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Stabilization of milk-sphingomyelin gel phases by glycosphingolipids: An *in-vitro* study on the characteristics of milk sphingolipid gel phases

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

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

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ABSTRACT

Sphingolipids constitute a class of bioactive lipids essential for the structural and functional integrity of milk fat globule membrane (MFGM). Milk sphingomyelin (milk-SM), as a key component of MFGM, contributes to the stability of milk fat emulsions. Milk-SM and other sphingolipids, like glycosphingolipids (GSL), coexist in the same outer bilayer of MFGM, suggesting significant role of their interaction in shaping the structural properties and functions of MFGM. In this study, using an *in-vitro* model membrane system, we investigated the impact of various GSLs, including cerebrosides and gangliosides, on the lateral segregation and phase behavior of milk-SM in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine bilayers. We also incorporated N-palmitoyl-D-erythro-ceramide for a comparative analysis of the impacts of sphingolipid head groups. The lateral segregation of sphingolipid gel phases was assessed using *trans*-parinaric acid (tPA) fluorescence lifetime analysis, and their thermostability was examined using steady-state fluorescence anisotropy of tPA. Additionally, we assessed the binary interactions between milk-SM and GSLs using the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH). The results indicate that GSLs promote the lateral segregation and stabilization of milk-SM-rich gel phases in the membrane bilayers. The size of the GSL head groups significantly influenced the degree of this stabilization, with larger head groups demonstrating diminished interactions with milk-SM. Our results provide valuable insights into the role of various sphingolipid structures in membrane phase behavior and organization. Comprehensive understanding of the interactions of these important sphingolipids in MFGM environment is crucial due to their structural and functional importance in dairy and nutritional applications.

1. Introduction

Sphingolipids (SLs) represent an important class of lipids characterized by the inclusion of a long-chain base, usually sphingosine ((2S,3 R,4*E*)-2-amino-4-octadecene-1,3-diol) (Slotte, 2013). Different functional groups can modify the long-chain base to produce distinct lipid species, including ceramide, sphingomyelin (SM), and both simple and complex glycosphingolipids (GSLs), including various gangliosides (Hakomori, 2008). In the biological membranes, sphingolipids provide distinct lateral asymmetric structure in the bilayers and are mostly abundant in the outer membrane leaflets (Van Meer et al., 2008). They also play a key role in significant biological functions, such as cell signaling, cellular processes, and disease mechanisms (Goñi and Alonso, 2006; Zheng et al., 2006; Bartke and Hannun, 2009).

Among biological membranes, the milk fat globule membrane (MFGM) has gained significant attention in recent years due to its distinct structure and functions. The MFGM is a unique biophysical system that stabilizes the fat globules in milk as an emulsion and enables lateral phase separation of milk lipids. MFGM surrounding the core of fat globules (mostly triglycerides) has a tri-trilayer membrane structure that are constituted of an inner monolayer, originated from the endoplasmic reticulum and an outer bilayer derived from the apical plasma membrane of mammalian epithelial cells (Mather and Keenan, 1998). The MFGM primarily consists of various phospholipids, sphingolipids, sterols, proteins and other essential bioactive compounds (Liu et al., 2023). Phosphatidylcholine (PC) is the primary glycerophospholipid, while milk sphingomyelin (milk-SM) is the most abundant sphingolipid species present in MFGM. Generally, PC, milk-SM, glycolipids, and cholesterol remain in the outer bilayer, while phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are abundant in the inner monolayer (Deeth, 1997; Lopez et al., 2010). The structure and composition of lipids significantly influence the physical structure,

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

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phase behavior, and overall biophysical properties of MFGM. Consequently, this impacts the digestion and absorption of milk lipids, along with the maintenance of milk's nutritional and physiological activities (Alshehab et al., 2019; Bourlieu et al., 2020; Dong et al., 2021).

It is well established that SLs promote the formation of highly ordered domains in the membrane due to their intrinsic ability to form hydrogen bonding networks with adjacent molecules (Pascher, 1976; Thompson and Tillack, 1985; Boggs, 1987a). Milk-SM, mostly consists of long-chain saturated fatty acids linked to the amino group of sphingosine by an amide bond and poses a high phase transition temperature, Tm ≈ 35 °C (Lopez et al., 2018). Milk-SM induced domain formation has been reported recently in MFGM model bilayer (Lopez et al., 2018; Murthy et al., 2015) and the MFGM in bovine milk and human milk (Lopez et al., 2010; Nguyen et al., 2015; Lopez and Ménard, 2011). Other minor sphingolipids such as ceramides, cerebrosides, and gangliosides, which are only found in small amounts in MFGM, serve as emulsifiers and are known to have important functions for human health (Liu et al., 2018). Galactosylceramide (GalCer), glucosylceramide (GluCer) and lactosylceramide (LacCer) are the most common cerebrosides, while GD3 and GM3 are the prevalent gangliosides in mammalian milk. Despite sharing structural similarities with SM, saturated ceramides and GSLs exhibit significantly higher transition temperatures than SM (Li et al., 2002, 2000). The differences in the molecular properties of the SLs are related to changes in their head group area, the saturation levels of their acyl chains, and how they form hydrogen bonding networks (Björkbom et al., 2010a; Morrow et al., 1992; Yasuda et al., 2016; Maunula et al., 2007). Due to their distinct biophysical properties, GSLs are thought to play an important role in the formation of lipid rafts in membranes (Hall et al., 2010; Hakomori, 2004). GSLs are also involved in numerous biological functions, such as regulating the immune system, supporting intestinal maturation, inhibiting gut pathogens, and promoting the development of the brain and nervous system (Aerts et al., 2019; Schnaar et al., 2014). Understanding the interplay of these sphingolipids, particularly their interaction with milk-SM, is crucial as they coexist in the same bilayer of the MFGM. Recently, palmitoyl ceramide-promoted milk-SM gel phase domain formation was reported in DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) bilayers (Murthy et al., 2018). In our earlier studies, we have demonstrated the impact of palmitoyl ceramide on lateral phase segregation of milk-SM in PC bilayers and also reported the effect of PC regioisomerism on this event (Sazzad et al., 2024). However, there is still an inadequate understanding of the interactions between different GSLs and milk-SM, as well as their potential impact on milk-SM gel phases in the MFGM. Such interactions might significantly alter the physicochemical properties of MFGM and potentially influence the digestion and absorption of milk lipids, as well as several biological processes, including infant gut health, immunological response, brain development, and metabolic health.

