Langmuir Film Balance Study of the Surface Properties of a Soluble Fraction of Milk Fat-Globule Membrane

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Viewed from its origin, composition, and physicochemical properties, milk fat-globule membrane (MFGM) appears to act as a good, natural emulsifier and should be also considered as a potential stabilizing agent in the preparation of certain foods, e.g. creams and emulsions, infant formulas, and reduced-fat products. The present work was undertaken in an attempt to gain further information on the physicochemical properties of MFGM. In particular, the purpose of this study was to determine surface properties of a soluble fraction of MFGM (SFMFGM) using a film balance and to investigate the changes in the surface activity at different temperatures, which may occur during milk cream ripening or during heat and mechanical treatments of dairy products. The results showed that film balance measurements at the air/water interface actually provided a useful method for the study of SFMFGM monolayers. It was deduced from the compression isotherms that the SFMFGM films spread at the air/water interface presented a different behavior when the temperature varied from 4 to 40 °C.

Keywords: *Milk fat-globule membrane; triglycerides; surface activity; surface pressure; monolayer*

INTRODUCTION

Milk fat-globule membrane (MFGM) is the complete layer that covers each fat globule in milk. This thin membrane (approximately 10 nm in cross section) consists of a complex mixture of proteins, phospholipids, glycoproteins, triglycerides, cholesterol, enzymes, and other minor components and acts as a natural emulsifying agent enabling the fat to remain dispersed throughout the aqueous phase of milk (McPherson et al., 1983). Proteins in the MFGM represent only about 1% of the total milk proteins. However, the important function of this small amount of proteins in helping to maintain the integrity of membrane appears to outweigh its relatively low level in milk. Isoelectric focusing studies in combination with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were used to elucidate the protein composition of MFGM. Forty separate polypeptides were observed, among which at least 8 were glycoproteins, ranging in molecular weight from 15 000 to 240 000 (McPherson et al., 1984). The major classes of polypeptides were found at molecular weights of 155 000, 63 000, and 44 000, and these were referred to as components 3, 12, and 16, respectively (Mather et al., 1975). The phospholipids of MFGM account for approximately 60% of the total milk phospholipids, but the major components of MFGM lipids are the neutral ones, which consist mainly of triglycerides. The fatty acid composition of the triglycerides is considerably different from that of milk fat. MFGM triglycerides contain a higher proportion of long-chain saturated fatty acids (e.g. C_{16}, C_{18}) and exhibit a higher melting point (Walstra et al., 1974; Wooding et al., 1975). Infrared spectroscopy studies have shown some differences between the lipids of isolated MFGM and core fat, in that the acyl chains of membrane lipids were more rigid than those of core fat triglycerides, which could contribute greatly to the stability of the intact membrane (Kitchen et al., 1977). While overall molecular organizations of the various fractions within this membrane around the intact fat globule have been postulated by many authors, there still remains no universally accepted structure that takes into account the vast amount of physicochemical data that have appeared in the literature (Kitchen, 1977). However, the structure of membrane was thought to be the result of the properties of their constituents, proteins, and lipids. Viewed from its origin, composition, and physicochemical properties MFGM should be also considered as a potential emulsifying agent for certain foods and other artificial emulsions (Kanno et al., 1989, 1991; Chazelas et al., 1995). In fact, the modern food-processing industry is placing more and more emphasis upon the utilization of natural ingredients to provide specific functional properties in a wide range of formulated foods. The MFGM is closely involved in natural processes in milk (e.g. creaming and agglutination) and is markedly affected by treatments such as cooling, heating, and homogenization of dairy products (Buchheim et al., 1986). Many properties of the final products, their stability and acceptability, are determined by the responses of the MFGM components to these processes (Keenan et al., 1983; Fink et al., 1985; Houlihan et al., 1992; Sharma et al., 1993). Thus, many current problems of the dairy industry are directly related to understanding of this unique membrane system. The objective of our investigation was to get more information on the surface and mechanical properties of the MFGM. For that purpose, the membrane material was isolated from the raw cream, and a Langmuir film balance measuring system was used to investigate the behavior of the soluble fraction of MFGM (SFMFGM) components in the water surface. Measurements were performed at different temperatures for

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Table 1. Composition of SFMFGM Purified by Washing Raw Cream Three Times with Phosphate Buffer (NaH_2PO_4/Na_2HPO_4 0.01 M, pH 7.2, 0.09% NaCl)

