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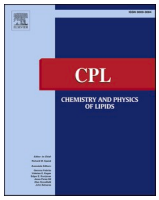

# Effect of phosphatidylcholine regioisomerism on lateral segregation of milk sphingomyelin in bilayer membranes

Md Abdullah Al Sazzad [a], Max Lönnfors [b], Baoru Yang [a,*]

[a] Food Sciences, Department of Life Technologies, University of Turku, FI-20500, Turku, Finland
[b] Cell Biology, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland

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ABSTRACT

Milk fat globule membrane (MFGM) promotes the lateral phase separation of milk lipids and stabilizes the fat globules in milk. The composition and structures of lipids have a significant impact on physicochemical properties of MFGM, which in turn influences the digestion and absorption of milk lipids. Phospholipids (PL), sphingolipids, and cholesterol are the major lipid constituents of MFGM. While the effects of the head-group and structure of the fatty acids (FAs) on membrane properties are commonly studied, little is known on the impact of PL regioisomerism. The present study investigated the impact of phosphatidylcholine (PC) regioisomerism on lateral segregation of milk-sphingomyelin (milk-SM) as well as the influence on the interaction of milk-SM with ceramide and cholesterol in simulated membrane systems. The regioisomer pairs of four molecular species PC 16:0/18:1n-9, PC 16:0/18:2n-6, PC 16:0/18:3n-3, and PC 16:0/20:4n-6 were included in this study. The lateral segregation was determined using lifetime analysis of *trans*-parinaric acid (*tPA*) fluorescence. Thermostability of the domains was detected using steady-state anisotropy of *tPA*. Our results demonstrated a clear impact of PC regioisomerism on membrane properties. PC regioisomers having the unsaturated FAs at the *sn*-2 position enhanced the lateral segregation of milk-SM with and without the presence of ceramide and cholesterol compared to the regioiosmers having 16:0 at the *sn*-2 position. Furthermore, the characteristics i. e. the acyl chain length and degree of unsaturation of *sn*-2 FA of the PCs had a major impact on the milk-SM gel phase and the intermolecular forces between milk-SM and ceramide/cholesterol. This work is the first investigation showing the effect of PL regioisomerism on milk-SM domains, which might have significant influence on functional properties of MFGM.

## 1. Introduction

Mammalian milk is one of the most ideal and complete natural food because of its dynamic role as a source of essential nutrients and bioactive compounds (Park, 2009; Gaucheron, 2011). In human milk, fats are secreted as lipid droplets known as milk fat globules (MFG), which serve as vehicles for triacylglycerols (TAGs) and fat-soluble bioactive molecules essential for the growth and development of infants (Zheng et al., 2019). These specialized colloidal assemblies are surrounded by a biological membrane called the milk fat globule membrane (MFGM). MFGM and its various components have a significant impact on the development of the brains of the infants, immune system, and intestines, as well as reducing the likelihood of infection in infants (Gila-Diaz et al., 2019). The complex structure of MFGM, which constitutes around 2–6 % of the entire fat globule, is composed of several bioactive molecules, including polar lipids, proteins, glycoproteins, cholesterol, enzymes, and other minor components (Dewettinck et al., 2008). In spite of their high importance for infants, the structure-functional role of lipids on the physiochemical properties of milk fat globule membranes is still a poorly understood aspect of milk.

The MFGM is a trilayer membrane that stabilizes the globules as an emulsion and promotes lateral phase separation of milk lipids. The composition and structure of lipids significantly influence the physical structure, phase behavior, and overall physicochemical properties of MFGM. This, in turn, affects the digestion and absorption of milk lipids, as well as the maintenance of the nutritional and physiological functions of milk (Alshehab et al., 2019; Bourlieu et al., 2020; Dong et al., 2021; Lin et al., 2021). The major lipid constituents of MFGM include various phospholipids (PLs), sphingolipids, and cholesterols. Phospholipids contain two fatty acids (FAs) linked to the *sn*-1 and *sn*-2 positions of their

* Corresponding author.
*E-mail address:* baoru.yang@utu.fi (B. Yang).

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glycerol backbone, and a polar head-group attached to the *sn*-3 position via a phosphodiester bond. Different head-groups can be associated at the *sn*-3 position, leading to distinct PL classes. For example phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) are the major PL found in MFGM. Generally PLs exhibit a low phase transition temperature and effectively maintain the permeability and fluidity of the membrane (Sánchez-Juanes et al., 2009). The structural implications of PLs have been extensively studied in terms of their head-group structure (Madrid and Horswell, 2013; Pal et al., 2018), hydrocarbon tail length, and chain unsaturation (Al Sazzad and Slotte, 2016; Shaikh et al., 2015; Williams et al., 2012; Shaikh et al., 2009; Niu and Litman, 2002; Emmelot and van Hoeven, 1975). However, additional structural variation in PLs can arise from regioisomerism, which results from swapping the relative positions of FAs on the glycerol backbone. In spite of the high biological importance of PL, the effect of PL regioisomers remains largely unexplored. It was observed in a recent study that human breast cancer and lung cancer cells have different ratios of phosphatidylcholine regioisomers compared to adjacent healthy tissue (Cao et al., 2020). It has been well documented that the regioisomerism in triacylglycerol may strongly influence the bioavailability, nutritional properties, physiological effects and physical properties of fats and oils in the diet (Michalski et al., 2013; Yaron et al., 2013; Lambert and Parks, 2012; Alfieri et al., 2018). The significance of different PL regioisomers in food and nutrition is still largely unknown, and even less is known about their impact on membrane physicochemical properties.

