1 **Title: Characterization of the nanoscale structure of milk fat**

2 **Authors:**

3 **Pere Randy R. Ramel** (pramel@uoguelph.ca)

4 **Fernanda Peyronel** (fsvaikau@uoguelph.ca)

5 **Alejandro G. Marangoni**^{*} (amarango@uoguelph.ca)

6 *Department of Food Science, University of Guelph, Guelph, ON, Canada, N1G 1Y2*

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^{*} Corresponding author: Tel: (519) 824-4120 x 54340 Fax: (519) 824-6631

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 (600nm x 200nm to 900nm x 300nm). Using small-angle X-ray scattering (SAXS), crystalline domain size, (*i.e.*, thickness of MF-CNPs), was determined (27nm (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.

Keywords: milk fat, nanostructure, milk fat-crystalline nanoplatelets (MF-CNP), cryogenic-transmission electron microscopy (Cryo-TEM), X-ray scattering

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 & 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; Robert G Jensen, 2002; C 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, 2L or triple, 3L (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 ($>1^{\circ}\text{C}/\text{min}$), and β' and β polymorphs at slow cooling rates (0.1 to $0.01^{\circ}\text{C}/\text{min}$), although co-existence of the different forms may occur due to the complexity in composition described above (Grotenhuis, Aken, Malssen, & Schenk, 1999; C Lopez, Lavigne, Lesieur, Bourgaux, et al., 2001; Christelle Lopez, Bourgaux, Lesieur, et

al., 2002). 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; C Lopez, Lavigne, Lesieur, Keller, & Ollivon, 2001; Christelle Lopez & Ollivon, 2009; Christelle Lopez, Bourgaux, Lesieur, 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, et al. (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).

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, Quinn, 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 and Marangoni, 1999; Marangoni, 2000; Marangoni and Rogers, 2003; Marangoni et al., 2012; Pink et al., 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, 1999a, 1999b, 1999c; 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 rheological 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, Quinn, 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 to 30,000 rpm for 8-10 minutes 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 minutes 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 (Branson 1210R-DTH, Branson Ultrasonic Corporation, Danbury, 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 5µL) 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 minutes. 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 4k 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 30mg of MF sample into a 2mL HPLC vial. For the MF-CNPs, after the second homogenization stage described in 2.2, 30mg of the sample was collected by filtering the mixture through a glass fiber filter under vacuum. After that, 600µL chloroform and 1mL 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, 5µm pore size, 4.6 x 250mm 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 on the sample. 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 (J. Ilavsky et al., 2012; Jan Ilavsky et al., 2009, 2012).

From SAXS patterns, d-spacings of 10-100Å 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 ~100~60000Å can be studied. Morphology and average sizes of CNPs are obtained from USAXS as well as the way that the CNPs aggregate to form larger structures.

Corresponding d-spacings were calculated using Bragg's law:

$$d = \frac{n\lambda}{2\sin\theta} \quad (1)$$

where d is the spacing between planes of atoms, n is an integer related to the order of reflection, usually set to 1.

The scattering theory uses the concept of "length scale", L rather than the concept of "d-spacings":

$$L = \frac{2\pi}{q} \quad (2)$$

where q is the scattering wave vector obtained as the difference between the direction of the incoming x-rays and the direction of the diffracted x-rays.

2.5.1. Small-angle x-ray scattering (SAXS)

SAXS analysis was performed to determine the thickness of the CNPs or crystalline domain size (ξ) using the Scherrer formula (Acevedo & Marangoni, 2010):

$$\xi = \frac{K\lambda}{FWHM \cos \theta} \quad (3)$$

where K is the shape factor, θ is the diffraction angle, FWHM is the full width at half of the maximum peak height in radians, and λ is the wavelength of the x-ray which is 1.54Å for copper. K is dimensionless and provides information about the “roundness” of the particle. A shape factor of 1 is given for spherical particles while a shape factor of less than 1 is given for other shapes. For crystallites of unknown shape, a value of 0.9 is usually used (Klug & Alexander, 1974).

