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Characterization of the nanoscale structure of milk fat

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ABSTRACT

The nanoscale structure of milk fat (MF) crystal networks is extensively described for the first time through the characterization of milk fat-crystalline nanoplatelets (MF-CNPs). Removing oil by washing with cold isobutanol and breaking-down crystal aggregates by controlled homogenization allowed for the extraction and visualization of individual MF-CNPs that are mainly composed of high melting triacylglycerols (TAGs). By image analysis, the length and width of MF-CNPs were measured (600 nm × 200 nm–900 nm × 300 nm). Using small-angle X-ray scattering (SAXS), crystalline domain size, (i.e., thickness of MF-CNPs), was determined (27 nm (d_{001})). Through interpretation of ultra-small-angle X-ray scattering (USAXS) patterns of MF using Unified Fit and Guinier-Porod models, structural properties of MF-CNPs (smooth surfaces) and MF-CNP aggregations were characterized (RLCA aggregation of MF-CNPs to form larger structures that present diffused surfaces). Elucidation of MF-CNPs provides a new dimension of analysis for describing MF crystal networks and opens-up opportunities for modifying MF properties through nanoengineering.

1. .Introduction

Characterization of milk fat (MF) microstructure at different length scales is important in the description and understanding of its function and importance in various food products. Having a view of the MF structure at all length scales allows for a comprehensive explanation of the effects of composition and different processing conditions on the structure and overall quality of MF-containing products. Previous work on USAXS have identified milk fat – crystalline nanoplatelets (MF-CNPs) as having smooth surfaces (Peyronel, Ilavsky, Pink, & Marangoni, 2014), however, visualization of CNPs using Acevedo and Marangoni (2010)'s technique was not performed. In this article therefore, a comprehensive description of the nanoscale structure of MF is provided through the extraction and image analysis of MF-CNPs and relating the results with X-ray scattering experiments.

MF is composed of 98% triacylglycerols (TAGs) of extreme heterogeneity that results in a complex thermal behavior (e.g., broad melting points of around −40 to 40 °C) and causes a major challenge in the characterization of its microstructure due to the presence of liquid oil (Dimick, Reddy, & Ziegler, 1996; Jensen, 2002; Lopez, Lavigne, Lesieur, Bourgaux, & Ollivon, 2001; Maleky, Smith, & Marangoni, 2011; Timms, 1980). During crystallization, TAGs pack in different polymorphs at the molecular level and stack epitaxially in a lamellar structure in a combination of different chain length structures – double, 2 L or triple, 3 L (D'Souza, DeMan, & DeMan, 1990; Marangoni et al., 2012; Sato, 2001). In MF, TAGs are frequently found to crystallize in the α form at fast cooling rates (>1 °C/min), and β′ and β polymorphs at slow cooling rates (0.1–0.01 °C/min), although co-existence of the different forms may occur due to the complexity in composition described above (Grotenhuis, Van Aken, Van Malssen, & Schenk, 1999; Lopez, Bourgaux, & Lesieur, 2002; Lopez et al., 2001). Most studies of MF structure and properties have focused on molecular arrangements in the solid state and how different factors such as cooling rate, TAG and fatty acid composition, and shear affect it (Bugeat et al., 2011, 2015; Campos, Narine, & Marangoni, 2002; Grotenhuis et al., 1999; Lopez, Lavigne, Lesieur, Keller, & Ollivon, 2001; Lopez & Ollivon, 2009; Lopez et al., 2002). MF microstructure has been extensively described in the macro-length scale using polarized light microscopy (PLM). However, few studies have been done to describe the structure of MF in between these two length scales, which is the nanostructure. Recently, Truong, Morgan, Bansal, Palmer, and Bhandari (2015) showed the stacking of TAG lamellar structures in nanoemulsions from fractionated milk fat fractions using cryo-TEM, while Acevedo and Marangoni (2010) showed that the large polycrystalline structures formed by fully hydrogenated canola oil (FHCO) in high oleic sunflower oil (HOSO) is brought about by the aggregation of nano-sized primary crystals (i.e., CNPs).

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The recently developed visualization method and characterization of CNPs could play a key role in describing fat functionality (Acevedo & Marangoni, 2015; Peyronel, Quinn, Marangoni, & Pink, 2014). CNPs are formed from the stacking of several TAG lamella. The aggregation of CNPs brings about the formation of a variety of meso- (i.e., submicron) and micro-scale structures. Light microscopy has been successful in elucidating the microscale, while ultra-small angle X-ray scattering (USAXS) has been proven useful in characterizing the meso-scale (Marangoni et al., 2012).

The X-ray scattering technique allows the characterization of CNPs and their aggregates. The Guinier-Porod and the Unified Fit models were shown to be of use in obtaining some parameters (Peyronel, Ilavsky, Mazzanti, Marangoni, & Pink, 2013; Peyronel et al., 2014). Computer modeling and simulations had shown that some of the parameters obtained from USAXS analysis can be interpreted in a fractal way (Pink, Quinn, Peyronel, & Marangoni, 2013; Quinn et al., 2014).

