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ARTICLE

Using Confocal Laser Scanning Microscopy To Probe the Milk Fat Globule Membrane and Associated Proteins

Sophie Gallier,[†,‡] Derek Gragson,[§] Rafael Jiménez-Flores,*[,‡] and David Everett[†]

[†]Department of Food Science, University of Otago, Dunedin, New Zealand, [‡]Dairy Products Technology Center, and [§]Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, California

The bovine milk fat globule membrane (MFGM) is an important, biologically relevant membrane due to its functional and health properties. Its composition has been thoroughly studied, but its structure, especially the lateral organization of its components, still remains unclear. We have used confocal laser scanning microscopy (CLSM) to investigate the surface structure of the MFGM in globules with different degrees of processing using two types of fluorescently labeled phospholipid probes and a protein dye. Using this technique, we have observed heterogeneities in the distribution of MFGM lipids and proteins relating to the processing and size of the globules. The effect of pretreating the milk (centrifugation, pasteurization−homogenization and churning) was studied by double-staining the surface of the milk fat globules, followed by observation using CLSM, and by determining the phospholipid profile of raw milk, raw cream, processed milk and buttermilk powder. Our findings agree with other techniques by showing that the composition of the MFGM changes with processing through the loss of phospholipids and the adsorption of caseins and whey proteins onto the surface.

KEYWORDS: Milk fat globule membrane; membrane structure; confocal laser scanning microscopy; phase coexistence; phospholipids

INTRODUCTION

Milk is an emulsion containing about 3.6% fat in the shape of a globule (varying from 0.1 to 20 μm in diameter) surrounded by a milk fat globule membrane (MFGM). The MFGM has been associated with several technological, nutritional and health properties (*1*−*3*). Its composition is well-known, but its structure remains poorly understood. Whereas the milk fat globule core contains mainly triglycerides, the membrane is made up of surface-active material consisting mostly of proteins, phospholipids, and other miscellaneous compounds such as cholesterol (*4*). The MFGM surrounding the lipid droplet originates from endoplasmic reticulum, however it is likely that between droplet formation, secretion and ultimate consumption the MFGM undergoes modification (*5*). The MFGM, through the lateral organization of its surface-active proteins and phospholipids, allows the fat to stay in suspension in the milk serum by encapsulating the lipid core. In the past few years some structural models of biological bilayers have been presented, including the fluid mosaic model introduced by Singer and Nicolson (*6*). These models are generally employed to describe cell membranes, with both the interior and exterior environments of the membrane being aqueous. Current models of the MFGM differ from bilayer models as the MFGM is thought to be composed of a phospholipid trilayer (*7*). The nonpolar environment resulting from the inner lipid core necessitates the formation of an inner phospholipid monolayer in addition to an outer phospholipid bilayer. It is also likely that there is a region of encapsulated cytoplasm, rich in protein, adsorbed between the inner monolayer and outer bilayer (*8*). Phospholipids compose the backbone of the MFGM in which the proteins are embedded and thus the organization of the phospholipids likely plays a crucial role in protein−lipid interactions. Deeth (*9*) used phospholipases to determine the asymmetrical distribution of the MFGM phospholipids. Phosphatidylcholine (PC) and sphingomyelin (SM) are mainly located on the external leaflet of the membrane, and phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are concentrated in the internal leaflet of the bilayer.

Different microscopy techniques have been used to elucidate the MFGM structure, such as electron microscopy, immunomicroscopic (*10*) and biochemical techniques (*11*), freeze-etching (*12*), and freeze-fracture immunocytochemistry (*8*). However, some of these techniques are time-consuming and might alter the MFGM structure or introduce artifacts (*8*). Recently, Evers (*13*) introduced the use of confocal laser scanning microscopy (CLSM) as a noninvasive technique to study the MFGM of cows' whole milk and human milk using lipophilic probes and lectins. Lopez et al. (*14*) used an exogenous fluorescent headgroup-labeled phospholipid analogue and two lectins to visualize the distribution of the phospholipids and the carbohydrate moieties within the bovine milk fat globule membrane.

Many researchers have used CLSM and fluorescent dye labeled phospholipids to elucidate the presence of lipid rafts, areas rich in cholesterol and sphingomyelin observed in cell

*Corresponding author. Fax: 805 756 2998. E-mail: rjimenez@calpoly.edu.

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membranes, and the lateral structure of phospholipids in model lipid bilayer systems by imaging the surface of giant unilamellar vesicles (GUVs) (*15*−*17*). Studies of ternary systems, composed of cholesterol, a saturated phospholipid and an unsaturated phospholipid, have been used to determine phase diagrams (*18*) showing regions where two distinct fluid phases coexist. Phase coexistence occurs under specific conditions of pressure, lipid concentration and temperature. It is generally accepted that one of these phases is more ordered and thus called the liquid-ordered (L_o) phase, whereas the other phase is less ordered, and called the liquid-disordered (L_d) phase. When these two phases coexist, they are separated into two distinct domains. The degree of order referred to in these two phases results mainly from the orientation of the fatty acid chains in the phospholipids. The liquid-ordered phase is more compact as the carbon−carbon bonds in the fatty acid chains adopt fewer gauche defects and thus can pack more tightly (*19*). Generally phospholipids that are saturated with longer and symmetric fatty acid chains more readily adopt the liquid-ordered structures. The ability to see this phase separation with CLSM results from the fact that the exogenous fluorescent dye labeled phospholipid partitions exclusively into the liquid-disordered phase, thus leaving the liquid-ordered domains "dark". This exclusive partitioning into the L_d region is a direct result of the large dye molecule that is attached to either the headgroup or fatty acid chain of the phospholipid thus preventing it from packing tightly enough to enter the liquid-ordered phase. The CLSM technique involving the use of exogenous probes and dyes at low concentration has been shown to not introduce significant perturbations of the native biological structure of the membrane (*20*).