2.5.2. Ultra-small angle x-ray scattering (USAXS)

The sample preparation for USAXS/SAXS analysis was carried out as follows. A melted AMF sample (up to 70°C) was pipetted into a circular silicon isolator 1 mm deep cavity and 13mm in diameter (Grace-Bio-Labs, Oregon, USA) with an attached 125µm thick microscope glass cover slip at the bottom. The sample was then allowed to crystallize in the isolator from 70 to 20°C at an average cooling rate of 0.1°C/min. A second cover slip was then placed on top of the sample to seal it in place. The sample was kept at 20-25°C for a total storage time of 1 week before the analysis.

Interpretation of USAXS results was performed using the Guinier-Porod and Unified Fit models as described by Beaucage, 1995, 1996; Hammouda, 2010; and Peyronel, 2014. Values of the parameters P and R_g (Unified Fit) and s (Guinier-Porod) were obtained from the fitting and determination of the slopes of the USAXS pattern using Irena running in Igor Pro 6.36 (Wavemetrics, Inc., USA) (Jan Ilavsky & Jemian, 2009).

The Unified Fit model returns a radius of gyration, R_g , and an exponent, P , for each structural level, using the following fitting equation:

$$I(q) = Ge^{\left(\frac{-q^2 R_{gi}^2}{3}\right)} + \frac{B}{q^P} e^{\left(\frac{-q^2 R_{gi-1}^2}{3}\right)} \left[\operatorname{erf}\left(\frac{-q R_{gi}}{6^{1/2}}\right) \right]^{3P} \quad (4)$$

The Unified Fit model, represents all scattering objects as approximately spherical (equation 2) as shown by the term $\exp(-q^2 R_g^2/3)$. The first term describes the Guinier region, valid for $q \leq q_1$, and the second term, the Porod power law region, is valid for $q \geq q_1$. G and B are the Guinier and Porod scale factors respectively; G is related to the volume of the scatterers while B contains specific surface area information. P is the Porod exponent that measures the internal structure of the aggregate (Schaefer, Kohls, & Feinblum, 2012).

It has been previously shown that for edible fat systems, the parameter P can be related to the fractality in the system (Peyronel et al., 2013; Peyronel, Quinn, et al., 2014). These authors showed that edible fats can contain up to three structural levels using Unified Fit. Following the model and computer simulation by Pink et al. (2013), Peyronel et al. (2013) interpreted the results as follows P_1 identified the morphology of the CNPs as a smooth, rough or fuzzy surface; P_2 described the aggregation of CNPs either into long rods, termed TAGwoods, or other forms; and P_3 described the aggregation of TAGwoods and other forms into even larger structures. The parameter R_g , on the other hand, is the average radius of gyration of the “scatterer” (*i.e.*, CNP or

of a larger structure, like TAGwood or their aggregation) for the particular structural level identified with P . Peyronel et al. (2013) identified R_{g1} that describes the average size of the CNPs, in the nanoscale range, while R_{g2} was identified with the average size of the aggregates, in the microscale range. Assuming spherical scatterers, the average size of the CNPs and the aggregates was computed using the equation:

$$d_{CNP} = 2\sqrt{\frac{5}{3}}R_g \quad (5)$$

Parameter s obtained from the Guinier-Porod model gives an indication of the shape of the scatterers. An s equal to 1 indicates the presence of long rod structures while 2 indicates the presence of platelet-shaped aggregates.

Porod's law shows that a $|P|=4$ values indicates a fractal dimension $D=2$, which is a smooth surface. If $3 \leq |P| < 4$, the fractal dimension is calculated by $6 - |P|$ which gives fractal values in the range of $2 < D \leq 3$. While, if $1 \leq |P| < 3$, then D is equal to the value of $|P|$.

2.6 Graph plotting and peak analysis

Data analysis and plotting were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel, and the peaks were analyzed using PeakFit v4.12.

