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Human Milk Fat Globules from Different Stages of Lactation: A Lipid Composition Analysis and Microstructure Characterization

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ABSTRACT: The physicochemical properties of human milk fat globules (MFG) at different lactation stages from Danish mothers and the microstructure changes of MFG membrane (MFGM) at varied temperatures were investigated, and the relationship between chemical composition and the microstructure of MFGM was elucidated. The fat content in MFG was found to be significantly increased as lactation progressed, and colostrum MFG had the largest mean diameter of 5.75 ± 0.81 μm and the lowest ζ potential of −5.60 ± 0.12 mV. Chemical composition analyses of MFG revealed the following: (i) Colostrum milk fat constituted higher content in PUFAs (ω-6, and long-chain ω-6 and ω-3) than transitional and mature milk fats, with the corresponding lower content of SFA in its sn-2 position. (ii) The content of polar lipids among total lipids varied during lactation course (maximized at transitional stage); however, in terms of subclasses of polar lipids, no significant change of the relative content of sphingomyelin was observed, while the content of phosphatidycholine in mature milk was higher than that in colostrum and transitional milk. (iii) Inspection of fatty acid composition in phospholipids from different lactation milk revealed no remarkable and regular changes could be generalized; and no obvious difference of the morphologies of MFGM at different lactation stages can be visualized. An investigation of the microstructure change of MFGM vs temperature demonstrated that the segregated domains became larger as temperature decreased to 4 °C, while it became smaller when increased to 37 °C. This phenomenon indicated that, in addition to sphingimyelin and cholesterol, phospholipids might also contribute to increasing the segregated domains at lower temperature, while, at elevated temperature, these domains could be diminished, most likely due to a restructuring or distributing of sphingimyelin and cholesterol.

KEYWORDS: *human milk fat globule, lactation, lipid composition, membrane, phospholipids, sphingomyelin, liquid-ordered phase, microstructure*

INTRODUCTION

Human milk provides the required dietary energy, essential nutrients, as well as physiologically active molecules for breast-fed infants. The lipids in the human milk are present in the form of colloidal assemblies called human milk fat globules (MFG). MFG have a special physical structure with a triacylglycerol (TAG) core inside and trilayer membrane outside, which is derived from a unique secretion mechanism. The lipid droplets synthesized in the endoplasmic reticulum are budded into cytoplasm with a diameter of <0.5 μm, termed microlipid droplets. These droplets grow in volume by fusing with each other, migrate to the apical pole of the cell, and finally are secreted.[1,2] During these courses, a trilayer membrane, commonly referred to as the milk fat globule membrane (MFGM), is formed, that is, a monolayer inner membrane with an electron-dense material composed of proteins and phospholipids derived from endoplasmic reticulum and a bilayer membrane from the apical plasma membrane of the epithelial cells that surround fat globules.[3] Due to the difference of lactation stages, dietary habits, and genetics, etc., the physiochemical properties of human MFG, including size distribution, fatty acid composition, and distribution of TAG, and polar lipids etc., are quite different. However, some common features can also be found by scientific sampling and analysis. The most important feature for human milk TAG is that 60−70% of palmitic acid is located at the sn-2 position, which is beneficial for the absorption of palmitic acid and calcium and has a great function in the development of infants.[4]

The human MFGM helps to stabilize the MFG in an emulsion within the aqueous environment of milk, which is mainly composed of cholesterol, polar lipids, protein, glycoproteins, gangliosides, and enzymes.[5] Some of the substances, such as glycoproteins, protect the infant from infection by acting as specific bacterial and viral ligands preventing the attachment of pathogens to the intestinal mucosa.[6] The polar lipids are the backbone of human MFGM constituted of phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM). The distribution of MFGM polar lipids is asymmetric, with PC and SM largely located in the outer layer of membrane and PE, PI, and PS concentrated in the inner surface.[7] This distribution has a great influence on the microstructure of MFGM. SM differs from other polar lipids in containing long-chain saturated fatty acids; thus, it has a higher melting temperature.[8] At room temperature, SM

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segregates from the glycerolphospholipids and packs them together with cholesterols to form some special domains with ordered molecular arrangement, named the liquid-ordered phase, and the surrounding glycerolphospholipids, mostly composed of unsaturated fatty acids with high fluidity, were in the liquid-disordered phase. The liquid-ordered domains are so tightly packed that other substances cannot insert into this area, whereas the liquid-disordered domains with high fluidity and disordered molecular arrangement can be easily inserted. This special character of the two different phases makes it possible to investigate the phase changes in the MFGM by using some fluorescent probes.[9−13] The special liquid-ordered domains on the MFGM are similar to the lipid rafts on the cell membrane, which serve as organized centers for assembly of signal molecules, influencing membrane protein trafficking and regulating neurotransmission and receptor trafficking.[14,15] However, whether or not these liquid-ordered domains in the human MFGM have some special functions still remains unknown.

Up until now, some literatures have reported the liquid-ordered domains and their formation mechanisms on bovine and human MFGM at room temperature. In addition to room temperature, the investigation of microstructures of MFGM at fridge temperature (4 °C, for storage purpose) and body temperature (37 °C, as the ultimate destination) is of scientific and practical interest, which may provide useful information about how liquid-ordered and liquid-disordered domains change in MFGM as a function of temperature, as well as how these changes are related to the lipid composition of MFGM. Therefore, this study was attempted to determine the physiochemical characteristics of human MFG at different stages of lactation and to investigate the microstructure variation of human MFG at different temperatures so as to further elucidate the relationship between the lipid composition of human MFGM and the variation of microstructure. The results might be useful to better understand the relationship of human MFGM structure and its function, as well as to advise regarding a more suitable infant milk formula.

MATERIALS AND METHODS

Samples and Reagents. Forty-five human milk samples at different stages of lactation (colostrum, 1−5 days; transitional milk, 6−15 days; mature milk, after 16 days) were individually donated by apparently healthy Danish mothers in Aarhus University Hospital, who had been well informed before participating in the project. The samples were characterized within 24 h after collection and then stored at −20 °C for further chemical analysis. The lipid-soluble Nile Red fluorescent dye (9-diethylamino-5H-benzoalpha-phenoxazine-5-one; Sigma-Aldrich, St. Louis, MO) was prepared at the concentration of 1 mg/mL in acetone and used to stain the triacylglycerol core of the human MFG. The fluorescent dye *N*-(lissamine rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rd-DOPE, 1 mg/mL in chloroform), used to label the phospholipid membrane, was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The phospholipid standards were supplied by Sigma-Aldrich, St. Louis, MO: PE (L-α-phosphatidylethanolamine, dioleoyl, purity 99%), PI (L-α-phosphatidylinositol ammonium salt from soybean; purity 98%), PS (1,2-diacyl-*sn*-glycero-3-phospho-L-serine from bovine brain; purity 97%), PC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; purity 99%), and SM (sphingomyelin from bovine brain; purity 97%). Ten percent of BF_3 methanol solution and silicic acid 60G TLC plates were purchased from Sigma-Aldrich, St. Louis, MO. The solvents used (methanol, chloroform, hexane, diethyl ether, heptane) were all of HPLC purity.

Size Distribution. The size distributions of human MFG were determined with integrated light scattering on a Mastersizer 2000 (Malvern Instruments Ltd., Malvern WR14 1XZ, U.K.). The refractive index for human MFG was 1.460 and 1.458 at 466 and 633 nm, respectively.[16] The casein micelles were dissociated by diluting the milk in 35 mM EDTA, pH 7 buffer, and the sample was diluted in the measurement cell to reach 10% obscuration. The size distribution was evaluated by volume-surface average diameter (d_{32}, defined as $\sum n_i d_i^3 / \sum n_i d_i^2$) and the volume-weighted average diameter (d_{43}, defined as $\sum n_i d_i^4 / \sum n_i d_i^3$), where n_i is the number of globules in a size class of diameter d_i. The span was defined as $(d_{0.9} - d_{0.1})/d_{0.5}$, where $d_{0.9}$ is the diameter below which lies 90% of the globule volume and 10% for $d_{0.1}$, 50% for $d_{0.5}$, respectively. The specific surface area was calculated by the software as $S = 6.\phi/d_{32}$, where ϕ is the volume fraction of milk fat.