Milk-SM mostly consists of long-chain saturated fatty acids that are linked to the amino group of sphingosine through an amide bond, resulting in a high phase transition temperature, Tm ≈ 35 °C (Lopez et al., 2018). Due to the intrinsic hydrogen bonding capacity of sphingomyelin, it closely associates with cholesterol and forms a densely packed liquid-ordered phase in the membrane (Ohvo-Rekilä et al., 2002; Slotte, 2013; Yasuda et al., 2016). Recent studies have shown the effect of cholesterol on phase behavior and mechanical properties of milk-SM in MFGM model bilayers (Lopez et al., 2018; Murthy et al., 2016; Cheng et al., 2017; Murthy et al., 2015). Sphingomyelin-cholesterol induced raft formation is well established in cellular membranes (Simons and Ikonen, 1997; Simons and Vaz, 2004) and the presence of lipid rafts in MFGM has been reported recently in bovine milk (Nguyen et al., 2015; Lopez et al., 2010) and human milk (Zou et al., 2015; Lopez and Ménard, 2011). Sphingomyelin-rich ordered phases as well as sphingomyelin-cholesterol interactions in different unsaturated phospholipid bilayers have been extensively investigated to understand the effect of phospholipid unsaturation on these phases (Williams et al., 2012; Kullberg et al., 2015; Engberg et al., 2016; Nyholm et al., 2019; Brzustowicz et al., 2002; Björkbom et al., 2007; Wassall and Stillwell, 2009). However, similar information is not available on how the properties of milk-SM can be influenced by the regioisomeric composition of PLs.

To fill this knowledge gap, we investigated how the lateral segregation of milk-SM is influenced by regioisomeric composition of distinct phosphatidylcholines using the regioisomer pairs of four molecular species PC 16:0/18:1n-9, PC 16:0/18:2n-6, PC 16:0/18:3n-3, and PC 16:0/20:4n-6. We have also examined how the presence of ceramide and cholesterol can affect the milk-SM-rich gel phases in the bilayer membrane consisting of PC regioisomers at different ratios. The fluorescence probe *trans*-parinaric acid (*tPA)* is a *trans* fatty acid which shows higher affinity for gel phase domains in membranes and can therefore be used as an efficient probe to detect lateral segregations and ordered phases in membrane (Al Sazzad and Slotte, 2016; Castro et al., 2007; Silva et al., 2006; Al Sazzad et al., 2019). In this study, lateral segregation of milk-SM gel phases was determined by lifetime analysis of *tPA* fluorescence as a function of milk-SM concentration in bilayer membrane. Thermostability of milk-SM rich gel phase domains were detected by steady-state fluorescence anisotropy of *tPA* as a function of temperature. To the best of our knowledge, this is the first study to systematically investigate how PLs regioisomers influence the properties of milk-SM, which has substantial implications for regulating the physicochemical properties of MGFM.

## 2. Material and methods

### 2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:1n-9), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 18:1n-9/16:0), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:2n-6), 1-linoleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 18:2n-6/16:0), 1-palmitoyl-2-octadecatrienoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:3n-3), 1-octadecatrienoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 18:3n-3/16:0), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PC 16:0/20:4n-6), 1-arachidonoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 20:4n-6/16:0), sphingomyelin (Milk, Bovine)/23:0 SMN-(tricosanoyl)-sphing-4-enine-1-phosphocholine (milk-SM), cholesterol, and palmitoyl ceramide (PCer) used in the study were obtained from Avanti Polar Lipids (Alabaster, AL) and Larodan (Stockholm, Sweden). The molecular structures of the lipids used in this study are presented in Figure S1. *Trans*-parinaric acid (*tPA*) was synthesized from the methyl ester of α-linolenic acid following the previously established procedure (Kuklev and Smith, 2004). The water used for sample preparation was purified by reverse osmosis followed by passage through a UF-Plus water purification system (Millipore, Billerica, MA), resulting in a final product with a resistivity of 18.2 MΩcm.

### 2.2. Experimental design

The lipids were dissolved in different solvents: cholesterol was dissolved in hexane-isopropanol (3:2 by volume); PCs, PCer and milk sphingomyelin were dissolved in methanol. All the experiments of this study were carried out in a model membrane system using multilammellar vesicles. In regioisomer bilayers, the lateral segregation of milk-SM rich gel phases was determined by *tPA* lifetime analysis and the thermostability of these domains was detected by steady-state fluorescence anisotropy of *tPA*. The characteristics of milk-SM gel phases were studied in the absence and presence of PCer and cholesterol.

### 2.3. Vesicle preparation

Multilamellar vesicles (100 nmol total lipid per 2 mL water leading to final lipid concentration of 0.1 mM) were prepared for fluorescence lifetime and anisotropy measurements. The vesicles were prepared in glass tubes by mixing the lipids at the desired molar ratios and evaporating the solvent under a stream of nitrogen. The dry lipid films were then hydrated with argon-purged MQ-water in a water bath at 70°C for 60 minutes. After this, the samples were vortexed and sonicated for 5 min in a water bath sonicator (FinnSonic M3 Bath Sonicator, FinnSonic Oy, Lahti, Finland) at 70°C. Immediately after sonication, *tPA* was added from a concentrated methanol stock solution (0.25 μM) to a final probe concentration of 1 mol%. The addition was done at constant stirring and after addition of *tPA*, the samples were purged with argon for 2 min. The vesicles were then cooled to room temperature for 30 min before the fluorescence measurements. The *tPA*-containing vesicles were not exposed to white light at any stage of the experiment.

### 2.4. Time-resolved fluorescence measurements

For fluorescence lifetime analysis of *tPA*, a FluoTime 100 spectrofluorometer with a TimeHarp260 pico time-correlated single-photon-counting module (PicoQuant, Berlin, Germany) was used. The *tPA* was excited with a 297 nm LED laser source (PLS300; PicoQuant), and the emission was collected through a 405 nm single-band-pass filter. Fluorescence decays were measured at a temperature of 23°C using a Peltier

device for temperature control. The samples were continuously stirred to maintain consistent mixing. Data were analyzed with FluoFit Pro software (PicoQuant). The decay behavior of *tPA* emissions in binary or more complex bilayers is often characterized by multiple lifetime components (Nyholm et al., 2011). The average lifetime was calculated as described in Lakowicz (Lakowicz, 2006).

### 2.5. Steady-state fluorescence anisotropy

Steady-state anisotropy of *tPA* in multilamellar vesicles was performed in quartz cuvettes on a PTI QuantaMaster 1 instrument (Photon Technology International, NJ, U.S.A.). The anisotropy of samples containing specified lipid compositions and 1 mol% *tPA* was measured throughout a temperature range of 10–60°C using a temperature ramp of 5 °C/min. The excitation and emission wavelengths for *tPA* were 305 nm and 405 nm, respectively. For each measurement point, the excitation polarizer was in the vertical position (0°), while the emission polarizers were switched between the vertical (0°) and horizontal (90°) positions. The G-factor, which represents the ratio of sensitivity of the detection system for vertically and horizontally polarized light, was obtained by placing the excitation polarizer in the horizontal position (90°). The steady-state anisotropy *r* was calculated using the Felix32 software (Photon Technology International) according to (Lakowicz, 2006):

$$r = (I_{VV} - GI_{VH})/(I_{VV}+2GI_{HV})$$

Where, I is intensity measured with vertical (V) or horizontal (H) polarizer plane (the first letter is for excitation polarizer, the second for emission polarizer).