3. Results and discussion

3.1 Milk fat-crystalline nanoplatelets (MF-CNPs)

Figure 1 shows a cryo-TEM micrograph of MF-CNPs extracted from slowly crystallized MF that was stored for more than 2 months at 7°C. The discernible platelet shape of MF-CNPs resemble the ones observed previously by many authors like Acevedo and co-workers (2010 & 2012) on blends of fully hydrogenated oils (e.g. canola oil, sunflower oil and soybean oil),

Maleky and co-workers, (2011) on cocoa butter, and Peyronel & Marangoni (2014) on hydrogenated palm oil stearin. It should be noted that the samples previously subjected to CNP extraction methods contain substantial amounts of symmetric TAGs such as tristearin (SSS) and triolein (OOO) and were found to crystallize mainly into the triclinic β polymorph. MF, on the other hand, also contains substantial amounts of saturated fatty acids, however, it is also rich in short chain fatty acids (*i.e.* butyric acid), and mono- and poly- unsaturated fatty acids (MUFA and PUFA), making it a complex system (R G Jensen, Ferris, & Lammi-Keefe, 1991). The wide melting range brought about by the complexity of MF thus required us to modify the extraction method for CNPs, for instance, by performing the experiment at lower temperatures and shorter shear time compared to the method developed by Acevedo and Marangoni (2010). After trial and error, we were successful in extracting and observing MF-CNPs. Using HPLC (Figure 2), these CNPs were found to be mainly formed by TAGs that have relatively higher melting points such as tripalmitin (PPP); palmitin-palmitin-stearin (PPS); palmitin-stearin-stearin (PSS); and tristearin (SSS) TAGs. This means that the lower melting TAGs (short chain and unsaturated fatty acids) that were present in milk fat at the start, were removed during the extraction process (*i.e.*, cold-washing with isobutanol, homogenization and filtration).

3.2 Crystal size distribution using Image J

Using Image J, the length and width of the extracted MF-CNPs were measured by determining the distance covered by a line (*i.e.*, edge-to-edge of the MF-CNPs) in pixels and scaling it to nanometers (Figure 3). We believe that due to complexity of the TAGs that make the MF, some CNPs do not show clear edges as the ones made by, for example FHCO or SSS which could mean that during the extraction process some of the CNPs with low-melting TAGs are

being melted as explained in the previous section. However, by setting a threshold on the images (*i.e.* adjusting contrast and brightness) and by analyzing numerous images, we were able to offset this. CNP dimensions are displayed as frequency distribution figures (%) of crystal length and width, in Figures 4a and 4b, respectively. Results show that the majority of MF-CNPs have lengths ranging from 600 to 900nm and widths of 200 to 300nm. Furthermore, it can be observed that the MF-CNPs extracted have a high aspect ratio of 1:3 (*i.e.*, >50% have widths less than 300nm and lengths greater than 900nm). According to Maleky et al. (2011) and Acevedo et al. (2012), the crystal size distribution of the CNPs are greatly affected by shear and cooling rate during crystallization. Those authors showed that as supersaturation is increased, the length and width of CNPs is decreased. Furthermore, they also showed that with the application of shear, a more uniform crystal size distribution is observed. However, for the purpose of revealing and characterizing MF-CNPs, we crystallized MF statically at low cooling rates.

3.3 Crystal domain size (*i.e.*, thickness of CNPs)

As SAXS analysis was performed at room temperature, it is expected that the scattering pattern observed comes mainly from crystals formed by higher melting TAGs. Therefore, the observed crystals under cryo-TEM could be correlated very well with the results of the X-ray scattering analyses as the TAGs that are being analyzed in both systems are similar. We determined the FWHM of the (001) Bragg peak (Figure 5, peak at $d=42.5\text{\AA}$) to be 0.012 radians. When using this value in the Scherrer formula (equation 3), the crystalline domain size (*i.e.*, thickness of a CNP) was calculated to be ~27 nm. It is known that during crystallization at slow cooling rate, MF preferentially crystallizes into the β' form with some traces of β (C Lopez, Lavigne, Lesieur, Keller, et al., 2001; Christelle Lopez, Bourgaux, & Lesieur, 2002). Taking the

