Effect of Processing Conditions on Physical Properties of a Milk Fat Model System: Microstructure

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ABSTRACT: The effect of processing conditions on the microstructure of three blends of 30, 40, and 50% high-melting fraction [Mettler dropping point (MDP) = 47.5°C] in the low-melting fraction (MDP = 16.5°C) of milk fat was studied. The effect of cooling and agitation rates, crystallization temperature, chemical composition of the blends, and storage time on crystalline microstructure (number, size, distribution, etc.) was investigated by confocal laser scanning microscopy (CLSM). To improve resolution, a mix of Nile blue and Nile red dyes was dissolved in the melted samples in proportions that did not modify the nucleation kinetics. Samples were then crystallized by cooling (0.2 or 5.5°C/min) to crystallization temperature (25, 27.5, and 30°C). After 2 h at crystallization temperature, a slurry was placed on a microscope slide and samples were stored 24 h at 10°C. During this period, more material crystallized. Slowly crystallized samples (0.2°C/min) formed different structures from rapidly crystallized samples (5.3°C/min). Crystals were sometimes diffuse and hard to distinguish from the liquid. Samples were darker as a result of this solid-mass distribution. However, rapidly crystallized samples had well-defined crystals and seemed to be separated by a distinct liquid phase. These crystals were not in touch with each other as was the case for slowly crystallized samples. Higher agitation rates led to smaller crystal size due to enhanced nucleation. Larger crystals were formed when crystallization occurred at higher temperatures. Storage time resulted in an increase of crystal size. Larger crystal size and structures with more evident links had a more elastic behavior with higher elastic modulus E'.

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KEY WORDS: Confocal laser scanning microscopy, microstructure, milk fat model system, processing conditions.

Many factors influence lipid crystallization, most notably the way in which the sample is cooled from the melt (cooling rate, initial and final temperatures, agitation rate) (1), aging, triacylglycerol (TAG) organization, and fatty acid composition (2). Larsson (3) stated that two factors are involved in the formation of a three-dimensional crystal structure: effective packing into unit layers and the stacking of these unit layers. The chemical and physical properties of a fat are linked by its microstructure. There is no conclusive single theory that relates all properties of fats. However, cognizance of microstructure (number, size, distribution, networks, etc.) is essential because the fat crystal network influences the macroscopic properties of fats, particularly the rheological properties (4). Narine and Marangoni reported the first approach to quantify the *in situ* microstructure of the fat crystal network (5,6).

Light microscopy is a well-developed and increasingly used technique for studying the microstructure and composition of food systems in relation to their physical properties and processing behavior (7). Good-quality high-resolution images of the internal structures of foods can only be obtained from thin sections of the sample. Procedures that apply substantial shear and compressive forces may destroy or damage structural elements, and sectioning is time-consuming and involves chemical processing steps that may introduce artifacts and make image interpretation difficult. Confocal laser scanning microscopy (CLSM) overcomes these problems. In this instrument, image formation does not depend on transmitting light through the specimen, and therefore, bulk specimens can be used for the first time in light microscopy. The instrument uses a focused, scanning laser to illuminate a subsurface layer of the specimen in such a way that information from this focal plane passes back through the specimen and is projected onto a pinhole (confocal aperture) in front of a detector. Only a focal plane image is produced, which is an optical slice. By moving the specimen up and down relative to the focused laser light, a large number of consecutive optical sections with improved lateral resolution (compared with conventional light microscopy) can be obtained with a minimum of sample preparation (8). Several reviews discussing the application of CLSM in microstructural studies of food products have been published recently (9–11). They showed the advantages of using CLSM over conventional techniques in studying the relation between the composition, processing, and final properties of these products. Comparatively few papers have been published that deal with food-related topics. The cellular structure of selected apple varieties was observed by this technique (12); the effect of processing on the structural organization of cereal grain was also investigated by this technique (13). Heertje *et al.* (14) developed a technique to study

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the stability of food emulsions, an important aspect of the processing and the shelf life of many food systems. Hagiwara *et al.* (15) analyzed the fractal structure of the aggregates in food protein gels. Lucey *et al.* (16,17) studied the rheological properties and microstructure of acid milk gels as affected by fat content and heat treatment.

The objective of this study was to observe the effect of processing conditions on the microstructure of a milk fat model system. Different blends of high-melting fractions in low-melting fractions of milk fat were studied by CLSM to observe the crystalline microstructure in a semisolid product.

MATERIALS AND METHODS

Starting blends. Three model systems were prepared by mixing 30, 40, and 50% of a high-melting fraction, with a low-melting fraction of milk fat. Fractions were obtained from Grassland Dairy (Greenwood, WI). The Mettler dropping points (MDP) and TAG composition of the milk fat fractions were reported previously (1), as well as the MDP and TAG composition of the blends.