In this study, we investigated the lateral segregation and phase behavior of milk sphingolipids in POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) bilayers. The study incorporated PGalCer (D-galactosyl-ß-1,1′ *N*-palmitoyl-D-*erythro*-sphingosine), PGlcCer (D-glucosyl-ß-1,1′ *N*-palmitoyl-D-*erythro*-sphingosine), PLacCer (D-lactosyl-ß-1,1′ N-palmitoyl-D-erythro-sphingosine), ganglioside GD3 and GM3 to investigate the impact of GSL molecular structure on their interaction with milk-SM and the resulting impact on milk-SM gel phases. PCer (*N*-palmitoyl-D-*erythro*-ceramide) was also included to enhance the understanding of the role of SL head groups in influencing milk-SM-rich phases. Lateral segregation of SL gel phases in POPC bilayer was determined by lifetime analysis of *trans*-parinaric acid (tPA) fluorescence. Thermostability of these gel domains was detected by steady-state fluorescence anisotropy of tPA. The fluorescent probe tPA is a *trans* fatty acid that exhibits a greater affinity for ordered domains and has been employed as an effective probe to detect lateral segregations and ordered phases in membranes (Sazzad et al., 2024; Al Sazzad et al., 2019; Al Sazzad and Slotte, 2016; Castro et al., 2007). The interaction and the phase behavior of the milk-SM/GSL binary mixture were studied using steady-state fluorescence anisotropy of DPH (1,6-diphenyl-1,3.5-hexatrienol), a probe that partitions equally between the liquid disordered phase and the liquid ordered phase in the membranes (Silva et al., 2007). Our results demonstrate that GSLs significantly promote the lateral segregation of the milk-SM gel phase and stabilize the domain formation in the membranes. The size of the GSL head group significantly influenced this event; as the head group increased in size, the interaction between GSL and milk-SM diminished. A schematic representation of the lipids utilized in this investigation is shown in Fig. 1.

2. Material and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), sphingomyelin (Milk, Bovine)/23:0 SMN-(tricosanoyl)-sphing-4-enine-1-phosphocholine (milk-SM), *N*-palmitoyl-*D-erythro*-ceramide (PCer), D-galactosyl-ß-1,1′ *N*-palmitoyl-D-*erythro*-sphingosine (PGalCer), D-glucosyl-ß-1,1′ *N*-palmitoyl-D-*erythro*-sphingosine (PGlcCer), D-lactosyl-ß-1,1′ N-palmitoyl-D-erythro-sphingosine (PLacCer), Ganglioside GD3 (Bovine Milk), and Ganglioside GM3 (Bovine Milk) used in the study were obtained from Avanti Polar Lipids (Alabaster, AL). *Trans*-parinaric acid (tPA) was synthesized in house from the methyl ester of α-linolenic acid following the previously established procedure (Kuklev and Smith, 2004). 1,6-diphenyl-1,3.5-hexatrienol (DPH) was purchased from Molecular Probes (Leiden, Netherlands). The fluorophores were stored at −80 °C in vials until dissolved in argon-purged methanol prior to use. The solvents used were of spectroscopic grade and all other inorganic and organic chemicals used were of highest available purity. Stock solutions of lipids were prepared in methanol or chloroform/methanol (1:1 by vol, GSLs) and kept at −20 °C. 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. 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 experiments. The vesicles were prepared in glass tubes by mixing the lipids at the desired molar ratios including 1 mol% of fluorescence probe. The solvent was evaporated under a stream of nitrogen at 40 °C. The dry lipid films were then hydrated with argon-purged MQ-water in a water bath at 70 °C for 60 min. 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. The vesicles were then cooled to room temperature for 30 min before the fluorescence experiments.

2.3. Time-resolved fluorescence measurements

The fluorescence lifetime analysis of tPA was performed using FluoTime 100 spectrofluorometer with a TimeHarp 260 pico time-correlated single-photon-counting module (PicoQuant, Berlin, Germany). 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. Using a Peltier device for temperature control, fluorescence decays were recorded at 23 °C. To ensure uniform mixing, the samples were constantly stirred. Data obtained from fluorescence lifetime analysis 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).

