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Composition and microstructure of colostrum and mature bovine milk fat globule membrane

Xiaoqiang Zou [a,b], Zheng Guo [b], Qingzhe Jin [a], Jianhua Huang [a], Lingzhi Cheong [b], Xuebing Xu [b,*], Xingguo Wang [a,*]

[a] State Key Laboratory of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, School of Food Science and Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, PR China
[b] Department of Engineering, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

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ABSTRACT

The microstructures of colostrum and mature bovine milk fat globule membrane (MFGM) were investigated using confocal laser scanning microscopy (CLSM) at different temperatures, and the relationships between microstructure variations and the chemical compositions of the MFGM were also examined. Using a fluorophore-labeled phospholipid probe, we found that non-fluorescent domains on the MFGM were positively correlated with the amount of sphingomyelin at both room (20 °C) and physiological (37 °C) temperatures. However, at the storage temperature (4 °C), there were more non-fluorescent domains on the MFGM. These results indicate that the heterogeneities in the MFGM are most likely to be the result of the lateral segregation of sphingomyelin at the room and physiological temperatures, and at the storage temperature, phospholipids with saturated fatty acids affect the formation of these domains.

1. Introduction

Milk is an oil-in-water emulsion that contains approximately 3.5–5% lipids in an aqueous environment (Jensen, Ferris, & Lammi-Keefe, 1991). Lipids in milk are present as milk fat globules (MFG) which are surrounded by a biological membrane (MFGM). The size of MFG naturally ranges in diameter from ~0.2 to 20 μm and the size of the distribution profile has been shown to vary with lactation stages (Briard, Leconte, Michel, & Michalski, 2003; Patton & Keenan, 1975). These globules are important delivery vehicles of triacylglycerols (TAGs), fat-soluble nutrients and bioactive molecules for neonates (German & Dillard, 2006).

Before 2010, the most widely used model of the MFGM was based on a trilayer that consisted of an inner monolayer composed of proteins and phospholipids derived from the endoplasmic reticulum and a bilayer from the apical plasma membrane of the epithelial cells. The structure of the bilayer membrane can be described by the fluid mosaic model, which suggests that the phospholipids are the backbone of the membrane and exist in a fluid state, while proteins are globular molecules that are partially embedded in or protruding from the membrane (Dewettinck et al., 2008). In 2011, Lopez's group proposed a new model for the MFGM, which has rigid lipid rafts surrounded by more fluid lipids composed of glycerophospholipids and glycoproteins (Lopez, 2011; Lopez, Madec, & Jimenez-Flores, 2010). The MFGM is composed of polar lipids, cholesterol, proteins, glycoproteins, gangliosides and enzymes (Dewettinck et al., 2008; Lopez, 2011). Analysis of the proteins in bovine MFGM by SDS–PAGE has demonstrated that the MFGM contains a number of polypeptides. Many of the proteins present in the MFGM are reported to have physiological functions, such as butyrophilin (which suppresses multiple sclerosis), xanthine oxidase (a bactericidal agent), fatty acid binding protein (a cell growth inhibitor), and beta-glucuronidase inhibitor (which inhibits colon cancer) (Ito, Kamata, Hayashi, & Ushiyama, 1993; Martin, Hancock, Salisbury, & Harrison, 2004; Mañá et al., 2004; Spitsberg, Matitashvili, & Gorewit, 1995). The lipids in the MFGM are mainly polar lipids, including phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) (Rombaut, Camp, & Dewettinck, 2005). The glycophospholipids with high saturated fatty acid contents play a major role in the fluidity of the MFGM (Lopez et al., 2010). SM is characterized by its ceramide core, which contains

* Corresponding authors. Tel.: +45 89425089; fax: +45 86123178 (X. Xu). Tel./fax: +86 510 85876799 (X. Wang).
E-mail addresses: xiaoqiangzou@163.com (X. Zou), guo@mb.au.dk (Z. Guo), jqzwuxi@163.com (Q. Jin), huangjianhua1124@sina.com.cn (J. Huang), lingzhicheong@yahoo.com (L. Cheong), xu@mb.au.dk (X. Xu), wxg1002@qq.com (X. Wang).

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sphingosine bonded with a molecule of fatty acid via an amide link, esterified with one polar head group that is either phosphocholine or phosphoethanolamine. SM mainly contains saturated fatty acids, which contribute to its high melting temperature (Fong, Norris, & MacGibbon, 2007). The distribution of the MFGM polar lipids is asymmetric, with PC and SM largely located in the outer layer of the membrane and PE, PI and PS concentrated in the inner surface (Deeth, 1997).