A. exptl values	g/100 g	B. range of values reported in the lit. for different methods of membrane extraction	g/100 g
proteins	19.0	proteins	25/60[a]
phospholipids	10.5	phospholipids	12/48[b]
neutral lipids	66.0	neutral lipids	20/80[c]
phospholipids/ proteins	0.55	phospholipids/ proteins	0.13/0.66[b]
phospholipids/ lipids	0.16	phospholipids/ lipids	0.20/0.48[c]

[a] Anderson (1974). [b] Kitchen (1974). [c] McPherson et al. (1983).

monitoring the influence of heat treatments, which may occur during the ripening of milk cream or during various food-processing operations, upon the surface properties of SFMFGM.

MATERIALS AND METHODS

Preparation of a SFMFGM. SFMFGM was prepared according to the procedure of Basch et al. (1985) with modifications. Raw cream, used as the starting material, was warmed to 40 °C, washed three times with phosphate buffer (NaH_2PO_4/Na_2HPO_4 0.01 M, pH 7.2, 0.09% NaCl), and then centrifuged for 10 min at 45000*g* to separate the aqueous phase. After centrifugation, the cream sample was chilled at 5 °C overnight and churned with a Polytron until fat and sera were separated. It was then warmed to 50 °C and centrifuged again for 10 min at 45000*g* to separate the water-soluble fraction that contained the MFGM. Finally the SFMFGM was stored at 4 °C after lyophilization.

Compositional Analysis. Protein was determined according to the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951), and the total phospholipid content was estimated from a determination of phospholipid phosphorus according to the method of Morrison (1964) as modified by Carboni et al. (1986).

Electrophoresis. Membrane proteins were examined by SDS−PAGE, using a 0.5 mm thin precast polyacrylamide gradient gel (gradient 8-18) Pharmacia Biotech. Membrane samples were treated with SDS (1%), dithiothreitol (5 mM), and bromophenol blue (0.01%) and heated at 100 °C for 5 min before electrophoresis. For molecular weight determination a molecular weight calibration kit (Pharmacia LKB) was used.

Thin-Layer Chromatography (TLC). Lipids were extracted according to the method of Folch et al. (1957). Triglycerides and phospholipids were isolated and identified by TLC. About 10−15 mg of lipid material in chloroform was applied as a thin band on a 0.25 mm TLC plate in silica gel with fluorescent indicator (Polygram SIL G/UV_{254}). Development was done with petroleum ether (bp 60−80 °C)/ethyl ether/formic acid (60:40:1.5 by volume). The plate was air-dried and visualized under violet light to locate the phospholipid and triglyceride bands.

Differential Scanning Calorimetry (DSC). A solvent system of chloroform/methanol (2:1, v/v) was used for the extraction of lipids from membrane (Folch et al., 1957). The solvent extracts were evaporated to dryness, and the residue was analyzed by DSC with a DSC7 Perkin-Elmer Instrument. The sample was first heated to 80 °C and then quickly cooled to −40 °C with a speed of 50 °C/min, and the DSC thermographs were recorded during melting from −40 to 80 °C, which was carried out with a heating speed of 5 °C/min.

Surface Properties of SFMFGM. Surface pressure (π) versus surface area (A) was measured on a FW-2 Langmuir film balance (Lauda GmbH, Königshofen, Germany). The SFMFGM sample was prepared by dissolving the lyophilized material in water (0.1% w/v); the solution was then stored at 4 °C overnight to obtain a good hydration and solubilization. A Hamilton microsyringe was used for spreading this solution (60 μL) on a citrate/phosphate saline buffer (8 mM, pH 6.6, 0.16 M NaCl), and a 15 min period was allowed for the formation and equilibration of the film in a maximum surface of the 927 cm^2. The π/A isotherms were obtained by compressing spread monolayer at a constant rate of 1.54 $cm^2\ s^{-1}$ at various temperatures.