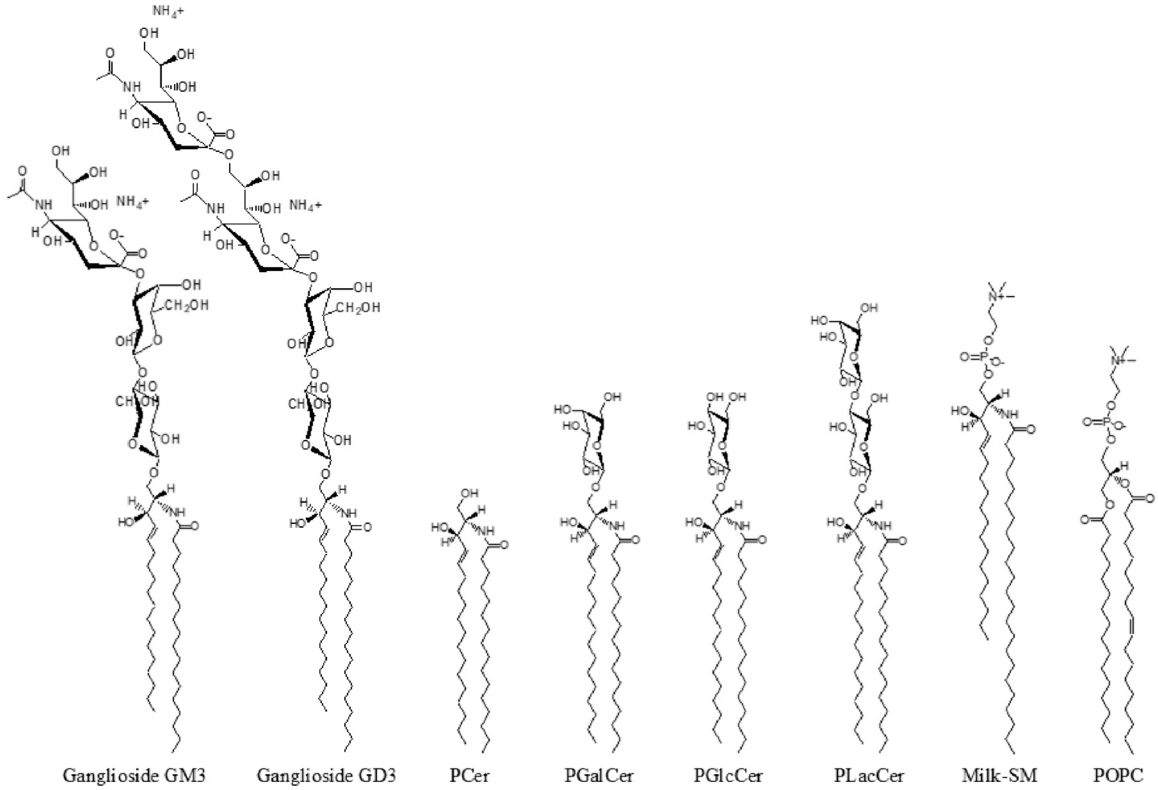

Fig. 1. Molecular structure of all the lipids used in this study. For the milk-SM lipid mixture the most abundant SM with acyl chain length (C23:0) is depicted.

2.4. Steady-state fluorescence anisotropy

Steady-state anisotropy of tPA and DPH 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 contained specified lipid compositions and 1 mol% fluorescence probe. The anisotropy analysis was performed throughout a temperature range of 5–80 °C depending on the lipid composition of the vesicles using a temperature ramp of 5 °C/min. The excitation/emission wavelengths for tPA were 305 nm/405 nm and for DPH 360 nm/430 nm, respectively. At each measurement point, the excitation polarizer was oriented vertically (0°), while the emission polarizers switched between the vertical (0°) and horizontal (90°) orientations. The G-factor, representing the ratio of the detection system's sensitivity to horizontally and vertically polarized emission light, was determined by positioning the excitation polarizer at 90° (horizontal orientation). To ensure consistency and reliability across all experimental conditions, the G-factor was calculated prior to each set of measurements in this investigation. The G-factor and the steady-state anisotropy (r) were determined utilizing the Felix32 software developed by Photon Technology International according to method described previously (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), and G is indicating the G-factor.

3. Results

3.1. Bilayer order profile of the sphingolipids in POPC

Fluorescence lifetime analysis of tPA was performed to investigate the potential ordered phases of sphingolipids in POPC bilayers at 23 °C. The lifetime analysis of tPA is a well-studied method and serves as an effective approach to identify the formation of ordered phases within a mixed bilayer, considering that the lifetime of tPA is sensitive to the packing properties in bilayers. In fluid bilayers, the lifetime of tPA is notably short, but very long in highly ordered gel or solid phases, while it is intermediate in a liquid-ordered phase (Nyholm et al., 2011; Castro et al., 2009). The intensity-weighted average lifetime of tPA in POPC/SLs bilayers, as represented in Fig. 2, indicates that in pure POPC (in the absence of any SL), the tPA lifetime is short (about 6 ns), suggesting the existence of a fluid phase within the membrane. As the SLs concentration increased in all the binary mixtures, the lifetime component of tPA increased considerably, indicating phase segregation in the membrane bilayers (including gel phases). The partition coefficient of