## 3. Results

### 3.1. Lateral segregation of milk-SM in phosphatidylcholine regioisomers bilayers

To understand the effect of PC regioisomers on lateral segregation of milk-SM, we prepared bilayer membranes by mixing an increasing amount of milk-SM in all the regioisomeric composition of PCs used in this study. The fluorescence lifetime measurements of *tPA* were performed to detect lateral segregation in the bilayers at 23°C. In *tPA* lifetime experiments the time-resolved fluorescence decay component of *tPA* indicates the packing properties of the mixture. When the probe partitions into a more ordered phase from a fluid phase, the lifetime component of *tPA* increases considerably, indicating phase segregation in the membrane bilayers (including gel phases) (Castro et al., 2007; Ekman et al., 2015). In contrast to disordered phases, the partition coefficient of *tPA* is significantly higher in ordered or gel phases (Sklar et al., 1977).

In Fig. 1, the intensity weighted average lifetime component of *tPA* fluorescence was plotted against milk-SM content in the PC bilayers. As shown in Fig. 1, the lifetime of *tPA* increased with the increasing milk-SM concentration, which is an indication of more ordered phase formation. Lateral segregation indicating the formation of gel phase was observed in all PC bilayers containing above 30 mol% of milk-SM content. When comparing the pattern of lateral segregation between the PC regioisomer pairs, in PC 16:0/18:1 and PC 18:1/16:0 pair, the behavior of milk-SM phase separation was mostly identical (Fig. 1 **A**). However, as the number of double bonds or chain length increased in the PCs, the differences in milk-SM phase segregation or gel phase formation were more apparent between the regioisomer pairs, as observed in the case of PC 16:0/18:2 and PC 18:2/16:0, PC 16:0/18:3 and 18:3/16:0, and PC 16:0/20:4 and PC 20:4/16:0 pairs (Fig. 1 **B, C, D**). In these cases, *tPA* lifetime was longer in bilayer system formed in the PC regioisosomers with the saturated fatty acid 16:0 at the *sn*-1 position and the unsaturated fatty acids containing 2–4 double bonds at the *sn*-2 position compared to the system consisting of the corresponding regioisomers, indicating formation of more ordered phase in these PC bilayers.

Fig. 2 represents how the changes of different FA in *sn*-1 or *sn*-2 position of PCs influence their membrane bilayer properties. Fig. 2**A** shows the *tPA* lifetime in PCs with palmitic acid in the *sn*-1 position and increase in length and unsaturation of the acyl chain in the *sn*-2 position, and Fig. 2**B** consists of the *tPA* life time in the system formed by corresponding regioisomers in order to compare the influence of structural features of *sn*-1 fatty acids. We observed that the onset of milk-SM gel phase formation occurred at much lower concentration in PC 16:0/20:4 (above 25 mol% of milk-SM) compared to PC 16:0/18:1 (above 30 mol % of milk-SM) (Fig. 2**A**). In contrast, no significant difference was observed for milk-SM phases when the corresponding changes in fatty

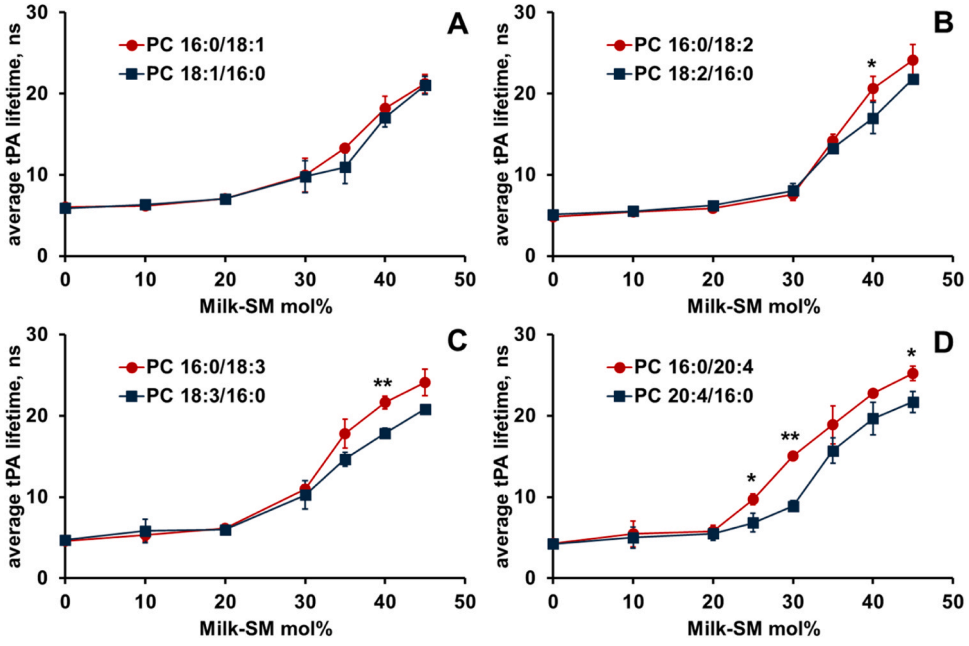


**Fig. 1.** *tPA* emission lifetime (ns) in milk-SM/PC complex showing the impact of PC regioisomers (at 23°C). Milk-SM segregation was assessed from the increase in average *tPA* emission lifetime (ns) as a function of milk-SM concentration in the bilayer. Each value is an average ± SD from n=3 (* = p<0,02 and **= p<0002).