ζ Potential. Samples were prepared by suspending 3−5 μL milk in 10 mL of buffer (20 mM imidazole, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.0),[16] and ζ potential was measured at 25 °C on a Malvern Zetasizer Nanoseries ZS (Malvern Instruments, Worcestershire, U.K.) in triplicate.

Lipid Analysis. *Extraction of Total Lipids.* Total lipids were extracted from the freeze-drying samples by homogenization with chloroform/methanol (2:1, vol/vol) as described by Folch et al.[17] The extract was shaken and equilibrated with one-fourth volume of a saline solution (NaCl 0.86%, w/w). The solvent phase was filtered and evaporated under vacuum, and the obtained total lipids were stored at −20 °C for further chemical analysis.

Analysis of Total Fatty Acid Composition. Fatty acid methyl esters (FAMEs) were prepared according to the AOCS method Ce-1b 89 (2007) and subsequently analyzed on a gas chromatograph (GC) (Thermo-Fisher Scientific, Waltham, MA) equipped with an autosampler, a flame ionization detector, and an ionic liquid capillary column (Supelco SLB-IL 100, 60 m × 0.25 mm × 0.2 μm, Sigma-Aldrich, St. Louis, MO). Helium was used as the carrier gas with a flow rate of 1 mL/min. The column oven temperature was kept at 170 °C, and the running time for each sample was 60 min. The injection port and detector temperatures were both set at 250 °C. The FAMEs were identified by comparing the retention time with the standards, and the relative contents expressed as mol % were then calculated.

Analysis of sn-2 Fatty Acid Composition. Hydrolysis of TAG to sn-2 monoacylglycerols (MAG) was carried out according to the method detailed by Luddy et al.[18] The hydrolytic products were separated on silica gel G TLC plates, and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The band corresponding to sn-2 MAG was scraped off and extracted twice with diethyl ether. The solvent was then removed by nitrogen, and the residue was methylated as described above and analyzed on a GC.

Analysis of Polar Lipids. Analysis of polar lipid classes was carried out on a high-performance liquid chromatograph (HPLC) equipped with an evaporative light scattering detector (ELSD) as described by Rombaut et al.[19] with some modification. Nitrogen was used as the nebulizing gas at a flow rate of 1 L/min, and the evaporating temperature was set at 85 °C. A silica column (4.6 mm × 250 mm, 5 μm particle size) conjugated with a precolumn was used. The elution program was isocratic conditions with 87.5:12:0.5 (vol/vol/vol) choloroform/methanol/triethylamine buffer (pH 3, 1 M formic acid) from 0 to 10 min and then a linear gradient with 87.5:12:0.5 (vol/vol/vol) at t = 11 min to 28:60:12 (vol/vol/vol) at t = 45 min. The mobile phase was brought back to the initial conditions at t = 47 min, and the column was allowed to equilibrate until the next injection at t = 55 min. The flow rate was maintained at 0.5 mL/min, the injection volume was 10 μL, and the samples and the column were equilibrated at 40 °C. The identification of polar lipids was carried out by comparison with the retention time of pure standards, and the quantitation of polar lipids was achieved by using calibration curves: PE, $y = 0.001x^{0.9735}$ ($r^2 = 0.9917$); PI, $y = 0.0016x^{0.9106}$ ($r^2 = 0.9976$); PS, $y = 0.0012x^{0.9719}$, ($r^2 = 0.9946$); PC, $y = 0.0024x^{0.8924}$ ($r^2 = 0.9951$); SM, $y = 0.0015x^{0.9459}$ ($r^2 = 0.9911$), which were obtained by injection of different amounts of PE (0.1−2 μg), PI (0.2−2 μg), PS (0.5−3 μg), PC (1−4 μg), and SM (1−6 μg).

Analysis of Fatty Acid Composition of Phospholipids. The polar lipids were separated from the total lipids by silica gel G TLC plates

with the developing solvent system of hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol). The polar lipids was scraped off from the baseline and extracted with 3 mL of a chloroform/methanol/water (5:5:1, vol/vol/vol) mixture.[20] After centrifugation at 4000 rpm for 10 min, organic phase was collected. The remaining water phase was extracted twice with the same method, and the organic solvent was pooled and evaporated. Three hundred microliters of BF_3 methanol solution was added for methylation, and the screw-capped tubes were kept at 100 °C for 90 min. Six hundred microliters of heptane and 500 μm of saturated NaCl solution were added. The mixture was centrifuged at 4000 rpm for 10 min at 20 °C, and the solvent phase was collected and dried by anhydrous sodium sulfate. After centrifugation, the upper layer was injected into GC for fatty acid analysis.

Microstructural Analysis. The neutral lipids were stained by Nile Red fluorescent probe by adding 10 μL of solution into 0.5 mL of human milk. The samples were mixed by gentle swirling and inversion of the vial until the even dispersion of the fluorescent probe. The mixed samples were kept at room temperature for 20 min before microstructural analysis. One hundred microliters of the stained milk was taken out into a vial, and 100 μL of agarose (10 g/L in deionized water, kept in 45 °C) was added. 25 μL of mixture was taken out to a slide, and a coverslip was then applied rapidly without excessive pressure. The phospholipids in the human MFGM were labeled by Rd-DOPE with 5 μL of solution into 0.5 mL of milk, and the latter procedures were the same as described above. The prepared slides were kept at room (20 °C), storage (4 °C), and body temperature (37 °C) for 30 min for redistribution of the fluorescent probe, respectively. The slides were then used for microstructural analysis.

The microstructure of human MFG was analyzed with a Zeiss LSM 510 Meta confocal microscope. A 63 × 1.4 oil immersion objective was used for all images. Nile red and Rh-DOPE were both exited with the 543 nm line of the He−Ne laser, and the emission was captured with the 560 to 620 nm band-pass filters.

Statistical Analysis. The data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System software (SAS, Cary, NC). The significance level being tested was $\alpha = 0.05$, and differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Size Distribution and Apparent ζ Potential of Human MFG. The size distributions of colostrum, transitional, and mature MFG, determined by laser light scattering, ranged from 0.63 to 45.71, 0.55 to 11.48, and 0.72 to 17.37 μm, respectively (Figure 1). The colostrum had a much broader size distribution

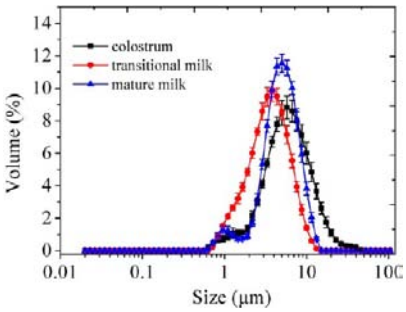

Figure 1. Size distribution of human MFG at different lactation stages determined using laser light scattering.

with a maximum volume % at 5.75 μm, and the transitional and mature milk had narrower size distributions with maximum volume % values at 3.80 and 5.01 μm, respectively. Both colostrum and mature milk had two peaks, which meant these two types of milk might contain two groups of MFG. Fat globules of different sizes in milk had different chemical compositions, especially some submicrometer globules as reported by Argov et al.[21] These nanoscale MFG, lacking significant TAG components analyzed by laser trapping confocal Raman spectroscopy, different from other TAG enriched globules acting mainly as TAG deliverers, might have a new mechanism of formation, and thus a new function. Therefore, the inconsistent size distributions of human MFG at different stages of lactation might be associated with their specific physiological functions for the growth of infants at each stage. The physicochemical characteristics of the fat globules from colostrum and transitional and mature milk are presented in Table 1. The average diameters (D_{43}) of MFG at different