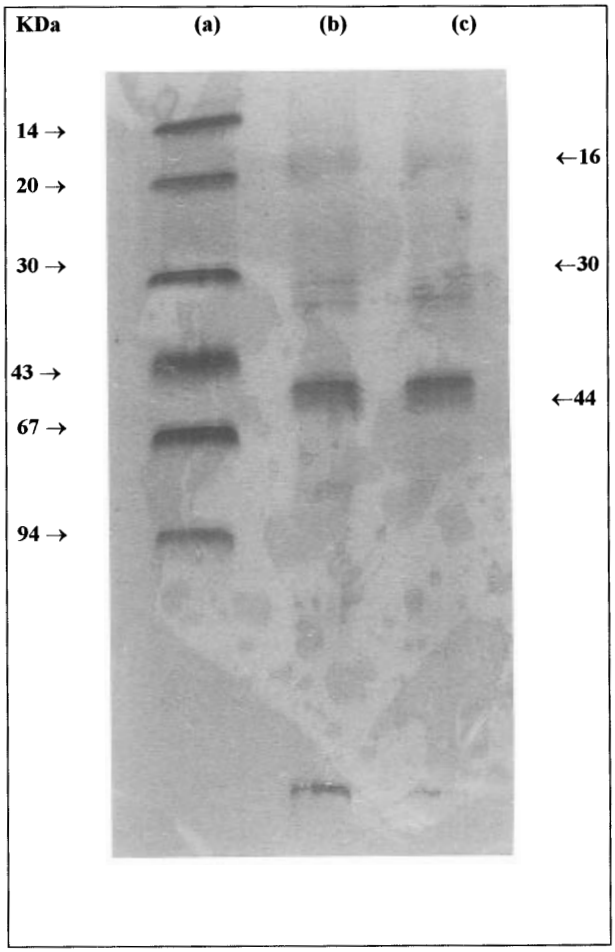

Figure 1. SDS−PAGE pattern of milk fat-globule membrane proteins: (a) standard purified proteins [phosphorylase (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (21 000), and α-lactalbumin (14 400); (b and c) SFMFGM lyophilized. The concentration of SFMFGM was 1.5 mg/mL, and 15 μL was loaded on the gel.

RESULTS AND DISCUSSION

Compositional Analysis of SFMFGM Material. Prior to interfacial characterization, compositional analyses on SFMFGM were performed. In Table 1, the protein, lipid, and phospholipid contents of SFMFGM prepared by washing raw cream three times with phosphate buffer are compared with the compositional patterns formerly reported by various works (Anderson, 1974; Kitchen, 1974; McPherson et al., 1983). Differences in composition were observed since many changes in the content of the MFGM individual components occurred during various milk-processing operations and since different methods of membrane extraction have been used (Kitchen, 1977).

The polypeptide pattern in SFMFGM determined by SDS−PAGE is illustrated in Figure 1. Three zones corresponding to molecular weights of 44 000, 30 000, and 16 000 on average are clearly observed on the SFMFGM profile. These major classes of polypeptides are in a range that compares favorably with the data of Mather and Keenan (1975) and most other workers (Anderson et al., 1974; McPherson et al., 1984). The present bands are most likely the following: 44K =

Table 2. Mean and Coefficient of Variation of Surface Area for Milligrams of Molecules (m^2 mg^{-1}) Calculated at Different Surface Pressures by Six Trials Made on SFMFGM Monolayer Spread on a Citrate/Phosphate Saline Buffer Surface (8 mM, pH 6.6, 0.16 M NaCl) at 25 °C

surface pressure (mN m^{-1})	mean (m^2 mg^{-1})	coefficient of variation (%)
7	0.510	1.84
14	0.423	1.72
21	0.367	1.56
28	0.253	3.97
35	0.193	3.32
42	0.152	4.29
49	0.108	12.56
56	0.076	5.02
63	0.063	3.55

