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Size-Dependent Lipid Content of Bovine Milk Fat Globule and Membrane Phospholipids

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Supporting Information

ABSTRACT: The mammary epithelial cell produces unique structures and a range of diversely sized lipid particles from tens of micrometers to less than 1 μm. The physical, chemical, and biological properties of the differently sized milk fat globules (MFGs) and their complex membranes are not well described. Six size fractions of MFGs were obtained by gravity-based separation and analyzed, and their partial lipidome was determined. The smallest MFGs had a higher concentration of polyunsaturated fatty acids (FAs). The FAs indicative of elongase activity were highest in the smallest MFGs, whereas those FAs indicative of desaturase activity did not differ between size groups. The phosphatidylinositol concentration was highest whereas the phosphatidylserine concentration was lowest in MFGs with an average diameter of 2 μm. Phosphatidylethanolamine and cholesterol concentrations were highest whereas that of sphingomyelin was lowest in MFGs with an average diameter of 3 μm. Phosphatidylcholine concentrations did not vary between the size groups. Results suggest that the assembly of milk fat globules that differ in size is not a homogeneous nor random process and that the differences in composition may reflect discrete biosynthetic routes.

KEYWORDS: fatty acid, lipid, milk fat globule, milk fat globule membrane, phospholipids, sphingolipids

INTRODUCTION

Traditionally, the milk fat globule (MFG) was considered to be exclusively a vehicle for fat delivery, despite its known diversity of sizes. Recently, however, the milk fat globule membrane (MFGM) has been recognized for the complexity of its bioactive components and the diversity of its structures.[1−3] Nonetheless, the dominant view of milk fat overlooks the fact that the MFG population is itself diverse with discrete particle sizes that span over 2 orders of magnitude, from 200 nm to 15 μm, depending on the species, lactation stage, and nutrition.[4,5]

MFG secretion follows a unique cellular pathway in which the apical membrane of the mammary gland epithelial cell envelops the emerging MFG, thereby forming the intact globule complete with MFGM, one of the most unique particles in biology.[6] The MFGM consists of complex structural lipids, including phospholipids, glycolipids, cholesterol, and sphingolipids, derived from the apical membrane of the secreting epithelial cells. Due to this unique secretion pathway, it is thought that all MFGs are enveloped by the same membrane composition. However, the simplifying assumption that all MFGMs are compositionally identical overlooks the possibility that the membrane composition is guided by cellular metabolic pathways and by the need to secrete and stabilize lipid−protein assemblies of different diameters. Different phospholipid membrane compositions have been shown to track the metabolic and differentiation status of mammary cells.[7] In addition, increasing the ratio of phosphotidylethanolamine (PE) to phosphotidylcholine (PC) in the membrane would tend to enhance membrane fusion, a mechanism that facilitates cellular vesicle formation and secretion[8] and may dictate MFG fusion. Perturbations in phospholipid assembly have been identified as causal to structural abnormalities in cell biology. For example, in hepatocytes, an aberrant pathway of PE to PC synthesis has been shown to compromise lipoprotein secretion and induce lipid accumulation by the liver cells.[9] Such examples clearly demonstrate that the composition of membrane compartments has important consequences to cellular and lipid metabolism, in situ. However, mammary gland epithelial cell membrane composition has never been studied in relation to milk fat globule formation, secretion, milk fat concentration, and MFG size distribution.

Milk lipid-related research to date has focused almost exclusively on understanding and eventually altering the concentrations of specific fatty acids within the fat fraction of mainly bovine,[10−12] but also human,[13] milk. Molecules such as unsaturated fatty acids and conjugated glycolipids have been the focus of an array of studies[14,15] due to their health properties. However, the alterations of specific compounds in the milk lipid fraction as a function of different MFG sizes have yet to be understood and technologically manipulated.

An indication for the association between MFG and MFGM structural−compositional differences and milk fat concentration was recently reported.[16] A higher endogenous plasma insulin concentration was found to be associated with lower milk fat percentages and a higher ratio of polar to neutral lipids. The elevated relative concentration of polar lipids suggests that this was accompanied by an altered MFG size distribution. Another study showed that increasing the content of linseed oil in dairy cow rations reduces the net milk fat concentration and alters the MFG size distribution within the cream fraction.[5] These

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modifications induced by diet were associated with changes in the MFGM phospholipid composition and thus provided the first evidence of an association between the membrane composition of mammary gland epithelial cells and the milk fat concentration. Nevertheless, that study, focused on the cream fraction of milk and its MFGM composition's response to diet. Other reports of the MFG structural−compositional interactions also focused on the largest MFGs, derived from cream.[5,17,18] Hence, the possibility that different compositions of mammary cell membranes alter the secretion of different MFG sizes was not addressed.

In this paper, we extend previous research that demonstrated different chemical fingerprints for differently sized MFGs in human milk.[19] The overall goal of the research is to map the biochemical pathways of lipid synthesis onto the mechanisms of globule secretion and ultimately to understand the cellular regulatory systems controlling fat globule size, structure, and composition. Here, we present the first evidence of size-dependent chemical and physical properties of bovine MFGs. MFGs derived from the skim milk fraction were analyzed quantitatively for fatty acid composition, and their MFGM was analyzed for its polar lipid composition.

