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Laminar Shear Effects on Crystalline Alignments and Nanostructure of a Triacylglycerol Crystal Network

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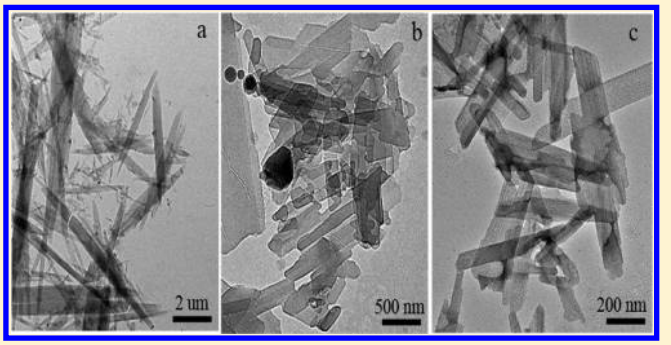
ABSTRACT: The effects of laminar shear on crystalline orientation and the nanostructure of triglyceride crystal networks were quantified by using different microscopic techniques. Cocoa butter (CB) was crystallized in the presence and absence of an external shear field. Two different dynamic samples were crystallized under a shear rate of approximately 340 s^{-1} by using a continuous Couette-type laminar shear crystallizer and by using a standard paddle mixer. To improve imaging resolution, liquid oil was removed from crystallized samples using isobutanol and aqueous solutions of different surfactants such as AOT, Teepol, and Fatsolve. Using cryogenic scanning electron microscopy (Cryo-SEM), oriented sheets of crystalline cocoa butter were observed in the sample obtained in the laminar shear crystallizer, while spherulitic structures were observed in the statically crystallized sample. The strong influence of the applied laminar shear on the nanoscale structure is demonstrated by characterization of CB platelet size using cryogenic transmission electron microscopy (Cryo-TEM). Shear crystallization caused a reduction in the platelets' length from 2000 to 300 nm and width from 165 to 130 nm. The platelets' thickness, obtained from Scherrer analysis of the 002 SAXS reflection, yielded a domain size of 54.8 nm for the specimen crystallized under laminar shear and 58.2 nm for the statically crystallized sample. This work demonstrates the large effects of shear during the crystallization process on the microstructure of polycrystalline materials.



INTRODUCTION

The study of microstructure in polycrystalline materials such as edible fats has become increasingly important since the macroscopic properties of such materials depend on the structure of their crystal networks.[1−7] Edible fats are a class of plastic materials composed of a continuous network of crystalline fat suspended in an oil phase.[8,9] It is widely held that the balance between van der Waals attractions and Brownian motion causes the suspended crystals to aggregate and form a three-dimensional (3-D) network via diffusion-limited cluster−cluster aggregation.[10,11] The properties of this 3-D network depend not only on the amount and distribution of the network mass but also on the properties of the individual particles. These include the size, shape, and arrangement of the crystals. A significant amount of research has shown that the habit of fat crystals is greatly affected by heat, mass, and momentum transfer conditions established during the crystallization process.[6,12−14] Therefore, by modifying processing conditions (i.e., crystallization temperature, cooling rate, and agitation rate), the crystal habit and the subsequent properties of the crystal network can be tailored. It has been shown that the crystallization temperature affects the packing of the triacylglycerol (TAG) molecules and thus influences the microstructure of the crystallized fats.[6,15,16] Marangoni and McGauley (2003) reported that while cocoa butter crystallized at different temperatures showed similar polymorphism, the resultant material exhibited dissimilar microstructure.[6] Several research groups have demonstrated the effects of cooling rate on the nucleation, polymorphism, and aggregation of small crystalline particles in fat materials.[5,17−20] They observed that the slow cooling of milk fat results in a small number of large crystals, whereas rapid cooling produces numerous small crystallites with a fairly uniform size distribution.

The effects of shear on the development of a fat crystal network have been widely studied. Several research groups have found that subjecting the melt to shear stress while it crystallizes accelerates the rate of solid-state phase transformations.[21−27] Furthermore, it has been found that the application of shear during crystallization hinders crystal aggregation such that the size of the formed crystallite clusters was reduced.[24,28,29] A few studies also reported that the application of shear during crystallization resulted in internal rearrangements of the crystalline lamella. This was confirmed by X-ray diffraction studies.[25,29−32]

Although the combined influence of these processing conditions on the microstructural properties of the network is relatively well understood, nanoscale characteristics have yet to be investigated in full.[33,34]

The purpose of the present study is to develop a suitable imaging technique for the visualization of the structure of crystallized fat at the meso- and nanoscale levels. Particular attention will be paid to the alignment and distribution of crystal mass within the network in addition to observing the habit of the crystals (size and morphology). These observations can furnish data for

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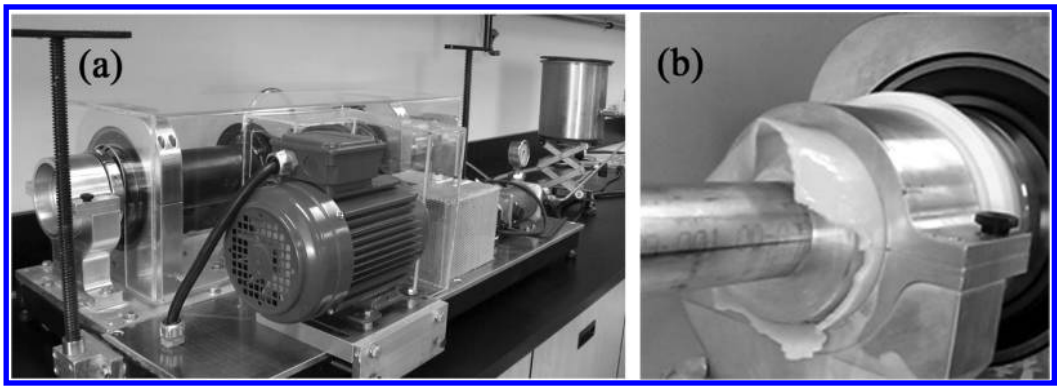

Figure 1. (a) The continuous laminar shear crystallizer. (b) A cocoa butter sheet was crystallized continuously in the laminar shear crystallizer.

further studies as the nano- and microstructure of fat crystal networks strongly influence the functional properties of fat-structured products.

MATERIAL AND METHODS

A. Materials. The cocoa butter used in this study was donated by Mars Chocolate North America (Hackettstown, NJ, USA), which had an average fatty acid composition of 27.0% palmitic, 35.7% stearic, 35.6% oleic, 0.64% linoleic, 1.03% linolenic acids, as determined by standard gas−liquid chromatographic methods. Cocoa butter was used as the lipid of choice for the study of laminar shear on the crystallization of triglyceride molecules. The cocoa butter was heated in an oven at 70 °C to ensure complete melting. The fat was held at this temperature for at least 30 min before use to ensure destruction of the crystal memory. The surfactants and organic solvents such as 2-methyl-1-propanol 99.5%, sodium dioctylsolfosuccinate (Aerosol OT, AOT), Teepol 610S were purchased from the Sigma-Aldrich Co. and Fatsolve VF21L was provided by Johnson Diversey, Inc. (ON, Canada).