De Almeida et al. (*21*) reviewed the use of binary and ternary model systems to study the domain formation induced by the presence of cholesterol, ceramide or proteins. The presence of cholesterol and/or sphingomyelin in the system induced a phase separation and, upon further addition of cholesterol or sphingomyelin, domain growth. The membrane is usually thicker in liquid-ordered domains, more laterally compressed and with less permeability. In the liquid-disordered phase, the acyl chains are mobile and the headgroups hydrated. Therefore the bilayer in these domains is thinner, more permeable and less packed. MFGM lipids contain about 0.3−2.3% sterols, 90% of which is cholesterol, and saturated and unsaturated phospholipids (*3*). Therefore it is likely to observe phase separation within the MFGM.

In this study, two different types of fluorescent phospholipid probes, similar in chemical structure to natural phospholipids, one headgroup-labeled and one fatty-acid-labeled, were used to stain the MFGM in situ and observe the distribution of the phospholipids at the surface of the milk fat globules. The staining of the proteins at the surface of the MFGM was carried out with the use of Fast Green FCF, a dye electrostatically attracted to charged groups on proteins (*22*). The spray-dried buttermilk powder aggregates were stained with a dye with an affinity for triglycerides and Fast Green FCF.

It is generally accepted that the structure and composition of the MFGM is altered under milk processing conditions. During the cream-making process, milk is centrifuged and MFGM phospholipids partition into the serum phase (*23*). Raw milk for commercial consumption is homogenized after pasteurization to reduce the size of the fat globules, preventing creaming. Pasteurization and homogenization of the cream result in a loss of native MFGM material, which is then partially replaced by caseins and whey proteins at the interface (*24*). During homogenization, the milk fat globule membrane is ruptured and the interfacial tension between the lipid core of the globule and the milk serum increases from 2 mN/m to 15 mN/m (*25*). The adsorption of caseins and whey proteins stabilizes back the interfacial tension at 3−4 mN/m. Heat treatment leads to the association of milk proteins with MFGM (*6*). In the buttermaking process, cream is churned, breaking completely the membrane structure and creating the phase separation of lipids and water-soluble components. The MFGM material is amorphous and recovered in the buttermilk, which is usually then spray-dried.

Our objective in this work was to optimize the CLSM technique for the observation of milk fat globules and the MFGM, gain knowledge about the structural properties of the MFGM, and investigate the effect of milk processing on lipid−protein interactions and the lateral organization of phospholipids in the MFGM. We varied the degree of processing by studying raw milk, raw cream, pasteurized and homogenized milk and reconstituted spray-dried buttermilk powder. The spray-dried buttermilk powder represents the most drastic processing in our study.

MATERIALS AND METHODS

Samples and Reagents. Bovine raw milk was collected from the bulk tank of milk from the dairy herd at the Dairy Product 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 140 bar in the first stage and 100 bar in the second). Buttermilk powder was provided by Land O'Lakes, Inc. (Arden Hills, MN). Deionized water was used unless otherwise specified. All solvents (chloroform, methanol, hexane, diethyl ether) were from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. The silica SI-1 normal phase SPE cartridges were purchased from Strata, Phenomenex (Torrance, CA). Nile Red, 9-diethylamino-5*H*-benzo[α]phenoxazine-5-one, was used to stain the lipid core of the milk fat globules (1 mg/mL in acetone, Aldrich Chemical Co., Milwaukee, WI). The fluorescent fatty-acid-labeled phospholipid analogues *N*-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sphingosine-1-phosphocholine (NBD-SM, 250 μg/mL in chloroform), 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-*sn*-glycero-3-phosphocholine (NBD-PC, 1 mg/mL in chloroform), 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl}-*sn*-glycero-3-phosphoethanolamine-(NBD-PE, 1 mg/mL in chloroform) and the fluorescent headgroup-labeled phospholipid analogue 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rd-DOPE, 1 mg/mL in chloroform) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Fast Green FCF (disodium2-[[4-[ethyl-[(3-sulfonatophenyl)methyl]-amino]phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]-cyclohexa-2,5-dien-1-ylidene]methyl]-5-hydroxybenzenesulfonate) (1 mg/mL in Milli-Q water, 18 MΩ·cm) was purchased from Sigma-Aldrich (St. Louis, MO).

Phospholipid Profile Determination. The phospholipid profile of the four dairy products was determined to probe the effect of milk processing on the phospholipid distribution.

Phospholipid Extraction. A total lipid extraction by the Folch method (*26*) was performed on freeze-dried raw milk, freeze-dried processed milk, raw cream (from raw milk centrifuged at 3000*g* for 5 min at 20 °C in a laboratory Eppendorf centrifuge) and buttermilk powder. The raw milk and processed milk samples contained 25% total fat on a dry matter basis, the raw cream 50% and the buttermilk powder 10%. After extraction with chloroform:methanol (2:1) and addition of a 8.76% NaCl solution, the dried total lipids were dissolved in hexane. As milk phospholipids are found in dairy products in very small proportions, it is advisible to remove the triglycerides and other neutral lipids prior to any quantitative analysis. A solid-phase extraction on a silica cartridge was carried out to separate the neutral lipids from the phospholipids following the Bitman procedure (*27*).

Phospholipid Analysis. A complete quantitative analysis of the phospholipid composition was carried out by the Kansas Lipidomics Research Center. The lipidomic profile was obtained by tandem mass spectrometry (Applied Biosystems API4000 and Applied Biosystems Q-TRAP).