MATERIALS AND METHODS

Chemicals and Reagents. For lipid extraction, methanol and chloroform (both analytical reagent grade) were purchased from Bio-Lab Ltd. (Jerusalem, Israel). For the high-performance liquid chromatography (HPLC) analysis, chloroform and ethanol (used at 97:3 (v/v), both analytical reagent grade) and methanol (HPLC grade) were purchased from Bio-Lab. The triglyceride triolein (>99% pure) was purchased from Supelco (Bellefonte, PA). The phospholipid standards were supplied by Sigma-Aldrich Ltd. Israel (Rehovot, Israel) and consisted of PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, 10 mg of phospholipid/mL of $CHCl_3$, purity 99%), PI (L-α-phosphatidylinositol ammonium salt, from bovine liver, purity 98%), PS (1,2-dioleoyl-*sn*-glycerol-3-phospho-L-serine sodium salt, purity 95%), PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, purity ≥99%), and SM (from bovine brain, purity 97%); conjugated linoleic acid was used (9-*cis*-11-*trans*-linoleic acid, purity >95%) as a standard for free fatty acids. For the gas chromatography (GC) analysis, methanol (analytical reagent grade) was purchased from Bio-Lab, petroleum ether (analytical reagent grade) was from Gadot Lab Supplies (Netanya, Israel), and sulfuric acid was from Bet Dekel (Ra'anana, Israel). Retention times were determined by injection of commercial standard mixes of phospholipids and triglycerides.

Animals. To avoid systematic physiological or metabolic effects that can result in an artifactual size-dependent composition, milk was collected from both primiparous and multiparous cows, either at very early stages of lactation or at lactation stages characterized by a transition to positive energy balance. Milk was collected from cows at first ($n = 6$) or second ($n = 3$) lactation, at different lactation stages; five cows were 9.4 ± 0.7 days (average ± SEM) postpartum, and four cows were 58 ± 2.1 days (average ± SEM) postpartum. To make sure that the milk samples used in the present study represent milk from the entire milking session and not a specific fraction of milk, 100 mL was collected throughout the milking session by using the milk sampling feature of the "Affimilk" system (Afikim, Israel).

Gravity-Based Separation Method. Milk was collected in a plastic vial and sent to the laboratory on ice. Immediately upon arrival to the laboratory, no longer than 2 h postcollection, 60 mL of the milk samples was transferred into glass tubes with a lower funnel for 20 h, at 17 °C. After 24 h, six 10 mL fractions were collected from the skim milk. The cream fraction that was located in the upper part of the tube was also collected for MFG size measurements. Different MFG fractions were designated F1 to F6 and cream. The lowest fraction collected from the tube was F6 and was expected to consist of the smallest MFGs, whereas the upper fraction of the skim milk, F1, was expected to consist of the largest MFGs remaining in the skim milk fraction and not aggregated in the cream fraction. MFG sizes were measured in fresh cream or skim milk samples in duplicate using a particle size analyzer with a single modular light-scattering system (Microtrac UPA, North Wales, U.K.). For each milk sample, the fat globule size was calculated as the mean diameter (μm) of the area distribution of the particles in solution obtained from two 180 s scans. Mean diameter = $\Sigma V_i/\Sigma(V^i/d^i)$, where V = volume percentage in the channel size and d = channel diameter (width of the light path). To prevent artifactual submicrometer-sized globules, each milk fraction consisting of the smallest MFGs (i.e., F6) was measured once diluted in 35 mM EDTA for casein micelle dissociation.

Milk Parameters. Fat, protein, and lactose percentage data were obtained from an "Affimilk" milking system (Affikim, Israel) equipped with a built-in detection system that collects online data during milking. Presented values are the means of the four last morning milking sessions prior to and including the sample-collection milking session.

To analyze possible interactions between milk fat, protein, and lactose concentrations and the lipid fraction in the MFG size fractions, milk parameters collected by the Affimilk system were split into two or three groups as follows and used as a nominal character: lactose percentage, two groups, ≥5% and <5%; protein percentage, two groups, ≥3% and <3%; fat percentage, three groups, <3%, 3−4%, and >4%; weight, three groups, <500, 500−600, and >600 kg; somatic cell count, three groups, 0−200000, 201000−800000, and >800000 cells/mL.

Extraction of Total Lipids. Total lipids were extracted from milk using a protocol adapted from the cold extraction procedure developed by Folch as previously described.[20] Each sample was extracted twice, once for lipid analysis by normal-phase LC and once for fatty acid analysis by GC. Total lipids were extracted from 0.5 mL of milk with methanol−chloroform−water (1:2:0.6, v/v). After overnight incubation at 4 °C, the upper phase was removed and the lower phase was collected and filtered through a 0.45 μm Teflon syringe filter (Axiva Sachem Biotech, India) into a new vial. After solvent was evaporated under vacuum, 100 μL of chloroform−ethanol (3%, v/v) was added. Total extracted lipids were stored at −20 °C until further analysis. For GC analysis, fatty acid methyl esters (FAMEs) were generated.

FAME Preparation. FAMEs were prepared from the lower phase of the Folch extraction of the milk samples. After evaporation of the lower phase of the Folch extract, 2.5 mL of 5% (v/v) H_2SO_4−methanol was added, the vials were sealed, and the tubes were sealed and placed in a water bath at 65 °C for 1 h. After incubation, the tubes were allowed to reach room temperature, and 1.9 mL of petroleum ether was added. After shaking, 3.5 mL of doubly distilled water was added. The upper phase was collected and evaporated under vacuum, and 100 μL of petroleum ether was added before injection for GC analysis.

GC Analysis. Chromatographic analysis was performed with a gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a fused-silica capillary column (60 m × 0.25 mm i.d., DB-23, Agilent) under the following conditions: The oven temperature was programmed from 130 to 170 °C at a rate of 27 °C/min and from 170 to 215 °C at a rate of 2 °C/min, held at 215 °C for 8 min, programmed from 215 to 250 °C at a rate of 40 °C/min, and held at 250 °C for 5 min. The run time was 37.9 min. Helium was used as the carrier gas at 2.21 mL/min. The flame ionization detector temperature was 270 °C, and the injector temperature was 280 °C. Air and hydrogen flows were adjusted to give maximal detector response. Peak identification was based on relative retention times of two external standards. Fatty acids were recorded as the percentage of total fatty acids within each sample (mol %). Fatty acids for which more than 20% of the data were missing or that were

below the limit of quantification were dropped from the analyses and considered undetermined in the results.