Crystallization procedure. Each sample (500 g) was melted in a water bath at 80°C and kept at this temperature for 40 min. A combination of 100 mg of Nile blue and 0.5 mg of Nile red was added, and the sample was stored in an oven at 60°C overnight to dissolve the dyes. The dye system did not modify nucleation kinetics when induction times were measured by turbidimetry (1). Nile red is fluorescent and allows the use of the laser source at 10% or less power, which prevents the dyes from burning or samples from melting. Moreover, crystal structure is more defined and air bubbles are easy to distinguish from crystals. However, Nile blue is also necessary to better distinguish the background from the crystals. Nile blue stain was used to negatively stain milk fat crystals. This lipophilic stain diffuses into the oil phase of a sample and generates a deep yellow fluorescence, whereas the solid fat does not fluoresce (14).

The melted fat samples were placed in a 1.0-L stainless-steel jacketed vessel with a 23-cm height and an 8.4-cm inner diameter. The samples were crystallized as reported previously (18). The following processing conditions were used: cooling rates of 0.2 and 5.5°C/min, agitation rates of 50, 100 and 200 rpm, and crystallization temperatures of 25, 27.5, and 30°C. After 2 h, samples were almost completely crystallized, and a slurry was placed between a slide and a cover slide. The slides were cooled and stored for 24 h at 10°C before observation in the microscope. During cooling and storage, additional fat crystallized to reach the final solid fat content (SFC) at that temperature. Samples received the same thermal treatments as were used for rheological studies. Thus, these images can be related to the rheological behavior of the samples as reported previously (18). Samples were also stored at 10°C for 3 wk to investigate the effect of aging. All experiments were run in duplicate.

Confocal microscopy. The Bio-Rad MRC-600 (Bio-Rad Laboratories, Hempsted, England) confocal laser scanning microscope with a krypton/argon mixed gas laser was used to collect the images. A 10× ocular was used, together with a 10× objective for a visual magnification of 100×. The laser intensity was 10%, and the confocal aperture was set at 2. Images were taken at increasing depths from the surface with increments of 3 μm. Images were recorded by using confocal assistant 4.02 software (Todd Clark, Brelje, MN), provided with the MRC-600 CLSM.

RESULTS AND DISCUSSION

Figure 1 shows the microstructure obtained when a 50–50% blend was slowly crystallized to 25°C at 50 rpm. Images at different depths were selected to show how the solid and liquid materials are distributed. The dyes are only soluble in the liquid phase; therefore, the white zone is the liquid and the different gray zones are the solid phase, which does not fluoresce. Black dots that appear in images are air bubbles incorporated during agitation. In every sample, slowly crystallized samples showed similar behavior. Wide crystal size distributions can be observed in these images, in agreement with the reported crystallization behavior found at these processing conditions (1). When these blends were crystallized slowly, nucleation and growth occurred together and, as a result, broad size distributions were found. There was a primary crystallization at the selected crystallization temperature (25, 27.5, or 30°C). After 2 h at crystallization temperature, samples were cooled to 10°C, and there was a secondary crystallization at this temperature. Most probably, the small and more diffuse crystals that appear in the background were formed during storage. Some of the primary crystals did not have a well-defined border, perhaps because they grew during cooling to 10°C.

For these conditions of crystallization, a distinct pattern of crystalline structure was observed. Primary crystals, formed at crystallization temperature, were generally more dense and distinct from the secondary crystalline structure formed during subsequent cooling of the slurry to 10°C. The primary and secondary crystals were dispersed in liquid fat, which remained uncrystallized at 10°C. The nature of this crystalline structure was dependent on crystallization conditions and led to different mechanical properties.

Figure 2 shows the effect of a fast cooling rate (5.5°C/min) on the crystalline microstructure. Small crystals in the background and some ill-defined large crystals were observed as the result of cooling to 10°C. However, crystal size was markedly smaller and more uniform than for a slow cooling rate. Liquid oil was more uniformly distributed between the crystals, and it might be expected that the crystals form weaker links between each other. Rapidly crystallized samples were always less dense, with more defined liquid and solid zones, despite having the same SFC as slowly crystallized samples (18).