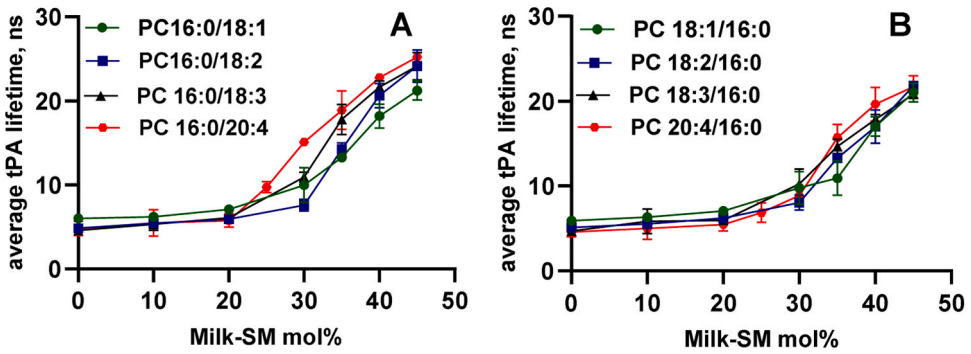


**Fig. 2.** Lateral segregation of milk-SM in PC bilayers at 23° C. Milk-SM segregation was assessed from the increase in average *tPA* emission lifetime (ns) as a function of milk-SM concentration. Each value is an average ± SD from n=3. (A) PCs containing palmitoyl chain at *sn*-1 position and modifications at *sn*-2 position. (B) PCs containing palmitoyl chain at *sn*-2 position and modifications at *sn*-1 position.

acids occurred at *sn*-1 position among the PCs (Fig. 2**B**). These results suggest that the *sn*-2 acyl chain of PCs had a larger effect on milk-SM-rich gel phase formation, as compared to the *sn*-1 acyl chain of the PC isomers.

### 3.2. Stabilization of milk-SM phases by palmitoyl ceramide in PC 16:0/18:1 bilayers

The impact of palmitoyl ceramide (PCer) on lateral segregation of milk-SM was examined in PC 16:0/18:1 PC bilayers using time resolved measurements of *tPA* at 23°C (Fig. 3). PCer is known to have relatively good miscibility with palmitoyl sphingomyelin (PSM), and has been shown to stabilize PSM-rich gel phase domains (Castro et al., 2007; Maula et al., 2015). MFGM contains trace amount of ceramide, and in order to understand the effect of ceramide content on milk-SM gel phases we have investigated the SM-gel phase formation in the presence of different PCer concentration in the bilayers. We prepared the bilayers containing PC 16:0/18:1/PCer at different molar ratios (95/5, 97.5/2.5, and 99/1) and increasing milk-SM amount in the bilayers. Fig. 3 demonstrated that the addition of PCer into PC 16:0/18:1 bilayers resulted in the stabilization of milk-SM gel phase domains. As indicated, less milk-SM was required for formation of gel phase domains when PCer was present, even at very lower concentration in the bilayers. In the absence of PCer, milk-SM formed the ordered phases above 30 mol% of milk-SM content whereas inclusion of small amount of ceramide (PC/PCer - 99/1 bilayer) moved the onset of ordered phase formation above 20 mol% of milk-SM content. As the PCer amount increased in the bilayer (PC/PCer - 97.5/2.5 and PC/PCer-95/5), the onset of gel phase formation dramatically started already at a much lower milk-SM content as indicated by the higher lifetime of *tPA*.

We also analyzed the thermostability of the milk-SM/PCer domains using steady-state anisotropy of *tPA*. Previously it has been demonstrated that the thermostability of gel or ordered phases obtained from steady-state anisotropy of *tPA* is in good agreement when compared to applicable results from Differential Scanning Calorimetry (DSC) (Al Sazzad and Slotte, 2016). As shown in Fig. 4, the gel phase melting temperature of 40 mol% of milk-SM in PC 16:0/18:1 was about 27°C. Inclusion of PCer at different molar concentration (1, 2.5 and 5 mol% PCer) in the bilayers containing 40 mol% of milk-SM in PC gradually increased the thermostability of the gel phases up to about 39°C. The average lifetime data, as observed in Fig. 3, showed similar patterns to the thermostability data obtained from *tPA* anisotropy measurements in Fig. 4. There was a clear correlation between thermostability and average lifetime of *tPA*.

### 3.3. Milk-SM-PCer-rich phases in absence or presence of cholesterol in PC regioisomer bilayers

Next, we investigated milk-SM domain stabilization by PCer in the PC regioisomer pairs to understand the impact of PC regiosiomers on this event. We also included cholesterol to investigate the effect of PC regiosiomers in a more complex system. *tPA* lifetime analysis (at 23°C) was used to investigate the lipid phases in the mixtures with various molar ratios. These mixtures include binary PC/milk-SM - 60/40, ternary PC/milk-SM/PCer - 55/40/5, and quaternary PC/milk-SM/PCer/Cholesterol - 50/40/5/5. As shown in Fig. 5, the milk-SM-rich phases (in binary mixtures) were more ordered when the PC regioisomer had more unsaturated and/or longer fatty acyl chain at the *sn*-2 position and saturated fatty acid (palmitic acid) in the *sn*-1 position. Addition of PCer stabilized the domains in all PC bilayers, as the *tPA* lifetime significantly increased in the ternary mixtures compared to the binary composition. Further, we examined the effect of cholesterol on

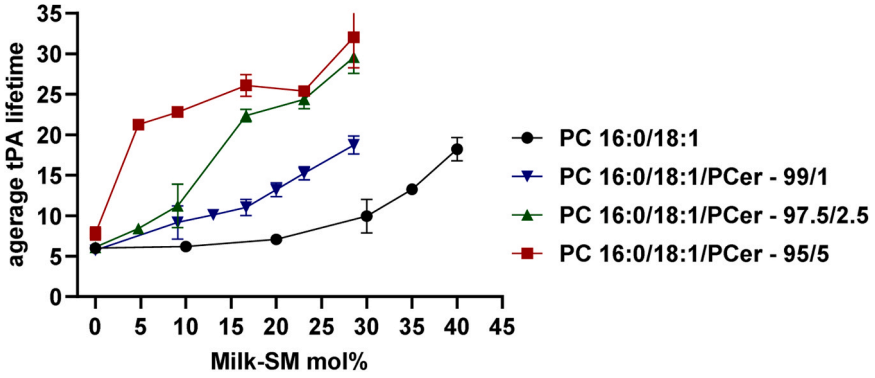


**Fig. 3.** Lateral segregation of milk-SM in PC 16:0/18:1 bilayers in the absence and presence of PCer at 23° C. When PCer was included, the PC/PCer molar ratio was 99/1, 97.5/2.5 and 95/5. The average *tPA* emission lifetime (ns) is plotted against the concentration of milk-SM. Each value is average ± SD from n=3.