Measurements of Fatty Acid Flux through Elongase and Desaturase Pathways. The lipid composition was analyzed for markers of de novo lipogenesis: desaturase and elongase. Desaturase markers included the monounsaturated fatty acids 16:1n7 and 18:1n9, as well as the 16:1n7/16:0 and 18:1n9/18:0 ratios, which are well-established markers of desaturase activity. Elongase markers included the metabolites 18:1n7, 22:1n9, 22:0, 24:0, and 22:5n3, measured as the ratio of product to substrate: 18:1n7/16:1n7, 20:1n9/18:1n9, and 20:0/18:0.

Analysis of Polar and Neutral Lipids. Polar and neutral lipids were identified and quantified by HPLC (HP 1200, Agilent) combined with an evaporative light-scattering detector (ELSD; Agilent) according to a previously described protocol for normal-phase lipid separation.[21] The separation process was managed by ChemStation software for data acquisition from the ELSD. Lipids were identified by the use of external standards: triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids, cholesterol, PE, PS, PI, PC, and SM. Two quantification methods were used: (1) area under the curve (AUC), to determine the relative proportions of each of the identified polar lipids from the total detectable glycerolphospholipids and sphingolipids in the sample; (2) weight percentage of each of the identified glycerolphosopholipids, sphingolipids, and cholesterol of the overall weight of the identified membrane components (i.e., cholesterol, PE, PS, PI, PC, and SM), as indicated below.

HPLC/ELSD Calibration. Phospholipids, SM, and cholesterol were identified by comparison with the retention time of pure standards: five calibration curves were determined from the AUC values obtained by injection of different amounts of standard: cholesterol (2−15 μg), free fatty acids (5−80 μg), PE (2−25 μg), PI (5−80 μg), PC (0.5−10 μg), and SM (5−80 μg). Calibration curves were calculated by applying the power model equations to the AUC and concentration values: cholesterol, $y = 0.1016X^{0.49}$ ($r^2 = 0.97$); free fatty acids, $y = 1.73X^{0.41}$ ($r^2 = 0.99$); PS, $y = 0.0824X^{0.7645}$ ($r^2 = 0.99$); PE, $y = 0.035X^{0.63}$ ($r^2 = 0.99$); PI, $y = 0.114X^{0.607}$ ($r^2 = 0.99$); PC, $y = 0.051X^{0.603}$ ($r^2 = 0.99$); SM, $y = 0.11X^{0.62}$ ($r^2 = 0.99$). The sum of the glycerolphospholipid, SM, and cholesterol concentrations was regarded as the total membrane weight (100%). The weight of each of the polar lipids in 1 mL of milk was calculated according to the standard curves, and its weight percentage of total membrane lipids (PE + PI + PC + SM + cholesterol) was determined. Relative concentrations of glycerolphospholipids, shingolipids, and cholesterol were also determined as the AUC percentage of the membrane polar lipids' total AUC. The sum of glycerolphospholipids, SM, and cholesterol concentrations was regarded as the total membrane AUC (100%). Each polar lipid AUC was determined as a percentage of the total membrane AUC.

Statistical Analysis. All statistical procedures were performed using JMP software, version 7 (SAS Institute, Cary, NC). The average fatty acid and polar lipid concentrations in every MFG size fraction were used for statistical assessments of size-dependent MFG and MFGM compositions. Protein, lactose, and fat concentrations and the somatic cell count were used to determine their effect on the size-dependent lipid composition of the MFGM.

To determine differences in the fatty acid and lipid compositions between different MFG size classes, analysis of variance (ANOVA) was performed using the "Fit Y by X" procedure of JMP. When the parameters were found to differ between the size groups, the Tukey honest significant difference (HSD) test was used to determine which groups differed. A paired sample t test was performed diagnostically to identify which pairs of size groups were significantly different (the results are presented in the Supporting Information).

The run model of ANOVA was used to determine the interaction between the "fraction" effect and "fat", "protein", "lactose", "SCC" (somatic cell count), "lactation number", and "days in milk" (DIM) on the fatty acid and lipid compositions of the MFG and MFGM, respectively.

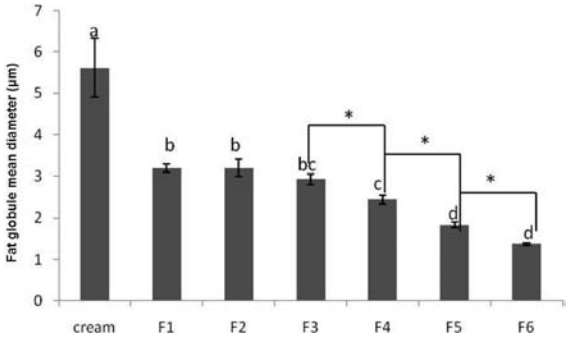

Figure 1. Mean diameters of milk fat globule size fractions after 24 h at 17 °C. The values represent the volumetric mean diameter corresponding to the highest ln % value in each size fraction ± standard error. Different letters indicate significant differences ($P = 0.05$) when all groups were analyzed for differences by ANOVA. The asterisks indicate different mean diameters when two size groups were paired by Student's t test.

Significance was set at $\alpha = 0.05$, and group responses that reached near significance are also reported herein. A tendency was reported at $\alpha = 0.1$.

RESULTS

Gravity-Based MFG Size Separation. Gravity-based separation of raw milk incubated at 17 °C for 24 h was used to stratify six size groups of fat globules (Figure 1). The protocol achieved the separation of four groups of MFGs differing in size. The free fatty acid (FFA) concentration was determined in each of the size groups to evaluate whether the incubation time and temperature induced fat degradation. Such degradation, if occurs, would increase the formation of FFAs and partial glycerides and bias the interpretation of all compositional data. No differences in free fatty acid AUC or weight percentages were found between MFG size groups.