B. Sample Preparation. *Crystallization Procedure Used to Induce Crystalline Orientation.* The molten sample was pumped through a continuous laminar shear crystallizer in the Couette-type configuration (two concentric cylinders — an inner bob with an outer cup, with the cup rotating) shown in Figure 1[25] at a flow rate of 30 mL/min. A shear rate of 340 s^{-1} was applied and the cocoa butter was crystallized from the melt (60 °C) to 20 °C. Reduction of temperature was achieved by three water jackets connected to a cooling water bath.[25] The resulting solid sheet of CB was stored for 7 days in an incubator set at 20 °C. This sample is curved with a 2.5 mm thickness and will be referred to as the "oriented sample" (OR) throughout the rest of this study.

Crystallization Procedure for Sheared and Static Samples. Sheared and nonsheared (static) samples with the same dimensions and shape as the oriented sample were crystallized in a different shear cell (Figure 2). This shear cell was in the Searle configuration (the inner bob rotates while the outer cup remains stationary). The shear cell consisted of two concentric aluminum cylinders with a 2.5 mm gap in between. The outer cylinder was designed such that it could be disassembled readily to aid in the extraction of the sample. To this end, the outer cylinder was halved lengthwise. A groove, to allow the fitting of an O-ring, was cut into the contact points between the two halves. The O-ring provided a tight seal between the two halves, preventing the leakage of sample. A groove was also cut into the bottom of the cup. A matching groove was also machined on the bottom of the bob. This groove allowed a bearing to be installed. This bearing would affect the rotation of the bob. The bob can be connected to a variable-speed motor so that shear could be applied if necessary. The applied shear rate was controlled by varying the angular velocity of the bob. A cooling jacket was also included in the design so that the sample may be cooled using an appropriate cooling regime.

Figure 2. The disassembled shear cell used for the preparation of the static and sheared samples.

The sheared specimen was created by crystallizing molten cocoa butter with (in a flask) inside a temperature-controlled water bath (Neslab RTE-111, Fisher Scientific, St. Louis, MO) from 60 to 20 °C. The crystallization process was carried out for 30 min. During cooling, a Lightnin mixer (Lightnin Labmaster LIU10F, Wytheville, VA, USA) was used to agitate the melt at a shear rate of approximately 340 s^{-1}. The partially crystallized cocoa butter was then transferred into the above-described shear cell kept at 20 °C to allow the material to set.

The static sample was made by transferring the molten cocoa butter into the heated (60 °C) shear cell. By adjusting the temperature of the water bath attached to the shear cell, the cocoa butter was cooled to 20 °C at a rate of 2 °C/min. The shear cell was then transferred to an incubator set at 20 °C to complete the crystallization process. The sheared and static samples were stored for 7 days in an incubator set to 20 °C before further study.

C. Microscopy. *Polarized Light Microscopy (PLM).* A Leica DM RXA2 deconvolution light microscope (Leica Microsystem, Toronto, Canada) equipped with a digital monochrome camera (Q Imaging Retiga 1300, Vancouver Canada) was used to take polarized light images of the oriented, sheared, and static samples. The crystallized fat samples were cut into very thin layers, placed onto a microscope slide and squeezed into thinner sections using a coverslip. Photomicrographs were

taken using a 50× objective lens corresponding to a magnification of 500×.

Cryogenic Scanning Electron Microscopy (Cryo-SEM). The crystallized samples were suspended in either 2-methyl-1-propanol (isobutanol), Teepol, sodium dioctylsolfosuccinate (AOT), or Fatsolve for the purpose of deoiling the sample such that surface characteristics could be observed using SEM. The crystallized cocoa butter was dispersed in isobutanol at a ratio of 1:25 (sample/solvent) and the mixture was allowed to stand for 24 h at 5 °C. The mixture was then vacuumed filtered through filter paper No. 4. to remove the solvent. Residual solvent was vaporized by placing the retentate on a dry piece of filter paper and allowing the solvent to evaporate overnight. Any potential artifacts due to sample recrystllization during solvent removal have not been accounted for.

The crystallized samples were dissolved in aqueous solutions of either 5% AOT, 30% Teepol, and 80% Fatsolve for 24 h at 20 °C before being observed under the electron microscope. The specimens deoiled in the detergents were rinsed with distilled water to remove residual detergent. The excess water was removed by placing the sample on a dry piece of filter paper. The samples were then cut and attached to the copper sample holder using the Tissue-Tek embedding medium. The sample holder was immersed in a liquid nitrogen slush (−207 °C) using an Emitech K1250x Cryo-preparation unit (Ashford Kent, UK). The liquid nitrogen slush was prepared by pulling a vacuum on liquid nitrogen. To prevent frost formation on the surface of the sample, the sample holder was withdrawn from the freezing chamber using an argon-filled chamber. The interior of the electron microscope is kept under a vacuum for the same reason. The samples were then sputter-coated with 30 nm of gold at a temperature below −130 °C using the Emitech K1250x cryo-preparation system. The holder was then transferred onto the scanning electron microscope's cold stage, which is kept at a temperature below −140 °C (Hitachi S-5700, Tokyo, Japan). The accelerating voltage of the electron beam was 10 kV. Images were captured digitally using the Quartz PCI imaging package (Quartz Imaging Corp., Vancouver, BC).

Cryogenic Transmission Electron Microscopy (cryo-TEM). The solvent needs to be removed from the matrix to enable observation of the microstructure. This is often achieved by freeze-drying and by solvent washing (when the solvent is hydrophobic).[33,34] Crystallized cocoa butter was dispersed in cold isobutanol (10 °C) at a ratio of 1:25 (sample/solvent). Dispersion was aided by a commercially available mixing apparatus (Rotostator, Power Gen 125, Fisher Scientific). Mixing was conducted at 30 000 rpm for 7 min. The solution was then vacuum filtered through a glass fiber filter with a pore size of 1 μm. The retentate was immersed once more in cold isobutanol to create a uniform dispersion of the nanocrystals. Dispersion of the crystals was aided by sonication using a sonicating bath (Bransonic 1210R-DTH, Branson Ultrasonic Corporation, Danburry, CT, USA) set at 60 Hz, 117 V, and 1.3 Amp. Sonication was conducted at 5 °C for 40 min. Five microliters of the crystal suspension was placed on a copper grid coated with a perforated carbon film (Canemco-Marivac, Quebec, Canada). Filter paper was used to blot the excess solvent. The sample was then stained with a 2% aqueous solution of uranyl acetate for the purpose of enhacing the contrast. The samples were transferred to the cryo chamber of the TEM (FEI Technai G2 F20, Eindhoven, The Netherlands). Using a Gatan 4K CCD camera, zero-loss energy-filtered images were taken of the nanocrystals. Digital Micrograph and Image J 1.42q software (USA) were employed to analyze the micrographs.

