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ORIGINAL PAPER

The Effect of Phospholipids on Milkfat Crystallization Behavior

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Abstract Depending on the production method, the fat component in butter can either be in a continuous phase or entrapped by milk fat globule membranes (MFGM). Phospholipids like those present in the MFGM are known to affect milk fat crystallization behavior. This study examines the effect of MFGM phospholipid concentration on butter crystal structure. Regular raw cream was first concentrated to 85% fat via centrifugation to produce a "plastic" cream, and was then blended with anhydrous milk fat (AMF) and skim milk in order to maintain the total fat-to-water ratio while altering the total amount of MFGM in the butter product. Whereas mixtures of AMF and skim milk contained large spherulites that had finer structures as the degree of supercooling was increased, the addition of globular fat (GF) broke up these structures, eventually producing smaller individual needle-like crystals. However, high levels of GF also lead to the coalescence of the aqueous phase, creating large water pockets that adversely affect sensory properties. Thus, the phospholipid concentration in the final butter product must be controlled in order to obtain optimal crystal structure and product quality.

Keywords Anhydrous milk fat · Butter · Phospholipids · Microstructure

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Introduction

The fat component of milk exists in the form of globules, which range in size from 0.1 to 10 μm [10]. These globules contain about 98% triacylglycerol (TAG) and are stabilized by cellular milk fat globule membranes (MFGM) [10]. The MFGM is a complex mixture of phospholipids (PL), proteins, glycoproteins, triglycerides, cholesterol (0.2–0.5% of total lipids), enzymes and other minor components [3]. Even though it is only 1-2% of the total lipid, the MFGM contains 50% of all the phospholipids present in milk [21].

In the most commonly used process of butter making, pasteurized cream containing 35% fat is first exposed to a specific time–temperature program in order to obtain the proper crystal structure [1]. Next, during the churning process, the globules are beaten in air, leading to the formation of butter grains and buttermilk. After buttermilk separation, the grains contain about 15% moisture but further working and cooling is required to produce the final butter product. From 5 to 30% of commercial butter can still be in the form of fat globules, with approximately 50–75% being free fat [21]. While a large portion of the MFGM is removed with the aqueous buttermilk phase, a substantial amount of phospholipid remains in the butter [10].

Alternative butter making processes have also been developed that first concentrate the cream to approximately 85% fat via centrifugation to produce a "plastic" cream, followed by phase inversion [12]. In fact, in 1966, 60% of USSR butter was produced by such a process [12]. However, this production method did not catch on in the West because the resulting butter tends to be harder at refrigeration temperatures [12]. With this process, all the MFGM remains in the butter since no buttermilk is produced.

It would appear that the MFGM PL concentration in butter can vary significantly depending on the particular

method and processing conditions used. However, no systematic study has examined the effect of this important component on butter functionality. In this work, blends will be prepared by combining high-fat-content plastic cream with anhydrous milk fat and various aqueous phases to alter phospholipid concentration and examine how it affects the properties of the resulting butter. This work focuses on the effect of MFGM PL on butterfat crystal structure whereas a subsequent publication will examine its effect on butter physical properties.

Materials and Methods

Materials. Anhydrous milk fat (AMF) was purchased from Gay Lea Foods, Guelph, ON. Pasteurized skim milk (0.1% MF), buttermilk (1% MF), and uncultured unsalted butter were purchased from a local grocery store. Raw milk was obtained from the University of Guelph Ponsonby Dairy Farm, Elora, ON. As a source of phospholipids, laboratory grade vegetable lecithin was purchased from Fisher Scientific (Nepean, ON). The manufacturer's specifications indicated that this lecithin contained 25% PC, 15% PI, 20% PE and 10% PA for a total phospholipid concentration of 70%.

Regular 35% cream was prepared by centrifuging raw milk at 1,500×*g* in a model IEC HN-SII centrifuge (Thermo Scientific, Waltham, MA). The resulting cream was further concentrated at 40,000×*g* in an Optima LE-8K ultracentrifuge (Beckman Coulter, Fullerton, CA) to produce a high fat content plastic cream.

Phospholipid analysis. The phosphorous content of AMF was determined using a spectrophotometric measurement that involves the formation of a blue phosphomolybdic acid complex as outlined in AOCS Official Method Ca 12–55 [13]. The resulting phosphorus concentration was then multiplied by 30 to obtain the equivalent wt% phosphatide.

Preparation of concentrated cream/AMF/skim milk mixtures. AMF was first heated to 80 °C to destroy any previous crystal history, and was subsequently cooled to 23 °C. Ultracentrifuged cream and skim milk (both at 23 °C) were then combined with the AMF in various combinations in order to alter the amount of globular to free milk fat ratio while maintaining the total lipid concentration at 80wt%. The mixtures were then blended with a spatula until a homogeneous product was obtained.