lamellar size for a β' polymorph as $\sim 4.2\text{nm}$ (Woodrow & deMan, 1968), the 27nm domain value indicates the stacking of about 6 to 7 lamellae per CNP. This shows an agreement with previous studies from Acevedo and Marangoni (2010) that showed the stacking of 7 to 10 platelets from systems containing high melting TAGs. Results from fractionating milk fat in nanoemulsions of stearin and olein crystallized slowly ($1^\circ\text{C}/\text{min}$) also showed a single lamellar thickness of $4.0\text{-}4.1\text{ nm}$, however stacking into CNPs were not determined. Factors like shear, cooling rate and undercooling are known to play a role in the domain size. For example Mazzanti and co-workers (2008 & 2009), showed that crystallization of MF under laminar shear resulted in a decrease of the domain size. Furthermore, the growth of the primary crystal was shown to occur only in the direction of the larger surface area side of the platelet-like crystallites (possibly CNPs). This effect of shear on the MF nanostructure could be further investigated using cryo-TEM if the MF-CNPs could be visualized laterally.

3.4 Characterization of MF-CNPs using USAXS

Figure 6 shows the USAXS pattern of MF obtained on a sample that was crystallized slowly and stored for one week at room temperature before the analysis. $|P_1| \sim 4$ obtained with Unified fit indicates that CNPs formed by high melting TAGs have smooth and clean surface. The $|P_2| \sim 2.1$ is in agreement with the statistical fractal aggregation called reaction limited cluster-cluster aggregation (RLCA) (Peyronel et al., 2013; Pink et al., 2013). This interpretation of the result is consistent with the idea that “a scatterer” had aggregated into larger structures which internal structure is described by RLCA. Most likely, the scatterer is the CNP. The $|P_3| \sim 4.3$ indicates that the larger structures formed have a diffused interface. Notice that P_2 covers the length scale from: 200 nm to 800 nm while P_3 covers the region from 1250 nm to 6200nm . We cannot

confirm the presence of TAGwoods as experiments were not performed under fast cooling rates and the data does not show a slope ~ -1 .

The Guinier-Porod model gave a value of $s \sim 2$ which is in agreement with previous work on MF that showed $s > 2$ (Peyronel, 2014). This s value indicates that the scatterer's shape is that of a platelet-like structure. It is not clear if the Guinier-Porod result points to larger CNPs or if the CNPs have aggregated in such a manner that they had become supra-platelets. The inherent sample preparation for cryo-TEM does not allow to confirm that supra-platelets were formed. Further experiments on MF prepared under different processing conditions and using both the USAXS and the cryo-TEM technique should help understand the structures formed in MF better.

Using the Unified Fit model and three hierarchical levels, two radius of gyrations were obtained (Figure 6: (1) $\sim 50\text{nm}$ and (2) $\sim 600\text{nm}$). When following the idea that each level seen with Unified Fit is a larger structure of the previous one, each radius of gyration can be used to identify an average size of the scatterer. The $R_{g1} \sim 50\text{nm}$ was used to compute the spherical average size (equation 5) of the CNP: $\sim 130\text{nm}$. The $R_{g2} \sim 440\text{nm}$ was used to compute an average size of the CNP aggregates, the one with an internal structure given by P_2 , which was $\sim 1.14\mu\text{m}$.

Using the Guinier-Porod model, only one radius of gyration was obtained at $\sim 20\text{ nm}$. The $s \sim 2$ obtained using this model indicates platelet-like structures.