The phospholipid composition and their fatty acid profile were evaluated in five replicates. Analysis of variance (ANOVA) was calculated

with the SPSS program (SPSS Inc., Chigago, IL, 14.0), and results were considered significantly different at $p < 0.05$.

Confocal Laser Scanning Microscopy. *Preparation of the Samples To Be Stained.* Raw milk was diluted 10-fold in PBS buffer, pH 7.2. Raw cream was prepared as follows: raw milk was centrifuged for 5 min at 3000 g and at 20 °C. The top layer was collected and reconstituted to a 20% fat content in PBS buffer, pH 7.2 and then diluted 10-fold. Processed cream was prepared the same way from processed milk but with a centrifugation time of 15 min and included the larger fat globules. Buttermilk powder was reconstituted at 1 g/10 mL in deionized water.

Preparation of the Slides. Processed cream was used in favor of processed milk for CLSM observation to avoid background interference from milk serum proteins, particularly in the double-staining experiment. Raw milk, raw cream and processed cream were stained to a ratio 1:100 (v/v) with the four fluorescent phospholipid dyes. The cream samples were double-stained with Rd-DOPE and Fast Green FCF (6:100 (v/v), for protein staining) and the reconstituted buttermilk powder with Nile Red (2:100 (v/v)) and Fast Green FCF (6:100 (v/v)).

Milk fat globules do not display an intrinsic fluorescence, requiring the labeling of the MFGM with an extrinsic fluorescent dye. Globules were stained with probes dissolved in chloroform, a lipophilic molecule that can penetrate and fit into a lipid bilayer. Turkyilmaz et al. (*28*) showed that chloroform can modify the lateral organization of the lipid components of a bilayer by loosening and rearranging phospholipids. The exposure of membranes to chloroform has a fluidizing effect and reduces the compactness of the lipid domains by increasing the number of gauche conformations. Therefore, chloroform was allowed to evaporate after deposition of the fluorescent probe onto a concave microscopic slide. Samples (25 μL) were then deposited onto the slide and left in contact with the probe for 20 min at room temperature. Agarose (50 μL, 0.5% w/v in deionized water) was added to fix the samples on the slides, improving the resolution of the 2D and 3D images. A coverslip was then applied rapidly without excessive pressure. Staining with Fast Green FCF and Nile Red was achieved by placing the sample in contact with the dye in an Eppendorf tube for 10 min.

CLSM Observation. An Olympus FV1000 inverted confocal laser scanning microscope (Olympus America Inc., Center Valley, PA) was used to probe the structure of the native MFGM. The CLSM is equipped with four lasers allowing the excitation of several fluorescent probes at the same time; this capability allows multi-imaging of a sample by selecting the correct excitation wavelength and filters to collect the emission light from a particular stain. A 60× oil-immersion objective (UPLFL60XO) was used for all images. Excitation of Rd-DOPE was achieved using the 559 nm emission from the diode laser and the emitted light was collected between 570 nm and 670 nm (570−625 nm when double-staining with Fast Green FCF). The NBD and Nile Red probes were excited with the 488 nm line from the Ar^+ laser and the filters set to collect the emitted light between 500 and 600 nm. Fast Green FCF was excited with the 633 nm line from the HeNe laser, and the filter used was BA655−755. Three-dimensional (3D) images were obtained by scanning the sample across a defined section along the *z*-axis. All images were acquired at 22 °C ± 1 °C.

RESULTS AND DISCUSSION

1. Comparison of the Phospholipid Composition in Raw Milk, Raw Cream, Processed Milk and Buttermilk Powder. About 60% of bovine milk phospholipids are located in the MFGM; residual MFGM material is found in skim milk, probably released during or after secretion of the lipid droplet (*5*). Centrifugation leads to a loss of phospholipids from the membrane surface (*23*). As SM and PC are mainly located in the outer leaflet of the bilayer (*9*), they are more likely to be released to the milk serum upon centrifugation. The lipidomics data (**Table 1**) confirmed this hypothesis since SM and PC are less abundant in raw cream than in raw milk. This has been reported by Christie et al. (*29*), who observed a higher proportion of SM in skim milk than in whole milk. Michalski et al. (*30*) found that small milk fat globules, separated by microfiltration, had different chemical and functional properties than large fat globules. The raw cream obtained after centrifugation consists mainly of the larger milk fat globules. After homogenization and pasteurization, a part of the MFGM rearranges at the surface of the globule with the absorbed whey proteins and caseins (*24*). The ratio of the different phospholipids present in raw milk and in processed milk does not seem significantly altered by these two processing conditions (**Table 1**). The relative composition of the buttermilk powder phospholipids has three times less PE than the other samples (**Table 1**). This phospholipid is more hydrophobic than PC and SM; it might be located on the layer surrounding the triglyceride core of the globule and on the inner leaflet of the bilayer, and thus partitions into the butter phase during butter manufacture. Buttermilk powder also contains more lysoPC (**Table 1**), meaning that PC is degraded during the churning process, during the spray-drying or over the storage of the buttermilk powder.

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

phospholipids	raw milk, mol %	raw cream, mol %	commercial milk, mol %	buttermilk powder, mol %
total LysoPC	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0	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 PE	22.6[c]	24.8[d]	20.8[b]	7.3[a]
total ePE	1.0[c]	0.9[bc]	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]

[a] Within a row entries with different letters differ ($p < 0.05$).