Preparation of lecithin/AMF/skim milk mixtures. AMF was first heated to 80 °C to destroy any previous crystal history. After cooling to 60 °C, lecithin was added and the solution was stirred until the granules had completely dissolved. Mixtures were first cooled to 23 °C and 20 wt% of either 23 °C skim milk or buttermilk was then added by continuous mixing with a spatula until a homogeneous product was obtained.

Fat mixture tempering. Butter mixtures were first placed in 2″ diameter aluminum dishes and then were put inside a MIR-153 programmable incubator (Sanyo, Tokyo, JP) where they were exposed to various time–temperature treatments.

Polarized light microscopy (PLM) analysis. An Olympus BX 60F5 (Olympus Optical, Tokyo, Japan) light microscope fitted with a Sensys HRD 060-NIK 0.60× camera (Photometrics, Tucson, AZ) was employed. Image-Pro® Plus, Version 4.5.1.29, (Media Cybernetics, Bethesda, MD) was used for the light microscopy image analysis.

Cryo-scanning electron microscopy analysis. A technique for cold-stage SEM developed by Schmidt et al. [15] and applied in dairy research by Kaláb [8] for the cold-stage SEM observation of cheese was used to study milk and milk fat product morphology. Images were taken with a HITACHI Type S-570 scanning electron microscope (Hitachi, Tokyo, Japan). An EMSCOPE SP2000A cooling unit (Canberra Packard, Meriden, USA), EMITECH K1250X cryogenic preparation system (Emitech, Ashford Kent, England) and EMITECH K550X turbo sputter coater (Emitech, Ashford Kent, England) were used for sample preparation.

Results

Phosphate analysis was conducted on both the AMF and high fat content (HFC) cream that had been washed three times with deionized water to remove all caseins and whey proteins. AMF and HFC cream were found to have phospholipid (PL) concentrations of 0.19 and 2.13%, respectively.

An SEM image of regular cream is shown in Fig. 1a. The fat globules range in size from 0.25 to 5 μm. In addition, white flakes are also present that are freeze-concentrated serum containing protein, salts and sugars, which are created during the cryo-preparation of the samples. In the unwashed HFC cream sample (Fig. 1b), the fat globules are no longer spherical because of the high degree of close-packing. The surface of the globules are also rougher, possibly a result of solids precipitating onto the surface of the MFGM.

The crystal structure of pure AMF depends on the temperature at which crystallization takes place. At relatively high levels of supercooling (e.g., a crystallization of temperature 18 °C), distinct spherulitic crystal structures can be seen in the SEM images (Fig. 2a). In systems experiencing highly nonequilibrium conditions, the growth of polycrystalline patterns is often observed [5]. When the

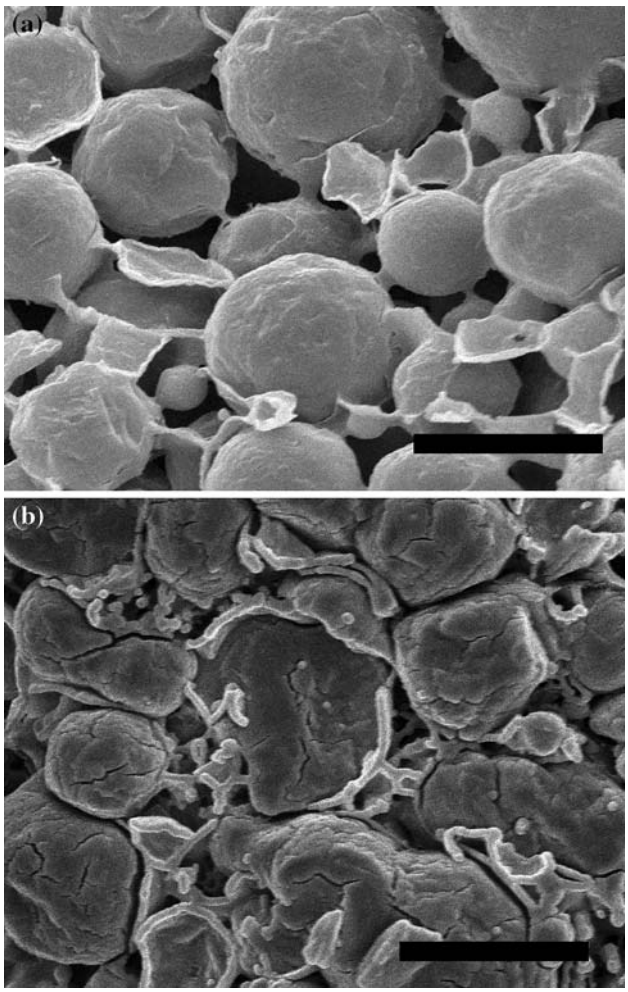

Fig. 1 Cryo-SEM images of cream at 4 °C: **a** 35 wt% milkfat; **b** 70 wt% milkfat (*black bar*, 3 μm)