D. Solid Fat Content Measurements. Solid fat content (SFC) was measured by means of pulsed nuclear magnetic resonance (p-NMR) using a Bruker Minispec spectrometer (Bruker Optics Ltd., ON, Canada). Glass NMR tubes (10 mm diameter, 1 mm thickness, and 180 mm height) were filled with approximately 3 g of the crystallized samples (samples were cut in small pieces and placed in the tube). Crystallized samples were kept at the crystallization temperature, 20 °C, for 7 days to monitor the SFC variation during storage.

E. Powder X-ray Diffraction. The polymorphism of the crystallized samples was determined through powder X-ray diffraction (XRD) techniques set at reflection mode using a Rigaku Multiplex powder X-ray diffractometer (Rigaku, Japan). The apparatus had a 1/2° divergence slit, a 1/2° scatter slit, and a 0.3 mm receiving slit. The accelerating voltage and current of the X-ray copper tube was set at 40 kV and 44 mA, respectively. The resolution of the instrument was determined to be 0.2 Å. Approximately 1 g of the crystallized sample was placed onto a prechilled glass X-ray slide. The glass slide was chilled by holding it in an incubator set to the crystallization temperature (20 °C). The filled slide was placed on the X-ray sample holder, which was also set to 20 °C. Scans were performed from 0 to 30 deg 2-theta at a scanning rate of 2°/min. The results were analyzed using MDI's Jade 9 powder X-ray analysis software package (Materials Data Incorporated, Livermore, California). The presence of crystalline orientation in the crystallized samples was analyzed by XRD using a Proteum pt-135, 2D detector (Bruker, Wisconsin. USA). The molybdenum tube was set at 40 mA and 35 kV and a wavelength of λ = 1.5418 Å was used. Experiments were done at both wide and small angle regions, with the detector located at both 225 and 75 mm from the cell. A small piece of crystallized fat (dimensions: 1 × 1 mm^2) was placed on the sample holder to study the crystalline orientation in *XZ*, *YZ*, and *YX* planes. Data were collected on each sample from two correlated images with 3 or 5 min exposure time for each. Datasqueeze 2.2 software (Wayne, Pennsylvania, USA) was used for the analysis of the images. Two determinations of four replications were done for the dynamic condition and samples in the static condition were run once.

F. Statistical Analysis. Prism 5 (GraphPad Software, Inc., CA, USA) was used to analyze all data obtained. The reported values correspond to the means ± standard errors. A one-way ANOVA with a 95% confidence interval was used to evaluate the statistical difference between the means.

RESULTS AND DISCUSSION

A detailed inspection of the effects of laminar shear on the phase transformations and crystalline orientations of cocoa butter during its crystallization has already been reported by Maleky and Marangoni.[25] In that work, it was demonstrated that the application of shear to crystallizing cocoa butter accelerated the transformation of thermodynamically metastable phases, α and β', to the more stable β_V phase. The application of shear accelerates polymorphic transformations which take days to achieve to less than 15 min. The orientation of fat crystallite layers was also noted. The crystallites were also found to be crystallographically oriented in a direction parallel to the external shear field.

Since changes in solid fat content influence the microstructure of TAG crystal networks,[35] the SFCs of all the samples were measured and monitored during 7 days storage period as shown in Figure 3A. It is evident that under dynamic crystallization conditions, the laminar shear applied to the samples accelerated the crystallization process relative to the static condition; however, statistical analysis confirmed that all samples had a similar solid fat content, 78%, after one day storage ($P > 0.5$). Since studies have shown that the properties of a material are often influenced by solid state polymorphism, powder XRD patterns of each sample were collected. Figure 3B shows an XRD pattern of the β (Form V) polymorphic form observed in all the cocoa butter samples. The extent of alignment of the crystalline material can be observed using small-angle X-ray scattering. Figure 4 depicts a characteristic small-angle X-ray Debye diffraction ring at the 002 reflection. The anisotropy in the scattering intensity around the ring indicates that X-rays are scattered unevenly by the sample

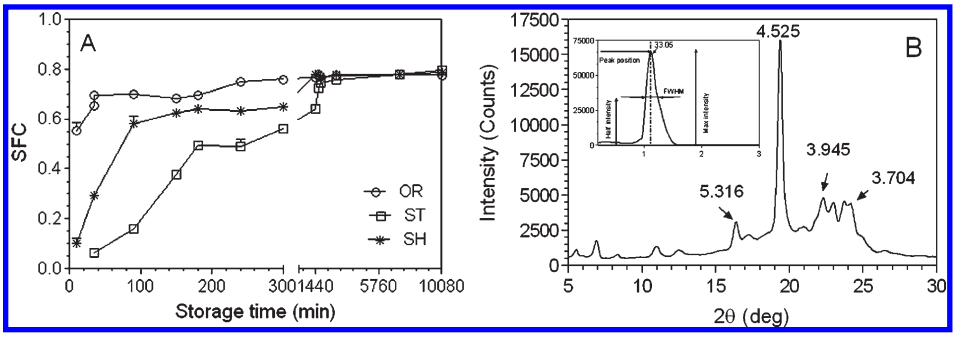

Figure 3. (A) Crystallization curves of the samples during 7 days of storage at 20 °C. (B) X-ray diffraction patterns in small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS) regions of CB after 7 days of storage at 20 °C.

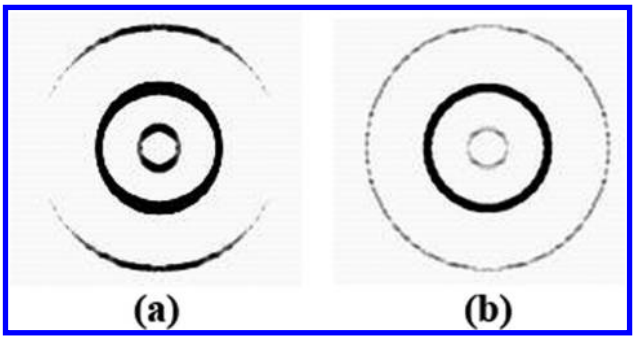

Figure 4. An example of X-ray diffraction pattern of all samples in the SAXS region: (a) oriented, (b) sheared/static. Patterns display a clear orientation of the crystals in the oriented sample, no orientation in the static and sheared samples.

crystallized in the continuous laminar shear crystallizer (Figure 4a). The uneven scattering around the Debye ring suggests an ordering of the crystalline material along a given axis/axes. A Debye ring of uniform intensity was obtained from the sheared and static samples (Figure 4b). This suggests that there was no orientation of the crystalline material in these samples ("random orientation") as the X-rays were scattered evenly in all directions.