Table 1. Size Distribution, Apparent ζ Potential, and Fat Content of Human MFG from Colostrum and Transitional and Mature Milk[a]

size parameter	colostrum	transitional milk	mature milk
D_{32} (μm)	3.51 ± 0.53 a	3.14 ± 0.62 a	3.25 ± 0.22 a
D_{43} (μm)	5.75 ± 0.81 a	4.01 ± 0.76 b	4.74 ± 0.33 c
span	1.74 ± 0.30 a	1.40 ± 0.19 b	1.57 ± 0.13 b
specific surface area (m^2/g fat)	1.81 ± 0.44 a	2.33 ± 0.55 b	2.05 ± 0.26 a
ζ potential (mV)	−5.60 ± 0.12 a	−6.72 ± 0.16 b	−7.25 ± 0.61 c
fat content (g/kg)	2.86 ± 0.22 a	3.15 ± 0.21 b	3.60 ± 0.33 c

[a]Means ± SD with the same letter are not significantly different at the 0.05 probability level.

stages of lactation were significantly different. The average diameter of fat globules in colostrum (5.75 ± 0.81 μm) was larger than those in transitional (4.01 ± 0.76 μm) and mature milk (4.74 ± 0.33 μm), and at the same time the average diameters of globules in colostrum varied significantly from more than 8 μm at the beginning of lactation to around 5 μm on the fifth day, whereas the average diameters of mature MFG were relatively stable at around 4.5 μm, although they gradually increased with lactation advancing. Similar results were also reported by Michalski et al.[22] However, Ruegg et al. [23] reported that the volume average diameters of human MFG increased with the duration of lactation from 1.74 μm in colostrum to 4.10 μm in mature milk. The different results might be due to differing dietary habits or genetics of different people. As seen in Table 1, the transitional milk globules had the smallest average diameter and, thus, had the highest specific surface area and the colostrum globules had a significantly larger span compared with transitional and mature milk, indicating a wider particle size distribution, as shown in Figure 1.

The composition of the human milk varies considerably during lactation, with the major changes usually occurring soon after the beginning of lactation. The amount of milk fat significantly increased with the duration of lactation. This result was similar to those of Boersma et al.[24] and Bitman et al.[25] The surface potential cannot be measured directly, but the ζ potential, which approximates the potential at a certain distance from the particle surface, can be measured electrokinetically.[26] The ζ potentials for colostrum, transitional, and mature MFG were −5.60 ± 0.12, −6.72 ± 0.16, and −7.25 ± 0.61 mV, respectively, indicating an increasing tendency as lactation progressed. Michalski et al.[16] also reported the ζ potential of mature MFG was −7.8 ± 0.1 mV, and Lopez et al.[11] reported it

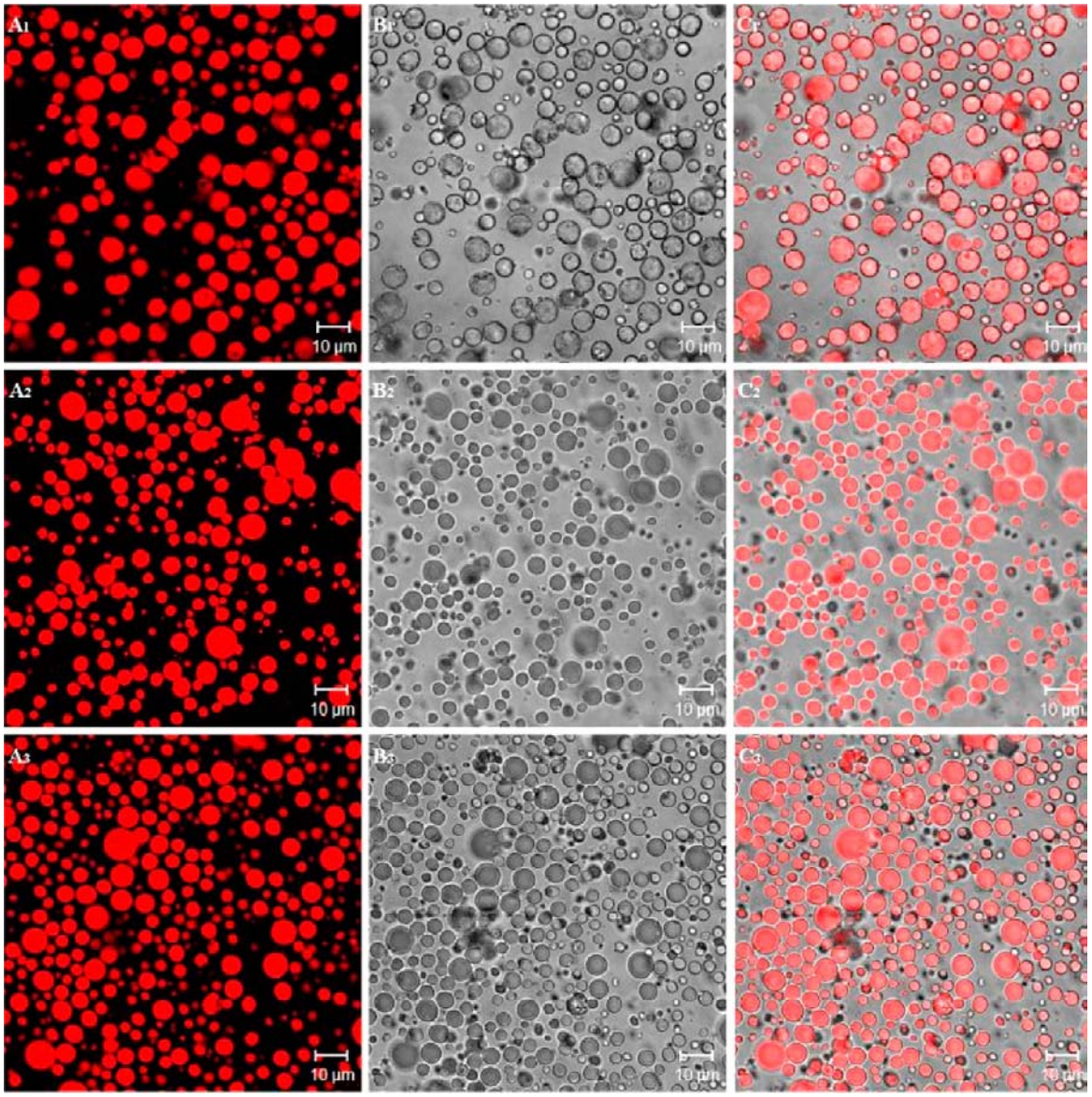

Figure 2. Microstructure of human MFG observed using CLSM with TAG stained by a Nile Red fluorescent probe (A_1, A_2, and A_3), optical microscopy with differential interferential contrast (B_1, B_2, and B_3), and the overlay images (C_1, C_2, and C_3). Colostrum (A_1, B_1, and C_1), transitional milk (A_2, B_2 and C_2), and mature milk (A_3, B_3, and C_3).

as −7.6 ± 0.5 mV. To our best knowledge, this was a first report about the change of ζ potentials of human MFG during the course of lactation. Compared with the ζ potential of MFG from other mammal animals such as cow MFG (−13.5 ± 0.9 mV)[16] and buffalo MFG (−11.0 ± 0.7 mV),[27] human MFG values were obviously lower than them. The differences of ζ potential of human MFG from different stages of lactation or from different species were largely due to the different composition of polar lipids and proteins in MFGM, and minerals present in the aqueous environment. From the colloidal point of view, MFG with high ζ potential are electrically stabilized while globules with low ζ potentials tend to coagulate or flocculate. However, the low ζ potential of human MFG may be of special benefit to the digestion and metabolism of human milk fat in infant.