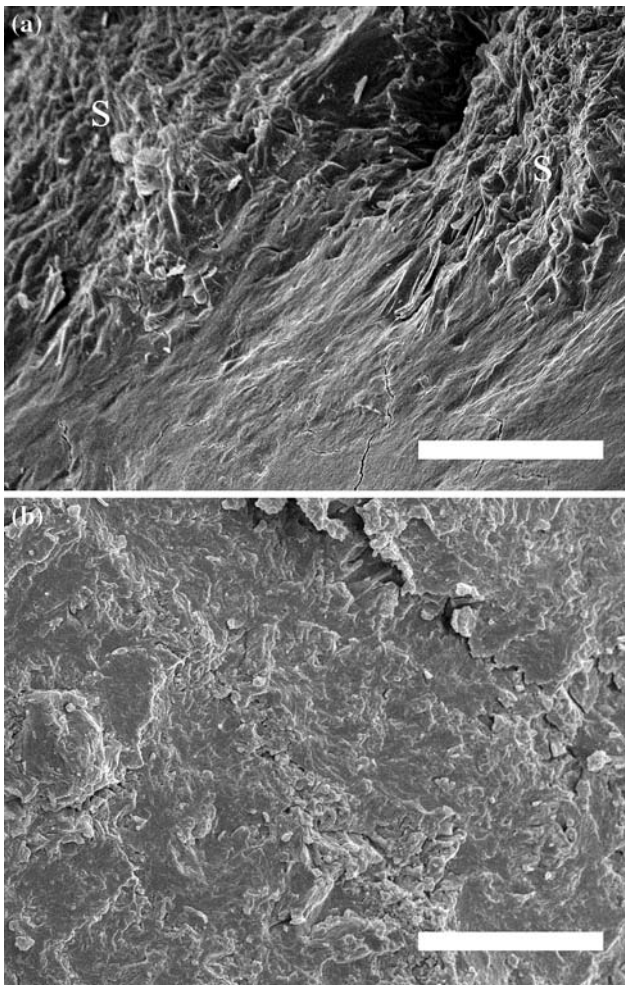

Fig. 2 Cryo-SEM images of AMF: **a** crystallized at 18 °C; **b** crystallized at 4 °C (*S*, spherulite; *white bar*, 10 μm)

crystallization temperature was reduced to 4 °C, the spherulites had smaller crystals and a finer structure (Fig. 2b).

Under polarized light, four main classes of solidified fat globules have been observed: O, N, L and M [22]. With type O, no crystals are seen whereas needle-like crystals are observed in the central region of the globule with type N (both these globule classes are transparent under polarized light conditions). Fat crystals form on the surface of the MFGM with both types L and M globules, creating a cross-like pattern under polarized-light conditions. The difference between the L and M forms is that L has a clear center whereas type M contains small crystals inside the central region much like what is observed with type N globules.

Figure 3a shows a PLM image of a butter spread crystallized at 18 °C that was produced by mixing AMF and skim milk. Not surprisingly, no fat globules are present. However, large dense spherulitic crystal structures with sizes similar to those observed in the SEM images (Fig. 3a) are evident. Similar images were obtained with mixtures of AMF and buttermilk (results not shown). Unlike skim milk that contains only 0.015% PL, buttermilk has on average 0.13% PL [21]. However, even this concentration would only produce a total PL level of 0.17% in this blend, which appears to be too low to effectively influence fat crystal structure.

When globular fat (GF) was added such that it made up 30% of the total fat present, this increased the PL concentration to 0.66%, and spherulite formation was inhibited (Fig. 3b); mostly small crystal aggregates and some intact type L fat globules are present. Evidently, the addition of the PL present in the MFGM of the HFC cream appears to disrupt the formation of spherulitic crystal structures. At 60% GF (1.29% PL), no spherulitic structures are present (Fig. 3c); some type L fat globules can be seen but crystals are more in the form of dense aggregates. With the 80% GF mixture (1.71% PL), more intact fat globules are apparent (Fig. 3d) and the crystal aggregate structure becomes more