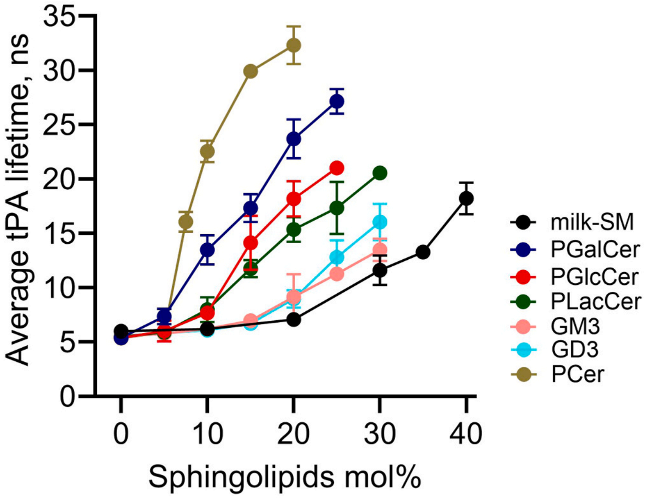

Fig. 2. tPA emission lifetime (ns) in POPC/SL binary composition showing the order profile of SLs in POPC bilayers (at 23 °C). SL segregation was assessed from the increase in average tPA emission lifetime (ns) as a function of SL concentration in the bilayer. Each value is an average + SD from n = 3.

tPA is considerably higher in ordered or gel phases compared to disordered phases (Sklar et al., 1977). Among the SLs, PCer was able to induce a highly ordered gel-like phase in the bilayers as shown; the onset of gel phase formation occurred at very low ceramide concentration (above 5 mol%). Less ordered phases were observed in the milk-SM-containing bilayer compared to other binary mixtures, since a higher amount of milk-SM was required for the commencement of the gel phase. Similar curves for PCer and milk-SM in POPC bilayers was also reported in our earlier study (Sazzad et al., 2024). As compared to the milk-SM, cerebrosides and gangliosides formed considerably more ordered phases in the bilayers. The phase behavior of the SLs was significantly influenced by the size of the head group they contained, which will be discussed in more detail later. Meanwhile, cerebrosides demonstrated a higher ability to induce ordered phases in bilayers relative to gangliosides. Overall, the binary compositions used in this study exhibited a consistent trend (in regards to their head group size) in the formation of gel phases (PCer>PGalCer>PGlcCer>PLacCer>GD3>GM3>milk-SM).

3.2. Thermostability of sphingolipid ordered phases in POPC bilayers

Next, we investigated the thermostability of SLs ordered phases in POPC bilayers using steady steady-state anisotropy of tPA as a function of temperature. The binary composition containing POPC/SLs – 85/15 was used to detect the end melting temperature of SLs ordered phases as presented in the Fig. 3. The representative anisotropy curves for each composition are presented in Figure S1. It has been reported in earlier studies that the thermostability of gel or ordered phases obtained from steady-state anisotropy of tPA aligns well with relevant findings from Differential Scanning Calorimetry (DSC) and there remains a clear correlation between thermostability and lifetime of tPA (Al Sazzad and Slotte, 2016). The domain melting temperature analysis shows that the milk-SM phases had the least thermostability compared to the other SLs, while PCer had the most thermostability among all. The melting temperature of the milk-SM gel phase was approximately 15 °C. In contrast, the gel phase melting temperature for the glycosphingolipids (GSLs) varied from 17 °C to 35 °C depending on the GLs composition, while PCer gel phase melting was around 40 °C. The thermostability of cerebrosides in POPC bilayers was relatively higher compared to that of gangliosides. Among cerebrosides, PGalcer exhibited the highest domain melting temperature, while the ganglioside, GD3 demonstrated a higher domain melting temperature than GM3. The thermostability of the SL gel phases in POPC bilayers was in good agreement with the order profile of the SLs derived from the tPA lifetime analysis, which followed a similar trend. This correlation suggests a close relationship between the molecular ordering within the bilayers and the stability of the SL gel phases.

3.3. The interactions between milk-SM and glycosphingolipids

The interactions between milk-SM and glycosphingolipids were investigated by studying the phase behavior of milk-SM/GSL binary mixtures (75/25 and 50/50 ratios) using DPH anisotropy (Fig. 4). DPH is able to equally partition into a fluid phase and an ordered phase; therefore, it has been used in this experiment to determine the mutual interactions and miscibility of milk-SM and GSLs. PCer was also included to better understand the structural impact of various SL headgroups on these interactions. It was observed that all the SLs interacted favorably with milk-SM, resulting in distinct gel-to-fluid phase transitions that arise from binary constituent compositions. The gel-to-fluid phase transition of pure milk-SM obtained from DPH anisotropy occurred about 35 °C which is in good agreement with previous report (Lopez et al., 2018). Inclusion of GSL or PCer with milk-SM, the phase transition temperature increased for both composition (75/25 and 50/50 ratios) compared to pure milk-SM phases. This melting transition is indicative of the presence of binary ingredient compositions in the mixture, where sphingolipids showed some miscibility. The presence of PCer at both molar ratios markedly enhanced the thermal stability of the binary mixtures, suggesting its more favorable interaction with milk-SM.

3.4. Milk-SM gel-phases promoted by glycosphingolipids in POPC bilayers

Next, to investigate the impact of GSLs on lateral segregation of milk-SM in POPC bilayers, tPA lifetime analysis was performed at 23 °C. For this investigation, the POPC bilayer containing 5 mol% of GSLs or PCer and an increasing amount of milk-SM was prepared. As shown in Fig. 5,