2. The Lateral Distribution of the Phospholipids Reveals Phase Coexistence within the MFGM through the Use of CLSM and Phospholipid Fluorescent Analogues. As discussed earlier milk fat globules were stained with Rd-DOPE, an exogenous dye. Unsaturated PE, such as DOPE, is one of the main MFGM phospholipids (*31*). The fluorophore is attached to the amine group of the phospholipid headgroup. Studies on phospholipid monolayers report that dyes used at a concentration as low as 1−2% do not perturb the system (*32*). As Rd-DOPE is a polar headgroup labeled phospholipid probe, it can be incorporated with minimal perturbation into the bilayer of the MFGM, which contains about 25−65% of phospholipids. Rd-DOPE has a high photostability (*15*) which makes it a suitable choice for CLSM imaging. The spatial resolution limit of the CLSM is on the order of one-half of the wavelength of the light used (here we excited the fluorophore at 559 nm). Since the MFGM is 10 to 20 nm thick (*7*), the thickness of the membrane is overestimated and it is not possible to observe the trilayer structure of the MFGM.

We note that the staining of the MFGM of raw milk (**Figure 1A**), raw cream (**Figures 1B** and **2**) and processed milk (**Figure 1C**) with Rd-DOPE is heterogeneous. Some dark regions are observed on the surface of the milk fat globules of raw milk, raw cream and processed cream. They indicate lateral heterogeneity in the distribution of the MFGM compounds, mainly the phospholipids. These images show that two phases coexist within the MFGM, a L_d phase into which Rd-DOPE partitions and a L_o phase where Rd-DOPE is absent and is thus dark. The L_o domains are analogous to lipid rafts of cell membranes. Lipid rafts of living cells are usually in the range 10−200 nm (*33*) but the lipid microdomains observed in GUVs, larger units than living cells of 30 to 100 μm, are micrometer-scale (*34*). Thus, in milk fat globules ranging from 0.1 to 20 μm, as expected we observe micrometer-scale liquid-ordered domains.

The time scale and the temperature of the observation (22 °C ± 1 °C) may explain why we did not observe any movement of the liquid-ordered domains at the surface of the milk fat globules. Bagatolli and Gratton (*34*) observed the growth and migration of

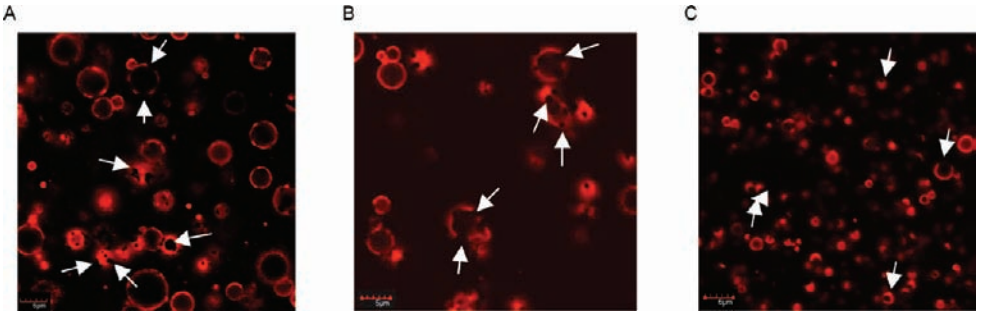

Figure 1. CLSM 2D images of milk fat globules from raw milk (**A**), raw cream (**B**) and processed milk (**C**) stained with Rd-DOPE (arrows pointing at liquid-ordered domains). Scale bar = 5 μm.

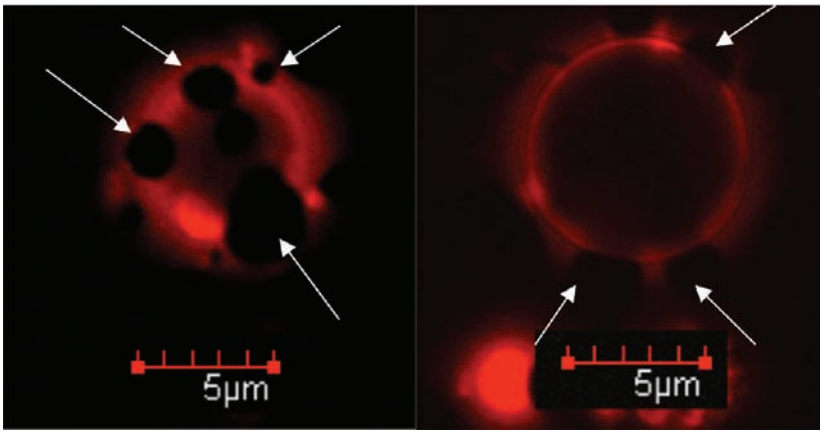

Figure 2. CLSM 2D zoomed-in images of a milk fat globule from raw cream stained with Rd-DOPE; images acquired at different *z*-depths: on the left top of the milk fat globule and on the right centered cross-section of the milk fat globule (arrows pointing at liquid-ordered domains).

lipid domains at the surface of phospholipid GUVs. They reported a decrease in the migration of the liquid-ordered domains at the surface of the GUVs with a decrease in temperature (55 to 28 °C for the first lipid system and 65 to 41 °C for the second lipid system) near the gel-fluid → gel phase transition, independent of the lipid mixtures used to form GUVs.

Raw milk fat globules (**Figure 1A**) and raw cream fat globules (**Figures 1B** and **2**) show heterogeneity of the liquid-ordered domains at the surface of the milk fat globule depending on their size but also within the same globule. The small size of the milk fat globules from processed cream hinders a clear observation of their surface. However it was possible to detect the presence of domains on the surface of the larger ones (**Figure 1C**).

