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Buffalo milk fat globules and their biological membrane: *in situ* structural investigations

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ABSTRACT

Milk fat globules and their surrounding biological membrane (the MFGM) are not well understood despite the importance of these milk components in human nutrition and the role of fat globules in determining the properties of dairy products. The objectives of this study were to investigate these unique colloidal assemblies and the microstructure of the MFGM in buffalo milk, which is the second largest global source of dairy products. *In-situ* structural investigations were performed at room temperature using confocal microscopy with multiple fluorescent probes (Nile Red, Rh-DOPE, the lectin WGA-488). Microscopic observations showed cytoplasmic crescents around fat globules and the heterogeneous distribution of glycosylated molecules and polar lipids with the occurrence of lipid domains. The lipid domains in the buffalo MFGM appear to form by the segregation of lipids with a high phase transition temperature (e.g. sphingomyelin and saturated phosphatidylcholine molecular species) and cholesterol resulting in a gel phase or a Lo phase forming circular domains. The structure of the buffalo MFGM results from a non-random mixing of components, consistent with observations for other species. Structural heterogeneities of the MFGM could affect the processability of buffalo fat globules and the bioavailability of milk lipids.

1. Introduction

Milk is an exceptionally complex biological fluid used for the manufacture of a wide range of dairy products. Numerous studies have focused on cow's milk although milks from other animal species such as buffalos, ewes, goats and camels are essential to the human diet in various parts of the world. Buffalo milk represents the second largest volume of milk produced globally after cow's milk with more than 97 million tons produced each year (FAOSTAT, 2012). Buffalo milk is also one of the richest milks from a compositional point of view (Ménard et al., 2010). Fat constitutes the main fraction of buffalo milk, with the almost twice the fat content of bovine milk (7.4–8.8% w/w vs. 3.6–4.7% w/w) (Solh, Staines, Honda, & Limley, 2007; Varricchio, Di Francia, Masucci, Romano, & Proto, 2007) and this fat is responsible for the high energetic and nutritive value of buffalo milk. Despite the nutritional value of milk fat and the influence of fat on dairy product properties, information about buffalo milk fat is scarce.

All milk fat is thought to be dispersed in colloidal assemblies called milk fat globules. Previous studies have demonstrated that buffalo milk fat globules are significantly larger in size (5.0 μm vs. 3.5 μm) and have a higher absolute zeta potential (−11.0 mV vs. −9.4 mV) compared to bovine milk fat globules (Ménard et al., 2010). The core of the milk fat globule is mainly composed of triacylglycerols (TAG, esters of fatty acids and glycerol; 98% of milk lipids). This core is surrounded by a biological membrane called the milk fat globule membrane (MFGM). The MFGM contains many bioactive compounds which are involved with several biological functions and health benefits, such as neonatal gut maturation, antibacterial infection, inhibition of colon development and lowering cholesterol absorption (Dewettinck et al., 2008; Lopez, 2011). Few authors have characterized the buffalo MFGM (Abou-Dawood, Moussaa, El-Demerdash, & Ahmed, 1988; D'Ambrosio et al., 2008; Ménard et al., 2010). This biological membrane contains mainly glycerophospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylserine, PS),

Abbreviations: MFGM, milk fat globule membrane; Lo phase, liquid ordered phase; CLSM, confocal laser scanning microscopy; DIC, differential interference contrast; TAG, triacylglycerol; SM, sphingomyelin; PC, phosphatidylcholine.

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sphingolipids (mainly sphingomyelin, SM), cholesterol and proteins (Keenan & Patton, 1995; Ménard et al., 2010). Polar lipids, which are mainly located in the MFGM, account for about 2.6 mg/g fat and about 190 mg/L of buffalo milk (Ménard et al., 2010), which is 28% higher than in bovine milk (Ménard et al., 2010). Also, buffalo milk contains less cholesterol than bovine milk (7.0–10.2 mg/100 mL vs. 10.5–19.8 mg/100 mL) (Strzalkowska, Jozwik, Baghnicka, Krzyzewski, & Horbanczuk, 2009; Talpur, Memom, & Bhanger, 2007; Zotos & Bampidis, 2014). The average content of membrane protein was reported to be less in buffalo MFGM compared to bovine milk, either when calculated as a percentage of fat (4.2% vs. 4.9%) or as a proportion of the total MFGM material (29.5% vs. 33.9%) (Abou-Dawood et al., 1988). A recent proteomic study identified 50 proteins within buffalo MFGM, with the major proteins being xanthine dehydrogenase/oxidase, butyrophilin, adipophilin, lactadherin and mucin, similar to bovine MFGM proteins (D'Ambrosio et al., 2008; Fong, Norris, & MacGibbon, 2007). Both the composition and the structure of the MFGM result from the mechanisms of secretion of fat globules from the epithelial cells of the mammary gland (Heid & Keenan, 2005). The compositional differences reported to date suggest there may be further underlying structural differences in the MFGM in buffalo milk and possible differences occurring during the *in vivo* secretion of these globules during milk production.