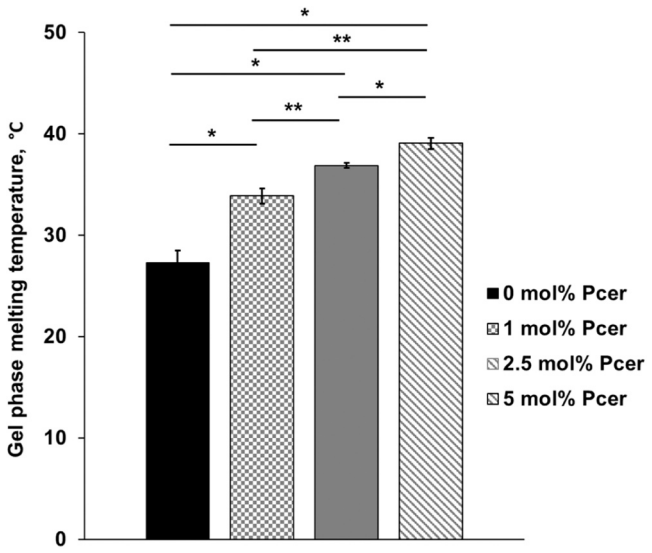


**Fig. 4.** End melting temperature of milk-SM rich gel phases in the absence and presence of PCer in PC 16:0/18:1 bilayers. All the bilayers contained 40 mol% of milk-SM. PCer was included at 1, 2.5 and 5 mol% in the bilayers. The end melting temperature of the milk-SM gel phases was determined from steady state anisotropy measurements of *tPA*. Each value of the gel phase melting is the average of at least three independently repeated experiments ± SD (* = $p<0001$ and ** = $p<0003$). The representative anisotropy curves for each composition are presented in Fig. S2.

these domains.

Cholesterol, like ceramide, has a high affinity for saturated sphingomyelins (SMs), resulting in ordered SM/cholesterol phases. When ceramide and cholesterol are present in the same bilayer, they may compete for interactions with SM, as a result cholesterol can destabilize the gel phase domains formed by PCer (Castro et al., 2009; García-Arribas et al., 2016). Similar behavior was observed from the *tPA* lifetime experiments, where average lifetime of *tPA* slightly decreased when cholesterol was included in the quaternary mixture (PC/milk-SM/PCer/Cholesterol - 50/40/5/5) compared to ternary PC/milk-SM/PCer. Our results suggest that cholesterol may partially solubilize PCer-rich domains in fluid phospholipid bilayers. The effect of PC regioisomerism was still observed by comparison within each of the regio-pair, showing longer average *tPA* lifetime in the PC regioisomers having more double bond or longer acyl chain at *sn*-2 position. The analysis of the individual lifetime components revealed no significant difference between the regioisomer compositions regarding the different lifetimes ($\tau_1$-$\tau_3$). However, the fractional amplitude and intensity for all lifetime components increased for the longer lifetimes when PCer was introduced. Adding cholesterol marginally shifted these values back towards shorter lifetimes for some of the lipid mixtures (Table S1).

### 3.4. Thermostability of the domains in PC regioisomer bilayers

We have further determined the thermostability of the lipid domains in PC regioisomer bilayers with similar lipid composition used for the lifetime time analysis as presented in Fig. 5. We observed that in the binary mixtures (PC/milk-SM - 60/40), thermostability of milk-SM domains increased with increasing double bond or chain length both at *sn*-1 or *sn*-2 position in the PCs. When compared between the PC regio-pairs, we observed these changes at *sn*-2 position in the PCs had more effect as the gel phase melting was comparatively higher in those PC bilayers. Presence of PCer significantly increased the thermostability of milk-SM-PCer-rich phases in all the PC bilayers. This effect was more prominent in the PC 16:0/18:1 and PC 18:1/16:0 regio-pairs, indicating PCer promoted more thermostability in a less stable gel phase (Fig. 6**A**). On the other hand, in PC 16:0/20:4 and PC 20:4/16:0 regio-pairs, where milk-SM already formed gel phase domains with high thermostability, addition of PCer had less noticeable effect on thermostability of milk-SM domains in these bilayers (Fig. 6**D**). Addition of cholesterol (quaternary PC/milk-SM/PCer/Cholesterol 50/40/5/5) did not show any significant effect on thermostability of these domains. As mentioned earlier, ceramide and cholesterol may compete each other to interact with milk-SM, therefore cholesterol and ceramide may coexist in the bilayer and inclusion of cholesterol does not significantly change the thermostability of the milk-SM-PCer gel phases in the quaternary mixtures.

## 4. Discussion

In recent years, there has been increasing interest in studying the physicochemical properties and phase behavior of membrane lipids in the MFGM. Milk-sphingomyelin is recognized as a crucial bioactive lipid due to its involvement in the formation of domains in glycerophospholipid bilayers in the outer membrane of MFGM and comprising about 40 % of the total polar lipid species in human MFGM. Phosphatiydylcholine is one of the major classes of milk glycerophospholipid with about 25 % of the total polar lipids in human MFGM. The proportion of these lipids in natural MFGM may vary depending on the source of different milk species (Zou et al., 2013). For our experiments, we used different PCs and milk-SM since they are the most abundant polar lipids found in human milk and the major component of MFGM. In the current study, we examined the interactions between milk-SM and phosphatidylcholines and how their interaction is affected by the regioisomerism of phosphatidylcholines. We performed a thorough investigation of the lateral segregation of milk-SM in multilamellar bilayers containing PC regioisomers with various chain lengths and degrees of unsaturation of fatty acids at the *sn*-1 or *sn*-2 positions. The impact of PC regioisomerism on the lateral segregation of milk-SM in phosphatidylcholine bilayers has not been previously investigated under precisely controlled conditions. MFGM naturally exists as a tri-layered structure. However, replicating this tri-layer configuration *in vitro* poses significant experimental challenges due to the absence of reliable methodologies to replicate such structures. We therefore used MLVs as the model system. Our investigation was primarily concentrated on the function and properties of the outermost bilayer of the MFGM tri-layer, which closely resembles a conventional bilayer structure.

