or kidney-bean shapes) after some hours when reaching equilibrium. It is surprising that these shapes appear with increasing temperature as crystal formation should be favored at low temperature. The fact that these shapes appear at higher temperature for the RC films might be related to a lower ratio of saturation to unsaturation (**Table 2**).

Despite care being taken during the extraction of the phospholipids, impurities (cholesterol and free fatty acids) might still be present (*19*). The presence of impurities increases the density of domains, and the boundaries become rough, leading to fractal structures (*12*). However, the boundaries may tend to smooth after a few hours. Impurities, exact lipid compositions, speed, and path taken to reach equilibrium are experimental parameters difficult to control but critically determine the size, shape, and growth of the domains.

AFM Images Show Differences among Phospholipid Films and Confirm the "Taller" Height of the l_o Domains. *LB Films*. The surface pressure and temperature at which the LB films were transferred onto a glass slide were chosen so that specific shapes and larger domains could be observed with imaging by AFM. Features of phase separation and molecular organization within the monolayer are retained in supported lipid membranes (*38*). Indeed, the LB films prepared here showed clear domain formation and looked similar at the air−water interface on the Langmuir trough (**Figures 3**−**6**) and deposited onto a glass slide (**Figure 12**).

AFM. BP, RC, RM, and PM phospholipid monolayers spontaneously form liquid-ordered domains that are detectable as raised features (**Figures 13**). The AFM study on supported monolayers showed thickening of the monolayer upon formation of ordered domains. Due to the higher *trans*/*gauche* ratio in a liquid-ordered phase, the liquid-ordered domains are thicker than the liquid-disordered phase (*7*).

AFM imaging of supported monolayer membranes demonstrated the presence of roughly round-shaped domains. Some domains seem to be linked to each other, forming aggregates of two to three domains (**Figure 13**). The AFM images (**Figure 13**)

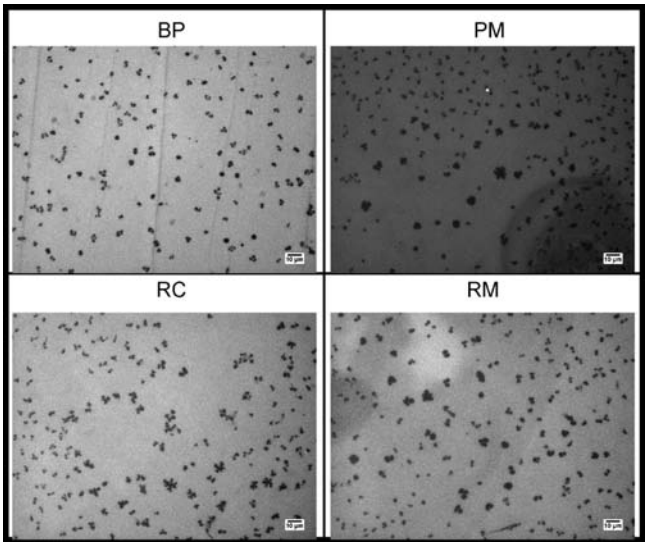

Figure 12. Fluorescence microscopic images of phospholipid monolayer LB films (BP: Surface pressure = 35 mN/m, T = 20 °C) (RC, RM, and PM: Surface pressure = 30 mN/m, T = 25 °C) deposited onto a glass slide. Scale bar = 10 μm.

confirmed that two fluid phases coexisted in the model systems under study. Very small amounts of impurities in the RM film were detected on the AFM contact mode images (white dots, **Figure 13**). In general, the l_o domains were observed to be about 1 nm taller than the l_d domains, which is in agreement with other studies on model membranes (*39*, *40*). AFM phase imaging provides information about the mechanical properties of a sample. It showed that the l_o domains were more rigid than the l_d domains.

Conclusions. Our motivation for studying monolayer films composed of milk phospholipids was to gain further insight into their fundamental properties as spread monolayers and to relate these properties to the biophysical properties of the native MFGM. Phospholipids from dairy products at various processing stages were studied as monolayer films, and conclusions were drawn regarding the effect processing has on both phospholipid composition and monolayer structure. Langmuir film balance, combined with epifluorescence microscopy, and AFM have proven to be efficient techniques for the exploring of the physicochemical characteristics of milk phospholipid monolayers. All milk samples produced monolayer films that showed the coexistence of two phases, confirming that the MFGM phospholipids are responsible for the phase separation at the surface of the native MFGM observed with CLSM. The morphological and physical behaviors of each phospholipid mixture were distinct,