The Nile Red fluorescent probe labeled human MFG from different stages of lactation was observed by confocal laser scanning microscopy (CLSM), shown in Figure 2. The TAG was present in the milk in the form of spherical droplets with polydispersed size distribution. In agreement with the measurement by laser light scanning, colostrum had more larger size fat globules and transitional milk had more smaller size ones. The milk fat droplets were budded out from the endoplasmic reticulum into the cytosolic compartment and secreted out of the cell. During these courses, the droplets were enveloped with a trilayer membrane.[1] Therefore, if the structures of the milk globules were not mechanically or thermally damaged, the TAG should be exclusively located in the core of the fat globules. This structure was proved by the overlapped images (Figure 2C_1, C_2, and C_3) from CLSM (Figure 2A_1, A_2, and A_3) and DIC (Figure 2B_1, B_2, and B_3). Additionally, Nile Red could pass through the human MFGM to stain the TAG in the core of the globules, indicating that the permeability of human MFGM allowed the transportation of some molecules.

Fatty Acid Composition and Positional Distribution of TAG in Human MFG. The fat globules from different phases

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Table 2. Fatty Acid Composition and Distribution of Human MFG from Colostrum and Transitional and Mature Milk[a]

	TAG			sn-2		
fatty acid[b]	colostrum	transitional	mature	colostrum	transitional	mature
C6:0	0.02 ± 0.01 a	0.04 ± 0.03 ab	0.07 ± 0.05 b	0.03 ± 0.02 d	0.05 ± 0.02 d	0.10 ± 0.06 e
C8:0	0.10 ± 0.01 a	0.17 ± 0.07 ab	0.24 ± 0.13 b	0.10 ± 0.03 d	0.14 ± 0.05 d	0.20 ± 0.08 e
C10:0	0.57 ± 0.21 a	1.56 ± 0.43 b	2.81 ± 0.93 c	0.43 ± 0.14 d	1.29 ± 0.41 e	1.55 ± 0.40 e
C12:0	3.56 ± 0.79 a	7.43 ± 1.71 b	9.84 ± 1.48 c	2.71 ± 1.07 d	7.32 ± 4.81 e	8.33 ± 2.73 e
C14:0	6.45 ± 0.57 a	9.01 ± 1.97 b	10.23 ± 1.39 b	8.44 ± 1.56 d	12.89 ± 2.43 e	14.47 ± 1.00 e
C14:1 ω-5	0.13 ± 0.03 a	0.34 ± 0.08 b	0.48 ± 0.01 c	0.44 ± 0.18 d	0.45 ± 0.27 d	0.52 ± 0.19 d
C16:0	26.59 ± 3.27 a	24.88 ± 4.49 ab	22.84 ± 2.30 b	55.31 ± 3.80 d	53.82 ± 3.10 d	52.63 ± 3.36 d
C16:1 ω-7	1.42 ± 0.48 a	2.35 ± 0.35 b	2.11 ± 0.44 b	1.90 ± 0.30 d	1.36 ± 0.78 d	2.12 ± 1.55 d
C18:0	8.13 ± 2.17 a	6.64 ± 1.30 b	5.64 ± 0.57 b	2.47 ± 0.36 d	1.35 ± 0.37 e	1.31 ± 0.16 e
C18:1 ω-9	36.21 ± 4.26 a	33.63 ± 3.13 ab	32.06 ± 4.55 b	13.25 ± 3.17 d	10.10 ± 2.3 e	9.25 ± 2.89 e
C18:2 ω-6	9.67 ± 0.95 a	8.63 ± 0.46 b	8.37 ± 0.74 b	8.53 ± 2.69 d	6.74 ± 1.38 e	5.63 ± 0.94 e
C18:3 ω-6	0.01 ± 0.01 a	0.04 ± 0.02 a	0.07 ± 0.03 b	0.01 ± 0.01 d	0.02 ± 0.01 d	0.04 ± 0.02 e
C18:3 ω-3	0.56 ± 0.09 a	0.62 ± 0.11 a	0.86 ± 0.17 b	0.70 ± 0.41 d	0.48 ± 0.24 de	0.31 ± 0.16 e
C20:0	0.45 ± 0.13 a	0.20 ± 0.05 b	0.17 ± 0.04 b	0.35 ± 0.14 d	0.24 ± 0.11 d	0.50 ± 0.14 e
C20:1 ω-9	0.19 ± 0.03 ab	0.26 ± 0.13 a	0.15 ± 0.03 b	0.29 ± 0.12 d	0.14 ± 0.05 e	0.15 ± 0.06 e
C20:2 ω-6	0.74 ± 0.09 a	0.54 ± 0.17 b	0.36 ± 0.10 c	0.46 ± 0.08 d	0.10 ± 0.04 e	0.09 ± 0.02 e
C20:3 ω-6	0.64 ± 0.25 a	0.52 ± 0.15 a	0.32 ± 0.12 b	0.39 ± 0.23 d	0.11 ± 0.03 e	0.18 ± 0.06 e
C20:4 ω-6	0.92 ± 0.40 a	0.41 ± 0.18 b	0.36 ± 0.14 b	0.93 ± 0.33 d	0.18 ± 0.08 e	0.25 ± 0.14 e
C20:5 ω-3	0.14 ± 0.05 a	0.20 ± 0.14 a	0.22 ± 0.10 a	0.17 ± 0.06 d	0.25 ± 0.15 de	0.31 ± 0.10 e
C22:0	0.39 ± 0.16 a	0.11 ± 0.05 b	0.06 ± 0.03 b	0.29 ± 0.05 d	0.21 ± 0.10 d	0.15 ± 0.05 e
C22:1 ω-9	0.36 ± 0.08 a	0.25 ± 0.09 b	0.18 ± 0.03 c	0.15 ± 0.02 d	0.06 ± 0.01 e	0.05 ± 0.01 e
C22:2 ω-6	0.15 ± 0.05 a	0.11 ± 0.08 a	0.05 ± 0.02 b	0.29 ± 0.14 d	0.13 ± 0.04 e	0.23 ± 0.09 d
C24:0	0.17 ± 0.06 a	0.12 ± 0.06 a	0.07 ± 0.04 b	0.09 ± 0.03 d	0.18 ± 0.07 e	0.05 ± 0.04 d
C24:1 ω-9	0.40 ± 0.10 a	0.18 ± 0.03 b	0.13 ± 0.05 c	0.56 ± 0.16 d	0.24 ± 0.05 e	0.18 ± 0.08 e
C22:4 ω-6	0.30 ± 0.02 a	0.23 ± 0.11 a	0.14 ± 0.07 b	0.39 ± 0.08 d	0.25 ± 0.12 e	0.10 ± 0.01 f
C22:5 ω-6	0.19 ± 0.04 a	0.13 ± 0.03 b	0.14 ± 0.09 ab	0.40 ± 0.11 d	0.25 ± 0.04 e	0.15 ± 0.07 f
C22:5 ω-3	0.34 ± 0.14 a	0.24 ± 0.09 a	0.26 ± 0.06 a	0.61 ± 0.24 d	0.48 ± 0.10 d	0.34 ± 0.12 e
C22:6 ω-3	0.71 ± 0.23 a	0.54 ± 0.29 ab	0.42 ± 0.18 b	0.80 ± 0.17 de	1.20 ± 0.80 d	0.66 ± 0.38 e
SFAs	47.13 ± 6.99 a	50.17 ± 2.75 a	50.56 ± 4.34 a	70.10 ± 6.09 d	77.48 ± 3.96 e	79.38 ± 6.73 e
MC-SFAs	4.41 ± 1.07 a	9.20 ± 1.34 b	12.96 ± 3.39 c	3.36 ± 0.85 d	8.79 ± 5.28 e	10.28 ± 2.43 e
LC-SFAs	42.73 ± 5.92 a	40.96 ± 3.56 a	37.61 ± 1.32 b	66.74 ± 5.25 d	68.69 ± 1.32 e	69.11 ± 4.31 e
MUFAs	38.59 ± 6.89 a	37.01 ± 3.22 a	35.11 ± 4.96 a	16.48 ± 3.23 d	12.35 ± 1.05 e	12.28 ± 4.19 e
PUFAs	14.16 ± 0.62 a	12.22 ± 0.34 b	11.57 ± 0.50 b	13.43 ± 3.18 d	10.17 ± 2.89 e	8.31 ± 1.53 e
PUFAs ω-3	1.75 ± 0.29 a	1.60 ± 0.44 a	1.76 ± 0.40 a	2.28 ± 0.30 de	2.41 ± 1.29 d	1.64 ± 0.31 e
LC-PUFAs ω-3	1.19 ± 0.38 a	0.98 ± 0.34 a	0.90 ± 0.29 a	1.58 ± 0.11 de	1.93 ± 1.05 d	1.30 ± 0.47 e
PUFAs ω-6	12.41 ± 0.57 a	10.62 ± 0.63 b	9.81 ± 0.49 b	11.15 ± 3.49 d	7.76 ± 1.61 e	6.67 ± 0.54 e
LC-PUFAs ω-6	2.89 ± 0.79 a	1.95 ± 0.19 b	1.37 ± 0.44 c	2.85 ± 0.80 d	1.00 ± 0.23 e	1.02 ± 0.41 e
LA/LnA	17.11 ± 1.32 a	13.36 ± 2.66 b	9.21 ± 2.39 c	10.62 ± 3.62 de	13.63 ± 1.92 d	15.43 ± 1.05 e
ω-6/ω-3	7.22 ± 1.30 a	7.08 ± 2.54 a	6.01 ± 2.98 a	4.90 ± 2.28 d	3.22 ± 0.62 e	3.06 ± 1.09 e
LC ω-6/LC ω-3	2.45 ± 0.12 a	2.25 ± 1.14 a	1.73 ± 1.11 a	1.81 ± 0.39 d	0.52 ± 0.16 e	0.79 ± 0.64 e