When these samples were analyzed for their rheological behavior, slowly crystallized samples always showed higher elastic modulus (E') than rapidly crystallized samples (18). Some models reported in the literature (19), to interpret rheological behavior of different systems, proposed that larger particles make connections between each other and thus, systems with

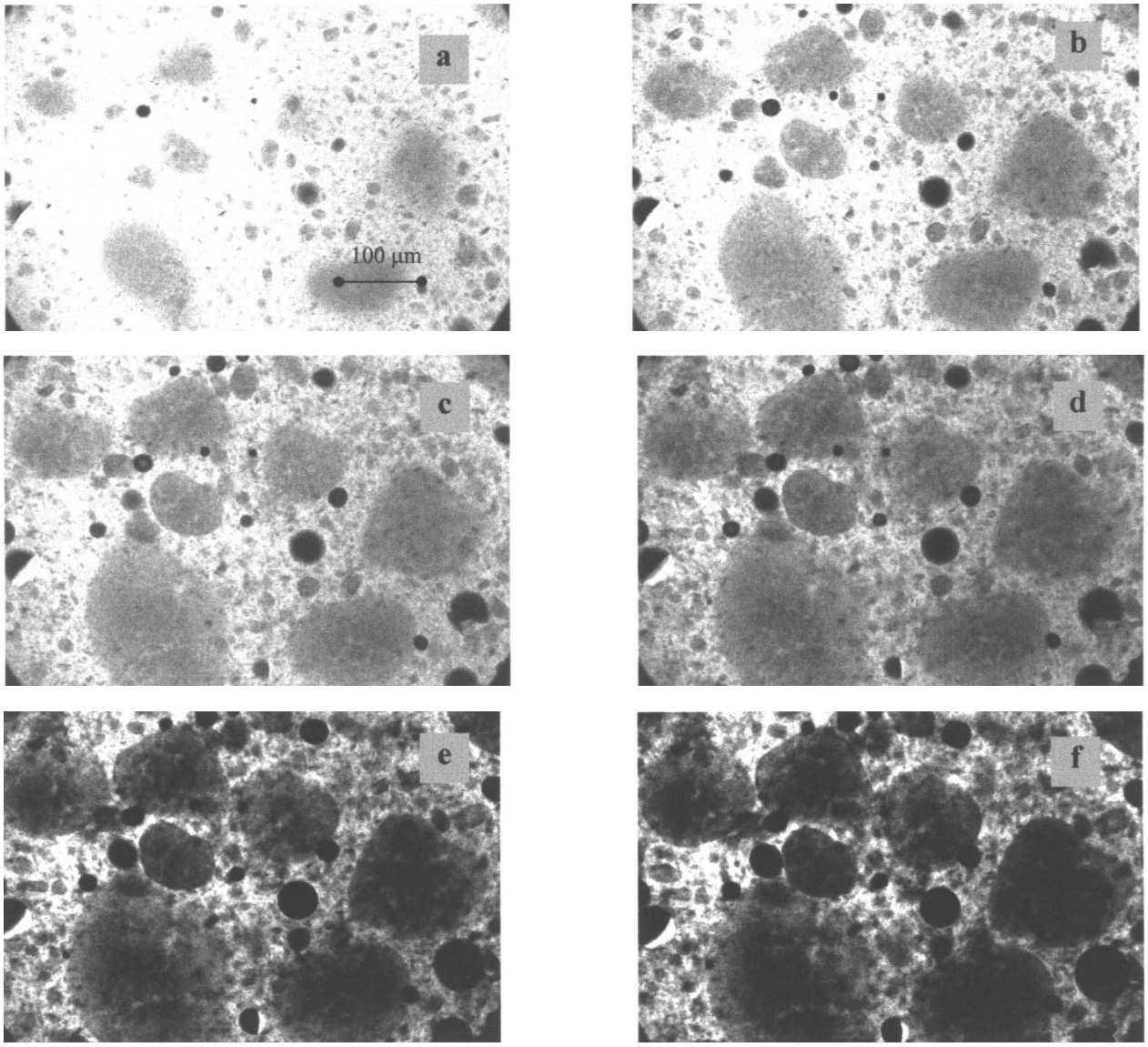

FIG. 1. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, agitation rate of 50 rpm, cooling rate of 0.2°C/min, and stored 24 h at 10°C. Images were taken at 3-μm increments from the surface: (a) surface; (b) 3 μm; (c) 6 μm; (d) 9 μm; (e) 12 μm; and (f) 15 μm.

larger particles have higher E' than those with smaller particles. However, the nature of the interactions between the primary and secondary crystal structures may also lead to differences in response of the material to compression forces.

The images obtained when a 50–50% blend was rapidly cooled to 25°C and agitated at three different agitation rates are shown in Figure 3. The most notable difference between these images is the size of the primary crystals. Increased agitation produced a marked decrease in crystal size. A nucleation study of these samples (1) showed that when the agitation rate was low, only a few initial crystals were formed, whereas when agitation was high, many small initial crystals were formed. With more nuclei formed, the final size is smaller for higher agitation rates. Figure 3 shows that, even after 24 h at 10°C, samples kept the characteristics of the primary nucleation. When these samples were analyzed for their rheological behavior (18), a decrease in E' with agitation rate was found. The value of E' is related not only to the solid mass but also to the crystal size. Smaller crystals build weaker links and these result in a decrease in E' (18).