Fatty Acid Profile of MFG Size Groups. The composition of lipids differed among the size-fractionated globules. The major fatty acids and their relative concentration differ between the size groups as indicated in Table 1 and in Figure 3. The smallest MFGs (F6) had the highest relative concentration of long-chain polyunsaturated fatty acids (PUFAs) and the lowest concentration of medium-chain saturated fatty acids. In contrast, the concentration of γ-linolenic acid was highest in the largest MFGs (F1). The lowest EPA (eicosapentaenoic acid; C20:5n3) concentration was found in F2 and F3. F3 also had the lowest arachidic acid (C20:0), while the highest lauric acid (C12:0) concentrations were found in the F5 size fraction. Vaccenic acid's (C18:1n7) relative concentration was lowest in the F1 and F2 groups compared with all other MFG size groups. To further study possible effectors that might modulate the milk fat globule lipid composition, variations in lipids were statistically correlated with other milk parameters.

Interactions between Milk Parameters and Fatty Acid Composition. No significant interactions were found between the fat, protein, or lactose percentage in milk and the concentrations of dietary fatty acids, for example, omega-3 fatty acids. However, the size-dependent relative concentration of de novo saturated fatty acid interacted significantly with the milk fat level ($P = 0.03$, Figure 3) and tended to interact with the milk protein and yield levels ($P = 0.07$ and 0.01, respectively). Therefore, pathways of fatty acid metabolism were defined and related to the milk fat globule size populations.

Table 1. Size-Dependent MFG Fatty Acid Composition[a]

fatty acid	F1	F2	F3	F4	F5	F6
C10:0	1.16 ± 0.4 ab	1.42 ± 0.45 a	1.04 ± 0.35 ab	0.52 ± 0.2 ab	0.56 ± 0.21 ab	0.04 ± 0.02 b
C12:0	11.07 ± 0.03 b	14.94 ± 0.03 ab	14.89 ± 0.02 ab	13.44 ± 0.02 ab	15.54 ± 0.03 a	3.33 ± 0.01 c
C18:1n7	1.15 ± 0.07 b	1.24 ± 0.07 b	1.73 ± 0.15 a	1.37 ± 0.08 a	1.43 ± 0.15 a	1.46 ± 0.06 a
C18:2n6	4.44 ± 0.12 b	4.58 ± 0.07 b	4.6 ± 0.11 b	4.72 ± 0.09 b	4.88 ± 0.09 b	5.41 ± 0.11 a
C18:3n6	0.09 ± 0.02 a	0.06 ± 0.01 ab	0.06 ± 0.01 b	0.04 ± 0 b	0.04 ± 0 b	0.04 ± 0 b
C20:0	0.1 ± 0.02 bc	0.12 ± 0.01 bc	0.1 ± 0.02 c	0.14 ± 0.01 abc	0.16 ± 0.01 ab	0.21 ± 0.02 a
C20:1n9	0.15 ± 0.01 ab	0.14 ± 0.01 b	0.14 ± 0.02 b	0.21 ± 0.02 a	0.14 ± 0.01 b	0.15 ± 0.02 ab
C20:4n6	0.23 ± 0.03 b	0.26 ± 0.03 b	0.24 ± 0.03 b	0.28 ± 0.01 b	0.31 ± 0.02 ab	0.38 ± 0.03 a
C20:5n3	0.06 ± 0.01 bc	0.03 ± 0.02 c	0.03 ± 0.02 c	0.05 ± 0.02 bc	0.12 ± 0.01 b	0.2 ± 0.02 a
C22:1	0.013 ± 0.008 b	0.014 ± 0.005 b	0.015 ± 0.0045 b	0.025 ± 0.003 ab	0.035 ± 0.004 a	0.046 ± 0.003 a

[a] Values are mole percent means ± standard error, and $n = 9$. Different letters indicate significant differences ($P < 0.05$).

Table 2. Elongase Markers, Indicating Flux of Fatty Acids through the Elongase Pathway[a]

	F1	F2	F3	F4	F5	F6
C22:1/C20:1	0.095 ± 0.025 c	0.083 ± 0.025 c	0.0822 ± 0.08 c	0.124 ± 0.02 bc	0.238 ± 0.021 bc	0.287 ± 0.035 a
C20:0/C18:0	0.009 ± 0.0005 c	0.0104 ± 0.0006 bc	0.0091 ± 0.0012 c	0.011 ± 0.0008 abc	0.013 ± 0.0009 bc	0.0148 ± 0.0007 a

[a] Values are mole percent means ± standard error, and $n = 9$. Different letters indicate significant differences ($P < 0.05$).

Table 3. MFGM Phospholipid (PL) Composition As Calculated by the AUC Percentage[a]

PL	F1	F2	F3	F4	F5	F6
PE	26.4 ± 2.74 a	11.96 ± 3.4 ab	14.82 ± 5.73 ab	12.43 ± 2.27 ab	1.68 ± 3.247 b	11.96 ± 3.15 ab
PI	2.78 ± 33 c	0.289 ± 0.289 c	5.5 ± 2.76 bc	12.99 ± 2.68 ab	16.35 ± 2.79 a	16.26 ± 2.72 a
PS	5.69 ± 1.49 ab	11,37 ± 2.73 a	7.86 ± 2.05 ab	2.84 ± 0.88 b	2.27 ± 065 b	2.31 ± 0.488 b
PC	42.03 ± 2.63 a	42.1 ± 1.57 a	39.86 ± 1.57 ab	37.46 ± 1.454 ab	37.63 ± 1.89 ab	33.88 ± 1.95 b
Slvl	23.07 ± 3.32 a	34.26 ± 4.17 a	36.79 ± 3.65 a	34.27 ± 2.66 a	33.05 ± 3.14 a	35.56 ± 2.54 a

[a] Values are the AUC percentage of each phospholipid from the sum of all identified polar lipids in the sample, means ± standard error, and $n = 9$. Different letters indicate significant differences ($P < 0.05$).