Sphingomyelin, due to its intensive hydrogen bonding properties, can induce lateral segregation in the membrane and promote domain formation. The sphingomyelins (SMs) act as hydrogen bond donors, specifically through the presence of amide group and hydroxyl group in the long-chain base. On the other hand, glycerophospholipids only serve as acceptors for hydrogen bonds. The glycerol-based structures at the interfaces of common glycerophospholipids do not usually have proton-donating polar functions. Therefore, milk-SM in the fluid PC bilayers form H-bonding network through the interaction between the FA chains of the PC and milk-SM. Using milk-SM instead of a single species of SM increases the system's complexity due to the variety of acyl chain lengths present. However, the lipid composition represented a closer simulation of the structure of natural MFGM. The primary contributor to membrane lateral stability, as mentioned earlier, is the formation of hydrogen bonds, which remains consistent across all milk-SM lipid species regardless of acyl chain length. Our results demonstrated that milk-SM at concentration above 30 mol% were able to form gel phase domains in all PC bilayers and there was a clear effect of chain length, degree of unsaturation and the *sn*-positioning of FA in the PCs. We observed that less milk-SM was needed for their lateral segregation in the membrane as the level of unsaturation or chain length increased in the PCs. These results are in good agreement with previous studies, which reported the influence of increasing unsaturation or chain length of PCs on the lateral segregation of SM (Kullberg et al., 2015) and ceramides (Al Sazzad and Slotte, 2016). Our most significant finding, however, was the difference in lateral segregation of SM resulting from regioisomerism in the PCs. Within each regioisomer pair, PC isomers

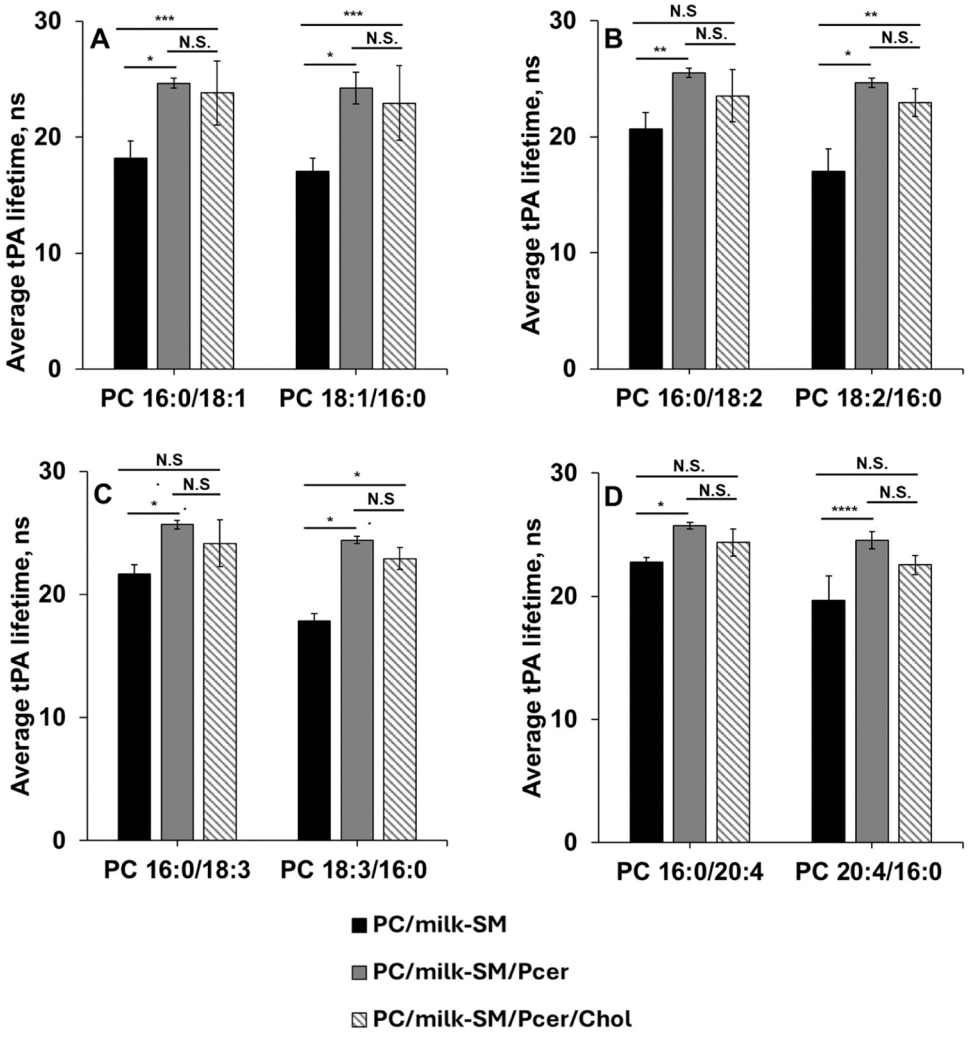


**Fig. 5.** The average *tPA* emission lifetime (ns) in binary (PC/milk-SM), ternary (PC/milk-SM/PCer) and quaternary (PC/milk-SM/PCer/Cholesterol) bilayers at 23° C. Each value is average ± SD from n=3 (* = p<0001, ** = p<0003, *** = p<0008, ****= p<0001).

having higher degree of unsaturation and longer acyl chain at the *sn*-2 position enhanced the lateral segregation of milk-SM domain compared to its isomer having identical fatty acids with reverse positioning between *sn*-1 and *sn*-2.