In **Figure 3**, very small globules (< 0.5 μm) were observed in raw cream. These very small vesicles are not likely to be Rd-DOPE vesicles as the CLSM observation of Rd-DOPE alone in PBS buffer did not show the presence of Rd-DOPE vesicles (result not shown). Waninge et al. (*35*) introduced the concept of isolated milk fat globule membrane vesicles in dairy products. These vesicles might originate from phospholipids released when the milk is cooled and centrifuged and from membrane material lost through processing such as homogenization and churning. Michalski et al. (*24*) found that very small fat droplets (< 500 nm) are present in milk at early processing stages (pumping and transporting) but not in milk analyzed right after milking, whereas Walstra (*36*) reported the presence of very small fat globules in milk after milking. Therefore we can suppose that the very small fat globules observed in **Figure 3** are a mix of very small native milk fat globules and residual MFGM material vesicles.

A qualitative observation derived from analysis of many images of the milk fat globules revealed that the number, size and shape of domains vary by type of product, within the same

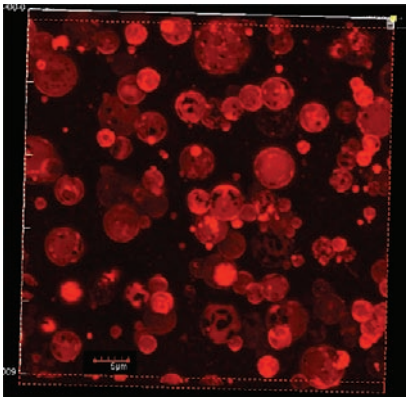

Figure 3. CLSM 3D image of milk fat globules from raw cream stained with Rd-DOPE. Scale bar = 5 μm.

sample, within the same globule and by size of the globules. The domains observed are circular (**Figure 4**), elongated or have a nondefined shape. The angular elongated shapes might be due to a partial crystallization of the lipids of the MFGM. The shape of the domains is due to a smoothing of the boundary by the line-tension at the liquid-ordered/liquid-disordered interface (*32*). The line tension is the interfacial energy per unit length between the coexisting phases (*32*). The electrostatic dipole−dipole interactions between the molecules at the interface of the domain and surrounding phospholipids also play a role in the shape of the domains (*20*). A domain tends to be circular to minimize its edge energy. The edge energy is the energy of the boundary of a domain and is proportional to the boundary length (*19*). It is possible that cholesterol, located inside the domains, is also located at the boundaries of the domains to act as an edge-active molecule and

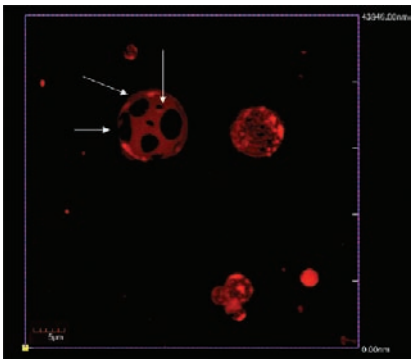

Figure 4. Cut section (focusing on the top surface) of a CLSM 3D image of milk fat globules from raw cream stained with Rd-DOPE (arrows pointing at liquid-ordered domains). Scale bar = 5 μm.

reduce the line tension between the L_d and L_o phases (*19*), leading to circular shapes. The MFGM proteins might also play the same role as cholesterol by smoothing the boundaries (*32*).

For each size class, a qualitative count of domains was performed on the images of fifteen globules. The small globules in raw milk and raw cream, under 2 μm, tend to have fewer domains, about three to four on average, but covering a larger fraction of the surface. The globules, with a diameter between 2 and 5 μm, have an average of five to six domains on their surface. The number of the domains on the surface of the large fat globules (>5 μm) is greatly variable. Some have many small (<1 μm) domains (about 12 on average) whereas some others have fewer domains (about six on average) but are larger (4 μm in diameter). Some of these globules can have a mix of small and large circular domains (about 10 on average). Those larger domains could be the result of coalescence of two or more smaller domains. Some of the large globules (15−20 μm) can have up to thirty small (2 μm in diameter) domains on their surface (**Figure 5**).

Being a headgroup-labeled probe, Rd-DOPE has a bulky headgroup that might hinder its incorporation into the inner leaflet of the bilayer and the phospholipid monolayer. To compare the effect of imaging the MFGM with a phospholipid probe molecule labeled with a fluorescent dye on the headgroup, as with Rd-DOPE, and with one labeled on the fatty acid chain, we used two fatty-acid-labeled fluorescent analogues, NBD-PE and NBD-PC. PE and PC are two major MFGM unsaturated phospholipids. The presence of the fluorescent dye molecule on the fatty acid chain prevents the phospholipid molecules from being able to pack tightly in the liquid-ordered phases, and thus these two probes distribute preferably in the L_d phase in a manner similar to the headgroup labeled Rd-DOPE. As expected, the fatty-acid-labeled probes show a similar distribution of the phospholipids at the surface of the globules (**Figures 6** and **7**) as headgroup-labeled probes. However, the NBD probes are very light-sensitive and photobleached in few seconds under the excitation light. It was therefore not possible to scan in the *z*-direction to obtain a 3D image as the dye intensity was fading quickly with time. Increasing the laser power would increase the background noise and the rate of photobleaching, which generates reactive oxygen species in the sample as a byproduct of fluorescence excitation (*37*). The rapid decrease in the dye intensity is related to a time-dependent decrease in the fluorophore concentration.