The effect of crystallization temperature (25, 27.5, or 30°C) on crystal structure for the 50–50% blend crystallized at the fast cooling rate with an agitation rate of 100 rpm is shown in Figure 4. Crystal morphology was similar for the three selected crystallization temperatures, but the size increased with temperature. This effect was found in all samples. A nucleation study of these samples (1) showed that, at high temperature, fewer initial crystals were formed. When supercooling was higher (25°C), nucleation was favored and more smaller crystals appeared. At 30°C, the E' was also higher (18), as expected for larger particles, whereas at 25°C, the elastic modulus was lower, in agreement with a smaller particle size, for all processing con-

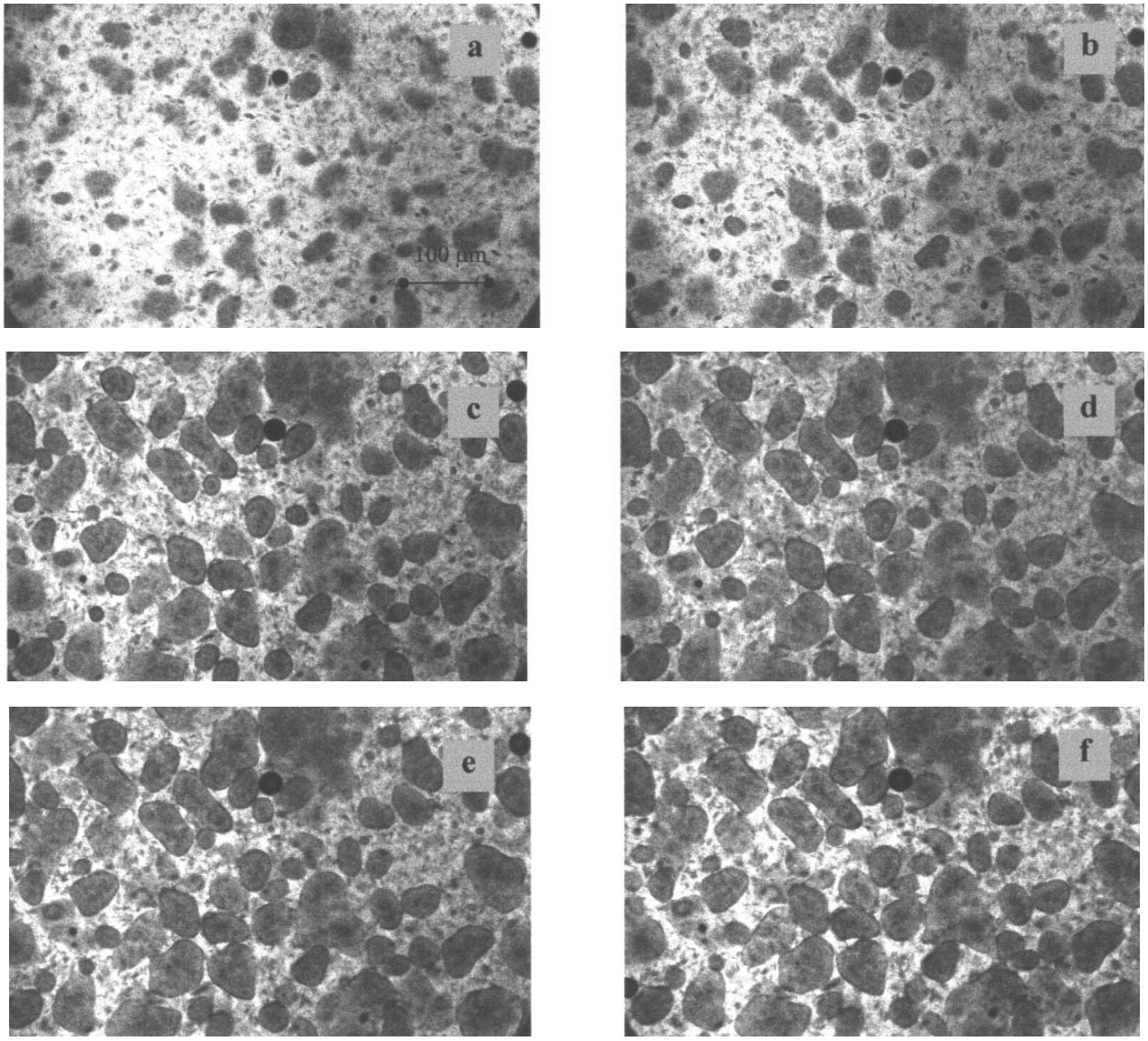

FIG. 2. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, agitation rate of 50 rpm, cooling rate of 5.5°C/min, and stored 24 h at 10°C. Images were taken at 3-μm increments at the same depths as Figure 1.

ditions studied. More air bubbles were incorporated during crystallization at 30°C due to the lower viscosity at this condition. These air bubbles may also affect mechanical properties, although the effect was not measured in this study. Future studies should investigate the mechanical properties of mixed structures (i.e., crystals, air cells, emulsions).