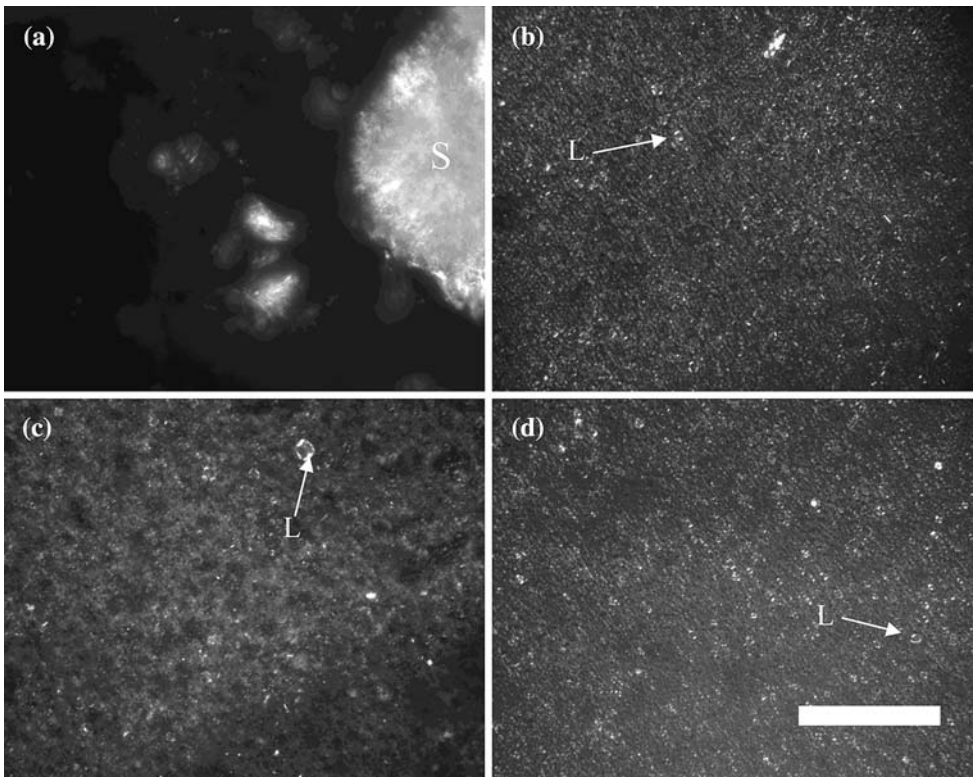

Fig. 3 PLM images of concentrated cream/AMF mixtures with a constant skim milk concentration of 15 wt% prepared at 23 °C and then stored at 18 °C for 24 h: **a** 0/100 wt%, **b** 30/70 wt%, **c** 60/40 wt%, **d** 80/20 wt% (*L*, type L fat globules; *white bar*, 50 μm)

compact. Further increases in GF resulted in structures very similar to those present in Fig. 3d.

The fine structure of the fat crystal network produced at 18 °C with AMF/concentrated cream blends can be better visualized using SEM at high magnification. At 30% GF, crystals are made up of a mixture of short chains and small spherical aggregates of sintered crystals (Fig. 4a). In addition, the aqueous phase is very finely dispersed. When the GF is increased to 60%, sintering is no longer linear, but more aggregated (Fig. 4b). In addition, gaps containing the aqueous phase start to become evident. At 80% GF, large crystal aggregates are present that are separated by voids containing the aqueous phase (Fig. 4c). Further increasing the GF content to 90% led to slightly smaller aggregates that were once again separated by the aqueous phase (Fig. 4d). None of the blends had much of a resemblance to concentrated cream (Fig. 1b).

The crystal structure of AMF/concentrated cream blends produced at a higher level of supercooling was also examined. Figure 5a shows a PLM image of an AMF/skim milk mixture crystallized at 4 °C. The spherulites are larger and have a more needle-like morphology as compared to those produced at 18 °C. Also, unlike what was observed at 18 °C, both the 30% (Fig. 5b) and 60% (Fig. 5c) GF blends had some spherulitic structures, but increasing the amount of MFGM did tend to repress their formation. In the 30% sample, many individual crystals are present but in the 60% blend, the crystal structure is more aggregated. At 80% GF (Fig. 5d), spherulitic growth was completely repressed and many intact type L fat globules can be observed. The crystal network was also mainly composed of clumped aggregates. Further increasing the GF beyond 80% produced images similar to Fig. 5d.

High magnification SEM images confirmed that the fine structure of the spherulites produced at 4 °C consisted of more linear chains of crystal aggregates (Fig. 6a) as compared those crystallized at 18 °C (Fig. 4a). Nevertheless, as was observed at 18 °C, increasing the amount of MFGM did result in less linear, more aggregated crystals (Fig. 6b). Increasing the amount of GF also resulted in the coalescence of the aqueous phase. At 80% GF, this coalescence phenomena lead to the formation of coral-like structures (Fig. 6c) that became more prevalent as GF was further increased to 90% (Fig. 6d).

In order to confirm that it was in fact the phospholipid present in the MFGM that was altering crystal morphology, the effect of soy lecithin addition on AMF crystal morphology at 18 °C was examined using PLM. The addition of lecithin up to 1.0 wt% (0.70% PL) stimulated spherulitic crystal formation (Fig. 7a–c). Also, increasing phospholipid concentration appeared to increase spherulite density until, at 2.0 wt% lecithin (1.4% PL), the spherulites broke up into a network made up of a mixture of individual needle-like and aggregated crystals (Fig. 7d). Interestingly, the disruption of spherulitic structures by MFGM was also observed in this same range of PL concentration (Fig. 3c).