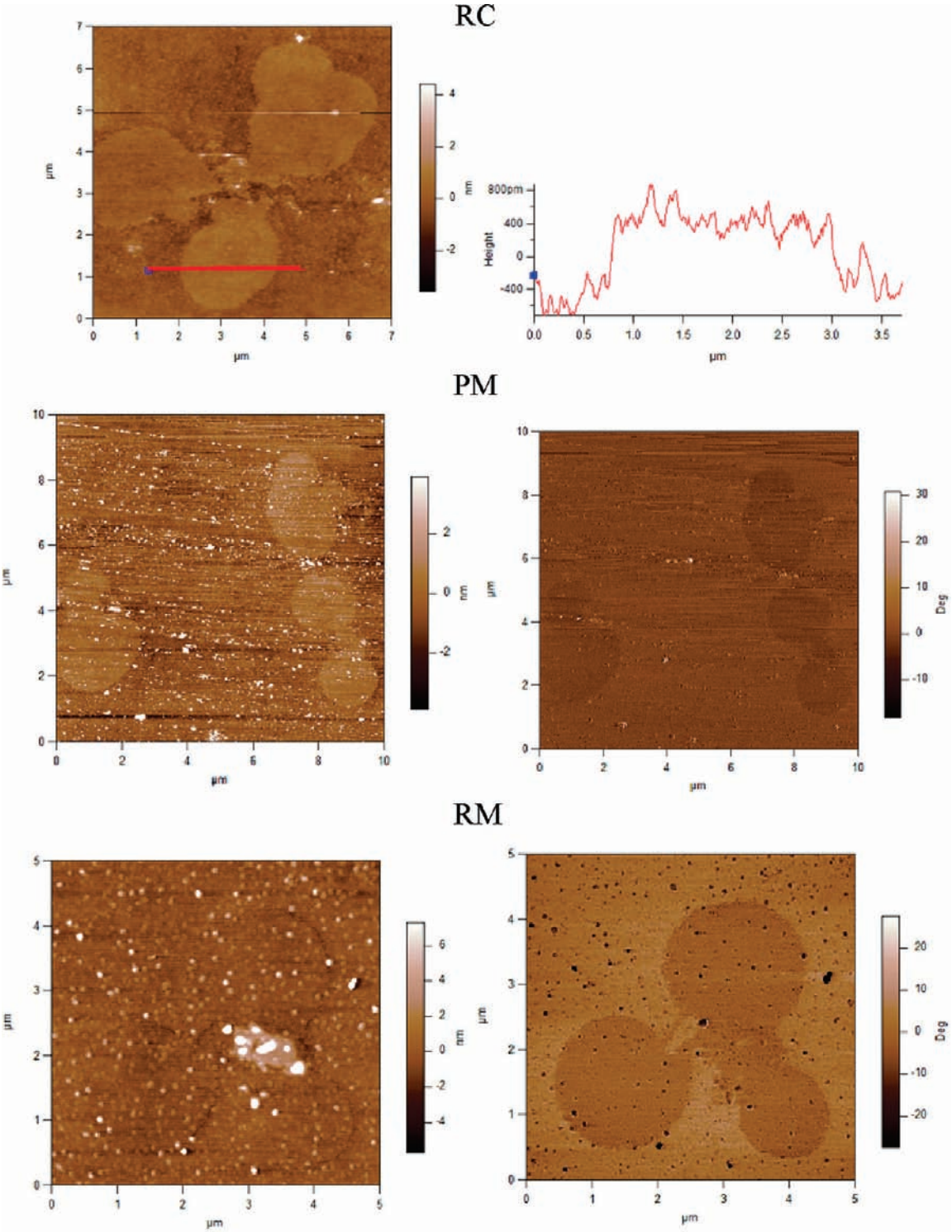

Figure 13. AFM images of RC (π = 30 mN/m, T = 25 °C, contact mode), PM (π = 30 mN/m, T = 25 °C, AC mode), and RM (π = 30 mN/m, T = 25 °C, AC mode) phospholipid monolayer films deposited onto a glass slide. For PM and RM phospholipid monolayer films, on the left are height images and on the right are corresponding phase images.

and the observed differences are likely the result of the phospholipid profile and the fatty acid distribution of the mixtures studied in our laboratory, with the buttermilk powder phospholipid mixture being the most different, probably due to a lower amount of phosphatidylethanolamine. The surface pressure and the temperature also had an impact on the physical properties of the films. The system under study, the milk fat globule membrane, is poorly understood for its biological role. The lipid−lipid interactions may play a key role in the physiological functions and emulsification properties of the MFGM. By processing milk, the phospholipid composition and the fatty acid distribution are altered, leading to different physicochemical properties as reported here.

ABBREVIATIONS USED

MFGM, milk fat globule membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; LysoPC, lysophosphatidylcholine;

LysoPE, lysophosphatidylethanolamine; ePC, ether phosphatidylcholine; ePE, ether phosphatidylethanolamine; PE-cer, phosphoethanolamine-ceramide; PL, phospholipids; ESI-MS/MS, electrospray tandem mass spectrometry; AFM, atomic force microscopy; RM, raw milk; RC, raw cream; BP, buttermilk powder; PM, processed milk; BCA, bicinchoninic acid.

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