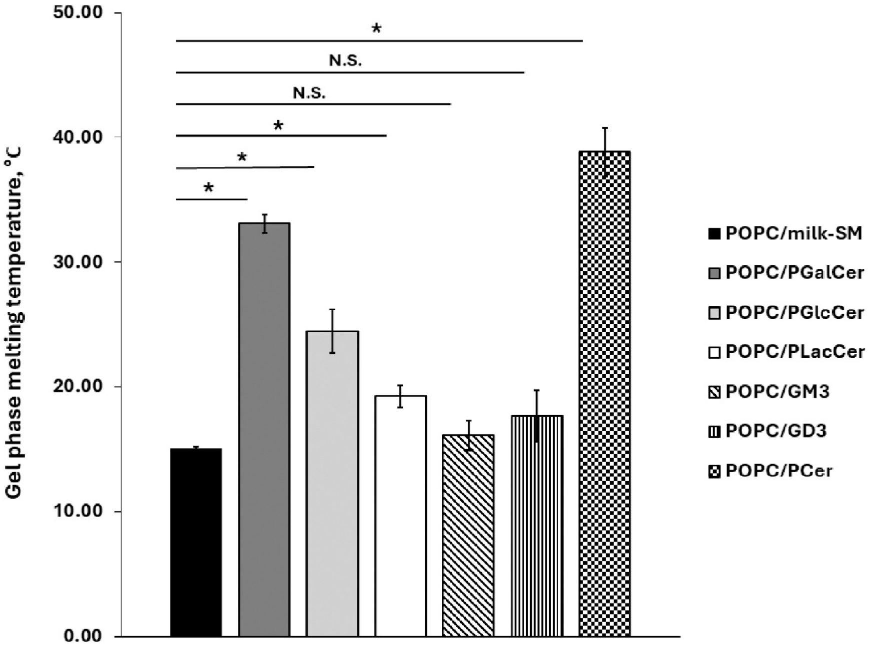

Fig. 3. End melting temperature of SL rich gel phases in binary POPC/SL - 85/15 bilayers obtained from steady state anisotropy measurements of tPA. Each value of the gel phase melting is the average of at least three independently repeated experiments + SD (* = $p < 0.002$). The representative anisotropy curves for each composition are presented in Fig. S1.

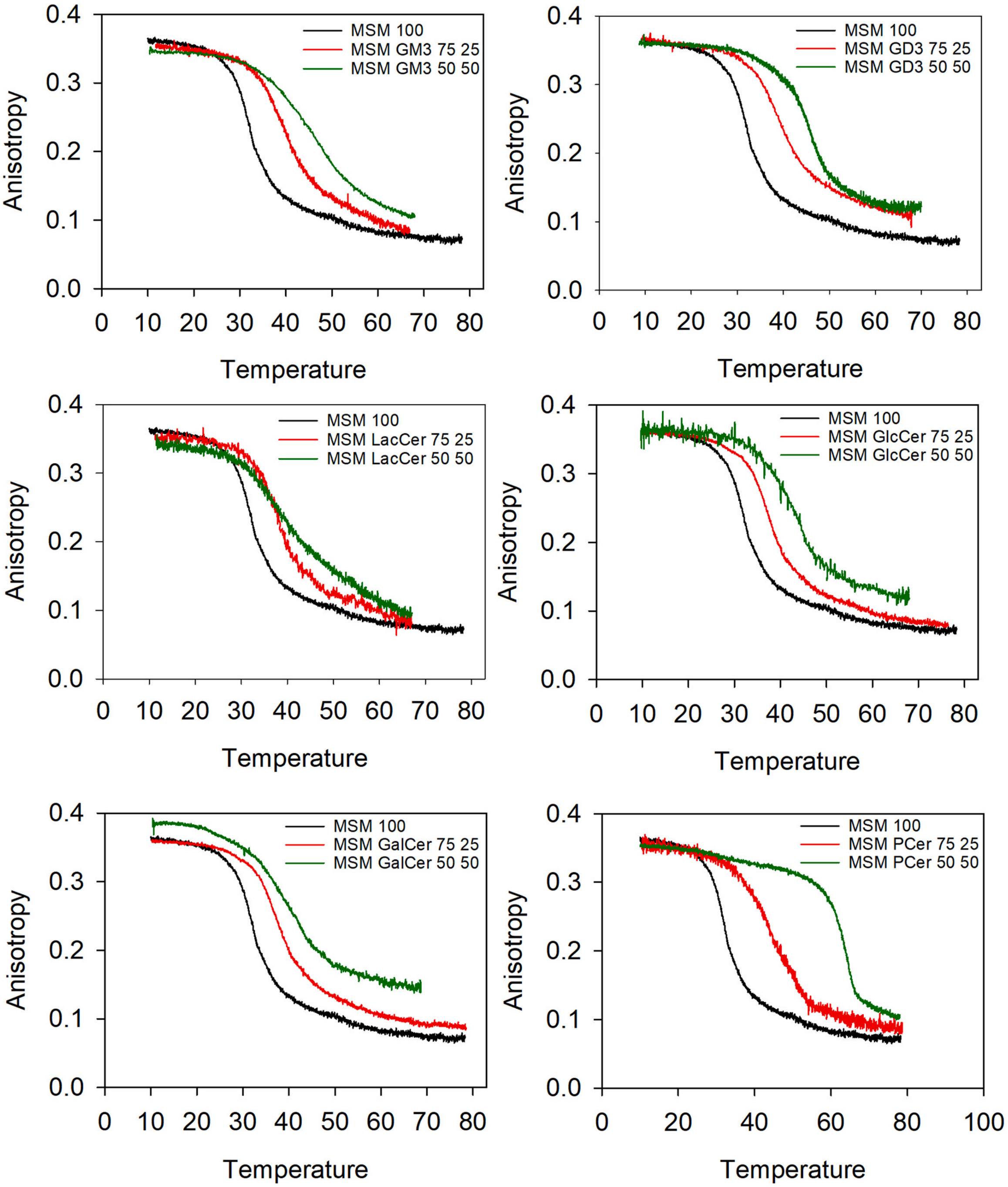

Fig. 4. Steady state anisotropy of DPH in milk-SM and milk-SM/GSL or milk-SM/PCer binary mixture. The scans are representative of at least three different experiments.