Elongation Markers. The fatty acids that are the precursors/products of the elongation enzymes and hence serve as surrogate markers showed a consistent relationship with the stratification of size. Elongation showed a pattern: higher concentrations of fatty acids fluxed through elongation activity in the small MFG group that decreased as the MFG diameter increased (Table 2). Elongation markers 20:0/18:0 and 22:1/20:1 were 1.5- and 3-fold higher, respectively, in the smallest (F6) compared with the largest (F1) MFGs.

MFGM Complex Phospholipid Composition: Area under the Curve Percentage. The stratification of MFGs by size revealed a diversity in composition of the MFGM for PI ($P < 0.0001$), PS ($P = 0.018$), PC ($P = 0.014$), SM ($P = 0.001$), and cholesterol ($P < 0.0001$). The AUC percentage of PE in the MFGM was least affected by the MFG size ($P = 0.22$). Analysis of variation using the MFG size did not detect significant interaction with the following parameters in its effect on the MFGM lipid composition as calculated by the phospholipid percentage: lactation number, DIM, and lactation number × DIM ($P = 0.99$, 0.95, and 0.98, respectively). The differences in lipid composition according to globule particle size were consistent across all of these lactation variables.

The relative concentration of PE varied between 1.7% in the small MFGs and 26% in the largest MFGs. PE was found to be equally distributed between the medium-size MFG fractions (Table 3). The milk yield was found to affect the PE relative concentration only in the largest MFG group ($P = 0.04$).

PS was found at the highest relative concentration in 3 μm MFGs (F2), whereas its relative concentration decreased by approximately 4-fold in the smaller MFG groups (1.3 and 2.4 μm in F6 and F5, respectively). The PS relative concentration in the medium-size MFGs (F3) tended to be associated with the milk yield ($P = 0.06$), whereas in the smallest MFGs (F6), it was associated with the weight and lactose percentage ($P = 0.06$ and 0.05, respectively).

The highest relative concentration of PI was found in MFGs of <2 μm diameter (F6 and F5) and was 5−30-fold less in MFGs >3 μm in diameter. The milk yield affected the PI relative concentration in the largest MFGs (F1, $P = 0.04$) and tended to affect the relative composition of PI in medium-size MFGs (F2). In the smallest MFGs (F6), the lactose percentage tended to affect the PI relative concentration in the MFGM ($P = 0.08$), whereas the somatic cell count significantly affected the relative PI concentration in the MFGM ($P = 0.04$).

The PC relative concentration varied between 33% and 42%, in MFGs >3 or <2 μm in diameter, respectively. The lactation number, milk yield, fat, protein, and lactose concentrations, and somatic cell count did not affect the PC relative concentration in the MFGM of any of the MFG size fractions.

The SM relative concentration was highest in the MFGM of MFGs <2 μm in diameter and decreased by approximately 30% in the largest MFG group (F1). The cows' weights were found to significantly affect the SM relative concentration in medium-size MFGs (F5, $P = 0.05$). The milk yield significantly affected the SM relative concentration in the MFGM of the smallest MFGs (F6).

The cholesterol relative concentration was highest in MFGs of >3 μm diameter (F1 and F2) in comparison with the smallest MFGs (F6). The milk yield and somatic cell count tended ($P = 0.09$) to interact with the cholesterol relative concentration in the largest and smallest MFGs (F1 and F6, respectively).

MFGM Lipid Composition: Weight Percentage. The relative concentrations of the free fatty acids did not differ between the six MFG size groups (data not shown) when calculated as weight percentage from the weight of all identified polar lipids in the sample.

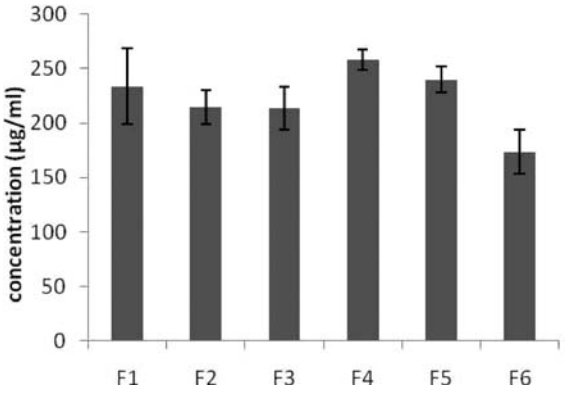

Figure 2. Membrane (sum of cholesterol, phospholipids, and sphingomyelin) concentration in milk fat globule size fractions. The values indicate the mean value of the membrane concentration in large milk fat globules (F1) through small milk fat globules (F6) ± standard error. Different letters indicate significantly different concentrations according to the HSD test ($P < 0.05$).

The MFGM concentration was determined as the sum of the weights of all identified polar lipids in the sample. The MFGM concentration did not differ between the size groups (Figure 2). To evaluate this result even further, a more sensitive statistical method was applied (i.e., the t test for paired size fractions). Using paired size fractions, we found only a tendency between the largest and smallest MFGs (F1 compared with F6, $P = 0.06$), and significant differences were found between the smaller fractions, i.e., F4 paired with F5 and F4 paired with F6 ($P = 0.01$ and 0.04, respectively; Supporting Information Table 1).

The PE weight percentage of membrane polar lipids varied between 5.5% in the smaller MFGs (but not the smallest fraction) and 11% in the largest MFG fraction (Figure 4A). When paired size fractions were analyzed for PE concentration differences, the largest MFGs were found to differ from the medium and smallest MFGs (F1 in comparison with F2 and F6; Supporting Information Table 2) at $P = 0.008$ and 0.02, respectively. The PE weight percentage tended to interact with the milk protein percentage in the F3 size fraction ($P = 0.08$) and significantly interacted with the milk fat concentration in the largest MFGs ($P = 0.002$). The PE weight percentage tended to interact with milk fat/protein concentrations in the F1 size fraction ($P = 0.1$).