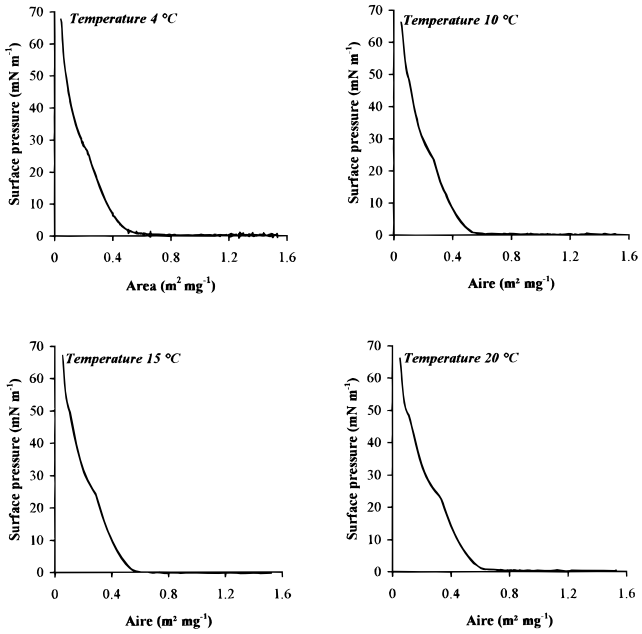

Figure 2. Isotherms of the SFMFGM on citrate/phosphate saline buffer (8 mM, pH 6.6, 0.16 M NaCl) at low temperatures (4, 10, 15, and 20 °C). All of the isotherms were run at least two times in the direction of increasing pressure with freshly prepared film from a 0.1% SFMFGM in water solution.

glycoprotein B; 30K = casein; 16K= peptides or fragments. It is easy to appreciate that the major fat-globule membrane protein, butryophillin, is absent from the preparation used in this study. The reason for this distribution of proteins can be seen in the method of MFGM extraction. Upon warming to 50 °C, the centrifugation (10 min at 45000*g*) spins down the major part of the MFGM, leaving behind the water soluble fraction; however, the butryophillin-rich fraction would not have spread very well at all, as it is insoluble under these conditions.

Total lipids were separated from SFMFGM with chloroform/methanol and the extract were run on a TLC plate. The TLC pattern shows only two different bands, corresponding to triglycerides and phospholipids, respectively.

Surface Properties of SFMFGM. Heat and mechanical treatments in the dairy industry lead to modifications in MFGM composition and structure. On the basis of this consideration, a Langmuir film balance was used to characterize SFMFGM monolayers. In fact, this apparatus allows one to evaluate by compression the mechanical properties of membrane material at the air/water interface. Moreover, isotherms at different

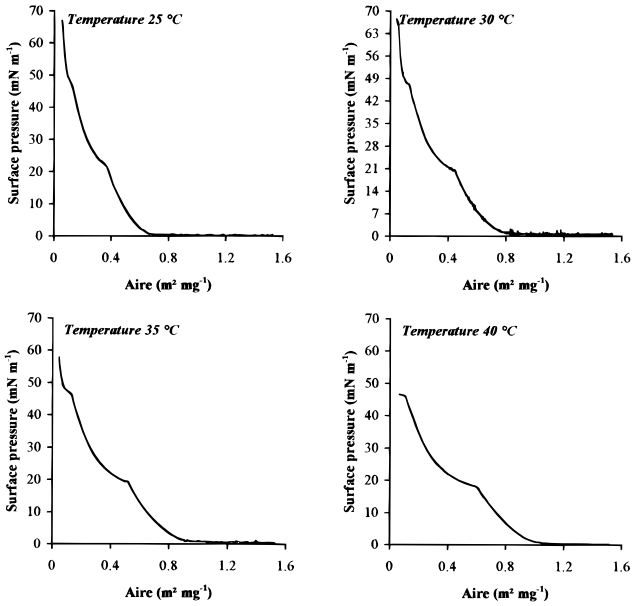

Figure 3. Isotherms of the SFMFGM on citrate/phosphate saline buffer (8 mM, pH 6.6, 0.16 M NaCl) at high temperatures (25, 30, 35, and 40 °C). All of the isotherms were run at least two times in the direction of increasing pressure with freshly prepared film from a 0.1% MFGM in water solution.

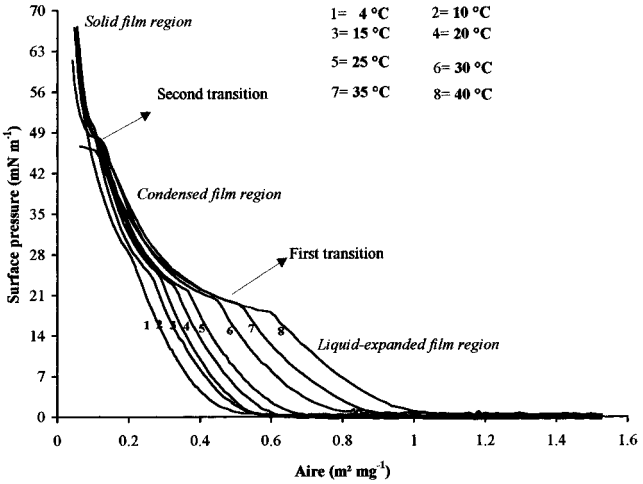

Figure 4. Isotherms of the SFMFGM on citrate/phosphate saline buffer (8 mM, pH 6.6, 0.16 M NaCl) at different temperatures (4/40 °C), obtained by compressing spread monolayer at a constant rate of 1.545 cm^2 s^{-1}.