[a]Means ± SD with the same letter are not significantly different at the 0.05 probability level. [b]SFAs, saturated fatty acid; MC-SFAs, medium-chain SFAs; LC-SFAs, long-chain SFAs; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LA, linoleic acid; LnA, linolenic acid.

of lactation have a substantial difference in the chemical composition. Although the fatty acid composition and distribution of milk fat have a minor impact on the microstructure of MFG, they are of great significance to infant nutrition. Some literatures reported the fatty acid composition and positional distribution of human milk fat in different countries.[24,28−30] However, due to the difference of dietary habits and genetics etc., the results were different. The fatty acid composition and positional distribution of fat globules from the Danish mothers are given in Table 2. Regarding the fatty acid composition of milk fat, the content of total SFAs did not vary significantly with the duration of lactation. Similar results were also reported by Wang et al.[31] As for the MUFAs, the total content did not significantly change during lactation course. Some studies reported that MFG sizes were highly associated with their composition in both bovine and human milk.[10,21,32,33] As reported before, the mean diameters of human MFG differed significantly among different stages of lactation, whereas the insignificance in the contents of total SFA and MUFA was observed in this study. These inconsistencies might result from factors such as sampling days postpartum, separation methods, genetics, etc. The colostrum contained a higher amount of PUFAs than the transitional and mature milk did. However, no significant difference of PUFAs content between transitional and mature milk was observed. The contents of PUFAs ω-6 and LC-PUFAs ω-6 varied significantly among colostrum and transitional and mature milk; however, PUFAs ω-3 and LC-PUFAs ω-3 did not. Colostrum contained a significantly higher amount of ω-6 fatty acids than traditional and mature milk did, and the content of LC-PUFAs ω-6 significantly increased as the lactation progressed. Among the two essential PUFAs, the colostrum

Table 3. Concentration of Polar Lipids (wt %) of Colostrum and Transitional and Mature Milk and Relative Proportion of Each Class of Polar Lipids[a]

polar lipids[b]	conc of polar lipids (mg polar lipids per total lipids)			relative proportion of polar lipids (% of polar lipids)		
	colostrum	transitional milk	mature milk	colostrum	transitional milk	mature milk
PE	0.41 ± 0.03 a	0.77 ± 0.12 b	0.76 ± 0.10 b	9.33 ± 1.09 a	13.16 ± 0.93 b	15.04 ± 1.47 b
PI	0.36 ± 0.02 a	0.40 ± 0.03 ab	0.41 ± 0.02 b	8.31 ± 0.98 a	6.81 ± 0.41 a	8.17 ± 1.17 a
PS	0.56 ± 0.03 a	0.81 ± 0.05 b	0.84 ± 0.06 b	12.67 ± 1.60 a	13.88 ± 1.58 a	16.71 ± 1.82 b
PC	1.26 ± 0.19 ab	1.50 ± 0.13 a	1.07 ± 0.11 b	28.46 ± 1.64 aa	25.73 ± 2.22 a	21.33 ± 2.36 b
SM	1.82 ± 0.26 a	2.37 ± 0.40 a	1.97 ± 0.33 a	41.23 ± 2.02 a	40.42 ± 3.28 a	38.75 ± 3.89 a
total	4.42 ± 0.40 a	5.86 ± 0.35 b	5.06 ± 0.38 c			

[a]Mean ± SD with the same letter are not significantly different at the 0.05 probability level. [b]PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

had significantly more content of C18:2 ω-6 than those of mature and transitional milk, whereas the content of C18:3 ω-3 in mature milk was significant more than those of colostrum and transitional milk, and the C18:2 ω-6/C18:3 ω-3 decreased with the duration of lactation. As for some individual PUFAs ω-3, which was important for the development of infant intelligence, the amount of C20:5 ω-3 was not significantly varied and the amount of C22:6 ω-3 in colostrum was more than that in mature milk. The ratios of ω-6/ω-3 and LC ω-6/LC ω-3 did not significantly change among colostrum and transitional and mature milk.

The special fatty acid positional distribution is the most important feature of human milk fat, which has a substantial influence on the assimilation and metabolism of the milk fat. Table 2 shows the Danish human milk fat has a high content of SFAs at the sn-2 position for colostrum and transitional and mature milk, and the content of total SFAs in mature and transitional milk is significantly higher than that in colostrum. The content of C16:0, which was the major SFA in milk fat, did not change significantly; thus, some other SFAs such as C10:0, C12:0, and C14:0 were responsible for this significance. The percentages of C16:0 at the sn-2 position, calculated as sn-2 FA × 100%/(3 × total FA), in colostrum and transitional and mature milk were 69.33 ± 5.12, 72.11 ± 5.41, and 76.81 ± 6.33%, respectively, increasing during lactation. This distribution was important for assimilation of palmitic acid and calcium. With regard to MUFAs and PUFAs at the sn-2 position, the colostrum had significantly higher contents than transitional and mature milk. For ω-6 fatty acids, the amounts of PUFAs ω-6 and LC-PUFAs ω-6 in colostrum were significant higher than those in transitional and mature milk, whereas, for ω-3 fatty acids, the amount of PUFAs ω-3 and LC-PUFAs ω-3 in traditional milk was significantly higher than that in mature milk. Other information regarding the sn-2 fatty acids such as C18:2 ω-6/C18:3 ω-3, ω-6/ω-3, and LC ω-6/LC ω-3 can be seen in Table 2.