A number of studies performed using confocal microscopy have revealed heterogeneities in the organization of the MFGM (Evers et al., 2008), in the localization of membrane proteins (Lopez, Madec, & Jimenez-Flores, 2010; Lopez & Ménard, 2011) and the presence of lipid domains in the membranes of bovine (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Lopez et al., 2010, 2011) and human milk (Lopez & Ménard, 2011; Zou et al., 2012). This heterogeneous distribution has been attributed to the phase separation of polar lipids according to their temperature of phase transition (Tm). A recent study performed by atomic force microscopy with a model membrane showed that milk SM segregates from the fluid phase of unsaturated polar lipids to form domains in the gel phase (Guyomarc'h et al., 2014). This study also showed that cholesterol has a large effect on the domains formed by milk SM; the shapes and nanomechanical properties of the domains formed in the gel phase in the absence of cholesterol or in the liquid-ordered (Lo) phase when cholesterol is present are different (Guyomarc'h et al., 2014). The lipid domains recently revealed in the MFGM could potentially be involved in several biological processes (e.g. digestion of milk fat globules, interaction with pathogens and viruses in the gut) and cellular functions. Also, the lipid domains present in the outer bilayer of the MFGM could be involved in functional properties of milk fat globules, important for dairy applications.

The objective of this study was to perform a structural analysis of fat globules and their biological membrane, *in situ* in buffalo milk. The microstructure was investigated using the combination of optical microscopy with differential interferential contrast together with confocal laser scanning microscopy (CLSM) with adapted fluorescent dyes able to label TAG, total proteins, polar lipids and glycosylated molecules located in the MFGM.

2. Materials and methods

2.1. Milk samples

The buffalo milks used in this study were a mixture of the individual milks produced by 30 buffaloes of the Mediterranean breed *Bubalus bubalis* and collected from evening and morning milking from Coopérative de Bufflonnes (Maurs, Cantal region, France). NaN_3 (0.02% w/v) was added to the milk to prevent the growth of bacteria. Milk samples were stored at ambient temperature before fat globule size measurements and CLSM experiments. The content of fat in the milk was determined using the Gerber method (IDF, 2008).

2.2. Fat globule size measurements

The fat globule size distributions were determined by laser light scattering, using a Mastersizer 2000 (Malvern Instruments, Malvern, U.K.) equipped with a He/Ne laser ($\lambda = 633$ nm) and an electroluminescent diode ($\lambda = 466$ nm). The refractive indexes of milk fat were set at 1.46 (at 466 nm) and 1.458 (at 633 nm) and the refractive index was set at 1.33 for water. The absorption coefficient used was 0.0001. The experiments were performed at room temperature. Aliquots of approximately 70 μL of whole milk were introduced into the measurement cell of the apparatus, which contained 100 ml of water, in order to reach 10% obscuration (optimal conditions for particle-size measurements with this apparatus). A solution of 35 mM EDTA/NaOH pH 7.0 buffer (>98% disodium salt dihydrate, Prolabo, Fortenay-sous-Bois, France) was added to the measurement cell to disrupt the casein micelles. All analyses were performed in triplicate with three replicate milk samples collected from different days, giving the total of nine measurements. The specific surface area and volume-weighted average diameter d_{43}, defined as $\Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of fat globules of diameter d_i, were calculated by the software.

2.3. Microstructural analysis

Microstructural analysis was performed using an inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France). Confocal laser scanning microscopy (CLSM) was performed using an argon laser operating at an excitation wavelength of 488 nm with emission detected between 500 nm and 530 nm, a He-Ne laser operating at 543 nm wavelength excitation with emission detected between 565 nm and 615 nm and a diode operating at 633 nm, detected with a long pass filter >650 nm. The observations were performed using a × 100 (numerical aperture NA 1.4) oil immersion objective.

The staining protocols followed previously described methods (Lopez & Ménard, 2011; Lopez et al., 2010). Briefly, Nile Red (5H-Benzo α-phenoxazine-5-one, 9-diethylamino, supplied by Sigma-Aldrich, St. Louis, USA) was prepared at a concentration of 42 μg/mL in propanediol and used to stain the triacylglycerol core of the fat globules. Fast Green FCF (Sigma-Aldrich, St. Louis, USA) was prepared at a concentration of 10 mg/mL in water and used to stain proteins. N-(Lissamine rhodamine B sulfonyl) dioleoylphosphatidyl ethanolamine (Rh-DOPE, Avanti polar lipids Inc., Birmingham, England) was provided at a concentration of 1 mg/mL in chloroform and used to label the phospholipids in the membrane surrounding buffalo milk fat globules. Wheat germ agglutinin Alexa fluor 488 (WGA488, Invitrogen, Cergy Pontoise, France) was prepared at a concentration of 1 mg/mL in phosphate saline buffer and used to label the glycosylated molecules in the membrane, i.e. carbohydrate moieties containing N-acetylglucosamine and N-acetyl neuraminic acid (sialic acid) residues.

The concentrations of the fluorescent dyes were adapted to the specific composition of buffalo milk. For observation of the fat globules, 100 μL of Nile Red and 10 μL of Fast Green FCF were added to 1 mL of buffalo milk samples. For observation of the MFGM polar lipids, 40 μL of the Rh-DOPE solution was placed in a glass vial and the chloroform was evaporated under nitrogen, to avoid the possible artefacts caused by this organic solvent. Then 1 mL of milk sample was introduced in the vial. For dual staining of the MFGM polar lipids and carbohydrate moieties, 10 μL of the WGA-488 solution was also added into the vial containing Rh-DOPE. Then, 1 mL of milk sample was added to the vial. The stained milk samples were kept at room temperature for at least 1 h prior to observation by CLSM. The microstructural analyses were performed at room temperature (19 ± 1 °C).

Optical microscopy using differential interference contrast (DIC, also called Nomarski, Cogswell and Sheppard (1992)) was also used to characterize buffalo milk fat globules. DIC images were superimposed on the fluorescent emission recorded in the CLSM images. The two dimensional images had a resolution of 512 × 512 pixels and the pixel scale values