the ternary mixture containing GSL or PCer stabilized the lateral segregation of milk-SM gel phases when compared with the binary POPC/milk-SM composition. Less milk-SM was needed for the onset of gel phase formation in the bilayers when GSLs or PCer were present. Among the SLs, PCer showed the most significant effect on stabilizing the milk-SM gel phase; similar PCer-induced stabilization of milk-SM phases has been reported previously (Sazzad et al., 2024). In binary POPC/milk-SM bilayers, the formation of the milk-SM gel phase occurred at approximately 30 mol% of the milk-SM content, whereas in the ternary mixture, the inclusion of 5 mol% of GSL in the bilayer shifted the onset of the ordered phase downward to a lowered concentration of milk-SM. This suggests that the mutual interaction between milk-SM and GSL as observed in the binary mixture (Fig. 4) also occurred in this case stabilizing the milk-SM gel phases in POPC bilayers.

3.5. *Thermostability of milk-SM gel-phases stabilized by glycosphingolipids in POPC bilayers*

To detect the impact of GSL on the thermostability of milk-SM gel phases in POPC bilayers, steady-state anisotropy of tPA was performed as a function of temperature. The binary mixture containing POPC/milk-SM 60/40 and the ternary mixture of POPC/milk-SM/GSLs 55/40/5 were used for this study. The end points of the gel phase melting temperature of the mixtures are presented in Fig. 6**.** As observed from the figure, the gel phase melting temperature for the binary POPC/milk-SM was about 25 °C, which is in agreement with our previous report (Sazzad

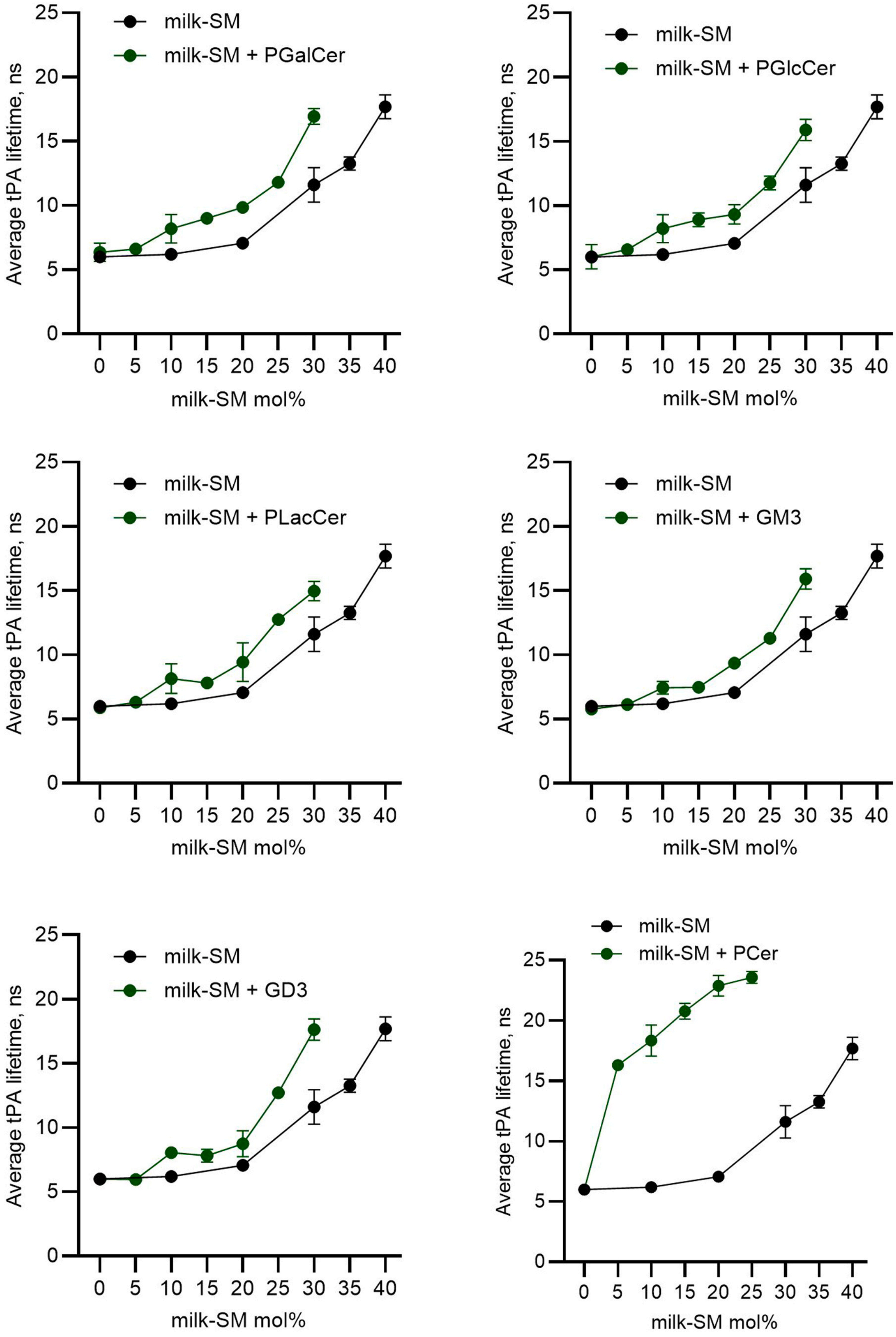

Fig. 5. Lateral segregation of milk-SM in POPC bilayers in the absence and presence of GSL or PCer at 23 ° C. GSL or PCer was included at 5 mol% in the bilayer. The average tPA emission lifetime (ns) is plotted against the concentration of milk-SM. Each value is average + SD from n = 3.