Figure 5 shows confocal images for the three blends crystallized with slow cooling to 25°C at 200 rpm. The 30–70% blend had larger crystals because, for this sample, 25°C is a relatively high temperature, with a MDP 4°C lower than for the 50–50% blend (18). This means the driving force for crystallization (supercooling) was lower in the 30–70% blend. This blend also showed a wider crystal size distribution. Although it had larger crystals, the E' was the lowest for the 30–70% blend at all processing conditions (18) because it had the lowest SFC. The 50–50% blend generally incorporated the most air bubbles, which were dissolved in the liquid oil (Fig. 5c) and could also contribute to the mechanical properties. Figure 6 shows the effect of composition for the fast cooling rate. The largest crystals again were found for the 30–70% blend, although the 50–50% blend again had the highest E' (18). As always happened when a fast cooling rate was used, crystals were more uniform in size, and it was easier to distinguish between solid and liquid phases.

Figure 7 shows the effect of storage time on a 50–50% blend crystallized at a rapid cooling rate to 25°C at 50 rpm. Crystals grew with time, as small crystals aggregated with larger ones. Figure 8 shows similar results for samples crystallized at a slow cooling rate. Crystals also aggregated in this case, but they appeared with a less defined border, more as shadows. Slowly and rapidly crystallized samples had different structures, and they remained different after 3 wk. When

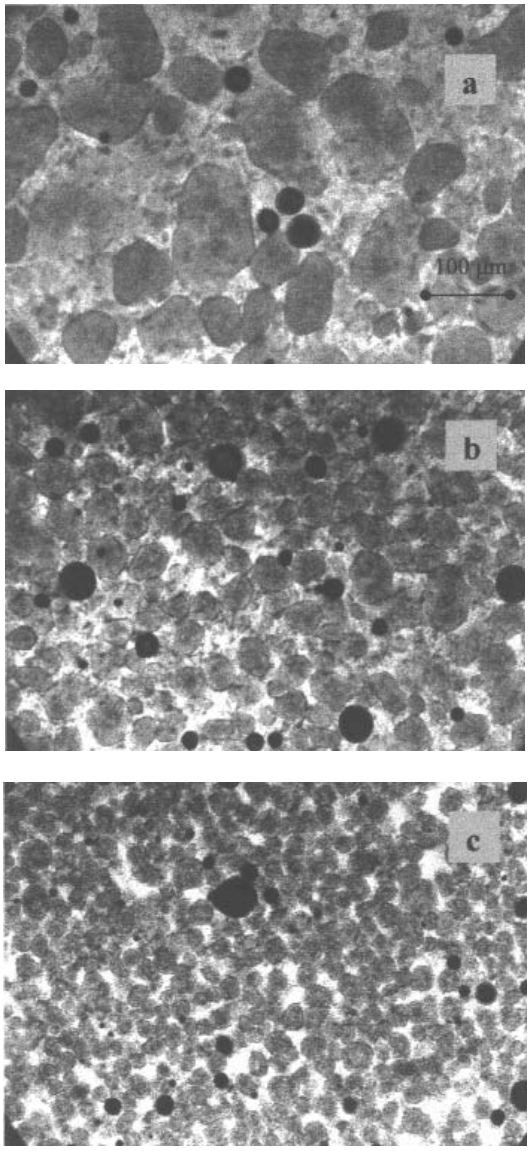

FIG. 3. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, cooling rate of 5.5°C/min and agitation rates of (a) 50 rpm; (b) 100 rpm; and (c) 200 rpm. Samples were stored 24 h at 10°C. Depth: 15 µm.

