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COMMUNICATION

Cryo-SEM images of native milk fat globule indicate small casein micelles are constituents of the membrane

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Using cryo scanning electron microscopy, smaller casein micelles (20–60nm) were found adsorbed onto the native milk fat globule membrane (MFGM). The casein exists as structural constituents of the native MFGM might participate in controlling the stability of fat globules. Whey protein at the surface of globules were also observed.

The milk fat globule membrane (MFGM), which has a cross-section of 10–20 nm, acts as an emulsifier and protects the globules from coalescence and enzymatic degradation.[1] The MFGM is formed during fat secretion when the fat droplets are enveloped by a three-layered phospholipid membrane.[2] By using confocal laser scanning microscopy, the structure of MFGM is now well-accepted as a non-random organization of polar lipids, proteins and glycoprotein.[3-5] However, the structure of the MFGM at the molecular level is unclear and the least well understood aspect of milk fat globules.[6]

A number of studies showed skim milk proteins (casein and whey protein) associated with MFGM proteins upon processing. High shear force has been reported to result in the adsorption of casein to the fat globule surface.[7] Treatment with heat and high-pressure are reported to cause denaturation of the MFGM proteins and interactions with whey proteins (*β*-lactoglobulin and *α*-lactalbumin) via sulfhydryl–disulfide interactions.[8,9] Part of the original MFGM remains on the globule but it is insufficient to cover the new surface, therefore casein semi-intact micelles and micellar fragments completely wrap the new surface and avoid the coalescence of fat globules.[10] *β*-lactoglobulin has also been found to change structure upon interacting with anionic surfactants and lipid vesicles under higher-pH conditions, which dues to electrostatic interaction between the negative lipid and positive protein and hydrophobic interaction with the lipid bilayer.[11-13]

During the isolation processes of MFGM, casein micelles and whey protein are usually still visible in MFGM material after three washes, but they are considered contamination caused by the MFGM damage.[14, 15] However, Morin et al. consider that skim milk proteins may interact strongly with MFGM even before milk is collected.[16] They suggest MFGM may capture and drag some of the casein with them into the pellet. There are only a few reports of skim milk protein interacting with the native MFGM. Su et al. observed the adsorption of isolated *κ*-casein onto isolated native globules and found the radius of native fat globules decreased by 15–20 nm after *κ*-casein adsorption, which might be caused by steric repulsion of aggregated particles.[17] Gallier et al. investigated the lipid–protein interactions at the surface of model phospholipid–protein monolayer films.[18] They found *β*-casein was associated mainly with the liquid-ordered domains and suggested the same protein–lipid interaction might occur at the surface of milk fat globules. However, there is no direct evidence for a protein–lipid / protein interaction at the surface of native milk fat globules.

The transmission electron microscopy (TEM) can provide a wealth of data about the morphology of nanosized objects, but has a major drawback in that it can be very difficult to prepare thin samples.[19] Fat globules may be damaged or misplaced during sectioning. In addition, pre-treatment to remove water can alter the nanomaterials organization.[20] Compared with TEM, cryogenic scanning electron microscopy (Cryo-SEM) enables the investigation of hydrated material without removing the water from the sample and offers the advantage of voxel resolutions may enable the investigation of well-defined cross-sections of the MFGM structural integrity. Cryo-SEM had been successfully used to observe the fat and protein matrix distribution changes that occur in the gel, curd and cheese during the manufacture.[21,

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[22] However, the Cryo-SEM have not yet been reported to image the membrane surface morphology of native fat globules.

In our study of the membrane surface morphology of native fat globules with different sizes by using Cryo-SEM, we unexpectedly found the MFGM was covered with numerous particles with a diameter of 20–60 nm, as shown in Fig. 1. The size distribution of the casein micelles on the surface of the MFGM was calculated by Image J software as shown in Fig.2.

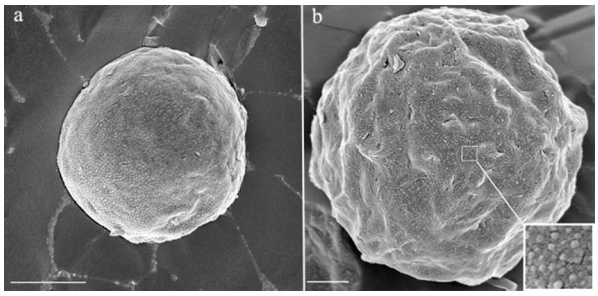

Fig. 1 Cryo-SEM image of native milk fat globules (a. native fat globules (diameter 4.5 μm), the scale bar represents 2 μm; b. native fat globules (diameter 6.5 μm) with boxed area magnified by 10-fold, the scale bar represents 1 μm).