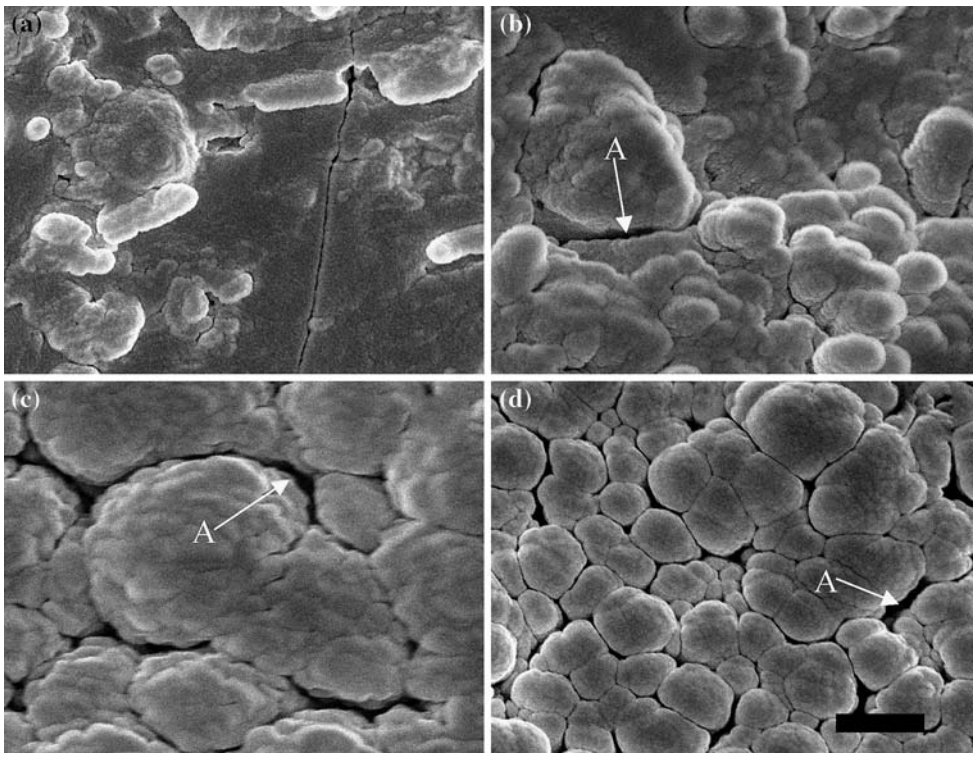

Fig. 4 Cryo-SEM images of concentrated cream/AMF mixtures with a constant skim milk concentration of 15 wt% prepared at 23 °C and then stored at 18 °C for 24 h: **a** 30/70 wt%, **b** 60/40 wt%, **c** 80/20 wt%, **d** 90/10 wt% (*A*, aqueous phase; *black bar*, 3 μm)