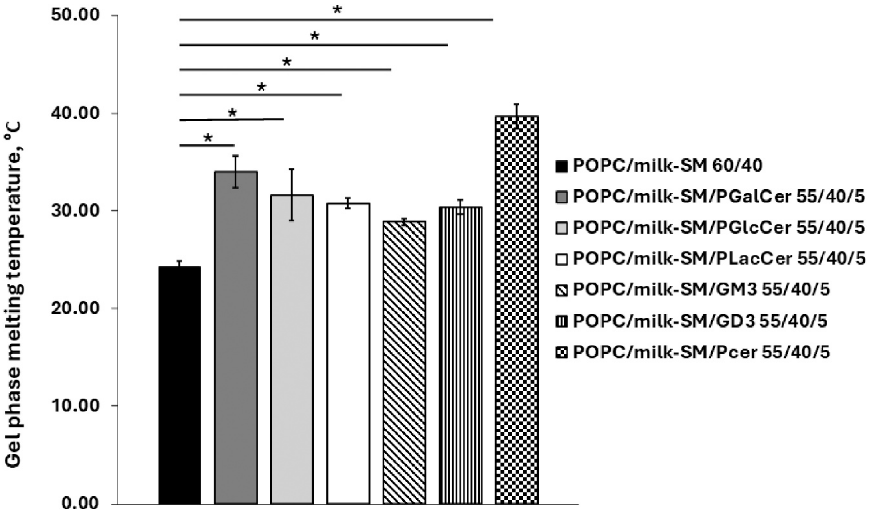

Fig. 6. End melting temperature of milk-SM rich gel phases in binary POPC/milk-SM and ternary POPC/milk-SM/GSL or POPC/milk-SM/PCer bilayers. Each value of the gel phase melting is the average of at least three independently repeated experiments + SD (*=$p < 0.01$). The representative anisotropy curves for each composition are presented in Fig. S2.

et al., 2024). Inclusion of GSLs in the mixture notably increased the thermostability of the ternary mixtures, indicating the stabilization of the milk-SM gel phases by GSLs in POPC bilayers. The gel phase melting temperature of the ternary mixtures increased about 5–10 °C depending on the GSL composition. The incorporation of PCer significantly raised the gel phase melting temperature of the ternary composition (melting about 40 °C) compared to the binary mixture (about 25 °C). These results are in good agreement with other experiments presented in this study, suggesting that GSLs influence the milk-SM gel phase properties in the bilayer, with PCer demonstrating the most significant impact among the SLs.

4. Discussion

The primary sphingolipid in milk fat globule membrane (MFGM), milk-SM, has been well studied in recent years for its role as important determinant of lipid phase separation in MFGM. MFGM also contains other sphingolipids like ceramide and glycosphingolipids, which are only present in small amounts but possess considerable biological significance. In human MFGM, milk-SM constitutes around 40 % of the total polar lipid species, whereas phosphatidylcholine, one of the major classes of milk glycerophospholipid, accounts for approximately 25 % of the total polar lipids (Zou et al., 2013). Depending on the source of various milk species, the proportion of the lipid composition in natural MFGM might vary (Zou et al., 2013). Naturally, MFGM exhibits a tri-layered architecture; nevertheless, replicating such structure in vitro presents significant challenges due to the lack of reliable methodologies. Therefore, by employing MLVs as the model system, our study focused primarily on the physiochemical characteristics of the outermost bilayer of the MFGM tri-layer, which mimics a traditional bilayer structure. In the current study, we investigated the interactions between milk-SM and glycosphingolipids and explored how differences in the structures of the glycosphingolipids influence such interactions. First, we conducted a comprehensive investigation of the lateral phase separation of all the sphingolipids in phosphatidylcholine bilayers to elucidate and to compare the characteristics of individual sphingolipids in gel phase formation. Next, we examined the binary interactions between milk-SM and GSL, and finally, to understand their interaction in a more complex MFGM-like environment, we investigated how the GSL affects the lateral phase separation of milk-SM in POPC bilayers. To examine the interactions among the selected lipid species, we utilized well-established fluorescence-based methods, including fluorescence anisotropy and fluorescence lifetime analysis. Sazzad et al., previously conducted a comparative investigation on lipid phase behavior applying fluorescence anisotropy, fluorescence lifetime analysis, and differential scanning calorimetry (DSC), demonstrating a strong correlation among these methodologies (Al Sazzad and Slotte, 2016). Their findings indicated that the gel phase melting temperatures of the examined lipid compositions (POPC/PCer - 9/1) were consistent with tPA-based anisotropy measurements and DSC data. In our present investigation, the gel phase melting temperature of milk-SM, determined using fluorescence anisotropy experiments, also closely aligned with the values published by Lopez et al., who evaluated the gel phase transition using DSC (Lopez et al., 2018).