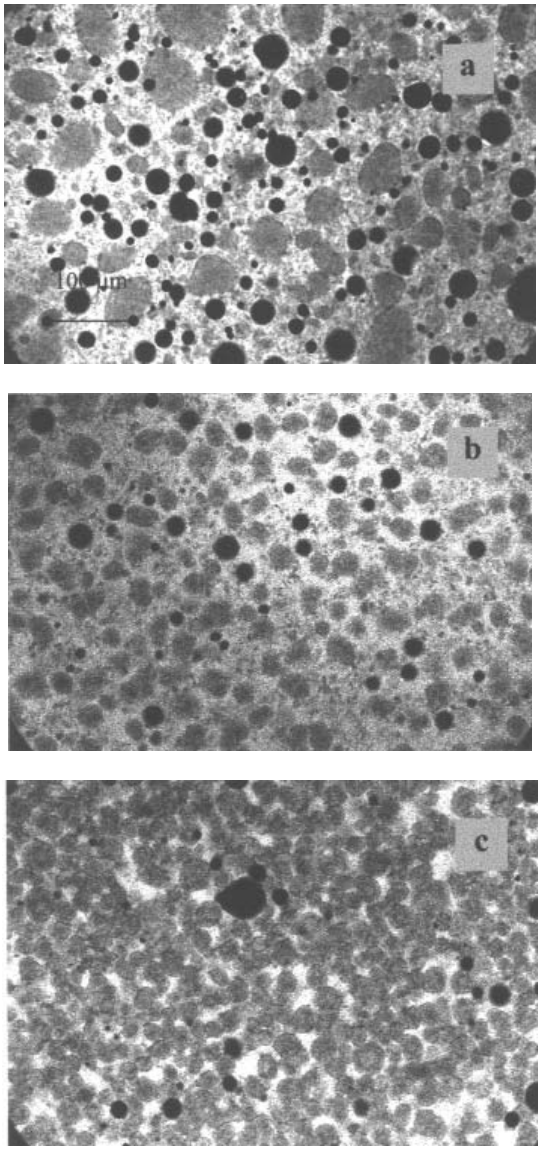

FIG. 4. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized with a cooling rate of 5.5°C/min and an agitation rate of 200 rpm at a crystallization temperature of (a) 30°C; (b) 27.5°C; and (c) 25°C. Samples were stored 24 h at 10°C. Depth: 15 µm.

the effect of storage time on rheological behavior of these samples was studied, we found that, in both samples, E' increased with storage time at least up to 3 wk (18). This was in agreement with the increase in crystal size.

Mechanical properties of fats are related to their SFC. However, samples with the same SFC had different E' (18) as a result of a different crystalline microstructure. Factors such as crystal size, crystal size distribution, density of crystal surface, primary and secondary structure, and liquid dispersion can also impact rheological properties. Larger particles could make stronger connections between each other and thus, systems with larger particles have higher E' than those with smaller particles. However, the nature of the interactions between the primary and secondary crystal structures may also lead to differences in response of the material to compression forces.

These images confirmed the correspondence between microstructure and rheological behavior in the three blends for the processing conditions used in this study. In general, for the same solid content, larger crystals with denser surface and more asymmetrical structures, in which solid and liquid phases were difficult to distinguish, led to higher values of E' and a more solid-like behavior (18). This kind of structure was obtained by cooling samples slowly, at slow agitation rate (50 rpm), at higher temperature, and at longer storage time.

The fractal dimension, calculated from rheological data (18), was useful in predicting the effect of agitation on E'. The main effect of increasing agitation rate was the decrease in crystal size (Fig. 3). However, no differences in fractal dimension were found due to changes in cooling rate (18) even

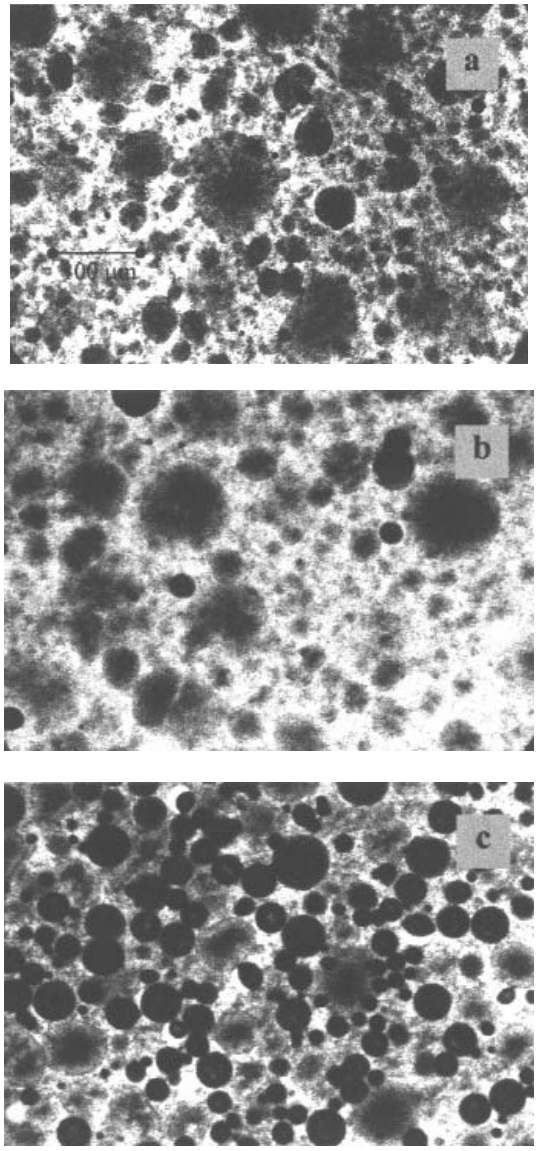

FIG. 5. Confocal images of (a) 30–70% blend; (b) 40–60% blend; and (c) 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, 200 rpm, and a cooling rate of 0.2°C/min. Samples were stored 24 h at 10°C. Depth: 12 µm.