Due to the size, quantity and uniformity, these small particles were first suspected to be ice crystals. In cryogenic techniques, ice crystal formation occurs regardless of the freezing rate. The freezing rate achieved using a nitrogen slush should produce ice crystals on the order of 50 nm and smaller, usually well below the resolution of the electron microscope for frozen hydrated samples.[23]

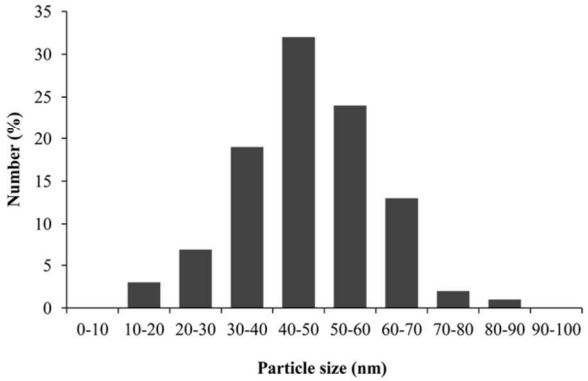

Fig. 2 The size distribution of the casein micelles adsorbed onto the MFGM as calculated by Image J software (n=100).

To distinguish whether the small particles might be the ice crystals caused by freezing process, we separated the fat globules from native milk through centrifugation and re-dispersed in SMUF to remove other milk constituents. As shown in Fig. 3b, very few small particles were observed on the surface compared to the native globule (Fig. 3a). The results confirm these small particles are from the native milk substrate and are not artefacts.

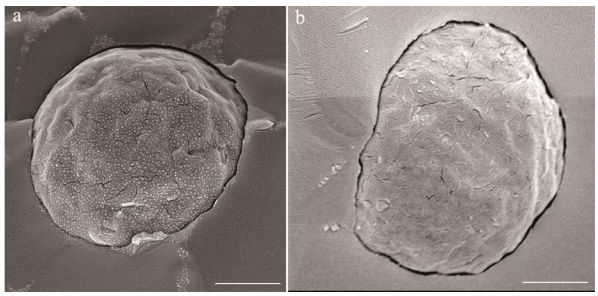

Fig. 3 Cryo-SEM image of fat globules separated from native milk (a. native fat globules (diameter 4.0 μm) as control; b. separated fat globules (diameter 5.2 μm)). The scale bar represents 2 μm.

These small particles were then suspected to be casein micelles due to the particle size. The diameter of casein micelles in bovine milk ranges from 20 to 500 nm.[24] Despite a average diameter of 150 nm, the smaller micelles (diameter <50 nm) account for 80% of particles by number.[24]

To identify whether these small particles are casein or not, the *κ*-casein, *α*-lactalbumin and *β*-lactoglobulin antibodies as well as FITC-labelled second antibody were used to study the native milk fat globules by immunofluorescence microscopy. Very little fluorescence was observed in the separated fat globules (Fig. 4a). The result is in accord with the result in Fig. 4a, which shows a absence of particles in the MFGM after centrifugation. Immunofluorescent images of native milk fat globules showed labelling of casein in a uniform circular pattern at the surface of the globules (Fig. 4b). The labelling of *α*-lactalbumin showed a heterogeneous and punctate ring-like pattern (Fig. 4c), while the fluorescence of *α*-lactalbumin was relatively weak and did not show an even dispersion in the milk substrate. The labelling of *β*-lactoglobulin showed a uniform circular pattern at the surface of the globules but also not well dispersed in the milk substrate (Fig. 4d). These results suggested the casein micelles and whey proteins were all exposed at the surface of the globule.

Many researches have reported a “new” membrane consisting of casein and whey protein that might be formed during the processing of dairy products. Heat treatment, homogenisation, agitation and other treatments can result in disruption of the MFGM integrity.[25] Part of the original MFGM remains on the globule but is not sufficient to cover the new surface. For that reason, casein semi-intact micelles and micellar fragments wrap the new surface completely and avoid the coalescence of fat globules.[10] Protein accounts for 25-60% of the mass of the membrane material.[1] It is widely accepted that MFGM isolation processes may induce MFGM material loss, especially the loosely bound peripheral proteins.[26] Casein and whey protein were usually still visible in MFGM material after three washes,[14] but they were considered contamination caused by the MFGM damage.[15] Consider of the intracellular origin and secretion process of milk fat globules, some casein micelles also can be seen in secretory vesicles underlying the partially secreted lipid droplet.[2] Caseins and other milk proteins are processed through the secretory pathway and are secreted with the aqueous phase of milk by either compond or simple exocytosis from secretory vesicles at the apical plasma membrane.[27] According to Figs. 1,

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3 and 4, the casein micelles and whey proteins might exist at the surface of native MFGM as structural constituents.

