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Supporting Information

1. Preparation of milk samples

Fresh milk was obtained from SanYuan Dairy Co (Beijing, China) and maintained at 4℃ for immediate use.

To adjust the protein amount adsorbed by the MFGM, the separated fat globules were mixed with different concentration of protein. The milk was centrifuged for 5 min at 3000 × g. The top cream layer was collected and reconstituted to a 20% fat content in simulated milk ultrafiltrate (SMUF) and then diluted ten-fold. To maintain the native state of the milk as much as possible, skim milk was separated by the same batch of milk by centrifuging at 4200 × g for 20 min. The protein content of skim milk were determined using the Kjeldahl method.[1] Then the skim milk was added to the SMUF which contain the fat globules as the proportion of 0.01, 0.1, 0.5 and 5 ($g_{\text{milk protein}}$ / $g_{\text{milk fat}}$).

2. Particle size distribution

A Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer (Beckman Coulter, USA) equipped with a 4mW helium/neon laser at a wavelength output of 633 nm was used to determine the average droplet size of milk samples. The refractive indices used were 1.458 and 1.460 for milk fat at 633 and 466 nm, respectively, and 1.333 for water. The samples were diluted in 100 ml of water directly in the measurement cell of the apparatus in order to reach 8% obscuration. From the size distribution, the average volume-weighted diameter, $d_{43}= \sum nidi^4/\sum nidi^3$ (where ni is the number of fat globules or casein in a size class of diameter di) was calculated by the instrument software.

3. Apparent ζ-potential of milk fractions

The ζ-potential values of each component and the interfacial and electrostatic properties between fat globules and skim milk protein were determined. The ζ-potential was measured by laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique using a Malvern Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Malvern, UK). Samples were diluted 100-fold in simulated milk ultrafiltrate and put in a capillary tube at 25℃. The apparent zeta-potential of milk samples was calculated as follows: the zeta-potential ζ of a particule is a value calculated from its electrophoretic mobility u, according to Henry's equation: $\zeta=(u\times 6\pi\eta/\varepsilon)/f(\kappa\alpha)$, where η and ε are, respectively, the viscosity and dielectric constant of the solution, at the temperature of the measurement. 1/κ is the Debye length and α is the radius of the particule. The Smoluchowski approximation, assuming $f(\kappa\alpha)=1.5$, was used. Ten readings from a freshly-diluted individual sample were collected and the measurements were run in triplicate on three independent milk samples.

The results are shown in the following Table:

Table 1. Apparent ζ-potential of milk fractions after centrifugation and reconstruction [1]

	Raw milk	Fat globules[2]	Skim milk	Milk 1[3]	Milk 2	Milk 3	Milk 4
ζ (mV)	-13.14±1.62[b]	-10.85±0.69[a]	-17.95±0.76[d]	-11.39±0.55[a]	-13.67±0.71[b]	-13.77±0.64[b]	-16.1±0.49[c]

[a] Means in a column with different superscripts are significantly different ($P < 0.05$).

[1] Results are mean ± SEM (n = 3).

[2] Means the cream layer collected after centrifugation and reconstituted in simulated milk ultrafiltrate

[3] Milk 1 means the skim milk added to the SMUF which contain the fat globules as the proportion of 0.01 g protein/g fat; Milk 2 means the skim milk added to the SMUF which contain the fat globules as the proportion of 0.1 g protein/g fat; Milk 3 means the skim milk added to the SMUF which contain the fat globules as the proportion of 0.5 g protein/g fat; Milk 4 means the skim milk added to the

SMUF which contain the fat globules as the proportion of 5 g protein/g fat.

4. Immunofluorescence Microscopy

The immunofluorescence microscopy method was adapted from Robenek et al.[2]. Confocal dish with a glass bottom was soaked in 0.01% v/v poly-L-lysine (PLL) solution (Sigma-Aldrich) to enhance the adhesion of milk fat. Then one part of native milk was fixed with seven parts of 2% paraformaldehyde on the dish for 20 min and the mixture was dried. Milk fat globule envelopes were blocked with 1% BSA in PBS for two hours. Then the milk samples were incubated with primary antibody in antibody diluents (CSN3 mouse monoclonal IgG2a, RR12, Santa Cruz Biotechnology; Rabbit Anti-alpha Lactalbumin antibody, bs-11131R, Beijing Biosynthesis Biotechnology; Rabbit Anti-Beta-Lactoglobulin antibody, bs-2064R, Beijing Biosynthesis Biotechnology) overnight at 4 ℃ and warmed at room temperature for 10 min before four 10-min PBS washes. Milk samples were incubated with secondary antibody (Cy2-conjugated donkey anti-mouse IgG-FITC, sc-2099, Santa Cruz Biotechnology; Goat anti-rabbit IgG/FITC antibody, bs-0295G-FITC, Beijing Biosynthesis Biotechnology) in antibody diluents for 1 hour in the dark and washed four times for 10 min in PBS. The immunofluorescence labeled samples were observed by confocal laser scanning microscope (NIKON Eclipse-A1-Rsi, Japan). The observations were performed using a × 100 (NA 1.4) oil immersion objectives. The confocal dish with FITC labeled samples were directly observed on the microscope (excitation laser = 488 nm). The microstructural analyses were performed at room temperature. Differential interferential contrast (DIC) microscopy was used to visualize fat globules (localization in milk, shape, size). The two-dimensional images had a resolution of 1024 × 1024 pixels and the pixel scale values were converted into micrometers using a scaling factor.

5. Cryo scanning electron microscope

Cryo scanning electron microscopy (Cryo-SEM, FEI Helios 600, 3 KV) to investigate the fat globules morphology according to an adapted method of Ong et al.[3]. The native milk and other samples(~20 μL) was prepared by mounting on a copper holder and being attached to the VTD that fits directly onto the slushing chamber of the Cryo unit (PP3000T, Quorum, UK). Milk were rapidly immersed into liquid nitrogen slush (a mixture of solid and liquid nitrogen at its freezing point (210℃). The liquid nitrogen used was freshly filled to prevent the presence of particulates that may provide nucleation for the growth of ice crystals. The samples was etched at -90℃ for 1.5 min then back to 180℃ and coated with sputtered gold (10 mA, 60 s). The detectors used for the SEM observation were an Everhart Thornley detector (ETD) and a solid state detector (SSD).

6. Statistical analysis

Quantitative measurements of Cryo-SEM images about the casein diameter and surface area were done using “Image J” software (version 1.47; National Institutes of Health, Bethesda, MD). The size distribution of the particles were determined by image analysis (n=100).

Reference

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3. L. Ong, R. R. Dagastine, S. E. Kentish and S. L. Gras, *LWT - Food Science and Technology*, 2011, 44, 1291-1302.