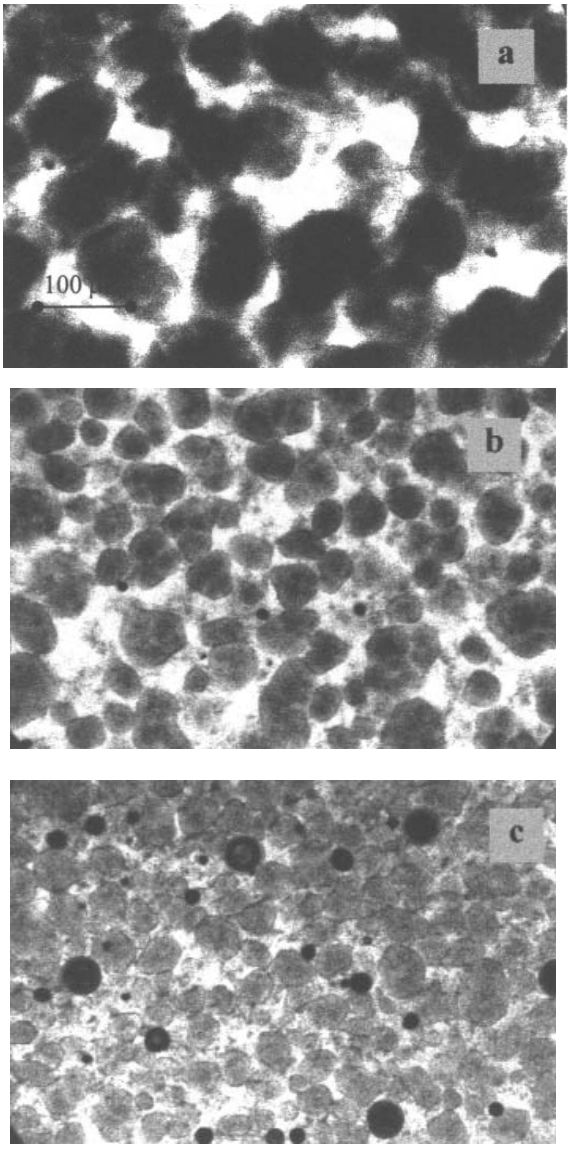

FIG. 6. Confocal images of (a) 30–70% blend; (b) 40–60% blend; and (c) 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, 200 rpm, and a cooling rate of 0.2°C/min. Samples were stored 24 h at 10°C. Depth: 12 µm.

though E' were significantly different. For cooling rate, the average crystal size was hardly different for fast and slow rates, but crystal size distribution and surface density were notably different (1).

Narine and Marangoni (5) calculated fractal dimensions for a variety of fat systems based on polarized light microscope images. Their samples were crystallized on a microscope slide without agitation. A particle-counting method, which assumed that the constituent particles (microstructural elements) of a particular microstructure were of the same average diameter, was used to calculate fractal dimension. This traditional assumption was valid for their crystallization procedure. Our system simulated an industrial process. Nucleation and growth occurred simultaneously, and as a result, crystals of different size were formed, especially at slow cooling rate (1). Thus, the assumption of uniform size is not valid for the processing conditions we used. In addition, samples were crystallized at 25, 27.5, and 30°C, and kept in the vessel for 2 h before cooling and storage at 10°C for 24 h. As can be observed from the images, during storage, many small crystals appeared in the background, and growth of the crystals formed at crystallization temperature also occurred. Crystals with different sizes, shapes, and surface characteristics appeared in the same image. In some samples, particularly those crystallized at slow cooling rate, ill-defined crystals appeared almost shadow-like. It is doubtful that these images had a truly fractal distribution and that their patterns were indeed self-similar over a considerable range of scales. This may explain why the fractal dimension calculated from rheological measurements gave the same fractal dimension for samples