The PS weight percentage of membrane polar lipids varied between 6% in the largest MFGs to more than 20% in the medium MFG fraction (Figure 4E). The small MFGs groups (F4 to F6) had the lowest amounts of PS. When paired size fractions were analyzed for PS concentration differences, the medium MFGs in the F3 fraction were found to significantly differ from the smaller MFGs (F3 in comparison with F4 to F6; Supporting Information Table 3). Using paired samples to compare the PS concentration also found that the larger MFGs tended to differ in their PS content; F2 had higher concentrations than F1 ($P = 0.06$). The PS weight percentage tended to interact with the lactose group in the F6 size fraction ($P = 0.08$) and significantly interacted with the milk yield group in the F5 size fraction ($P = 0.001$).

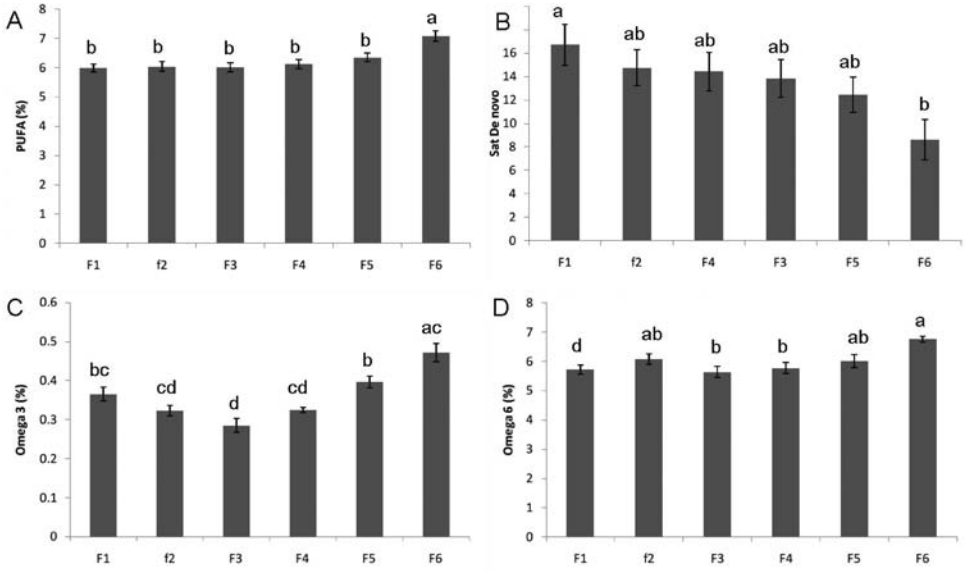

Figure 3. Relative concentration of fatty acid species in milk fat globule size fractions (F6, smallest; F1, largest). The concentration of each milk fatty acid species was calculated, and the values illustrate the relative concentration of PUFA (A), de novo synthesized saturated fatty acids (B), omega-3 fatty acids (C), and omega-6 fatty acids (D) as the percentage of all identified fatty acids in the sample. Values indicate the mean fatty acid species concentration ± standard error. Different letters show that the concentrations were significantly different according to the HSD test ($P < 0.05$).