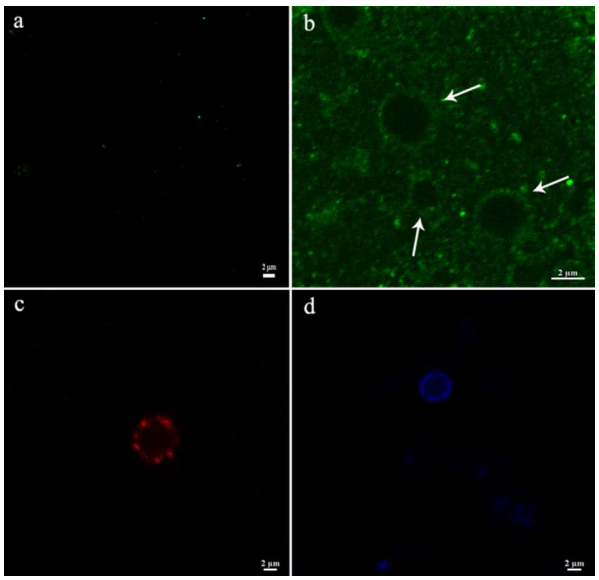

Fig. 4 Immunofluorescence microscopy reveals casein and whey protein on the surface of native fat globules (a. separated fat globules with FITC-labelled κ-casein, α-lactalbumin and β-lactoglobulin as control; b. native fat globules with FITC-labelled κ-casein; c. native fat globules with α-lactalbumin; d. FITC-labelled β-lactoglobulin).

Further, the results of Fig. 3 indicate the association between surface granules and the MFGM is not via covalent bonds. The interaction might be the combined effect of hydrogen bonds, hydrophobic interactions and electrostatic interactions.[18] Butyrophilin comprises over 40% by weight of the total protein in MFGM, which is a typical transmembrane protein with some amino acid sequences outward beyond the bilayered membrane.[28] Owing to the hydrophobic property of butyrophilin and the electrostatic interaction of the polar lipids, proteins and glycoproteins contained in the MFGM and the hydrogen bonds, the casein and whey proteins tend to be adsorbed onto the surface of native MFGM rather than distributed in the milk. Fat globules with adsorbed casein micelles could behave as large casein micelles and interact with casein micelles and whey proteins during further processing.

In addition, whey proteins dissolved in native milk at the molecular level, with a diameter of 3–6 nm.[29] Owing to the resolution limitation of Cryo-SEM (~5 nm), whey protein in the MFGM could not be observed (Fig. 1). According to the average diameter (~50 nm) in Fig. 2 calculated using Image J software, it can be concluded that it is the numerous small casein micelles are observed in the MFGM.

First, the selective absorption of smaller casein micelles might be the result of the selective binding modes and binding sites between small casein micelles and membrane phospholipids/proteins. In a model system of protein-membrane interaction, it is thought that the insertion of a hydrophobic peptide or membrane protein segment into a lipid bilayer can be spontaneous, driven mainly by van der Waals attractions between the hydrophobic segments of the peptides and the hydrocarbon chains of the membranes. On the other hand, the interaction between water-soluble proteins and membranes mainly depends on the overall electrostatic interactions between the anionic lipid head groups and positively charged protein residues.[12] The electrostatic interaction between the negative lipid and positive protein and hydrophobic interaction with the lipid bilayer is found to induce a conformational change of *β*-Lactoglobulin at neutral pH.[11-13] Therefore, we suppose there might be a similar conformational change mechanism happened to casein micelles. At some condition (suitable electrostatic interaction between the negative lipid and positive protein), the hydrophobic bonds become stronger, which causes the micelle structure to become closer and less porous and the micelle diameter to become smaller.

Second, the selective absorption of smaller casein micelles onto native MFGM might be a mode similar to the Pickering emulsion. A Pickering emulsion is an emulsion that is stabilized by solid particles which adsorbed onto the interface between the two phases. If oil and water are mixed and small oil droplets are formed and dispersed throughout the water, eventually the droplets will coalesce to decrease the amount of energy in the system. However, if solid particles are added to the mixture, they will bind to the surface of the interface and prevent the droplets from coalescing thus causing the emulsion to be more stable.[30, 31] Therefore, we suspect that when there are absence of fat globules in milk, casein molecules together with calcium phosphate form aggregates of several thousand individual protein molecules with average diameters of 150 to 200 nm, known as casein micelles; when fat globules are in the milk emulsion, the caseins molecules exist as nanoclusters, which composed of aggregated calcium phosphate, and adsorbed to the surface of fat globules to prevent the droplets from coalescing. Nanoclusters is formed by the binding of the phosphorylated regions of the caseins to small domains of calcium phosphate with the average diameters of 20-50 nm.[32, 33] Though details of the mechanism remain to be elucidated, to our knowledge, this is the first report of selective adsorption of smaller casein micelles onto native MFGM.