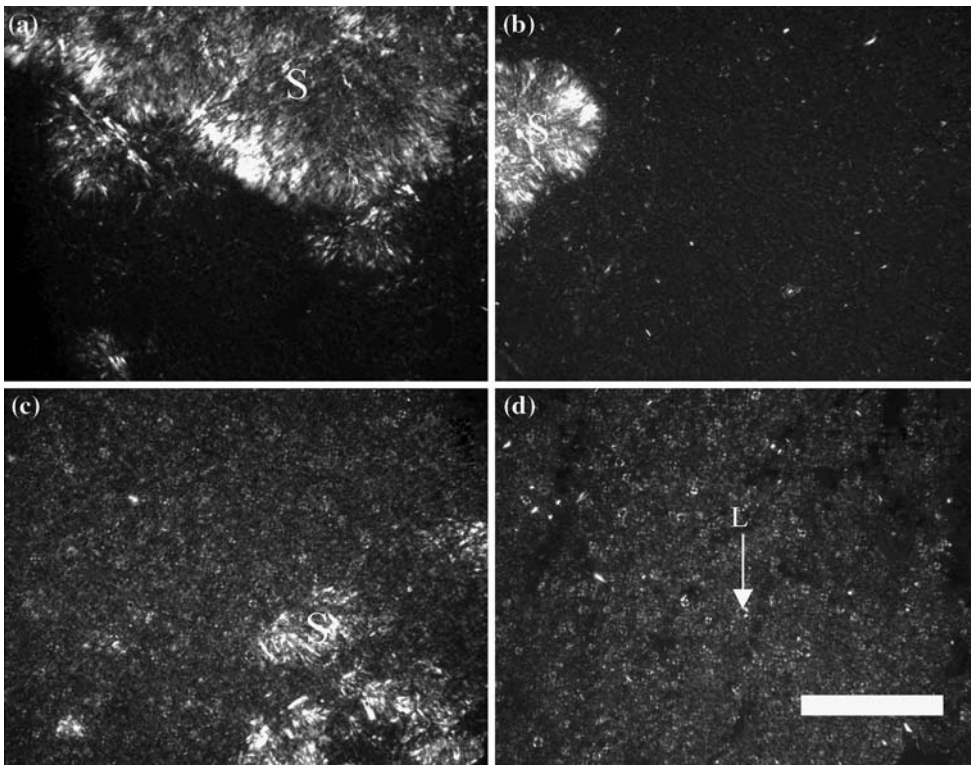

Fig. 5 PLM images of concentrated cream/AMF mixtures with a constant skim milk concentration of 15 wt% prepared at 23 °C and then stored at 4 °C for 24 h: **a** 0/100 wt%, **b** 30/70 wt%, **c** 60/40 wt%, **d** 80/20 wt% (*S*, spherulite; *L*, type L fat globules; *white bar*, 50 μm)

Discussion

As with all crystalline solids, AMF must be supercooled to well below its equilibrium melting point in order to instigate crystallization [19]. Just below the equilibrium melting curve is a metastable zone where crystallization proceeds only in the presence of appropriate seeds, whereas at lower temperatures below the supersolubility curve, spontaneous generation of nuclei can occur [11]. During the tempering process, cream is first rapidly cooled to

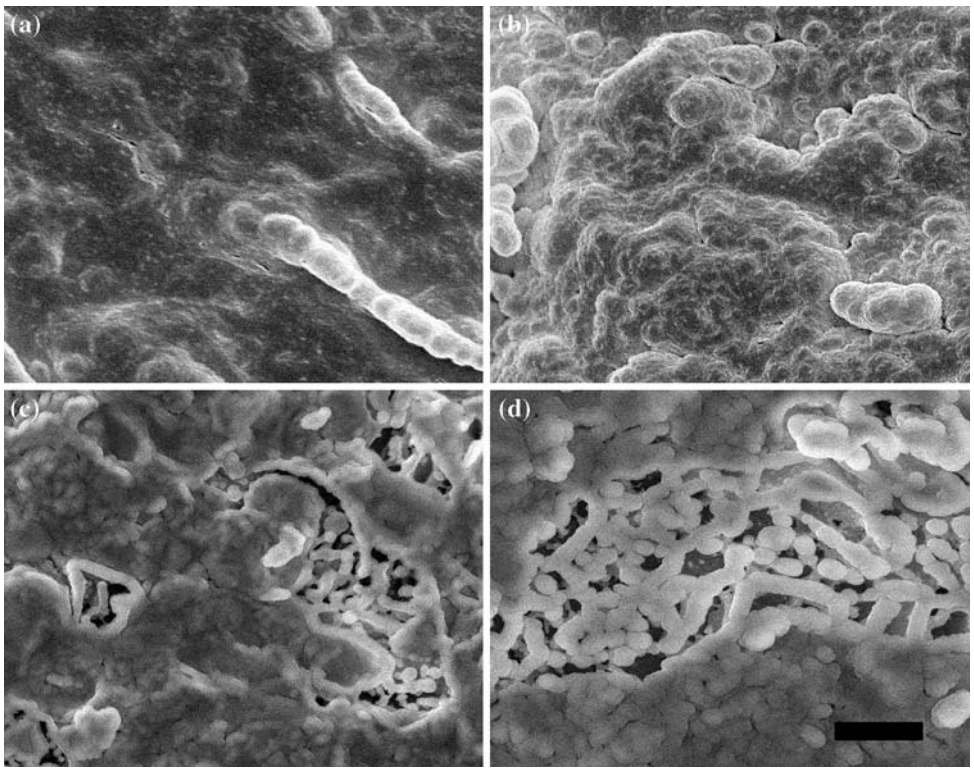

Fig. 6 Cryo-SEM images of concentrated cream/AMF mixtures with a constant skim milk concentration of 15% prepared at 23 °C and then stored at 4 °C for 24 h: **a** 30/70 wt%, **b** 60/40 wt%, **c** 80/20 wt%, **d** 90/10 wt% (*black bar*, 3 μm)