Polarized Light Microscopy (PLM). Polarized light microscopy was used to observe the microstructure of the fat materials. PLM is well-suited for the study of crystalline materials as individual fat crystals and their aggregates are birefringent and appear bright under crossed polarizers, whereas the amorphous oil phase appears dark.[36−38] PLM images representative of each of the crystallized samples are presented in Figure 5. Figure 5A shows the fat aggregates that were formed under static conditions. Crystal clusters with a size between 40 to 50 μm are formed via the agglomeration and/or secondary nucleation of the smaller crystallites. These crystalline clusters do not show a radial distribution characteristic of spherulites. Rather, a random orientation of the crystallites was observed. Figure 5B depicts the microstructure of a sheared sample. The microstructure suggested by Figure 5B is different from that described in Figure 5A. The crystalline clusters observed in Figure 5B are smaller than those observed in Figure 5A. This suggests that the size of the clusters was reduced by the applied shear. Similarly, laminar shear could have prevented the growth of clusters or the further aggregation of crystallites into clusters. We do not have evidence at this moment of which mechanism is responsible for the observed effects.

The microstructure of the sample crystallized within the laminar shear crystallizer is displayed in Figure 5C. The presence

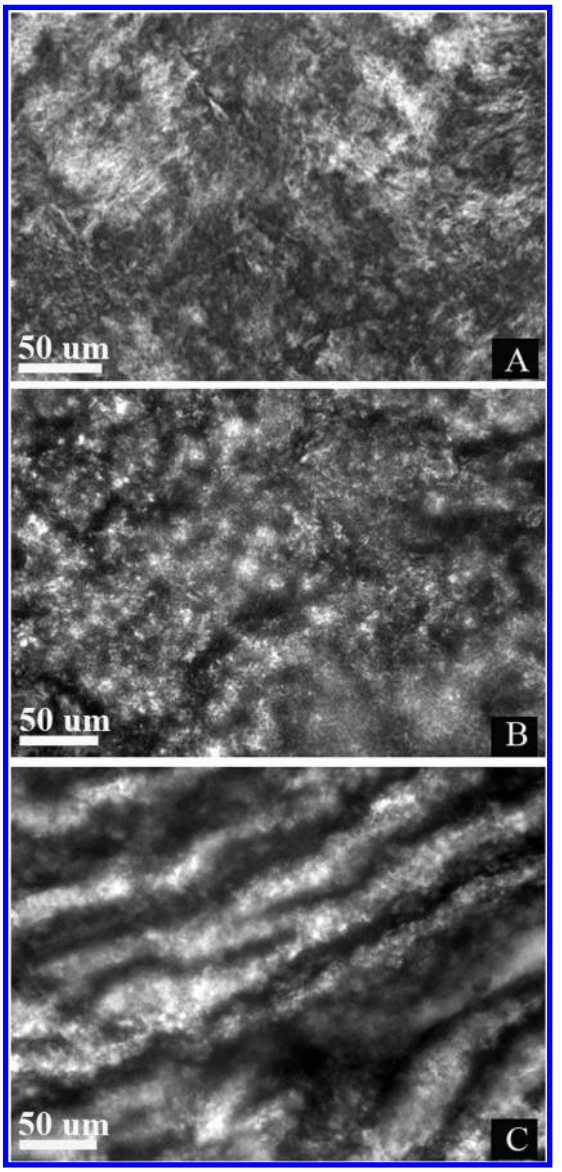

Figure 5. Polarized light micrographs of static (A), sheared (B), and oriented (C) samples.

of small spherulites within this sample strengthens the notion that the application of shear will reduce the size of the crystalline structures, mainly due to an increase in the nucleation rate of the