It has been observed that the differences in acyl chain lengths at the *sn*-1 or *sn*-2 positions of the PCs influence their thermotropic properties. These changes at *sn*-2 position showed a bigger effect on increasing the melting temperature of PCs compared to the similar changes at the *sn*-1 position (Marsh, 2013). For example, PC 14:0/18:0 has a gel phase to a liquid crystalline phase transition temperature of 38°C, while PC 18:0/14:0 has a transition temperature of 30°C (Koynova and Caffrey, 1998). We observed a similar effect as the gel phase melting temperature of milk-SM domains in PC bilayers were higher when the chain length or the number of double bonds increased in *sn*-2 position of the PCs compared to identical change in *sn*-1 position (Fig. 6). The gel phase separation of milk-SM in different PC regioisomer bilayers also revealed similar influences of fatty acids in PCs (Figs. 1 and 2). The possible difference of *sn*-1 and *sn*-2 acyl chain of PCs in their interaction with milk-SM may come from their different orientation pattern in the bilayer. It has been demonstrated that *sn*-1 and *sn*-2 acyl chains insert into the bilayer at varying depths, with *sn*-2 chain inserting less deeply into the bilayer than the *sn*-1 acyl chain (Gennis, 1989). This likely result in distinct effects of the regioisomers on lipid packing properties. Due to the lower degree of insertion of the *sn*-2 acyl chain into the PC bilayers, a marginally larger area of the acyl chain is exposed to the bilayer interface. This may lead to a more favorable interaction with milk-SM chains at the bilayer interface. Therefore, our results demonstrated a similar effect where the changes in *sn*-2 chain of PCs had major impact on determining the membrane properties of milk-SM. Increasing the acyl chain length and *cis* double bonds in PCs enhanced the lateral segregation of milk-SM in PC bilayers. This could be the outcome of a more advantageous hydrogen bonding network between PCs and milk-SM, as well as between milk-SM molecules themselves.

Additionally, we have studied the phase behaviors of milk-SM domains in the presence of ceramide and cholesterol in PC regioisomer bilayers to understand the effect of PC regioisomers in a more complex system. Though ceramides are present in small amount in MFGM (1.5–5 mol% of total phospholipid), they are highly important bioactive molecules (Rombaut et al., 2007). Cholesterol is another important class of lipid in human milk with concentration of 9–15 mg/100 mL (Jensen et al., 1978). Both ceramide and cholesterol lack large head-groups in their molecular structure; therefore, they cannot form stable bilayers on their own and must rely on bilayer-forming phospholipids to be present in the membrane. Although cholesterol and ceramide are amphiphilic molecules with small head-groups at the water interface, their hydrogen-bonding characteristics differ, resulting in distinct interactions with co-lipids in the membrane (Yasuda et al., 2016).

Our results from lateral segregation of milk-SM in PC bilayers (Fig. 3) demonstrated that the presence of PCer considerably promoted the milk-SM ordered phase formation even at very low PCer content.

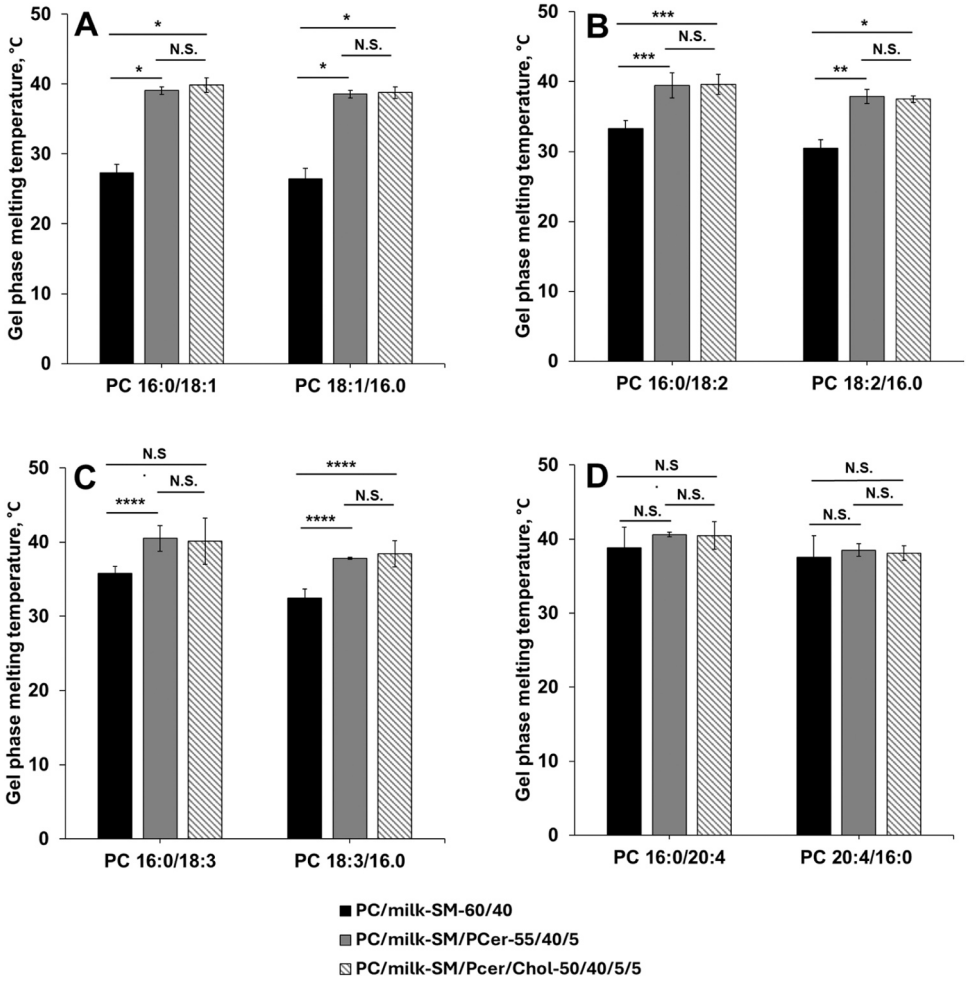


**Fig. 6.** End melting temperature of milk-SM rich gel phases in binary (PC/milk-SM), ternary (PC/milk-SM/PCer) and quaternary (PC/milk-SM/PCer/Cholesterol) bilayers. Each value of the gel phase melting is the average of at least three independently repeated experiments ± SD (* = p<0001, ** = p<0003, *** = p<0008, ****= p<0001). The representative anisotropy curves for each composition are presented in Fig. S3.