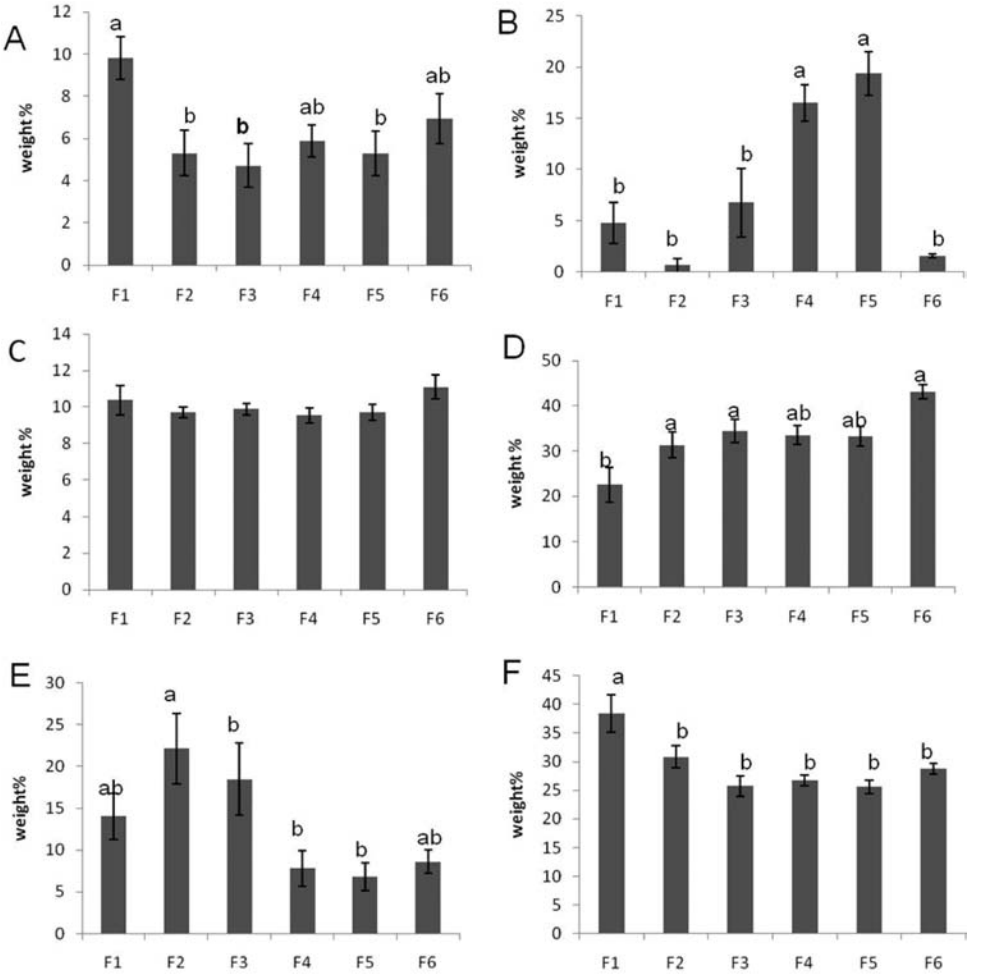

Figure 4. Relative concentration of each polar lipid in milk fat globule size fractions (F6, smallest; F1, largest). The concentration of each of the phospholipids and sphingomyelin was calculated, and the values illustrate the relative concentrations of phosphatidylethanolamine (a), phosphatidylinositol (b), phosphatidylcholine (c), sphingomyelin (d), phosphatydilserin (e), and cholesterol (f) as percentages of the sum of all membrane materials (phospholipids, sphingomyelin, and cholesterol). The values indicate the mean value of the membrane concentration ± standard error. Different letters show that the concentrations were significantly different according to the HSD test ($P < 0.05$).

The highest weight percentage of PI was found in MFG sizes of around 2 μm (F4 and F5), reaching 20% of the membrane lipids (Figure 4B). In the largest MFGs, the PI weight percentage was 4-fold lower and, in the smallest MFGs (F6), its weight percentage dropped 20-fold in comparison with that of the medium-size MFGs. When paired size fractions were analyzed for PI concentration differences, medium-size MFGs differed in their PI concentrations (F2 in comparison with F3; Supporting Information Table 3; $P = 0.02$) and tended to differ from the smallest MFGs (F3 in comparison with F6; Supporting Information Table 3; $P = 0.06$). The PI weight percentage of membrane polar lipids was associated with the milk protein percentage ($P = 0.03$) and fat concentration ($P = 0.04$) only in the F5 group. PI interacted with the fat/protein milk percentage in both F1 and F2 ($P = 0.01$ and 0.0006, respectively).

The PC weight percentage of total detectable membrane lipids did not differ between the size groups and varied between 10% and 13% (Figure 4C). When paired size fractions were analyzed for PC concentration differences, larger MFGs had higher PC concentrations relative to smaller MFGs (F2 in comparison with F4 and F5; Supporting Information Table 4; $P = 0.065$ and 0.055, respectively). The PC weight percentage tended to interact with the animal's weight ($P = 0.054$) and milk protein percentage ($P = 0.09$) in the F2 size fraction. On the other hand, the milk fat concentration was associated with the PC weight percentage in F1 ($P = 0.05$), F2 ($P = 0.03$), and F5 ($P = 0.04$) and tended to interact in F3 ($P = 0.08$).

The SM weight percentage of total detectable membrane lipids was highest in the small MFGMs, where it reached 42%, and gradually dropped with elevation in MFG diameter to 26% in the largest MFGs measured (Figure 4D). When paired size fractions were analyzed for SM concentration differences, the smallest MFGs had relatively higher SM concentrations than the medium-size MFGs (F6 in comparison with F3; Supporting Information Table 5; $P = 0.055$). The SM weight concentration tended to interact with the milk protein percentage ($P = 0.07$) in the F6 group, whereas significant interaction between the SM percentage and milk fat concentration was found in F4 ($P = 0.02$) and F5 ($P = 0.0003$). SM tended to interact with the fat/protein milk concentration in F3, F4, and F6 ($P = 0.09$, 0.06, and 0.08, respectively), while a significant interaction was found in F5 ($P = 0.02$).

The cholesterol weight percentage was highest in the two groups of largest MFGs (F1 and F2), reaching 44% of the membrane lipids, whereas in the three groups of smaller MFGs, its relative weight dropped to 27% (Figure 4). When paired size fractions were analyzed for cholesterol concentration differences, we found that larger MFGs had higher relative cholesterol than medium MFGs (F1 and F2 in comparison with F4; Supporting Information Table 6; $P = 0.006$ and 0.007, respectively). Cholesterol tended to interact with the milk yield ($P = 0.07$) in the F3 group and tended to interact with the protein percentage in the F4 ($P = 0.09$) and F6 ($P = 0.1$) groups. The cholesterol weight percentage was associated with the animal's weight ($P = 0.03$) in the F4 group and with the milk fat percentage in F4 ($P = 0.02$) and F5 ($P = 0.03$) and again tended to interact with milk fat in F6 ($P = 0.07$). Cholesterol interacted with the milk fat/protein concentration in the F5 and F6 MFG size fractions ($P = 0.03$ and 0.05, respectively).

DISCUSSION

The structural and compositional dynamics in the mammary gland epithelial cells which facilitates excretion of differently sized fat globules has been little studied. From biological, nutritional, and industrial points of view, elucidating the regulatory mechanism controlling the MFG size, and thus the milk fat composition and concentration, is of great importance.[22] In the current study, we present the unique chemical and physical properties of bovine MFGs of different sizes. Unlike our previous study that demonstrated chemical fingerprints of human MFGs,[19] here we present evidence for compositional modifications of (1) the fatty acid profile and 2) the polar lipid envelope of the MFG (i.e., MFGM) associated with the MFG diameter.

In an effort to avoid any controlled physiological parameter that might bias MFG composition−size interactions, we used milk from cows that differed in physiological and hormonal status: lactation number (primiparous and multiparous), lactation stage, and milk protein, fat, and lactose concentrations. The goal of this design was to answer the question of composition and particle size in the broadest sense. If the composition of the particles is an innate property of size, then the other lactation variables should be minor.