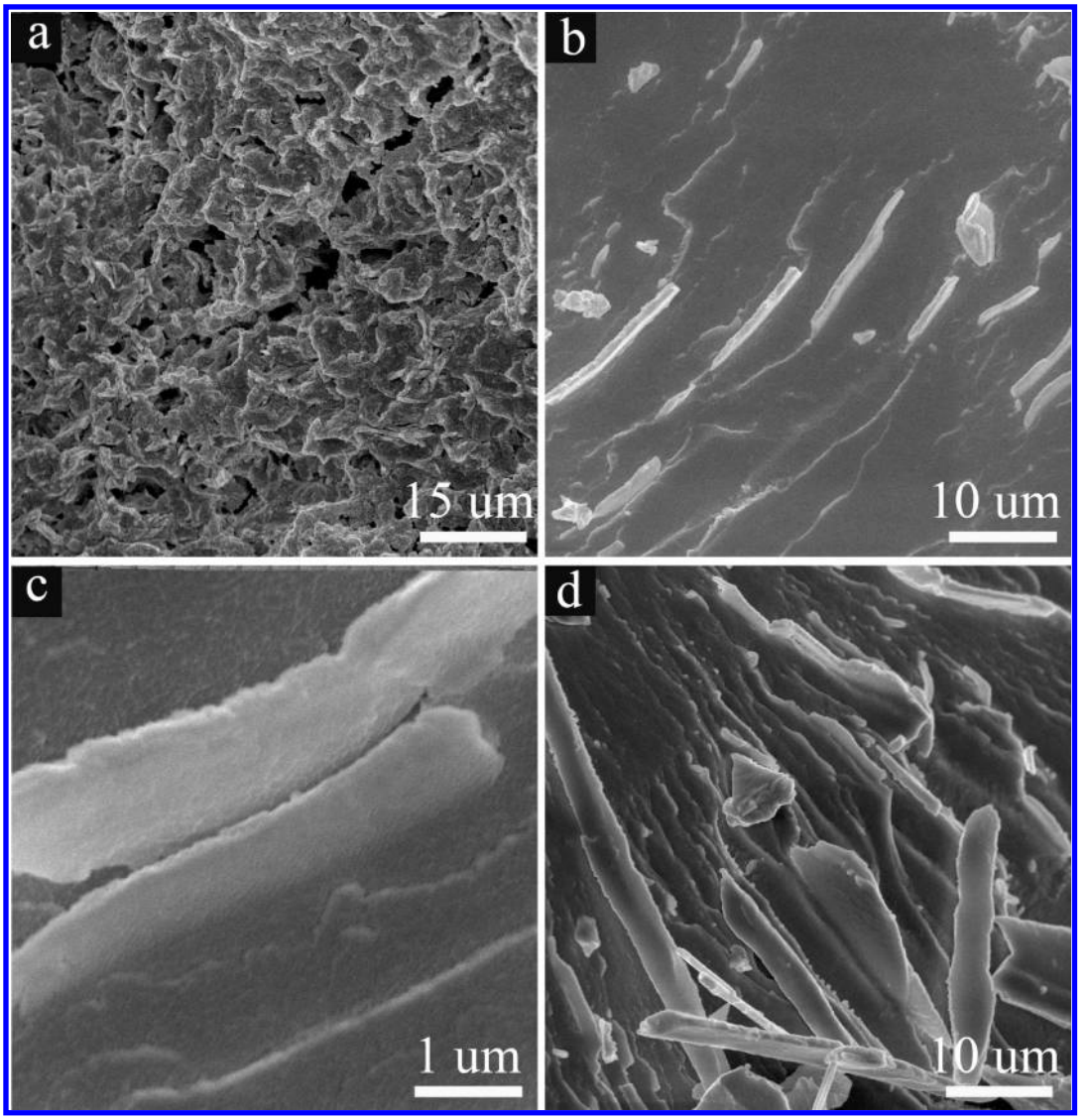

Figure 6. Cryo-scanning electron micrograph of samples after washing with isobutanol: static sample (a) and oriented samples (b, c); after washing with AOT: oriented sample (d).

material under shear. By comparing Figure 5, panels B and C side-by-side, it can be observed that the arrangement of the crystalline aggregates in the oriented sample is less random than the distribution of such aggregates in the sheared sample. This observation suggests that a measure of orientation is introduced when applying a specific type of shear during crystallization. This observation is in-line with the conclusions drawn from the anisotropic Debye rings obtained via small-angle X-ray scattering. Care must be taken, however, in drawing such conclusions on the basis of light micrographs alone as such methods are inevitably limited by the resolution of visible light. While PLM provides adequate information about the nature of the aggregates, PLM does not provide detailed information about the fine structure of the network formed by these aggregates.

Cryogenic Scanning Electron Microscopy (Cryo-SEM). Cryo-SEM was used to image the network structure at a higher magnification in the hope that information about the basic building blocks of the crystal aggregates could be obtained.[39−41] However, imaging by cryo-SEM is hampered by the presence of significant amounts of oil in the fat crystal network. The studied samples have a solid fat content of 78% with the remainder being liquid oil. The low temperatures used in cryo-SEM can crystallize the oil, which will hamper the ability to discern the crystalline structures which would be present at noncryogenic temperatures, when the oil is liquid. Moreover, the presence of oil in general hampers the observation of the original crystals formed. The oil must therefore be removed.

Various sample preparation techniques intended to remove the liquid phase from a fat crystal network have been developed and are widely reported in the literature. Some studies suggest treating the fat material with an organic solvent.[38,42] According to these studies, washing with isobutanol can remove the liquid phase without seriously affecting the network microstructure.[38,39,42] It has also been found that aqueous solutions of detergents such as Teepol[43] and Aerosol OT, AOT,[44] can be used to deoil fat samples. Disruption of the structure in a sample can be prevented through the use of a fixing agent. Mostafa et al (1985) reported that the application of osmium tetroxide to fat crystal isolates fixed the solid fat crystals leaving the network structure relatively untouched during deoiling.[45] Osmium tetroxide forms cross-links between the

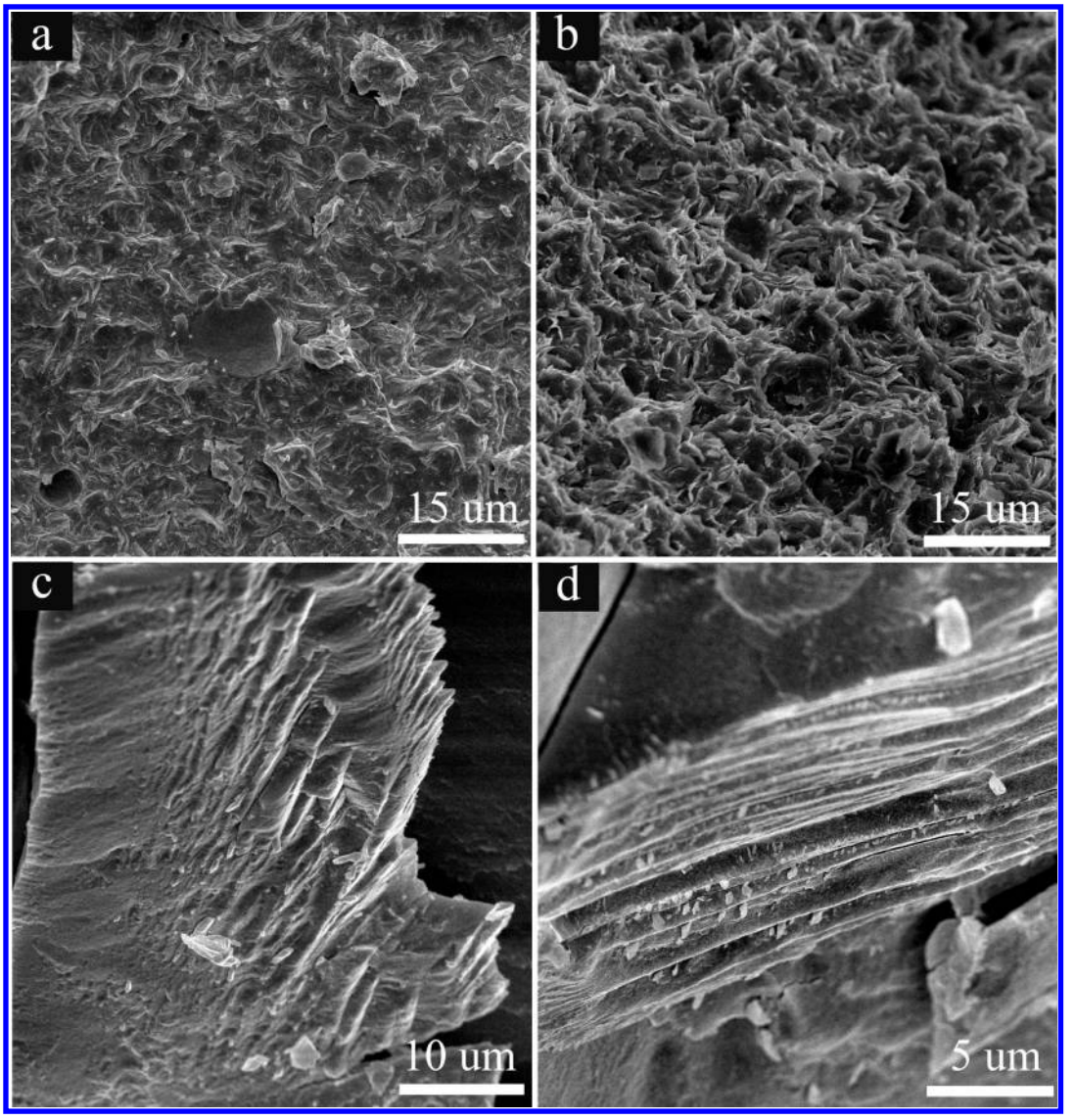

Figure 7. Cryo-scanning electron micrograph of samples after washing with Teepol: static (a), sheared (b), oriented samples (c, d).

double bonds of unsaturated fatty acids.[46,47] This method was originally developed for use in fats. Rogers et al. recently demonstrated that treating organogels with osmium tetroxide prior to washing with isobutanol improved the removal of the oil from the gel.[41]

Regardless of the method selected, optimal deoiling must be homogeneous across the sample. Furthermore, an optimal deoiling method will employ low temperatures as this will reduce the solubility of the triglycerides making up the solid phase and thus reduce dissolution of the solid crystalline particles. To determine which solvent was best suited to deoiling cocoa butter, all of the above-mentioned solvents and detergents were used.