3. NBD-SM as a Probe of the MFGM Surface. Model membrane studies have shown that liquid-ordered domains are rich in sphingomyelin, and thus we stained milk fat globules with fatty-acid-labeled NBD-SM, a fluorescent sphingomyelin analogue. Sphingomyelin is a long-chain, highly saturated phospholipid. The images of the milk fat globules (**Figure 8**) showed

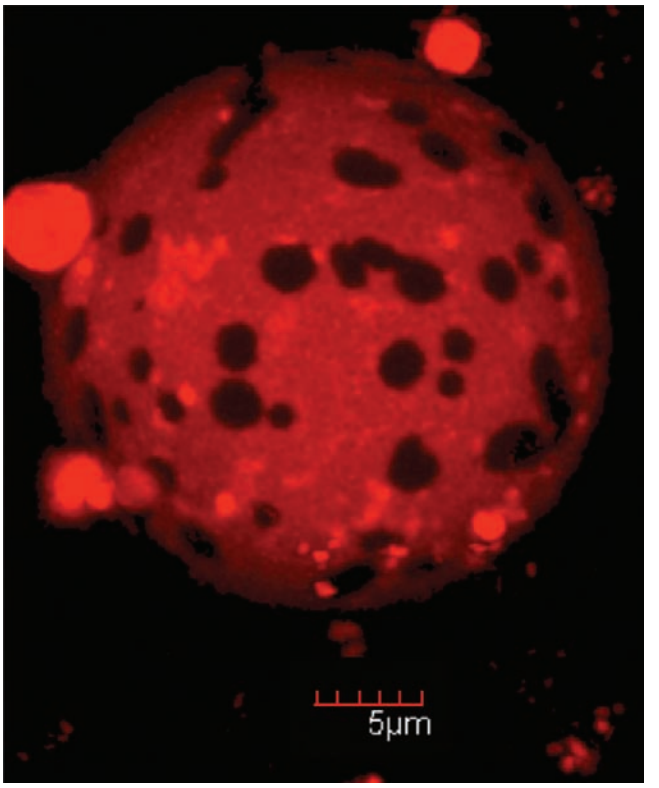

Figure 5. CLSM 3D image of a large milk fat globule from raw cream stained with Rd-DOPE.

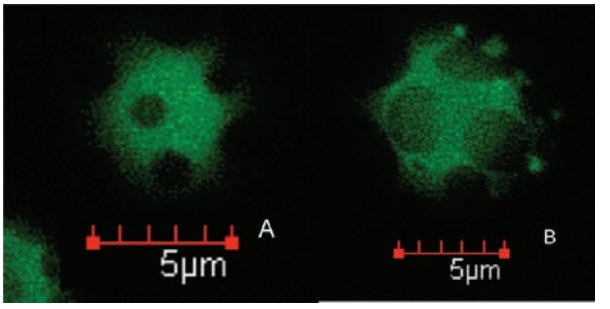

Figure 6. CLSM 2D images (focusing on the top surface) of milk fat globules from raw cream stained with NBD-PE. **A** has smaller liquid-ordered domains at the surface compared to **B**, possibly due to a different phospholipid composition.

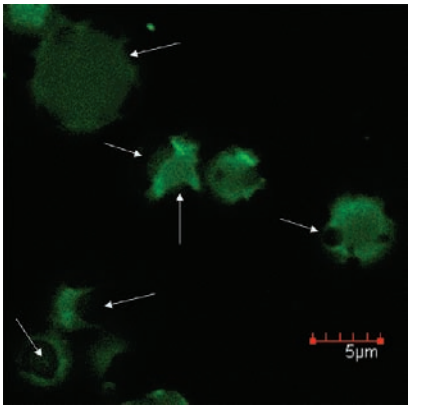

Figure 7. CLSM 2D image of milk fat globules from raw cream stained with NBD-PC (arrows pointing at liquid-ordered domains).

staining with NBD-SM was similar to the other fluorescent phospholipid analogues used above, that is we observed phase separation between the liquid-ordered and liquid-disordered phases. Again this observation is the result of the bulky fluorophore attached to the fatty acid of the sphingomyelin molecule thus limiting its ability to pack tightly and enter the liquid-ordered phase.

4. Dual-Staining of Milk Fat Globules from Cream Samples Reveals a Higher Amount of Proteins on the Surface of Processed Cream Globules. Cream samples were used instead of milk samples in the dual-labeling experiment as Fast Green FCF also stains the proteins of the milk serum. It was possible to observe the surface of few larger fat globules from processed cream. The shape of the domains did not differ from those at the surface of the raw cream fat globules. The major difference was the higher amount of proteins, probably caseins and whey proteins, attached to the surface of the processed cream fat globules (**Figures 9A**,**B**). It is important to note that this method allowed the observation of the protein added to the surface due to homogenization and pasteurization during processing, and at the same time, we can also observe the domains with similarity to the raw milk globules.

Keenan et al. (*38*) reported the effect of homogenization of milk fat globules and compared these with unprocessed milk fat globules. They reported a loss of total lipids, phospholipids and cholesterol, but the phospholipid distribution remained the same. Even though a large proportion of the MFGM remained at the surface of the globules, they observed casein micelles attached to the surface of the milk fat globules. Only a few Fast Green-stained casein micelles appear at the surface of the milk fat globules in raw cream (**Figure 9A**). In **Figure 9B**, we observe Fast Green-stained casein micelles, and possibly Fast Green-stained whey proteins, attached to the milk fat globules of processed cream. The protein:lipid ratio at the surface of the milk fat globules is thus higher in processed cream, involving major changes in functionality, such as different hydrophobicity of the surface and different interactions with the surrounding medium, as caseins, whey proteins and MFGM compounds possess different functional properties.