Content and Fatty Acid Composition of Polar Lipid in Human MFG. The total amount and relative proportion of polar lipids in colostrum and transitional and mature milk are presented in Table 3. The contents of polar lipids at different stages of lactation were significantly different, with the content ranging from 0.4 to 0.6%, which were in agreement with the previous reported data (0.3−1%).[2] The difference in the contents of polar lipids was largely related to the sizes of the MFG. Lopez et al.[10] established the relationship between the polar lipid contents and diameters by using bovine MFG, which proved that the contents of polar lipids were negatively correlated with the diameter of MFG. The mean diameters of human MFG arranged in order from small to large size were transitional milk, mature milk, and colostrum, and the contents of polar lipids in these milks were negatively correlated with the diameter, which is in accordance with the results reported by Lopez et al. The main polar lipid classes in human MFGM were PC and SM (>20%), irrespective of the lactation, which were in agreement with the reported studies.[25,30,34] SM, which was reported to have great influence on the microstructure of MFGM, was the highest content species in human milk, and the difference of contents of SM at different stages of lactation was not significant. In terms of the content of PC, mature milk contained significantly less PC than the amounts in colostrum and transitional milk. Both SM and PC contained choline, which was required for the rapid growth of organ and membrane biosynthesis, and thus, the high content of PC and SM in human milk was of great importance for the development of infants. As for other polar lipids, the content of PS in mature milk was significantly more than those in colostrum and transitional milk, the content of PE in colostrum was significantly less than those in transitional and mature milk, and there were no significant difference in PI content among different kinds of milk.

The degree of unsaturation of fatty acids in polar lipids has great influence on the membrane fluidity.[35−37] Unsaturated lipids create a kink, preventing the fatty acids from packing together tightly, thus decreasing the melting temperature (increasing the fluidity) of the membrane. According to some literatures,[10−12] SM in the MFGM together with cholesterol is responsible for the phase separation of the membrane, which is mainly composed of SFAs.[19,38] For better understanding of the roles of phospholipids (PC, PE, PI, PS) in the microstructure variation of MFGM, the fatty acid composition of phospholipids was analyzed and presented in Table 4. The fatty acids of phospholipids from human MFGM at different phases of lactation had a high amount of SFAs, but with no significant difference. However, when the contents of SFAs were individually compared, mature milk had a significantly higher amount of MCFAs than colostrum and transitional milk, whereas the content of C16:0 was lower, and at the same time, colostrum contained a significantly lower content of C18:0 than transitional and mature milk. As for LC-SFAs with high melting temperature, the mature milk contained a significantly higher amount. For the contents of total MUFAs and PUFAs, the milks from different stages of lactation were not significantly different. However, the contents of PUFAs ω-3 in mature and traditional milk were significantly higher than that in colostrum, and the contents of LC-PUFAs ω-3 increased significantly with the duration of lactation. The content of LC-PUFAs ω-6 in mature milk was significantly higher whereas the difference of contents of PUFAs ω-6 in milk at different lactation stages was

Table 4. Fatty Acid Composition of Phospholipids from Human MFGM[a]

fatty acid[b]	colostrum	transitional milk	mature milk
C6:0	0.05 ± 0.03 a	0.06 ± 0.03 a	0.07 ± 0.02 a
C8:0	0.17 ± 0.08 a	0.18 ± 0.07 a	0.47 ± 0.15 b
C10:0	0.29 ± 0.11 a	0.26 ± 0.15 a	0.84 ± 0.18 b
C12:0	0.65 ± 0.17 a	1.14 ± 0.55 a	1.95 ± 0.56 b
C14:0	9.16 ± 1.50 ab	10.43 ± 1.89 a	6.98 ± 3.54 b
C14:1 ω-5	0.56 ± 0.21 a	0.78 ± 0.10 b	0.36 ± 0.11 c
C16:0	45.44 ± 3.46 a	42.69 ± 4.95 ab	37.36 ± 8.51 b
C16:1 ω-7	0.22 ± 0.12 a	0.61 ± 0.24 b	0.97 ± 0.40 c
C18:0	10.59 ± 0.65 a	13.43 ± 1.10 b	14.84 ± 2.78 b
C18:1 ω-9	14.64 ± 2.36 a	11.76 ± 4.54 a	13.63 ± 2.11 a
C18:2 ω-6	12.54 ± 1.10 a	11.32 ± 4.87 a	12.32 ± 3.97 a
C18:3 ω-6	0.03 ± 0.01 a	0.04 ± 0.01 ab	0.05 ± 0.02 b
C18:3 ω-3	0.46 ± 0.21 a	0.97 ± 0.20 b	1.09 ± 0.53 b
C20:0	0.12 ± 0.03 a	0.32 ± 0.13 b	0.61 ± 0.12 c
C20:1 ω-9	0.71 ± 0.14 a	0.61 ± 0.15 a	0.39 ± 0.13 b
C20:2 ω-6	0.26 ± 0.07 b	0.48 ± 0.16 a	0.18 ± 0.08 b
C20:3 ω-6	0.41 ± 0.11 a	0.67 ± 0.26 a	1.27 ± 0.48 b
C20:4 ω-6	0.99 ± 0.18 a	1.36 ± 0.35 ab	1.70 ± 0.35 b
C20:5 ω-3	0.18 ± 0.05	0.42 ± 0.07 a	0.53 ± 0.21 b
C22:0	0.10 ± 0.02 a	0.16 ± 0.07 b	0.20 ± 0.10 b
C22:1 ω-9	0.14 ± 0.04 a	0.12 ± 0.02 a	0.18 ± 0.04 b
C22:2 ω-6	0.27 ± 0.09 a	0.29 ± 0.05 a	0.69 ± 0.32 b
C24:0	0.37 ± 0.08 a	0.44 ± 0.18 ab	0.59 ± 0.27 b
C24:1 ω-9	0.28 ± 0.09 a	0.50 ± 0.23 b	0.69 ± 0.13 c
C22:4 ω-6	0.17 ± 0.09 a	0.19 ± 0.06 a	0.14 ± 0.04 a
C22:5 ω-6	0.18 ± 0.05 a	0.23 ± 0.02 b	0.27 ± 0.06 c
C22:5 ω-3	0.28 ± 0.09 a	0.31 ± 0.14 a	0.57 ± 0.13 b
C22:6 ω-3	0.33 ± 0.18 a	0.73 ± 0.10 b	1.03 ± 0.27 c
SFAs	67.36 ± 3.28 a	69.09 ± 8.63 a	63.92 ± 9.21 a
MC SFAs	1.27 ± 0.21 a	1.63 ± 0.31 a	3.10 ± 1.18 b
LC SFAs	66.09 ± 3.07 ab	67.46 ± 8.33 a	60.82 ± 9.15 b
MUFAs	16.56 ± 2.36 a	14.38 ± 4.13 a	15.90 ± 2.02 a
PUFAs	16.08 ± 0.92 a	17.00 ± 4.97 a	19.65 ± 6.63 a
PUFAs ω-3	1.20 ± 0.24 a	2.53 ± 0.41 b	3.02 ± 1.66 b
LC-PUFAs ω-3	0.74 ± 0.03 a	1.35 ± 0.90 b	2.13 ± 0.19 c
PUFAs ω-6	14.88 ± 1.76 a	14.57 ± 4.95 a	16.63 ± 4.99 a
LC-PUFAs ω-6	2.26 ± 0.35 a	2.93 ± 0.10 a	4.25 ± 1.01 b
LA/LnA	28.01 ± 10.18 a	10.89 ± 2.54 b	11.27 ± 1.84 b
ω-6/ω-3	12.53 ± 1.87 a	5.98 ± 2.91 b	6.02 ± 1.55 b
LC ω-6/LC ω-3	3.07 ± 0.60 a	2.75 ± 1.75 ab	2.00 ± 0.44 b