To determine the influence of different protein concentration on the size of casein micelles adsorbed onto the MFGM, the skim milk was added to the SMUF which contain the same fat content, as the proportion of 0.01, 0.1, 0.5 and 5 ($g_{\text{milk protein}}$ / $g_{\text{milk fat}}$). To verify the nature of each component and the interfacial and electrostatic properties between fat globules and skim milk protein the ζ-potential values were determined. The ζ-potential results also demonstrated the adsorption of skim milk protein onto the surface of native fat globules (See supporting information).

As shown in Fig. 5a, when the proportion of protein to fat in milk is 0.01 g protein/g fat, very little protein is observed on the surface of the MFGM. When the proportion is increased to 0.1 g protein/g fat, casein micelles with an average diameter of ~140 nm are well distributed on the surface of the MFGM (Fig. 5b). As the proportion of protein to fat increases (Fig. 5c and 5d), the casein micelles adsorbed have an average diameter of ~50 nm.

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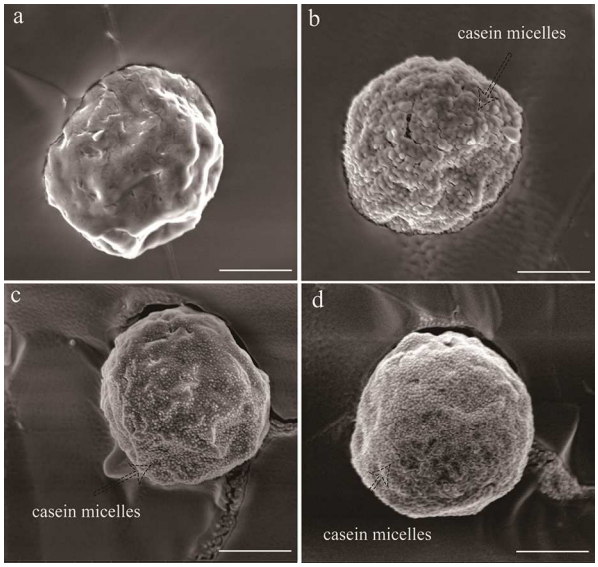

Fig. 5 Cryo-SEM image of fat globules from milk with different proportions of protein to fat (a. 0.01 g protein/g fat; b. 0.1 g protein/g fat; c. 0.5 g protein/g fat; d. 5 g protein/g fat. The scale bar represents 1 μm.

When the proportion of protein to fat is 0.01 g protein/g fat, if the critical micelle concentration is not reached, the casein micelles might be disassociated into casein monomers, which are not in the visible range of Cryo-SEM. Once the proportion increases tenfold, the surface of fat globules is covered with a compact and continuous coat of protein. Interestingly, the size of casein micelles at a proportion of 0.01 g protein/g fat in milk is significantly larger compared to a higher protein to fat proportion. The size distribution of casein micelles under various concentrations were measured but no significant difference was observed (results not shown). The size difference might be caused by the difference of binding modes between casein micelles and MFGM components at different concentrations of protein. However, details of the mechanism underlying the change of casein size micelles remain to be elucidated.

Conclusions

The application of modern microscope techniques in this study helped to gain new understanding of casein micelles and whey proteins as structural constituents of MFGM, which could affect the functionality of MFGM in the native state. Moreover, it is the small casein micelles that are adsorbed selectively onto the native MFGM. The size of casein micelles adsorbed onto MFGM is affected by the concentration of skim milk proteins. Understanding MFGM properties could help to explain the mechanism underlying MFGM variations during dairy processing, as well as developments of dairy products of specific interest.

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Notes and references

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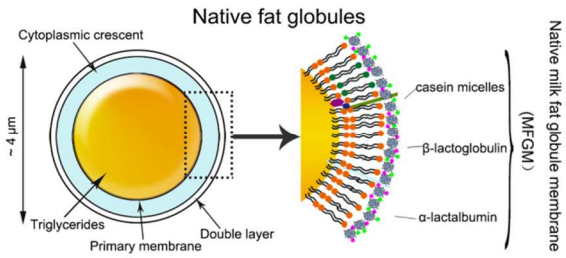

[Table of contents] Schematic representation of the native milk fat globule membrane: casein micelles and whey proteins on the surface of the membrane.

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