temperatures provide information on the influence of heat treatments. First, the validity of the method was confirmed by a test of reproducibility at 25 °C. The coefficients of variations calculated according to six different measurements are reported in Table 2. On average, the coefficients of variation are sufficiently low for all surface pressures considered.

Compression isotherms (surface pressure against surface area) of the SFMFGM monolayer were then examined at different temperatures (Figures 2 and 3). All of these curves were run at least twice to check the measurement reproducibility and the accuracy of the system under each temperature condition was good. The profiles of surface pressure against surface area per milligram of SFMFGM show a gradual increase in surface pressure while the film is compressed. However, it is interesting to note that this increase in surface pressure changes when the temperature of the monolayer is raised from 4 to 40 °C (Figure 4). Starting from 25 °C, all isotherms exhibit two transition phases, which

Table 3. Surface Area for Milligrams of Molecules (m^2 mg^{-1}) and Surface Pressure (mN m^{-1}) Values Corresponding at the Collapse of the Structure and the Film Transition Phases, Calculated by Isotherms at Different Temperatures

	collapse of film		transition phase I		transition phase II	
temp (°C)	surface area (m^2 mg^{-1})	surface pressure (mN m^{-1})	surface area (m^2 mg^{-1})	surface pressure (mN m^{-1})	surface area (m^2 mg^{-1})	surface pressure (mN m^{-1})
4	0.046	67.0				
10	0.046	67.0				
15	0.052	67.0	0.280	23.9	0.102	50.0
20	0.052	66.0	0.324	23.0	0.114	48.0
25	0.046	67.0	0.358	22.0	0.116	48.0
30	0.056	66.0	0.450	20.0	0.128	46.5
35	0.044	57.8	0.516	19.0	0.118	47.0
40	0.063	46.7	0.602	18.0	0.106	46

correspond to discontinuities (change of slope) in the π/A curves. The first discontinuity is attributed to a transition from a so-called liquid expanded film to a condensed film, with an expanded−condensed transition region; the second one expresses instead the change from a condensed phase to a solid film (Figure 4). When the temperature increases, the transition phases are more stressed and they occur at lower surface pressure values (Table 3). At the lowest temperatures (4 and 10 °C) there are no clear phase changes and the compressibility is nearly constant. Thus, it is deduced from the compression isotherms that SFMFGM films spread at the air/water interface at lower temperatures (under 15 °C) exhibit mechanical properties different from those of the films spread at higher temperatures. In the liquid expanded film region the surface pressure, at the same area, is greater at high than at low temperatures. For instance, at a surface coverage of 0.4 m^2 mg^{-1}, the surface pressure is 2.9 mN m^{-1} at 4 °C, but 8.4 mN m^{-1} at 25 °C; at 0.7 m^2 mg^{-1}, the surface pressure is 3 mN m^{-1} at 30 °C, but 12 mN m^{-1} at 40 °C. These results suggest that at lower temperatures the surface activity of the SFMFGM appears to be smaller if the film expanded region is considered. Moreover, the collapse of the film always occurs at the same surface area/milligram value for the lowest temperatures considered, and the maximum pressure achieved is about 67 mN/m. However, at 35 and 40 °C, the collapse appears at a surface pressures of 57.8 and 46.7 mN/m, respectively (Table 3). The different behaviors of the SFMFGM as a function of temperature may be partly explained by the presence of a high percentage of high-melting triglycerides, which have a different crystallization level with rising temperatures. At high temperature, the important percentage of triglycerides in melted state may explain the broader liquid expanded region. However, at higher pressures, the compressibility of the monolayers is strongly reduced, as the triglycerides are in a solid state (Iwahashi et al., 1985). At the lowest temperatures there is no phase change at higher pressures, probably since the triglycerides are already in crystalline state. The DSC technique was used to study the crystalline transition of the SFMFGM lipids. Figures 5 and 6 report the thermograph traces given by lipids extracted from a dried milk sample and the SFMFGM, respectively. Both melting curves show two endothermic peaks caused by the transition of low-melting and high-melting triglycerides, respectively, but the high-melting and low-melting triglycerides ratio of SFMFGM is very different from that of lipids in milk. This is further illustrated in Table 4, in which the percentages of the high-melting and low-melting triglycerides for SFMFGM and dried milk lipids, calculated by measurement of the area underneath the endothermic peaks, are reported. The SFMFGM contains a higher proportion of high-melting triglycerides,