Isobutanol is the solvent of choice for deoiling fat materials as isobutanol is nonpolar enough such that it will dissolve the oil fraction, while at the same time is not too nonpolar so as to rapidly dissolve the fat and oil completely.[37,38,48] Solvent deoiling inevitably leads to some loss of solid material; however, if carried out carefully by judicious choice of time−temperature combinations, this can be minimized. Solid fat content measurements revealed that the SFC of crystallized cocoa butter samples immersed in isobutanol for 24 h decreased significantly. The data show that treatment with isobutanol resulted in a reduction of 8% and 14% in the solid fraction of the static and dynamic (sheared) samples, respectively. The greater reduction of solid content in the dynamically crystallized samples can be explained by the smaller crystalline particles in the dynamically crystallized samples. Chawla et al. (1990) claimed that the removal of the liquid phase from the material will result in the loss of significant amounts of the solid phase if the crystals are small.[38]

An electron micrograph image of the surface of the fat particles washed with isobutanol is shown in Figure 6. In particular, Figure 6 illustrates the differences between the structures of cocoa butter crystallized statically, Figure 6a, and under the influence of shear (Figure 6b,c). Layers of crystals aligned along a single direction were observed in the oriented sample. Treating the samples with osmium tetroxide before washing with isobutanol did not enhance the quality of the micrographs either. A more aggressive deoiling method was needed.

The first detergent examined was an aqueous solution of 5% AOT. AOT is an anionic surfactant which is fairly miscible with

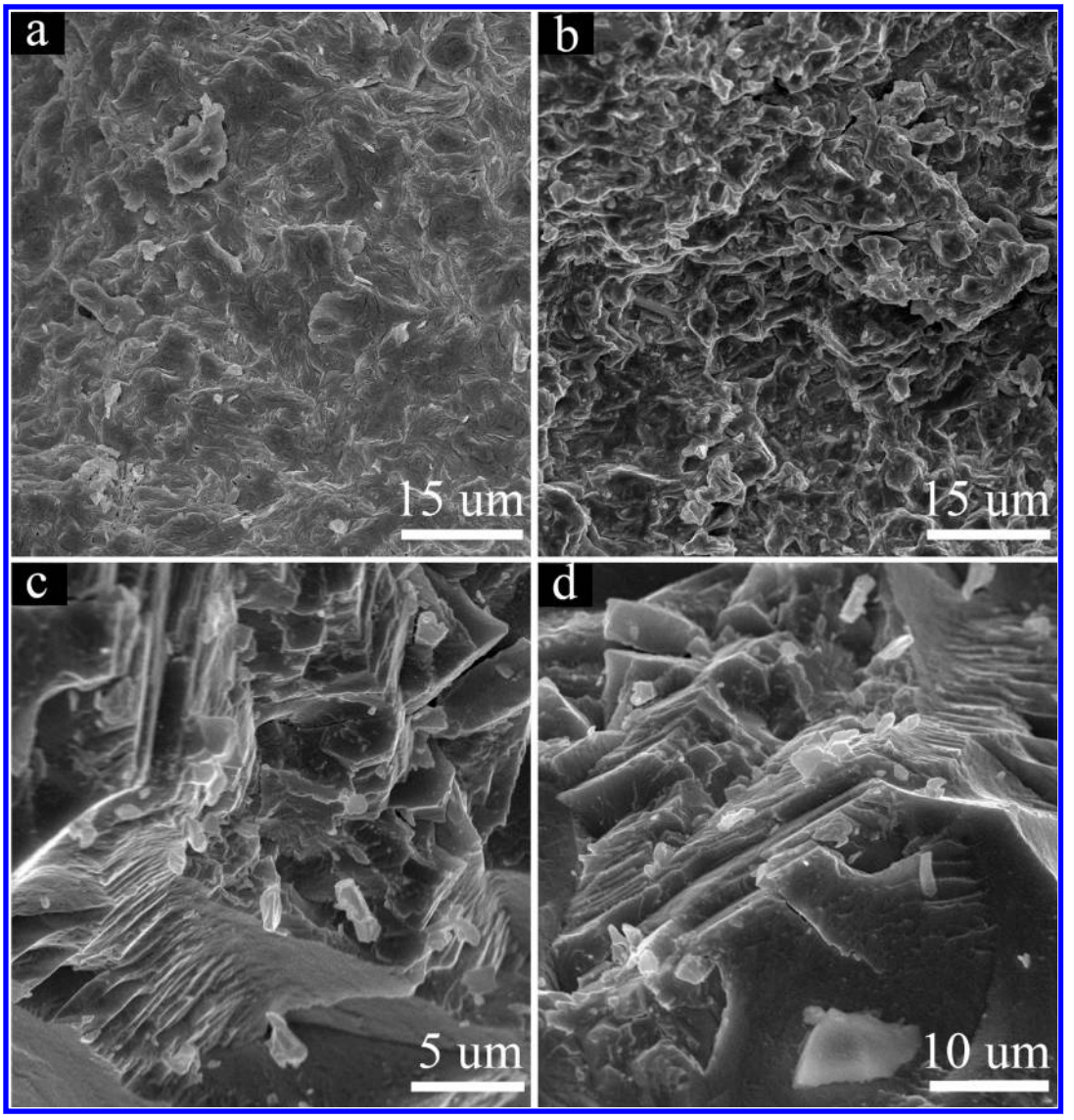

Figure 8. Cryo-scanning electron micrograph of samples after washing with Fatsolve: static (a); sheared (b), oriented samples (c, d).

liquid oil. It provides a high degree of oil removal from fat materials without causing dissolution of the solid fraction.[49−51] Although the EM images obtained from the AOT-washed static sample were similar to the one presented in Figure 6a, the use of AOT allowed for a better visualization of the growth of crystalline layers under the influence of laminar shear (Figure 6d). This micrograph showed that surfactant solutions can provide a better separation between the solid and liquid phases; consequently, the use of detergents was continued rather than solvents.