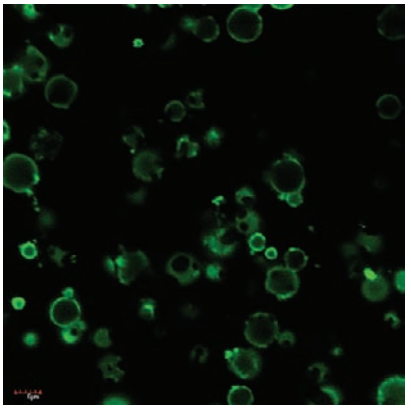

Figure 8. CLSM 2D image of milk fat globules from raw cream stained with NBD-SM. Scale bar = 5 μm.

Fast Green FCF is used as a dye for protein staining in isoelectrofocusing, native polyacrylamide gel electrophoresis (PAGE) and SDS−PAGE. It easily stains proteins without pretreatments when the sample has a high concentration of proteins, such as the buttermilk powder, as shown in **Figure 10**. Nile Red is an oxazone dye used to stain lipid droplets (*39*). It is specific to triglycerides. The double-staining of the reconstituted buttermilk powder with Fast Green FCF and Nile Red (**Figure 10**) shows aggregation of the proteins and the location of some lipid vesicles inside the aggregates. The aggregates are between 15 to 30 μm in length and width.

Fast Green FCF was rather unsuccessful at staining the proteins at the surface of the milk fat globules, possibly because the protein concentration was too low. Indeed, most of the dyes used to stain proteins in gels are less sensitive to membrane proteins (*22*). Additionally, Fast Green FCF might not be able to penetrate the MFGM and stain the proteins located on the layer surrounding the fat core and the inner leaflet of the bilayer.

The work by Evers (*13*) and Lopez and co-workers (*14*) investigating milk globules with CLSM shows similar dark regions on the surface of the globules as the ones we observe in our work here. Evers (*13*) associated the non-stained areas with a lack of a bilayer at the surface of the fat globules. According to Evers' MFGM model, the lack of a bilayer exposes the proteinaceous coat surrounding the surface-active layer of the intracellular fat droplet. However, he proposed the use of fluorescent WGA

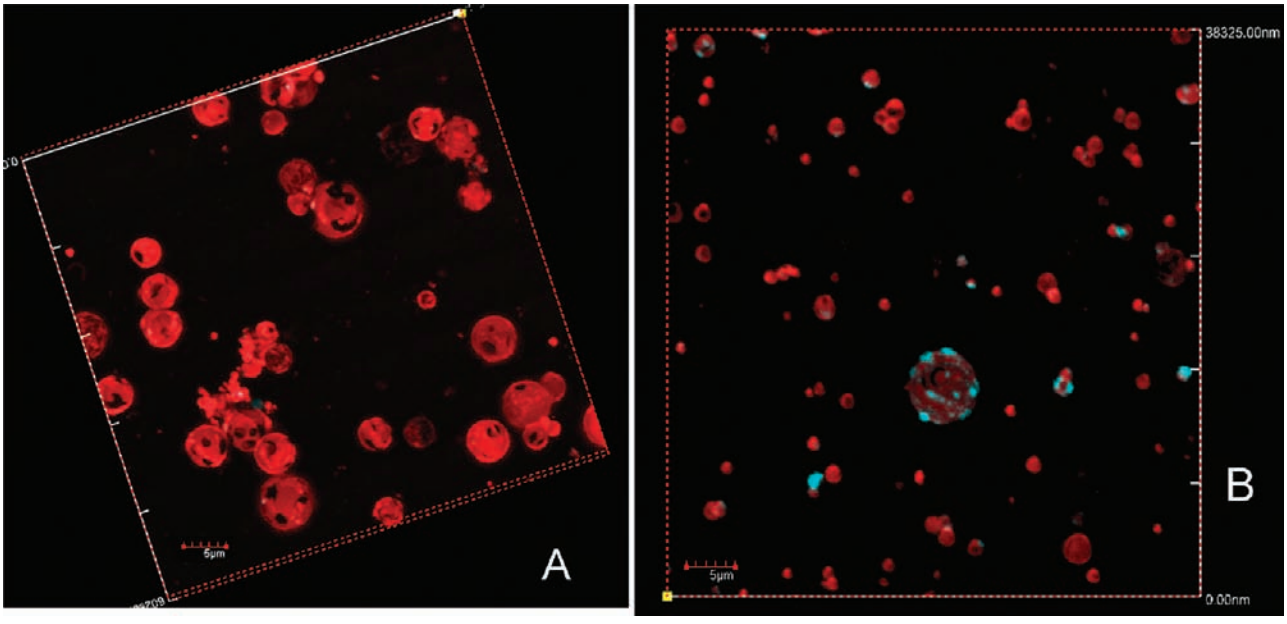

Figure 9. CLSM 2D image of milk fat globules from raw cream (**A**) and processed cream (**B**) stained with Rd-DOPE (red) and Fast Green FCF (blue). Scale bar = 5 μm.

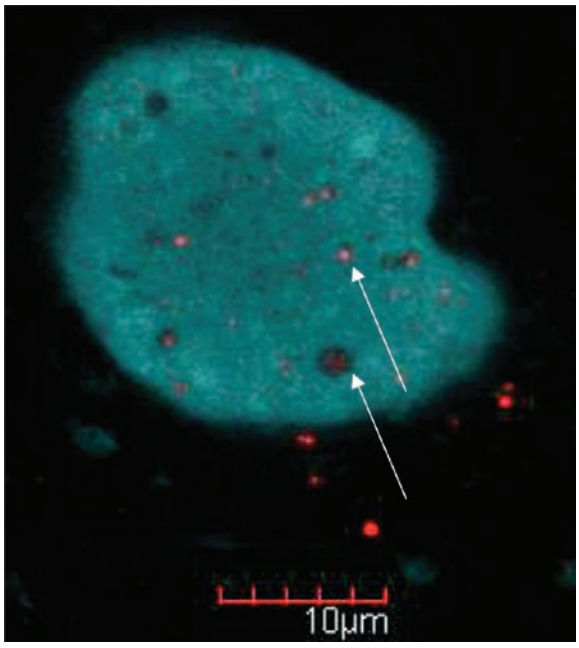

Figure 10. Dual-staining of reconstituted buttermilk powder with Nile Red (red) and Fast Green FCF (blue) (arrows pointing at lipid vesicles trapped in the protein aggregates).