[a]Means ± SD with the same letter are not significantly different at the 0.05 probability level. [b]SFAs, saturated fatty acids; MC-SFAs, medium-chain SFAs; LC-SFAs, long-chain SFAs; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LA, linoleic acid; LnA, linolenic acid.

not statistically significant, which was due to the insignificant amount of C18:2 ω-6. Some studies also reported the fatty acid composition of phospholipids in human milk fat. Sala-Vila et al.[28] reported there were about 57% SFAs and 17% MUFA in the human milk membrane phospholipids, and the differences among total SFAs, MUFAs, and LC-PUFAs of different stages of lactation were not significant. Harzer et al.[30] reported around 50% SFAs existed in the human MFGM of different lactation stages, and the content of C18:2 ω-6 in mature milk was significantly higher than those in colostrum and transitional milk, with the value of about 23%, and increased as the lactation proceeded. Meanwhile, higher contents of LC-PUFAs were found in membrane phospholipids, among which the amount of C24:4 was obviously higher, with values of 7.6%, 6.7%, and 6.1% for colostrum and transitional and mature milk, respectively. Bitman et al.[38] also reported about 53% SFAs was found in phospholipids, and the amount of C18:2 ω-6 was about 20% for phospholipids at different stages of lactation. The difference in fatty acid composition of phospholipids was probably due to the different diets, genetics, and environment, etc. Compared with the fatty acid composition of milk fat, a significant difference could be observed in the contents of most fatty acids, which again indicated that the polar lipids and milk fat were of different origins, and this difference might be of biological importance for the development of infants.

Because of the high content of SFAs in phospholipids, there should be some phospholipids with SFAs esterified at the sn-1, 2 positions, which have substantial influences on the rigidity of the membrane together with SM and cholesterols. The phospholipids with UFAs esterified at the sn-1, 2 positions are advantageous to the fluidity of the membrane, and the phospholipids with SFAs and UFAs might play both roles, depending on conditions. Therefore, the fatty acid composition of phospholipids might have a great impact on the microstructure of the membrane. Due to the higher content of SFAs in SM that was not checked in this study, it could be predicted that the content of SFAs in the MFGM was even higher. The SM mainly composed of LC-SFAs had a higher melting temperature and could easily separate from other phospholipids in the human MFGM at room temperature, detected by CLSM labeled with fluorescent probes; however, how the other phospholipids performed was unknown.

Microstructure of Human MFG at Varied Temperatures. The microstructure of human MFG was associated with the chemical compositions of the globule membrane such as the classes of polar lipids and their fatty acid composition. At first, the fat globules from different stages of lactation labeled with a Rh-DOPE fluorescent probe were observed under CLSM. However, an insignificant difference regarding membrane microstructure was found among them, which was probably due to the minor difference of the chemical composition of polar lipids and their saturation degree of fatty acids. Therefore, the mature MFG were selected to investigate their microstructure variation at different temperatures.

The microstructure variations of human MFGM labeled by Rh-DOPE were analyzed at different temperatures, shown in Figure 3. Rh-DOPE was a headgroup labeled phospholipid fluorescent probe with two oleic acids, by which this fluorescent probe had a low melting temperature, and also might not incorporate into the inner layer of the membrane and the phospholipid monolayer. Figure 3A_1, B_1, and C_1 shows the 2D images of MFG at 4, 20, and 37 °C. The globules in these images were observed at different *z*-depths from the top to bottom. There existed dark areas absent of the extrinsic fluorescent probe on the surface of globules at different temperatures, which were marked by the white arrows. These areas might be absent of bilayer of phospholipids[9] or be domains that the fluorescence were not allowed to integrate.[10] According to the theory reported by Lopez et al.,[11] the dark areas are the heterogeneities of phase separation of polar lipids in the plane of the MFGM with the lateral segregation of SM together with cholesterols, in liquid-ordered domains surrounded by the matrix of phospholipids (PC, PI, PE, PS) in the liquid-disordered phase. They also assumed that the liquid-disordered domains in the MFGM were like lipid rafts in the

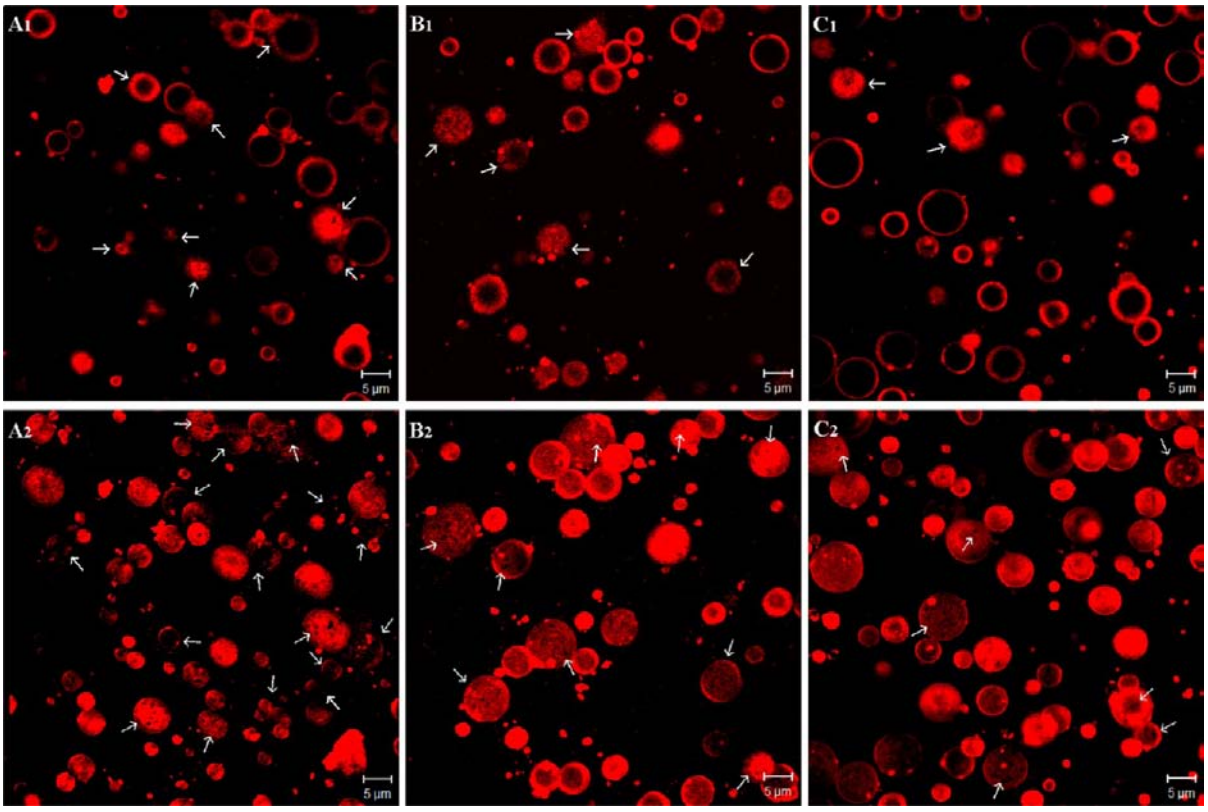

Figure 3. 2D and 3D images of human MFG at 4 °C (A_1, A_2), 20 °C (B_1, B_2), and 37 °C (C_1, C_2) observed by CLSM with Rh-DOPE fluorescent as a label probe.

cell membrane. Since the milk globules were secreted by epithelial cells of mammal glands and enveloped with the apical plasma membrane, it was of great possibility for the MFG to have these lipid rafts.