Jewell and Meara's research suggested that a 10−35% Teepol solution (a commercially available anionic detergent) in water was effective in deoiling a fat, so much so that a highly crystalline sample is usually the result.[43] The suitability of Teepol washing for deoiling cocoa butter was thus investigated. The samples were exposed to a 30% aqueous solution of Teepol at 20 °C for 24 h. The recovery of the solid and liquid fractions after washing with Teepol indicates that Teepol is an effective detergent for the removal of oil from fat matrices. Following the treatment with Teepol, samples were prepared for cryo-SEM imaging. The resulting images demonstrate that while Teepol is an effective deoiling agent, it does not adversely affect the morphology of the crystal network.

Figure 7, panels a and b are images of the sheared and static cocoa butter samples washed with a 30% aqueous Teepol solution. Both appeared to have fat clusters arranged in a random manner. The alignment of fat crystalline material becomes more pronounced in the electron micrograph of the oriented cocoa butter sample, Figure 7c. In this image, a well-defined layered structure of sheet-like arrangements can be clearly observed. A higher magnification of these layered structures is given in the last micrograph (Figure 7d).

Since this study is concerned with the structure of the fat crystal network crystallized under laminar shear, it is important to verify that the crystal alignments observed in these micrographs actually correspond to aligned crystals and not an imaging artifact caused by the washing of the fat material. The samples will be re-examined under the electron microscope using another deoiling agent. Fatsolve is a commercial aqueous detergent made from 2-butoxyethanol (1−5%), anionic surfactants (5−10%), triethanolamine (1−5%), and sodium hydroxide (1−5%). It was

employed as the third deoiling agent in this study. Figure 8 is a micrograph of the structure of an oriented sample washed with Fatsolve. The observed layering of the crystallites provides further evidence of the orienting effect of laminar shear on the structure of a fat crystal network.

For the first time, this research illustrates the 3-D structure of the aligned layers of crystalline particles in a fat material. The micrographs obtained showed the same aligned microstructure irrespective of the solvent or detergent used to wash away the liquid. The reproducibility of the images indicates that the observed particles are actually real crystals and not an artifact borne out of a particular preparation method.

These images clearly show that laminar shear applied in a Couette-type device can promote the orientation of crystallites in a direction parallel to shear field. The alignment effect is absent if the crystallites are merely "mixed" as occurs in the shearing of the melt in a beaker. The velocity gradient developed in the gap of the laminar shear crystallizer will orient the material as it crystallizes.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Recently, Acevedo and Marangoni[33,34] demonstrated that cooling rate and the shear have a profound effect on the crystallization behavior and the resulting nanostructure of a TAG crystal network. Nanocrystals of solid lipids were imaged using cryo-TEM. Their methodology will be adopted in this study to study the effects of laminar shear on the nanostructure of a cocoa butter fat crystal network.

TEM is a powerful tool for the study of crystalline materials. Compared to SEM, it allows a much higher magnification of the microstructure of a crystalline material without the loss of resolution. The magnification of TEM is so high that it can allow for the elucidation of crystal structures, crystal symmetry, crystal thickness, and the variation of the lattice parameters.[49] As was the case in SEM, the large volume of oil in a fat material hampered the imaging of the solid fat crystals. The samples were deoiled using all of the deoiling methods described in the cryo-SEM section. It was found that dispersing the cocoa butter in isobutanol provided the best cryo-TEM images. The images were of such quality that the dimensions of the individual nanoparticles could be quantified.

The nanostructure of the static, sheared, and oriented samples, as revealed by cryo-TEM, is shown in Figure 9. Platelet-like crystals are clearly discernible in all of the images. The difference in platelet size between the different samples is also noticeable. Larger particles can be observed in the static sample while much smaller particles are evident in the sheared and oriented samples. Using the ImageJ image analysis package, the dimensions of the nanocrystals were measured and plotted as a distribution. The frequency distributions of the length and width of the crystals are given in Figure 10. The sheared samples show a narrow crystal size distribution while the sheared samples show a broad distribution. A possible explanation for this behavior is that mass transfer is uniform in a melt that is under the influence of shear. Thus, the crystallites will grow under similar external fields (i.e., heat, momentum, and mass transfer) and supersaturation conditions. In the samples crystallized under static conditions, on the other hand, local variations in the supersaturation of the melt, as well as inhomogeneities in the external fields will result in different growth regimes for the nanocrystals and will thus result in a broader range of sizes. This will result in more homogeneous crystal compositions for sheared samples and more heterogeneous composition for statically crystallized samples.[26,29,52,53]

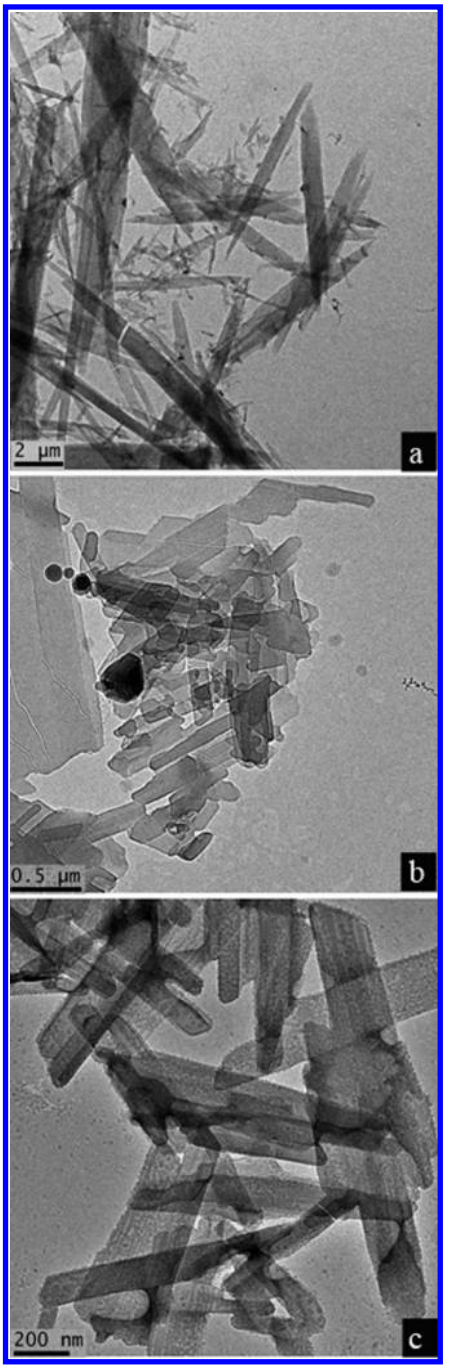

Figure 9. Cryo-transmission electron micrographs showing the nanoscale structure of static (a), sheared (b), and oriented (c) samples.