The fractal nature of TAG crystal networks is described as the result of the self-repetition and aggregation of primary crystals (i.e., CNPs) into larger structures (Narine & Marangoni, 1999a–c; Marangoni, 2002; Marangoni and Rogers, 2003; Marangoni et al., 2012; Pink, Peyronel, Quinn, Singh, & Marangoni, 2015). The fractality of TAG networks has been measured by rheological fractal dimension, D_r, which is inversely related to the shear stress modulus, G'. The higher the D_r, value, the higher the order of the distribution of solids, and the lower the G'. A lower G' subsequently indicates a softer material (Narine & Marangoni, 1999–c; Wright, Scanlon, Hartel, & Marangoni, 2001). For example, Wright and co-workers found a value of $D_r = 2.45$ for MF samples crystallized under slow cooling (0.1 °C/min) and $D_r = 1.95$ for MF samples crystallized at fast cooling rates (5 °C/min). Model and simulations paired up with rheogolical and USAXS experiments have shown that D_r can be matched to a mass fractal dimension D at a particular length scale when samples are prepared under specific crystallization conditions (Pink et al., 2013).

Since MF is added into food products for the characteristic rheological and sensorial attributes it provides, it is therefore advantageous to describe MF properties according to the morphological characteristics of its CNPs as well as their aggregates. The morphology and average sizes of MF-CNPs and the sizes of its aggregates will be described based on previous work in our lab (Peyronel et al., 2014).

2. Materials and methods

2.1. Anhydrous milk fat

Anhydrous milk fat (AMF) was obtained from Kraft Foods Group, Inc. (Glenview, Illinois, USA). AMF was melted to 70 °C, cooled down slowly at approximately 0.1 °C/min to 5 °C and then stored inside the refrigerator (5–7 °C) for more than two months before the analysis.

2.2. Observation of milk fat – crystalline nanoplatelets (MF-CNPs) by cryogenic-transmission electron microscopy (Cryo-TEM)

In order to observe MF-CNPs, preparation of AMF was carried out inside a walk-in freezer (approximately −22 °C) as follows: AMF, previously crystallized (~0.1 °C/min) was suspended in cold isobutanol at a ratio of 1:50 in order to wash off oil. The mixture was then homogenized at 25,000–30,000 rpm for 8–10 min using a rotostator (Polytron® 1300 PT, Kinematica AG, Switzerland). The mixture was then vacuum filtrated through a glass fiber filter of 1.0 μm pore size and the crystals were collected by scraping them from the glass fiber filter using a spatula. The recovered crystals were then re-suspended in cold isobutanol (1:50) and re-homogenized for 5–8 min using the rotostator. This was done in order to disperse crystals sufficiently. After re-homogenization, the mixture was sonicated for 60 min using an ultrasonic processor (Bransonic 1210R-DTH, Branson Ultrasonic Corporation, Danburry, CT, USA). Temperature control in the sonicator was set at 5 °C and any increase in temperature was prevented by putting ice packs in the water bath. Sonication was done to enhance dispersion and to prevent the aggregation of the crystals without melting them before observation under cryo-TEM.

Keeping conditions at refrigeration temperatures, a drop of the mixture (approximately 5 μL) was placed in a carbon grid with perforated carbon film (Canemco-Marivac, Quebec, Canada). Excess liquid was blotted using a filter paper and the solvent was allowed to evaporate for 6 min. A drop of 2% of uranyl acetate was then added in order to enhance contrast of the crystals against the solvent. Excess solution was again blotted using a filter paper. The grid was then immediately dipped into liquid nitrogen and transferred to a cryo holder (Gatan Inc., Pleasanton, CA, USA) for direct observation at −176 °C in a FEI Tecnai G2 F20 energy-filtered Cryo-TEM operated at 200 kV in low dose mode. Micrograph images obtained using a Gatan 4 k CCD camera, were then processed using Adobe Photoshop, stored, and analyzed.

2.3. Triacylglycerol analysis using HPLC

Sample preparation for TAG analysis was carried out by weighing 30 mg of MF sample into a 2 mL HPLC vial. For the MF-CNPs, after the second homogenization stage described in 2.2, 30 mg of the sample was collected by filtering the mixture through a glass fiber filter under vacuum. After that, 600 μL chloroform and 1 mL 60:40 HPLC-grade acetone:acetonitrile solution were added to completely dissolve the samples.

TAG components were then quantified using HPLC (Alliance Model 2690 Separation Module, Waters Corporation, MA, USA) with a reverse phase column (Xbridge C18, 5 μm pore size, 4.6 × 250 mm column, Waters Corporation, MA, USA) and a refractive index detector (Waters model 2410 RID, Waters Corporation, MA, USA). TAGs were then identified using standards that were run in the same column using the same mobile phase (60:40 v/v HPLC-grade acetone:acetonitrile).

2.4. Image analysis using Image J software

The dimensions (i.e., length and width) of MF-CNPs were determined semi-automatically by image analysis using Image J software 1.42q (National Institutes of Health, USA). Cryo-TEM micrographs were processed and examined. Crystal size was then determined by measuring the length of the lines drawn from one point to another in CNPs with visible edges and scaling pixels to nanometers (nm).

2.5. X-ray scattering

Sample preparation for the X-ray scattering experiments is described below. It should be noted that SAXS and USAXS were performed sequentially, one after the other with the X-ray on the same position. The SAXS detector was moved in front of the USAXS detector right after the USAXS measurement was finished. These experiments were performed at the Advanced Photon Source (APS), Argonne National Laboratory (Illinois, USA) using USAXS/SAXS instrument (Ilavsky et al., 2009; Ilavsky et al., 2012a,b).

From SAXS patterns, d-spacings of 10–100 Å can be calculated from the Bragg's peak positions that are measured. These d values, elucidate chain length and lamellar thickness. On the other hand, from USAXS patterns, larger structures in the range from ~100 to