As observed from the 2D images at different temperatures, these nonfluorescent domains had no significant difference. However, a significant difference was observed from the 3D images of human MFG in Figure 3A_2, B_2, and C_2. The number and area of nonfluorescent domains on the surface of globules deceased with the increase of the temperature from 4 to 37 °C, which indicated that these domains were associated with the variations of temperature and not exclusively composed of SM and cholesterol. Some substances at low temperature could pack them together and could also return back to the liquid-disordered domains at high temperature.

The variation of the nonfluorescent domains was closely related to the chemical composition of the outer layer of the membrane, including the polar lipid composition and the fatty acid composition of the phospholipids. The SM rich in SFAs has a higher melting temperature and special structures compared with phospholipids, which lead to a separation from the phospholipids and packing to form a liquid-ordered domain with cholesterol as reported in literatures. According to the fatty acid composition of phospholipids, there were about 60% saturated fatty acids in the globule membrane phospholipids. Therefore, there was great possibility for some phospholipids constituted of saturated fatty acids. These phospholipids with higher melting temperature could pack themself together to form a liquid-ordered phase at low or room temperature and dismiss themselves to a liquid-disordered phase at higher temperature. During the course of packing and dismissing, the fluorescent probe was reallocated on the surface of globules, which appeared as the variation of nonfluorescent domains when observed under CLSM.

The heterogeneities of the organization of polar lipids are most likely due to the lipid−lipid interaction, which occur among lipids with different structure and composition. These differences could be clearly observed under CLSM with zoom-in images which are presented in Figure 4. When the samples were kept at 20 °C, the liquid-ordered domains on the surface of globules were in a circular shape and evenly distributed. When the globules were kept at 37 °C, the number and area of the liquid-ordered domains were decreased. When the globules were kept at 4 °C, the domains of some fat globules were noticeably enlarged and had undefined shapes. The shape of the nonfluorescent domains is the result of line tension between the liquid-ordered and liquid-disordered boundaries and dipole−dipole interactions. Hereby, the shape of the domains tends to be circular to minimize edge energy, corresponding to the liquid-ordered phase.[13] However, when the milk was kept at 4 °C, the shapes became irregular, which indicated that the domains, at this moment, corresponded to the gel phase in which lipids were semicrystallized. The fluorescent probe used in the experiments was composed of two oleic acids and had relatively lower melting temperature. During the course of the decrease of temperature, the phospholipids with SFAs formed the liquid-ordered domains and the fluorescent probe was excluded out of these domains, which could explain the increase of the size of the nonfluorescent domains. However, due to the low storage temperature, the partial crystallization of the lipids in the membrane was formed, and thus, these irregular shapes of nonfluorescent domains were formed on the membrane. It can be deduced that if the decreasing rate was slow enough and the decreasing temperature was suitable, it was possible to get

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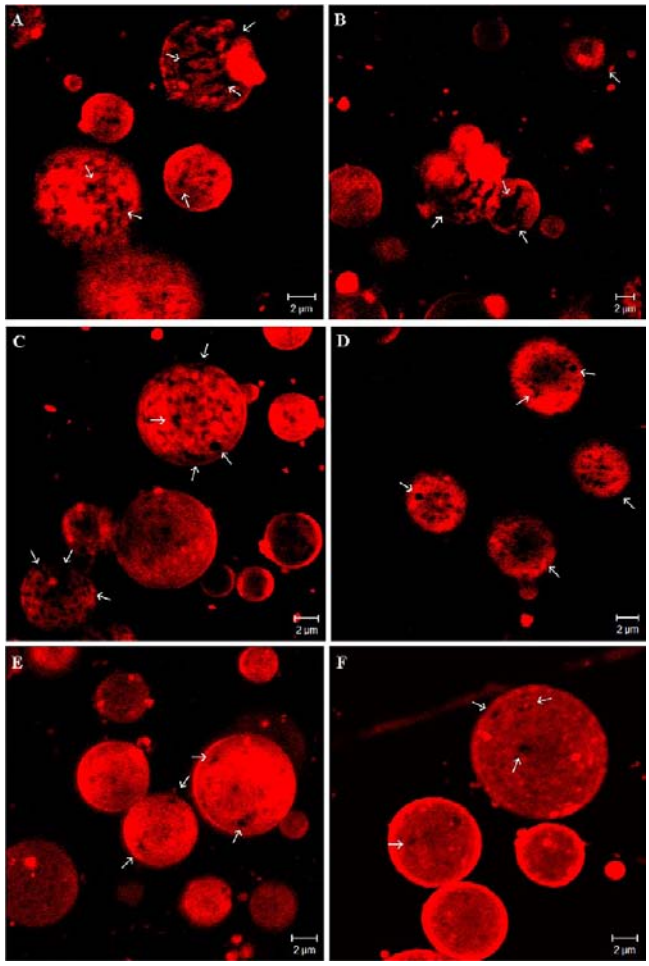

Figure 4. 3D zoom-in images of human MFG at 4 °C (A, B), 20 °C (C, D), and 37 °C (E, F) observed by CLSM with Rh-DOPE fluorescent as a label probe.

circular liquid-ordered domains with large areas. On the contrary, when the samples were kept at 37 °C, the liquid-ordered domains formed from the phospholipids with SFAs around the SM changed to the liquid-disordered domains, and the fluorescent probe could integrate and the nonfluorescent domains become smaller or even disappear. Because of the good fluidity of the membrane at higher temperature, the liquid-ordered domains still appeared in a circular shape. When the globules at 4 and 37 °C were kept at room temperature, the dark areas recovered to the normal state, which adequately implied that the reallocation of the fluorescent probe happened during the change of the temperature and how importantly the temperature affected the microstructure of the MFGM. Therefore, the MFGM had a special structure which delicately changed with the increase and decrease of temperature.

From the 2D and 3D images, some very small fat droplets can be observed under CLSM, which was also reported by Gallier et al.[13] These fat droplets might be derived from membrane materials lost through processing or the native small fat globules. Because the investigated mature milk did not go through any processing before observation, these small droplets should be the native small fat globules, and this result was in agreement with the size distribution reported above.

In conclusion, the physicochemical properties of human MFG from Danish mothers at different stages of lactation and microstructure variation at different temperatures were investigated, and the relationship between the chemical composition and microstructure variation was elucidated. As for the lipid composition, several results were revealed: (i) Colostrum milk fat constituted a higher content in PUFAs (ω-6, and long-chain ω-6 and ω-3) than transitional and mature milk fats, with the corresponding lower content of SFAs in its sn-2 position. (ii) The content of polar lipids among total lipids varied during lactation course (maximized at the transitional stage); however, in terms of subclasses of polar lipids, no significant change of the relative content of SM was observed, while the content of PC in mature milk was higher than those in colostrum and transitional milk. (iii) Inspection of fatty acid composition in phospholipids from different lactation stages revealed that no remarkable and regular changes could be generalized; and no obvious difference of the morphologies of MFGM at different lactation stages could be visualized. Microstructure analysis revealed that lateral segregation of polar lipids in the liquid-ordered phase occurred on the MFGM from different stages of lactation. However, the difference was not significant due to the minor chemical composition. Further investigation of microstructure variation through mature MFGM at different temperatures showed that the segregated domains became larger as the temperature decreased to 4 °C, while they became smaller when increased to 37 °C, indicating that, in addition to SM and cholesterol, some phospholipids with SFAs played important roles in these variations. Human milk provides the necessary energy and essential nutrients for infants, and the chemical composition of human MFG has been extensively studied. However, whether or not the microstructure variation of MFGM has any influence on the digestion should be further investigated in order to provide the infants with food similar to natural milk not only in chemical composition but also in physical structure.

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Notes

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