Following Ma's procedure for gravity-dependent MFG size separation,[23] MFGs were separated into six fractions, four of which showed size differences. Ma's protocol was chosen because it does not include any centrifugation steps. This is an important aspect of the separation process due to the fractionations that may occur upon even short-term, slow centrifugation, which enhances SM and PC release from the external leaflet of the MFGM.[17] The downside of using a relatively long procedure, which requires incubation of raw milk at 17 °C, is the possible hydrolysis facilitated by naturally occurring milk lipases. Triglyceride and phospholipid hydrolysis can increase the FFA concentration in the sample and introduce artifacts into the lipid analysis. Therefore, FFA weight and AUC percentages were monitored in the different size groups. Since no significant differences were found in the FFA concentrations, it appears that lipase activity did not alter the genuine compositional differences between the various sizes of MFGs.

Fatty acid profile analysis revealed that MFGs of all size groups have the same relative concentration of saturated fatty acids (data not shown). This result is interesting and might indicate a physical property that is kept constant regardless of the MFG size. This result concurs with our previous study on human MFG size differences, in which the saturated fatty acid AUC percentage was constant in MFGs of 1, 5, and 10 μm diameter,[19] and with a previous study that looked at the fatty acid composition of small vs large MFGs.[24] Although the overall saturated fatty acid concentration did not differ between the groups in the present study, their length and hence their alleged origin did. For example, higher concentrations of saturated fatty acids with a chain length of <16 carbons (i.e., de novo synthesized saturated fatty acid) were found in the large MFGs. These fatty acids are considered to be locally synthesized in the mammary gland.[25] The fact that medium chain length, saturated fatty acid concentrations were higher in larger milk fat globules coincides with a previous study that compared large and small milk fat globules (6.5 and 1.6 μm, respectively) and found higher concentrations of c12:0 and c14:0 in small milk fat globules.[26] On the other hand, the concentration of PUFAs that derived from the circulation was higher in smaller MFGs, in accordance with the previous study.[26] A higher PUFA concentration in small MFGs was also found in milk from humans[19] and cows[27] and can be accounted for by the higher content of phospholipids, which naturally contain more PUFAs. [28]

Distinguishing between the different PUFA families, we found that the trends in size-dependent omega-6 and omega-3 fatty acid concentrations are not necessarily identical. For example, the EPA (omega-3) concentration was lowest in the medium-sized MFG groups (F2 and F3) in comparison with the largest and smallest groups (F1 and F6, respectively), whereas arachidonic acid (omega-6) relative concentrations were constant throughout the large and medium MFG groups (F1 to F4) and only higher in the smallest MFGs (F6). Thus, it appears that the mechanistic control of the PUFA composition in MFGs of different sizes is not random and is not due only to the fatty acids' degree of unsaturation.

One of the most powerful factors in milk that was associated with an altered lipid composition of almost all phospholipids in almost all size fractions was the milk protein concentration. Additionally, the milk protein-to-fat ratio also influenced the size-dependent MFGM composition. One possible explanation for this is the fact that both factors reflect the animal's energy balance. Energy balance is also tightly associated with lipogenic activity of the mammary gland and therefore can be associated with the triglyceride synthesis level and consequently the MFG size. Hence, the association between the MFG size and polar lipid composition may explain the association between the protein-to-fat ratio in milk and polar lipid composition of the size groups.

One observation is perplexing and may indicate even further complexity of the milk globule assembly: the sum of the weights of the membrane lipid components, detectable by our method, did not differ between the size groups. It should be noted that the milk fat globule membrane consists of other chemical components such as proteins and glycolipids[29] that were not monitored in this study. Hence, the constant concentration of the lipid components in all size groups does not necessarily imply that the entire, intact, membrane concentration is constant and equal in all MFG sizes.

The fact that the cellular lipogenic and secretion activities are affected by the membrane composition has been shown in various cell types and in different models. For instance, it was demonstrated that the activity of amphitropic enzymes, including signal transducers and lipogenic enzymes, is modified by their association with membranes.[30,31] Moreover, recent papers have determined that the rate-limiting enzyme in PC synthesis (CTP, phosphocholine cytidyltransferase, a member of the amphitropic enzyme family) is regulated by physical membrane properties

such as the surface density and lipid composition.[31] Although these studies clearly illustrate that the cell's ability to respond to environmental signals, as well as its lipogenic status, is influenced by the membrane composition, this issue was never studied in relation to the regulation of the milk fat concentration and MFG size distribution.

The major membrane lipid constituent that is mentioned in relation to modulation of the vesicle diameter and secretion activity is PE. Ishii and Nii[32] showed that vesicles formed from a mixture of PC and PE are larger than those spontaneously formed by PC alone. In *Escherichia coli* it was demonstrated that PE is an essential membrane component that facilitates vesicle secretion.[33] These studies illustrate physical as well as biological effects of the PE membrane composition on the formation and secretion of vesicles. Interestingly, we found that the PE relative concentration was highest in the largest and smallest MFGs (F1 and F6, respectively) while its concentration in the middle size groups was lower. Hence, the PE content in the membrane should be further studied in regard to its involvement in the lipogenic capacity of the mammary gland epithelial cells and in determining MFG size postsecretion.

Although some principals apply to both synthetic vesicles formed spontaneously by a well-controlled lipid mixture and the size-dependent MFGM composition, it should be noted that the structure of the MFG is much more complex and that the MFGM does not consist of a simple mixture of phospholipids. Therefore, our ability to extrapolate from studies with synthetic vesicles on the role of the MFGM in determining the MFG diameter, and thus the milk fat content, is challenged. Nonetheless, understanding how the MFGM composition changes in relation to the MFG diameter and thus the milk fat content can shed light on our understanding of lipid metabolism in the mammary gland. Future studies may even determine the nature of the physiological conditions which will allow precise production of the MFGM to enhance specifically desired MFG size distributions.

■ ASSOCIATED CONTENT

Supporting Information. Results of diagnostically paired sample *t* tests to identify which pairs of size groups were significantly different for each of the membrane components that were identified and analyzed in the present study (Tables 1−7). This material is available free of charge via the Internet at http://pubs.acs.org.

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