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Nanoscale observation of the natural structure of milk-fat globules and casein micelles in the liquid condition using a scanning electron assisted dielectric microscopy

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ABSTRACT

Recently, aqueous nanoparticles have been used in drug-delivery systems for new type medicines. In particular, milk-casein micelles have been used as drug nanocarriers for targeting cancer cells. Therefore, nanostructure observation of particles and micelles in their native liquid condition is indispensable for analysing their function and mechanisms. However, traditional optical and scanning electron microscopy have difficulty observing the nanostructures of aqueous micelles. Recently, we developed a novel imaging technique called scanning electron-assisted dielectric microscopy (SE-ADM) that enables observation of various biological specimens in water with very little radiation damage and high-contrast imaging without staining or fixation at an 8-nm spatial resolution. In this study, for the first time, we show that the SE-ADM system is capable of high-resolution observation of whole-milk specimens in their natural state. Moreover, we successfully observe the casein micelles and milk-fat globules in an intact liquid condition. Our SE-ADM system can be applied to various biological particles and micelles in a native liquid state.

1. Introduction

To analyse the functions of biological and organic specimens, it is important to observe samples in their natural state in a liquid condition [1–3]. Although traditional optical microscopy (OM) is capable of imaging liquid specimens [3], its spatial resolution is limited to 200 nm by the diffraction limit of light. On the contrary, conventional scanning electron microscopy (SEM) achieved a resolution of a few nanometres using biological specimens under vacuum conditions [4,5]. Moreover, the atmospheric-sample holder enables observation of aqueous biological nanoparticles during nanoscale imaging [6,7]. However, because the electron beam (EB) is directly irradiated upon the biological specimens in the liquid condition, it causes damage to the samples [7,8]. Therefore, the electron dose has to be kept very low such that the specimen can be investigated over a reasonable time with minimal damage [8]. Such low-dose SEM observations result in very low contrast and a low signal-to-noise ratio. Therefore, a high-contrast and low-damage imaging method for biological specimens in the liquid condition is required in this field.

In recent studies, we have developed a novel imaging technique called scanning electron-assisted dielectric microscopy (SE-ADM), which enables observation of intact cells, bacteria and protein particles in water with very little radiation damage, as well as high-contrast imaging without staining or fixation at a spatial resolution of 8 nm [9–11]. Our system is capable of producing high-contrast images of untreated biological specimens in aqueous conditions. Biological samples are enclosed in a liquid holder composed of a tungsten (W)-coated silicon nitride (SiN) film and are not directly exposed to the EB; thus, the system can minimise electron-radiation damage [9–11].

In this report, we show for the first time that the SE-ADM system is capable of high-resolution observation of whole-milk specimens in their natural state. Moreover, we successfully observe casein micelles and milk-fat globules in an intact liquid condition. In this case, whole-milk specimens are useful for comparing the abilities of imaging methods in observing organic materials. Our SE-ADM system can obtain higher resolution and contrast compared to other traditional imaging methods using OM or a wet-SEM-sample holder.

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2. Materials and methods

2.1. Metal coating on the SiN film

A 50-nm-thick SiN film supported by a 0.4 mm × 0.4 mm square window in a Si frame (4 × 4 mm^2, thickness = 0.381 mm; Silson Ltd., UK) was coated with tungsten using a magnetron-sputtering machine (Model MSP-30T, Vacuum Device Inc., Japan). The W-sputter conditions were 1.1 Pa, 300 mA and 15 s. The distance between the sputter target and the SiN films was 50 mm.

2.2. Sample preparation

The whole-milk specimens were prepared using the 'Meiji oishii-gyunyu' commercial product in a 200-mL-bottle pack obtained from Meiji Co., Ltd. (Tokyo, Japan). A skimmed-milk solution was obtained after the removal of the supernatant by centrifugation of the whole-milk specimens at 12,000 rpm for 5 min at 28 °C.

2.3. Atmospheric holder

The newly developed sample holder maintained the sample solution at atmospheric pressure in the space between a W-coated 50-nm-thick SiN film and another, uncoated 50-nm-thick SiN film [9,10]. The two SiN films incorporating the whole-milk sample were sealed using two sample-holding parts with an O-ring, and the holding pieces were the sealed using four screws. The sample holder comprised an upper aluminium part and a lower acrylic-resin part. The upper part was connected to a bias voltage of −9 V, allowing conduction to the metal layer on the SiN film. The lower part, made of resin, had a high resistivity; hence, measurement at the terminal on the underside of the holder was insulated from the metal-coated SiN film [10].