The measured mean length and width of the platelets, as well as the crystals' aspect ratio (i.e., the ratio of the length and width for a disk-like platelet) are presented in Table 1. Statistical analysis shows that shear processing resulted in a significant reduction in the size of the nanoparticles. These results are consistent with the reported effects of processing conditions (shearing and mixing) on the characteristics of crystalline particles.[24,32,33]

It is difficult to quantify the thickness of the platelets by Cyro-TEM due to the random orientation of the crystals during the preparation of the sample. Instead, we decided to determine the domain size of the 001 plane using small-angle X-ray scattering

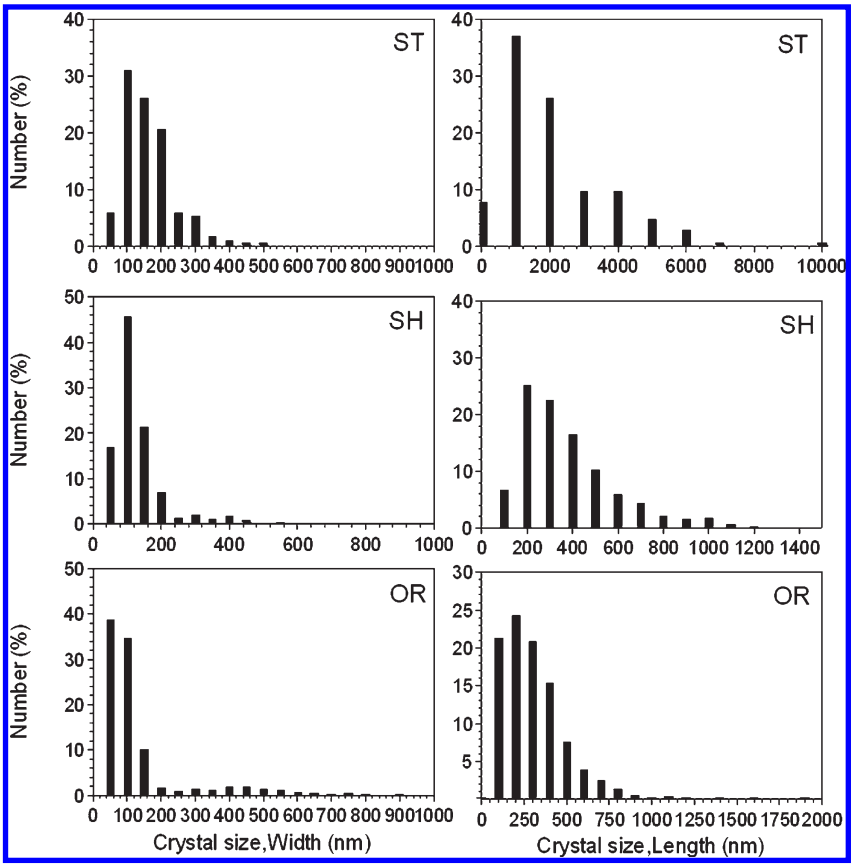

Figure 10. Crystal size distribution for the length and width of nanocrystals obtained from static (ST), sheared (SH), and oriented (OR) samples.

Table 1. Cryogenic Transmission Electron Microscopy Analysis Reporting the Dimensions of Platelet-Like Structure at the Nanoscale[a]

	crystal size		
sample	length (nm)	width (nm)	aspect ratio
static (ST)	2085 ± 128[a]	164 ± 6.2[a]	12.6
sheared (SH)	386 ± 12.6[b]	132 ± 4.0[b]	2.9
oriented (OR)	310 ± 7.3[b]	138 ± 5.3[b]	2.3

[a] The same superscript letters indicate no statistically significant differences at the 95% confidence level within a given column.

Table 2. The Number of Crystals Analyzed by Cryogenic Transmission Electron Microscopy and the Corresponding Derived Dimensions, the FWHM, *d*-spacing, and Domain Size Obtained from the X-ray Diffraction Pattern[a]

sample (CB)	static (ST)	sheared (SH)	oriented (OR)
number of crystals	536	765	931
fwhm	0.161 ± 0.001[a]	0.170 ± 0.001[b]	0.168 ± 0.001[b]
domain size (nm)	58.2 ± 0.05[a]	54.8 ± 0.16[b]	55.7 ± 0.25[b]
d (nm)	6.40	6.58	6.45

[a] The same superscript letters indicate no statistically significant differences at the 95% confidence level within a given row.

(SAXS). SAXS is an accurate and nondestructive technique that requires only minimal sample preparation. SAXS data can be used to estimate the crystallite thickness, which has been previously shown to correspond to the crystalline domain size.[33,34]

Since the shape of the reflection corresponding to the 001 peak is somewhat distorted, we used the 002 reflection instead, shown in the inset of Figure 3. Analysis of the 002 reflection yielded a width of the peak at half of the maximum peak height. This full-width half-maximum (fwhm), *B*, can be related to the crystalline domain size of the crystallite using the Scherrer equation:[54]

$$t = \frac{0.9\lambda}{B \cos \theta_B} \quad (1)$$

where *t* is the domain size of the crystal, λ is the X-ray wavelength, and θ_B is the Bragg angle. The Scherrer equation is based on the assumption that a larger domain size will result in more coherent scattering and will thus result in a narrower peak (i.e., lower width at half-maximum peak height). As stated above, in our case, this domain size value obtained from the SAXS reflections corresponds to the thickness of the nanoplatelets.

The *d* value and the average single-domain thickness obtained from the Scherrer equation are presented in Table 2. The reported *d* values are in agreement with the values found by Van Mechelen et al.[55,56] who provided structural details of β polymorph of several mono unsaturated TAGs as well as cocoa butter. The trends observed in the SAXS experiments are consistent with the trends observed in the length and width of the nanocrystals, as determined by TEM. The results suggest that the samples crystallized under shear have a smaller domain size. Comparison of the domain size of the sheared samples with the domain size of the nonsheared samples shows that the thickness of the lamella in the sheared sample is smaller than the thickness

of the lamella of the static samples by an amount that is roughly equivalent to one lamellar layer.

CONCLUSION

The use of deoiling agents is an essential step in the preparation of fat samples for observation under the electron microscope. Once deoiled, a wealth of information can be gleaned from images of the micro/nanostructure of the fat crystal network. The use of both solvent (isobutanol) and aqueous solution of surfactants (AOT and Teepol) resulted in comparable results and allowed for a good differentiation between fat crystals and liquid oil. The microscopic techniques employed allowed reliable quantification of the crystal morphology and habit, allowing the study of shear-crystallized cocoa butter. Data from cryo-SEM, cryo-TEM, and SAXS all provided evidence that laminar shear affected the crystalline distribution, morphology, and size of the crystalline particles in the fat crystal network.

Further investigation is necessary to determine whether or not the observed changes will impact the functional properties of the crystalline matrices. It is not inconceivable that by tailoring nanoplatelet size and distribution using laminar shear, the strength and permeability of the material can be modified.

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