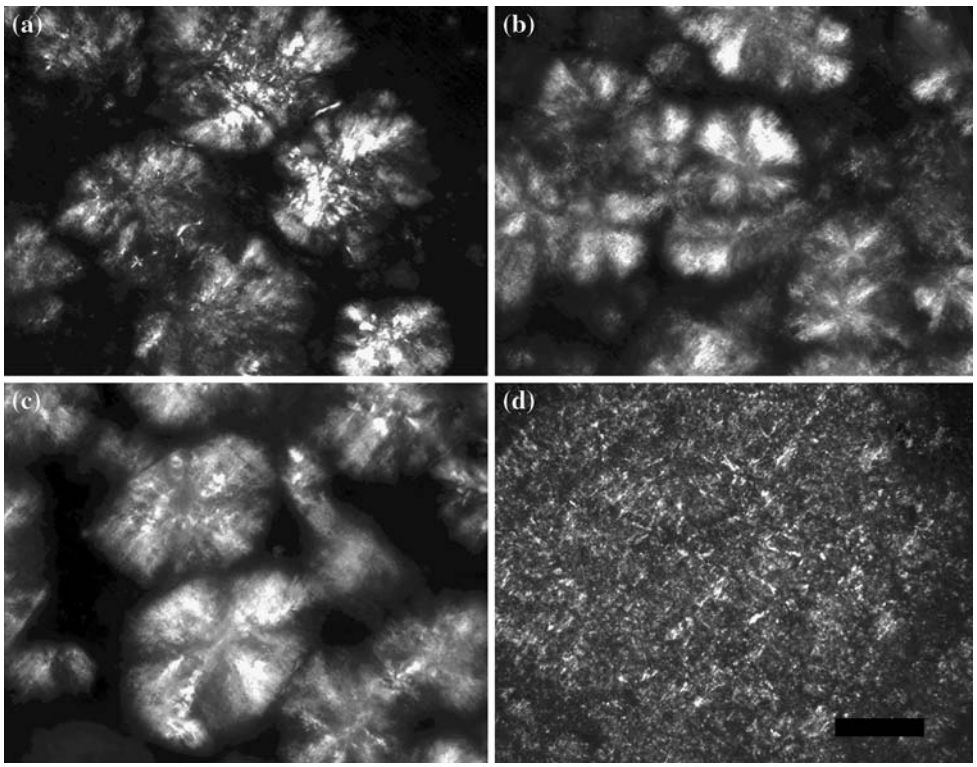

Fig. 7 PLM images of AMF/lecithin mixtures with a constant skim milk concentration of 15 wt% prepared at 23 °C and then stored at 4 °C for 24 h: **a** 0.1 wt%, **b** 0.5 wt%, **c** 1.0 wt%, **d** 2.0 wt% lecithin (*black bar*, 50 μm)

between 6 and 8 °C in order to produce crystal nuclei and then gently heated to between 15 and 21 °C to encourage the growth of the large crystals needed to instigate proper butter grain formation during churning [1].

The resulting crystal morphology is strongly influenced by mass and heat transfer phenomena occurring during the crystallization process. If the melt is in the metastable zone, crystal growth can still proceed if seeds are added.

Under conditions very close to the equilibrium melting curve, crystal growth will be very slow. As a result, the transfer of crystallizing species to the crystal surface or the transfer of heat away from the crystal will not be rate limiting. In this environment, spherical crystal growth patterns are typically observed [5]. However, as the system moves farther away from equilibrium, the solid–liquid interface at which growth occurs becomes unstable, and the crystallization pattern breaks up into fingered structures that are commonly referred to as "seaweed" [5]. This type of growth pattern is favored under these conditions because the non-crystallizing molecules are pushed to the side, creating heat and mass reservoirs.

If, within the system, mass and/or thermal gradients are anisotropic (which is usually the case—especially in quiescent systems), crystals tend to grow in a more needle-like, dendritic shape [5]. At even higher degrees of supersaturation, growth front nucleation starts to become significant, leading to polycrystalline structure formation [7]. At first, these structures have a branched dendritic appearance, but at very high degrees of supersaturation, spherulitic structures are created [5]. Spherulites have a random local crystal orientation but still retain spherical symmetry at large scales.

With AMF, a relatively high degree of supersaturation is required in order to obtain reasonable crystallization rates. Although significant crystal growth can occur at 35 °C, as stated previously, cream is usually tempered at temperatures below 21 °C before churning in order to obtain proper crystal formation [1]. The two crystallization temperatures examined in this study (18 and 4 °C) would be considered very non-equilibrium conditions. Thus, it is not surprising that AMF formed large spherulites at both these temperatures. With spherulitic growth, the fineness of the internal structure tends to increase with increasing degree of supersaturation [5]; our results are consistent with this finding since the 4 °C spherulites were much finer than those produced at 18 °C (Fig. 2).