2.4. SEM and the SE-ADM imaging system

The field-emission (FE)-SEM (JSM-7000F, JEOL, Tokyo, Japan)-based high-resolution SE-ADM imaging system was previously reported [10]. The liquid-sample holder was mounted onto the SEM stage, and the detector terminal was connected to a pre-amplifier under the holder [10]. The electrical signal from the pre-amplifier was fed into the analogue-to-digital (AD) converter after low-pass filtering (LPF), as previously described [11,12]. The LPF and EB-scanning signals were logged by a PC through an AD converter at a sampling frequency of 50 kHz. SEM images (1280 × 1020 pixels) were captured at 5000–50,000× magnification with a scanning time of 80 s, a working distance of 7 mm, an EB-acceleration voltage of 4 kV, and a current of 10 pA.

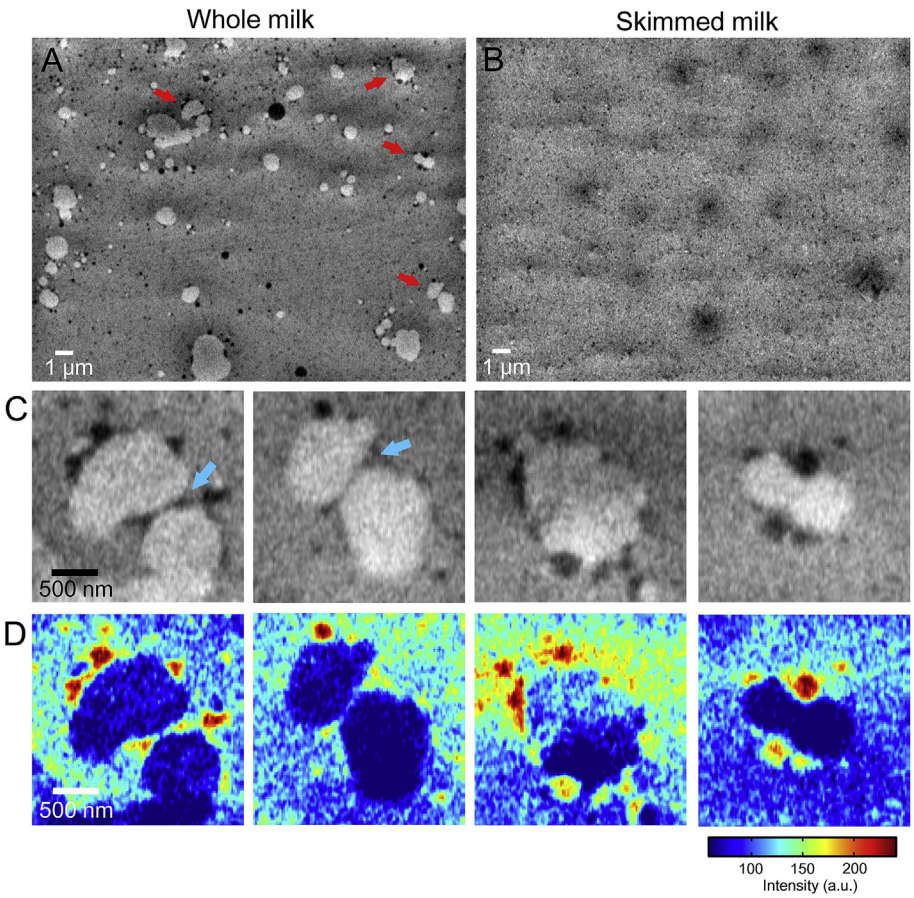

Fig. 1. Images of milk specimens obtained by the SE-ADM system.
(A) A dielectric-impedance image of untreated whole-milk in the liquid condition, taken with a 4-kV EB, 5000× magnification and −9-V bias. The milk specimens comprise large white spheres and small black particles. (B) The skimmed-milk image in liquid condition, taken with a 4-kB EB at 5000× magnification. The large white spheres disappeared in the skimmed milk; therefore, we conclude that they are milk-fat globules. (C) Four expanded images of the milk-fat globules indicated by red arrows in (A). The black casein-micelle particles adhered around the milk-fat globules. Moreover, the connected membranes between two milk-fat globules are clearly shown by blue arrows. (D) Pseudo-colour maps of (C) after intensity inversion. Scale bars are 1 μm in (A) and (B) and 500 nm in (C) and (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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