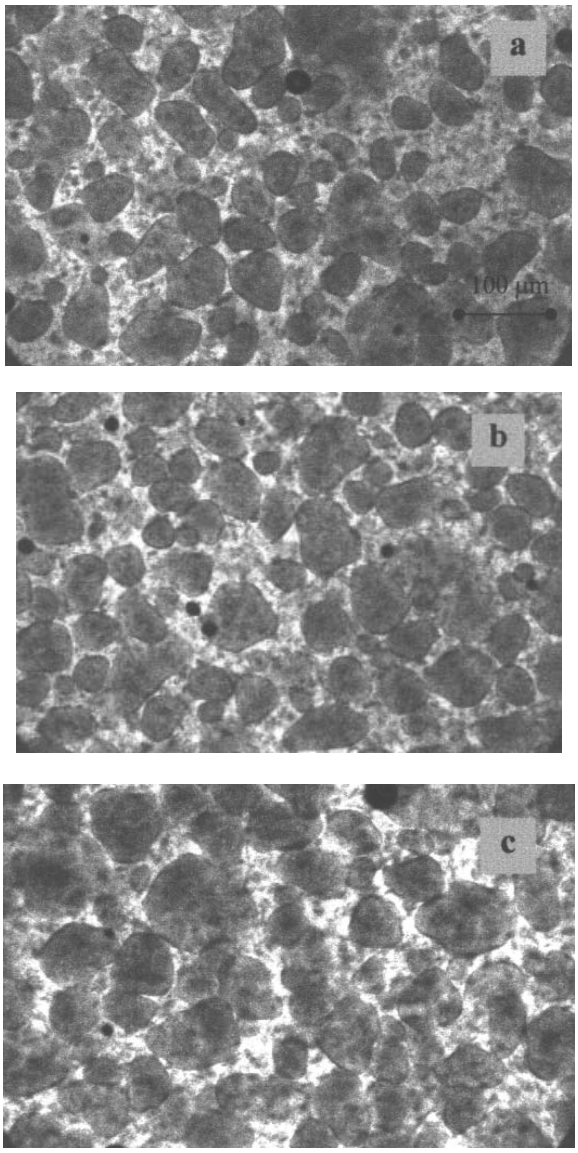

FIG. 7. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, 50 rpm, and a cooling rate of 5.5°C/min, and stored for (a) 24 h; (b) 1 wk; and (c) 3 wk at 10°C. Depth: 9 µm.

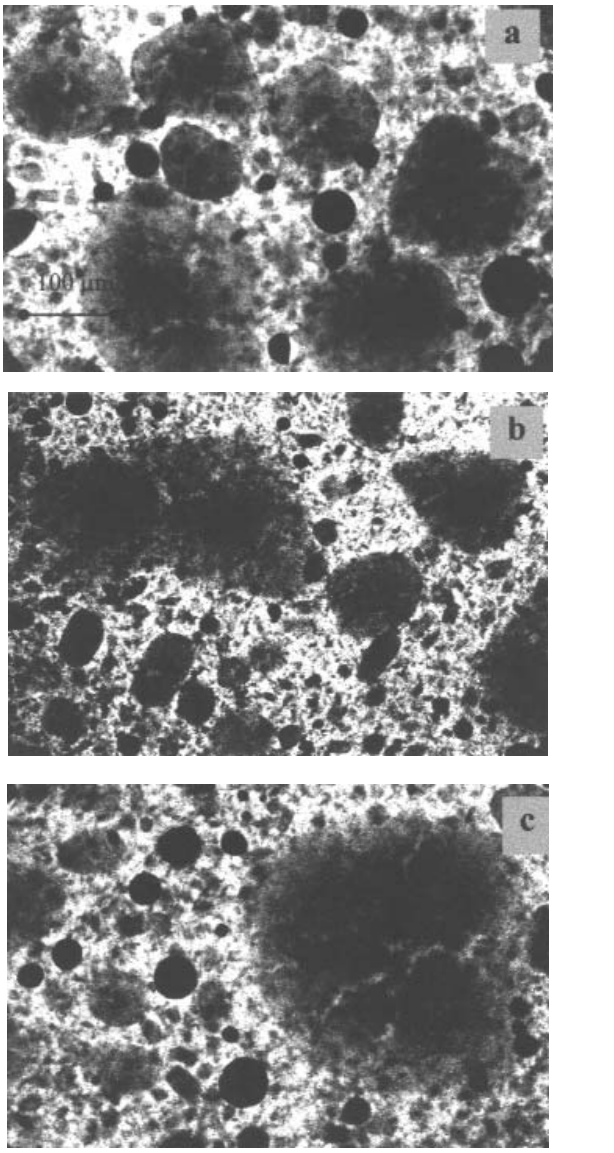

FIG. 8. Confocal images of a 50–50% blend of high-melting fraction in low-melting fraction of milk fat crystallized at 25°C, 50 rpm, and at a cooling rate of 0.2°C/min, and stored for (a) 24 h; (b) 1 wk; and (c) 3 wk at 10°C. Depth: 9 µm.

crystallized at slow and fast cooling rate, even though the E' were different (18).

Based on these results, techniques other than fractal analysis are needed to correlate crystalline microstructure with mechanical properties. Any technique must account for differences in crystal size, size distribution, shape, surface structure, density and mass distribution, as well as SFC. Furthermore, differences in polymorphic behavior also need to be considered for more complex lipid crystallization systems.

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