Minor polar lipids such as diacylglycerols, monoacylglycerols, free fatty acids, sterols, and phospholipids are known to have a significant influence on fat crystallization behavior [6]. A considerable amount of work has been conducted on the effect of DAG on fat crystallization. At low levels of supercooling, 1.0 wt% concentrations of both *sn*-1,3 and *sn*-1,2 dipalmitin DAG significantly increased the crystallization induction time of palm oil [16]. However, crystal growth was only inhibited by high concentrations of these DAG (e.g., 10 wt%). Wright et al. [23] also found that, with AMF, the addition of *sn*-1,2 dipalmitin DAG delayed crystallization and inhibited growth rates whereas a racemic mixture of dipalmitin DAG had little effect at a concentration of 0.1 wt%. Foubert et al. [4] observed that 0.5 wt% racemic distearin DAG increased the induction time of AMF, and began to also retard crystal growth when the concentration was increased to 1.0 wt%. These researchers also noted that 1.0 wt% racemic diolein DAG could actually decrease induction time while at the same time accelerate crystallization of AMF. Evidently, both the type of fatty acids and their relative location on the DAG has a strong influence on fat crystallization behavior.

Vanhoutte et al. [20] observed that the addition of 0.07 wt% phospholipid (PL) in the form of lecithin delayed AMF crystallization. On the other hand, this effect could be counterbalanced if lecithin was combined with water before being added to AMF. This is not surprising since PL morphology is strongly influenced by the presence of water [17]. For example, PC can form lamellar crystalline (L_c), laminar β' ($L_{\beta'}$), laminar α (L_α) or oblique β' ($P_{\beta'}$) structures depending on the temperature and water concentration. Without water addition, the PL in lecithin would be in the crystalline form at temperatures below 60 °C. However, the addition of 20% water would transform the PL in lecithin into a L_α conformation [17].

Soy lecithin is a mixture of several different types of PL, containing approximately 28 wt% PC, 30 wt% PI, 26 wt% PE and 16 wt% lyophosphatidyl choline (LPC) [2]. Smith [18] has shown that the effect of PL on the crystal growth of palm oil and trilaurin is quite structure-specific, with PE much more significantly retarding growth rates as compared to PC. Similar specificity effects were observed with cocoa butter, where PI and LPC were much more effective at increasing induction times as compared to PC [14]. These researchers speculated that the observed differences in PL behavior were due to the fact that, in chocolate, PC tends to form an inverse hexagonal H_{II} phase (i.e., with the fatty acids pointing outward) while PI and LPC orientation is in the normal hexagonal H_I phase (i.e., with the polar head groups pointing outward). The principle PL components in MFGM are: PC, 32 wt%; PE, 31 wt%; sphingomeylin (SM), 20 wt%; PI, 7 wt%; PS, 5 wt%; and lactosyl-cerebroside (LS), 5 wt% [9]. Because of their relatively low HLB values, PC and PE tend to stabilize water-in-oil emulsions whereas SM, PI, PS and LS tend to form oil-in-water emulsions [17].

In this work, increasing the amount of GF versus AMF in the fat phase of the butter blends had the same effect as increasing the crystallization temperature from 4 to 18 °C: the spherulites became smaller and courser and eventually broke up into needle-like crystals. Smith [18] observed similar behavior when certain PL were added to palm oil, where the addition of PE, PI or LPE increased spherulite coarseness. In contrast, adding PC reduced spherulite size but did not change morphology. Smith [18] speculated that PC decreased spherulite diameter by acting as a nucleating agent whereas PE, PI and LPE retarded crystal growth,

leading to a courser structure. The PC component of the MFGM could also be acting as a nucleating agent for butterfat, producing smaller crystals. MFGM also contains PE and PI, which could be slowing growth in much the same manner as a decrease in supersaturation, thus creating courser spherulites, and eventually smaller individual crystals. This hypothesis was indirectly confirmed by the observation that lecithin addition had a similar effect on AMF crystallization since it contains high concentrations of PC, PE and PI.

Unlike previous studies in the literature where moisture levels were relatively low, the fat globule/AMF blends examined in this work contained a large excess of water. As previously discussed, PL can display a wide variety of solution behaviors that are very structure specific [17]. In buttermilk, the PL is very dilute and are in the form of liquid crystals [17]. As a result, it would be very difficult for these molecules to reenter the oil phase and significantly interact with fat crystallization. This is likely why substituting buttermilk for skim milk had little effect on crystal structure in the butter blends examined. We also observed that with skim milk/AMF mixtures, water droplets were so finely dispersed that they were not observed at the levels of SEM magnification used (Figs. 4a, 6a). However, as the amount of MFGM increased, significant coalescence of the water phase occurred, eventually creating a more oil-in-water like microstructure (Fig. 4d) or very large pockets of water (Fig. 6d). Therefore, too much PL can have a negative effect on butter functionality. In fact, one of the main problems with the Russian butter produced from the inversion of high fat content plastic cream was poor water dispersion [12]. This effect was likely due to high concentrations of PI and SM in the MFGM that tend to stabilize water-in-oil emulsions.

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