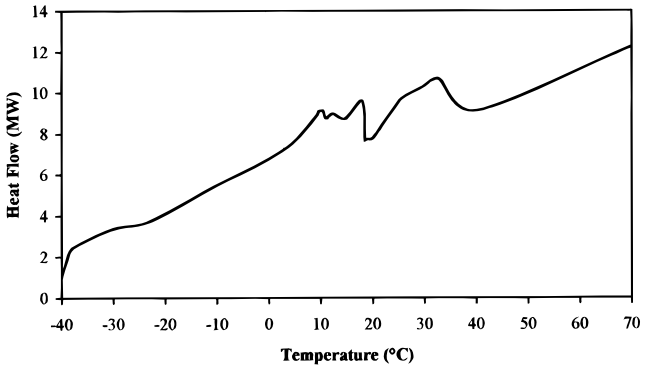

Figure 5. DSC curves of the dried milk lipids, registered during the melting of sample from −40 to 80 °C with a heating speed of 5 °C/min.

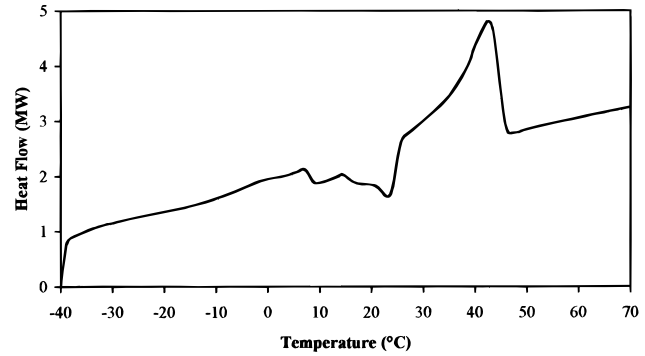

Figure 6. DSC curves of the SFMFGM lipids, registered during the melting of sample from −40 to 80 °C with a heating speed of 5 °C/min.

Table 4. Percentages of the High-Melting and Low-Melting Triglycerides (Grams/100 g of Total Lipids) for SFMFGM and Dried Milk Lipids, Calculated by Melting Curves

	low-melting triglycerides (g/100 g)	high-melting triglycerides (g/100 g)
freeze-dried MFGM	30.41	69.59
dried milk fat	58.33	41.67

and it has been suggested that these triglycerides are derived from fat crystals which contaminate the membrane during cooling and churning process (McPherson et al., 1983). The crystallization rate of the SFMFGM triglycerides (grams of crystalline triglycerides per 100 g of total triglycerides) at different temperatures adopted in Langmuir film balance measurements was determined by thermograph traces (Table 5). It can be seen that even for small temperature changes, significant variations in the crystalline triglycerides percentage are observed. In particular, in the temperature range considered, the percentage of crystalline triglycerides changes from 86.5% at 4 °C to 28.6% at 40 °C, and these

Table 5. Percentages of the Solid Triglycerides (Grams/100 g of Total Triglycerides) of the SFMFGM at the Different Temperatures, Determined by DSC Curve

temp (°C)	solid triglycerides (g/100 g)	temp (°C)	solid triglycerides (g/100 g)
4	86.5	25	70.7
10	78.5	30	60.6
15	73.3	35	47.1
20	72.0	40	28.6

different crystallization rates certainly influence the comportment of SFMFGM monolayers and then the surface activity.

Conclusions. In conclusion, the film balance studies at the air/water interface actually provide a useful method for evaluating the mechanical properties of membrane material and for studying the influence of different temperatures on the SFMFGM monolayers. As shown, the MFGM films spread at lower temperatures exhibit different properties in comparison with the films spread at higher temperatures. This result may then indicate the importance of the temperature on the physical properties of SFMFGM, which could be exploited commercially as an ingredient in the manufacture of new and improved foods.

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