lectins to visualize the relative locations of MFGM proteins. The glycoproteins and glycolipids were heterogeneously distributed on the surface of the fat globules, revealing the outer glycocalyx of the MFGM (*14*). Unfortunately, the dark patches, which appeared after staining with lipophilic probes, were not stained either by the lectins. Moreover, in our study (**Figure 9**), Fast Green FCF did not stain the dark patches, which appeared after staining the globule with Rd-DOPE. So it is unlikely that the dark patches correspond to areas lacking bilayer structure, thus exposing proteins. Further we observe nonstained areas with Rd-DOPE on the surface of fat globules in raw milk where there has been no processing. The lectins have an affinity for carbohydrate moieties, thereby staining glycolipids and glycoproteins. It is thus difficult to draw conclusions about the organization of proteins as not all the MFGM proteins are glycoproteins and MFGM lipids contain glycolipids. Furthermore, from studies on detergent-resistant membranes (DRM), lipid rafts are thought to contain also glycolipids (*40*). However Evers (*13*) and Lopez and coworkers (*14*) did not report fluorescence from the lectin probe inside the domains when observing the MFGM using CLSM suggesting that the glycolipids could be located at the boundary of the domains and therefore carried away with the domain material during the extraction with nonionic detergents in DRM experiments.

Membrane proteins play a complex role in domain organization. Robenek et al. (*8*) presented a very illustrative work on protein localization of three major MFGM proteins, butyrophilin, xanthine oxidase and adipophilin by freeze-fracture immunocytochemistry and protein gold-labeling. This study showed the triple phospholipid layer structure of the MFGM, a monolayer surrounding the lipid core and a continuous bilayer. Xanthine oxidase, which represents 20% of the total MFGM proteins (*3*), is located in the monolayer surrounding the fat core; adipophilin was found in the monolayer and in the inner leaflet of the bilayer; butyrophilin, which accounts for 20 to 40% of the total MFGM proteins depending on the cow breed (*3*), was found in both the monolayer and the bilayer but with more abundance in the monolayer. The staining of the MFGM glycan-containing compounds with lectins performed by Evers (*13*) and Lopez and co-workers (*14*) reveals, among glycolipids and other glycoproteins, the location of butyrophilin in the outer leaflet of the bilayer.

Membrane proteins may act as a stabilizer of domain boundaries. The incorporation of trans-membrane proteins in the lipid rafts seems unlikely as they could create local perturbations in the ordering of the L_o domains. However some membrane proteins have been shown to partition preferentially into the lipid rafts, playing a role in signal trafficking (*40*). Ongoing work in our laboratory on model systems derived from the MFGM is currently underway to determine the location of the proteins within the MFGM and to further understand physicochemical aspects of lipid−protein interactions. We are also investigating the phase coexistence in the MFGM at physiological and milk storage temperatures. We have found that phase separation occurs also at temperatures higher than the physiological temperature (results not published), providing evidence that lipid microdomains are present at physiological temperature and that the presence of dark regions in the above CLSM images is the result of a phase separation phenomenon and not due to crystallization of phospholipids upon cooling.

Whether or not lipid microdomains are present on both sides of the bilayer leaflet is still an arguable point. Lopez et al. (*14*) provided a schematic representation of the MFGM structure showing the presence of microdomains of sphingomyelin and cholesterol on both sides of the bilayer. However, the distribution of the MFGM phospholipids is asymmetric, with PC and SM mostly on the outer leaflet and PE, PI and PS on the inner leaflet (*9*); thus the presence of lipid rafts in both sides of the bilayer remains questionable. However, few studies on supported lipid bilayers show that they actually do exist in both leaflets and superpose (*41*−*43*).

Conclusions. CLSM is a powerful tool, which can be employed to observe the MFGM in situ and in real time. It was possible to detect the lateral organization of the components of the outer leaflet of the MFGM bilayer. The phospholipids are organized as a liquid-disordered phase coexisting with a liquid-ordered phase, the latter likely being rich in sphingomyelin and cholesterol. Domains observed on the outer surface of the fat globules showed differences in the structural properties of the surface upon milk processing, size of the globules and phospholipid composition of the MFGM. This comparative study on native membranes of milk fat globules also showed the association of milk proteins with MFGM material upon processing. An unexpected observation was made on processed cream, where, besides the addition of milk proteins to the surface of the membrane, it was possible to observe similar organization of phospholipids as in native globules. This argues to the possibility of membrane component reorganization after the drastic change of homogenization. Further research should focus on the potential loss of functional and nutritional qualities when milk is processed, with concomitant altered milk fat globule membrane structure. The milk fat globule membrane plays an important functional role in dairy and other food products through the suspension of the lipid droplet and the interactions with the surrounding medium. Whether the MFGM is associated or not with milk proteins will affect its functionality. New products could also be developed by selecting milk fat globules according to their size.

ABBREVIATIONS USED

MFGM, milk fat globule membrane; CLSM, confocal laser scanning microscopy/microscope; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; GUV, giant unilamellar vesicle; 2/3-D, two/three-dimension; DIC, differential interference contrast; DRM, detergent resistant membrane.

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