The MFGM structure has been investigated with microscopy techniques. Wooding (1971) used electron microscopy to observe a dense-staining layer of 10–20 nm between the fat globule and the plasma membrane when the fat globule approached the plasma membrane. Horisberger, Rosset, and Vonlanthen (1977) observed that glycoproteins of bovine and human milk fat globules were clustered and uniformly distributed over the external membrane surface. Confocal laser scanning microscopy (CLSM) has been used to observe the heterogeneous organization of the MFGM using lipophilic probes and lectin (Evers et al., 2008) and to reveal the distribution of polar lipids, membrane proteins, glycolipids and glycoproteins in the bovine and human MFGM (Lopez & Ménard, 2011; Lopez et al., 2010, 2011). The heterogeneities in the MFGM are due to the lateral segregation of SM, cholesterol and highly saturated phospholipids in rigid liquid-ordered domains surrounded by a fluid matrix of liquid-disordered glycophospholipids (Lopez et al., 2010; Zheng, Jiménez-Flores, Gragson, & Everett, 2014). Lopez et al. (2011) illustrated that these heterogeneities were exclusive of proteins. The reason for the observation of phase separation under CLSM is that the liquid-ordered domains are so tightly packed that the fluorescent probes cannot insert into this area, whereas they insert easily into the liquid-disordered domains, which have high fluidity and a disordered molecular arrangement (Lopez et al., 2010).

The major component of the MFGM is polar lipids, whose relative proportions and fatty acid compositions vary with factors such as the lactation stage, diet, genetics and MFG sizes. Whether and how the composition of the polar lipids affects the microstructure of the MFGM remains unknown. Lopez et al. (2011) hypothesized that the non-fluorescent domains observed under CLSM were mainly composed of SM, on the basis of its special properties. Gallier, Gragson, Jiménez-Flores, and Everett (2010) used fluorophore-labeled SM to stain the SM-rich domains, but the images obtained under CLSM were similar to other phospholipid-based fluorescent probes. Furthermore, studies on the microstructure of the MFGM have been conducted mainly at the room temperature (20 °C), and the microstructure changes under the storage (4 °C) and physiological temperatures (37 °C) have rarely been reported, except that Lopez and Ménard (2011) investigated the human MFGM at the physiological temperature. Based on the above-mentioned considerations, colostrum and mature bovine milk, which have significant differences in polar lipid proportions and their fatty acid compositions, were used to investigate the relationship between the lipid composition of the MFGM and its microstructure at different temperatures.

2. Materials and methods

2.1. Samples and reagents

Bovine colostrum (1–5 days, $n = 10$) and mature (after 16 days, $n = 10$) milk samples were obtained from a local Danish dairy farm. Lipid-soluble Nile Red fluorescent dye (9-diethylamino-5H-benzoalpha-phenoxazine-5-one), bought from Sigma–Aldrich Corp. (St Louis, MO, USA), was prepared at 1 mg/mL in acetone and used to stain the triacylglycerol core of the bovine MFG. The fluorescent dye N-(lissamine rhodamine B sulfonyl) di-oleoyl-phosphatidylethanolamine (Rd-DOPE, 1 mg/mL in chloroform) was used to label the phospholipid membrane, purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Phospholipid standards were supplied by Sigma–Aldrich Corp.: 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 (SM from bovine brain; purity 97%). Silicic acid 60G TLC plates and BF_3 methanol solution (10% w/v) were purchased from Sigma–Aldrich Corp. Methanol, chloroform, hexane, diethyl ether and heptanes were all of high-performance liquid chromatography purity.

2.2. Size distribution

The MFG size distributions were determined with integrated light scattering using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern WR14 1XZ, UK). The refractive index for bovine milk fat is 1.460 and 1.458 at 466 and 633 nm, respectively (Michalski, Michel, Sainmont, & Briard, 2002). The casein micelles were dissociated by diluting the milk in 35 mM EDTA buffer (pH 7) and the sample was diluted in the measurement cell to reach 10% obscuration. The size distribution was evaluated by the 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.

2.3. Lipid analysis

2.3.1. Extraction of total lipids

Total lipids were extracted from the freeze-dried samples by homogenization with chloroform/methanol (2:1, v/v), as described by Folch, Lees, and Sloane-Stanley (1957). 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.

2.3.2. Analysis of the fatty acid composition of polar lipids

The polar lipids were separated from the total lipids with silica gel G TLC plates using a developing solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The polar lipids were scraped off the baseline and extracted with 3 mL chloroform: methanol:water (5:5:1, v/v/v) mixture (Benoit et al., 2010). After centrifugation at 4000 rpm for 10 min, the 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 microlitres of BF_3 methanol solution was added for methylation and the screw-capped tubes were kept at 100 °C for 90 min. Next, 600 μL of heptane and 500 μL of saturated NaCl solution were added. The mixture was centrifuged at 4000 rpm for 10 min at 20 °C. The solvent phase was collected and dried with anhydrous sodium sulfate. After centrifugation, the upper layer was injected into a gas chromatograph for fatty acid analysis.

Fatty acid methyl esters were analyzed on a gas chromatograph (Thermo Trace GC Ultra, USA) equipped with an autosampler, a flame ionization detector and an ionic liquid capillary column (SLB-IL 100, 60 m × 0.25 mm × 0.2 μm). Helium was used as the carrier gas at 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 fatty acid methyl esters were identified