Thermostability of milk-SM domains also significantly increased by the presence of PCer in the bilayer (Figs. 4 and 6). Our results are in agreement with the previous findings where stabilization of milk-SM domains were demonstrated in the presence of PCer in DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) bilayers (Murthy et al., 2018). Since ceramides have higher affinity for the gel phase domains, it is possible that ceramides interact with milk-SM more preferably over PCs and form milk-SM-PCer rich gel phases. This preference may arise due to the ordered acyl chain of milk-SM that could interact with the saturated acyl chain of ceramides and also could facilitate increased hydrogen-bond formation between these two lipids. The ability of ceramides to produce a gel phase in lipid membranes is linked to their distinctive molecular structure. Ceramides are able to form strong hydrogen bonding network with the neighboring lipids in membranes. This strong hydrogen bonding network capacity is facilitated by the polar functional groups of ceramides that includes hydroxyl group at C1 and C3; an amide group at C2 position that is linked with the acyl chain which is typical of sphingolipids (Boggs, 1987).

Presence of cholesterol in such milk-SM-ceramide rich domains is also significant since ceramide and cholesterol both have high affinity for SM and they can compete to interact with SM (Castro et al., 2009; Sot et al., 2008). Consequently, the subsequent effects that ceramides and cholesterol have on each other in membrane bilayers are extremely complex and highly dependent on their concentration as well as the composition of the membrane (García-Arribas et al., 2016; Alonso and Goñi, 2018). At high cholesterol content in the membrane, cholesterol may induce destabilization and solubilization of ceramide-rich phases. Whereas ceramide-induced displacement of cholesterol from ordered (e.g., SM-rich) phases have been observed at lower cholesterol content in membranes (Sot et al., 2008; Grossert et al., 2014; Megha and London, 2004; Alanko et al., 2005). Our results demonstrated that PCer significantly stabilized the milk-SM domains in the PC bilayers and addition of cholesterol did not significantly destabilize or solubilize these gel domains as observed from the *tPA* lifetime experiments (Fig. 5). Also, no significant difference was observed in gel phase melting temperature for cholesterol containing bilayers compared to those without cholesterol (Fig. 6). In some of the tested lipid mixtures the addition of cholesterol did increase the fractional amplitude and intensity for the intermediate lifetime, indicating some solubilizing effect on the gel phase domains (Table S1.) In our experiments, we used comparatively low and equal molar ratio of ceramide and cholesterol (5 mol%). To better understand the ceramide-cholesterol interplay on milk-SM-rich phases more extensive investigation should be performed in future that could establish a ternary phase diagram containing Milk-SM, ceramide and cholesterol. Such investigations would provide information about affinities and intermolecular interactions among these lipids; this particular parameter will be significantly relevant for numerous biological processes. Overall, when comparing all our results from the PC regioisomers, the effect of the regioisomeric structure of PC was apparent in all the bilayer compositions where the degree of unsaturation and length of *sn*-2 acyl chain of PC showed major effect on membrane properties (binary, ternary and quaternary mixtures). However, the effect was marginally

mitigated in a more complex system (quaternary mixtures).

Our findings present important background knowledge to understand the effect of PC regioisomers on milk-SM domains in the absence or presence of ceramide and cholesterol. The phase behavior of milk-SM in MFGM is vital in regulating many biological processes; for example, casein micelles do not interact with milk-SM in gel or ordered phase, whereas a favorable interaction was observed in liquid disordered phase (Obeid et al., 2019). Furthermore, milk-SM significantly influence the hydrolysis of gastric lipase, as the hydrolysis rate increases with increasing level of milk-SM (Lopez et al., 2019). Human milk has a highly complex profile consisting of a large variety of fatty acids regio- and stereo-specifically distributed in the lipid molecules. Nevertheless, the impact of different isomeric structures of milk lipids on their physiological roles remains mainly unknown and requires further investigation. MFGM is a tri-layer structure consisting of an inner mono layer phospholipid membrane derived from endoplasmic reticulum membrane and an outer bilayer plasma membrane. The physicochemical properties of the latter play an important role in regulating the fat digestion. Therefore, studying the influence of these structural isomers on physiochemical properties of MFGM is very crucial, which ultimately determines the nutritional and physiological functions of milk. The results of our study will provide valuable insights for future research on lipid digestion, such as understanding the hydrolysis of milk-SM by sphingomyelinase, the process that leads to digestion of milk fat globules, and also investigating the formation of milk-SM/cholesterol complexes in the gastrointestinal tract during digestion of milk lipids. Moreover, our results demonstrating the impact of PL regioisomers on milk-SM gel phase segregation and the role of ceramide and cholesterol on stabilizing or destabilizing these gel phases are highly relevant and will be beneficial information for the future development of MFGM storage and stability in infant formulas.

## 5. Conclusions

In the present study, we demonstrated how regioisomeric composition of phosphodydicholine influences the lateral phase segregation of milk-SM. To understand its impact in more complex system, we have also examined how the phase properties of milk-SM can be influenced in the presence of ceramide and cholesterol. Our results demonstrated clear impact of PC regioisomerism on membrane properties. PC regioisomers having the unsaturated FAs at the *sn*-2 position enhanced the lateral segregation of milk-SM with and without the presence of ceramide and cholesterol compared to the regioiosmers having 16:0 at the *sn*-2 position. Furthermore, the characteristics of the fatty acid chain attached to the *sn*-2 position of glycerophospholipids had a greater influence than the structural features of the fatty acid at the *sn*-1 position in regulating the lateral segregation of the milk-SM-rich gel phase and the intermolecular interactions therein. Thermostability of the milk-SM gel phases were also higher when the chain length and unsaturation level increased in fatty acids in the *sn*-2 position of the PC regioisomers. This study provide a solid foundation to understand the role of PC regioisomers and their consequences on membrane properties, which has important biological significance. In fact, the physiochemical properties and lateral phase separation of milk polar lipids in the MFGM might have significant impacts on their nutritional functions and the mechanisms of lipid digestion. The results of this study were obtained from a more simplified model membrane system compared to the complex composition of human MFGM. Therefore, our results may partially characterize the lipid interactions of more complex MFGM, where numerous molecular species coexist and interact in intricate manners.

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## Supporting information

This article contains supporting information.

## CRediT authorship contribution statement

**Max Lönnfors:** Writing – review & editing, Visualization, Resources. **Baoru Yang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Md Abdullah Al Sazzad:** Writing – original draft, Visualization, Validation, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemphyslip.2024.105445.

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