4. Conclusions

We had shown the structure of MF at different length scales: from few angstroms to few micrometers. Cryo-TEM as well as USAXS-SAXS results confirmed the existence of smooth crystalline nano-platelets. CNPS obtained from MF at 7°C were determined to be composed

mainly of high melting TAGs that are also the TAGs being studied under USAXS-SAXS performed at room temperature. These CNPs have dimensions of 600-900nm x 200-300nm x 27nm (length x width x thickness). The Unified fit model identified MF-CNPs with smooth surfaces and average sizes of 130nm. It is to note here that the R_g is a phenomenological parameter (equation 4) and as such, its value is not to be taken as an exact value. The Unified Fit also provided a second radius of gyration that gave an average size of the scatterer of 1.14 μm . This value was interpreted as the size of an aggregate formed by the aggregation of CNPs, which showed an internal structure in accord to RLCA. At even larger scales, larger aggregates were formed and all that can be said is that they showed a diffused surface.

Using the Guinier-Porod model, all we can say is that the $s=2$ value identified platelet-like structures. It is not clear at this point how the Guinier-Porod model and the Unified Fit model results can be brought together to make for one big picture.

Work needs to be done in improving the sample preparation method to be able to extract MF-CNPs that show sharp edges under cryo-TEM visualization. More importantly, experiments under different processing conditions must be carried out in order to see how the morphology and sizes of MF-CNP is affected, as well as to understand the structures that are formed in the meso-scale. Elucidation and characterization of MF-CNPs is important as it provides a new dimension for analyzing and improving MF crystallization behavior, structure formation, and functionality.

Acknowledgements

The authors would like to thank Dr. David Pink (St. Francis Xavier University, Nova Scotia, Canada) for the fruitful discussions on USAXS, Mr. Robert Harris (Electron Microscopy Unit,

University of Guelph, Ontario, Canada) for his help with the use of cryo-TEM, Mr. Saeed Ghazani for his help with the HPLC, and Dr. Jan Ilavsky and his team at beam line 9ID for their invaluable support and help through the data collection and analysis. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

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FIGURE CAPTIONS

Figure 1. Crystalline nanoplatelets (CNPs) extracted from milk fat (MF) that was slowly cooled and stored for more than 2 months at 7°C. Samples imaged by cryogenic-transmission electron microscopy (cryo-TEM). The scale bar corresponds to 0.2µm

Figure 2. HPLC chromatograms showing the triacylglycerol composition of (a) milk fat (MF) and (b) crystal nanoplatelets that were extracted from milk fat (MF-CNPs). Bu – butyric; Cp – Caprylic; La – Lauric; M – Myristic; P – Palmitic; S – Stearic; O – Oleic ; Le – Linoleic.

Figure 3. Image analysis of cryogenic-transmission electron microscope (cryo-TEM) micrographs of extracted milk fat-crystal nanoplatelets (MF-CNPs) using Image J software. The lengths and widths are indicated by arrows. The scale bar corresponds to 0.2µm

Figure 4. Frequency distribution (%) of the crystal length, a, and width, b in nanometers of extracted milk fat-crystal nanoplatelets (MF-CNPs). Values were obtained by analyzing cryogenic-transmission electron micrographs (cryo-TEM) using Image J software.

Figure 5. Small-angle x-ray scattering (SAXS) pattern of milk fat (MF) cooled slowly and stored for 1 week at room temperature (20°C). A lamellar thickness of 42Å (d_{001}) indicates a double chain length (2L) stacking of triacylglycerols. The other two peaks (d_{002} and d_{003}) shows the second and third order of the 2L lamellar structure. The full-width-half-maximum width (FWHM) is indicated by arrows.

524 **Figure 6.** Ultra-small-angle x-ray scattering (USAX) pattern of milk fat (MF) cooled slowly and
525 stored for 1 week at room temperature (20°C). Parameters of the Guinier-Porod and Unified Fit
526 models were determined by the measuring the slope of the lines using Irena (Igor Pro 6.36,
527 Wavemetrics, Inc., USA) and are indicated in the graph.

528

Figure 1

Figure 2

Figure 3

Figure 4