Sphingolipids, owing to their distinct capacity to establish hydrogen bonding networks with adjacent lipid molecules, can induce a highly ordered gel phase within bilayers. This phenomenon was also apparent in our studies, where all sphingolipids examined exhibited lateral phase separation in phosphatidylcholine bilayers. The characteristics of the SL gel phases in the bilayers exhibited significant differences, which were mainly influenced by the molecular structure of the SLs. Interfacial hydrogen bonding properties of the SLs are all likely to be very similar since they all share the same ceramide backbone structure, but the main determinant of the degree of lipid packing properties lies in the head group region. Among the SLs, PCer induced highly ordered gel phases, while milk-SM-rich phases were less stable, as demonstrated by the tPA lifetime and anisotropy experiments (Fig. 2 and Fig. 3). The tendency for lateral segregation of the SLs appeared to correlate with the presence of head group size in the carbon 1 position of the ceramide moiety. Milk-SM possesses a large phosphocholine head group in its molecular structure, whereas PCer lacks any significant head group and contains only a hydroxyl group at the C1 position. In the case of GSLs, cerebrosides, or gangliosides with one or more sugar moieties in their head group structure, the size of the head groups still remains smaller compared to the phosphocholine head group in milk-SM. The size of the head groups significantly influences the environment (like polarity, electrostatics, hydration, and pH) at the water-lipid interface and facilitates substantial selective interactions among membrane components (Björkbom et al., 2010b). The large head group moiety could hinder close interactions, whereas a smaller head group makes it easier for molecules to come in close contact and increases intermolecular interactions through van der Waals forces, leading to more ordered membranes and higher Tm. Therefore, as compared to the milk-SM and PCer, lateral phase separation and the thermostability properties of the GSLs in POPC bilayers displayed an intermediate value (Fig. 2 and Fig. 3).

The significant hydration ability of the phosphocholine residue, resulting from the ionic phosphate and amine groups, along with its more parallel alignment to the interfacial region, enhances steric bulkiness in relation to adjacent lipids. This could limit both the intra- and intermolecular hydrogen-bonding capacity of SM, thereby effectively restricting their interactions within membrane bilayers. Fully hydrated glycosphingolipids exhibit a complex thermotropic phase behavior and high phase transition temperatures (85 °C for PGalCer, 87 °C for PGlcCer, and 78 °C for PLacCer) in contrast to milk-SM (35 °C) (Lopez et al., 2018; Li et al., 2002; Ruocco et al., 1981; Saxena et al., 1999). In this study, the phase transition temperature of the pure GSLs was not characterized using fluorescence spectroscopy methodologies. This is due to the high tendency of GSLs, such as GalCer and GlcCer, to form crystal-like structures, similar to ceramide (Pinto et al., 2011; Dicko et al., 2003). The densely packed crystal structure could potentially result in the exclusion of fluorescent probes at high GSL content. When examining the binary interactions between milk-SM and the GSLs, we observed a favorable interaction between the lipid molecules (Fig. 4). The gel-to-fluid phase transition temperature of the binary mixtures (milk-SM/GSLs) exhibited a notable increase in Tm compared to the Tm of the pure milk-SM hydrated bilayer. The influence of head group size in the binary mixture was also apparent as increased phase transition was observed with the smaller head groups of the GSLs. When we studied the interaction between milk-SM and GSLs in the POPC bilayers, we observed that the presence of even small amounts of GSLs (5 mol%) in the bilayer can significantly promote the gel phase separation of milk-SM (Fig. 5) and increase the thermostability of the milk-SM gel phases (Fig. 6), suggesting mutual interactions between the sphingolipids. As demonstrated, GSLs are prone to forming ordered domains in fluid bilayers (Fig. 2 and Fig. 3) and interacting extensively with milk-SM (Fig. 4). Therefore, in the ternary bilayer, it is likely that they interact with milk-SM more favorably than with POPC, leading to the formation of milk-SM-GSL-rich gel phases (Fig. 5 and Fig. 6).

The stabilization of the milk-SM gel phase by PCer has been demonstrated by Murthy et al. (Murthy et al., 2018) as well as in our previous studies (Sazzad et al., 2024). In the present work, we showed how the GSL stabilized the milk-SM gel phases, though they behaved differently compared to ceramide. The hydration of glycolipids significantly differs from that of ceramides due to variations in the head group structure (Westerlund and Slotte, 2009). A delicate balance between the repulsive forces of the big head groups and the favorable interactions, such as intermolecular hydrogen bonding and hydrophobic interactions among the saturated hydrophobic regions of these molecules, regulates their phase behavior. The interaction between water and GSL head groups in the hydrated bilayer led to a significant water ordering effect due to the formation of a spherical hydration shell surrounding the head group (Arnulphi et al., 1997). As the size of the head group increases, it results in extensive hydrogen bonding with neighboring water molecules. Consequently, with the increasing number of sugar residues, a large hydration shell is observed for glycosphingolipids (Zaraiskaya and Jeffrey, 2005). In case of the cerebrosides, the structural difference between PGalCer and PGlcCer is attributed to the sugar part linked to the ceramide backbone. Despite this, the orientation of the two head groups with respect to the bilayer normal is almost identical. The primary structural distinction is that GalCer contains galactose, whereas GlcCer incorporates glucose as the sugar moiety. The stereochemical differences in the sugar moiety clearly influenced the phase behavior of PGalCer and PGlcCer; their interactions with milk-SM as demonstrated in our study. PGalCer can establish a strong inter-lipid hydrogen bonding network, while the glucosyl moiety of PGlcCer primarily forms hydrogen bonds with surrounding water molecules, resulting in a more extensive hydration (Boggs, 1987b). This could result in the PGlcCer domains being more loosely packed, which may lead to reduced thermostability compared to PGalCer. In case of the PLac-Cer, the addition of one more sugar in comparison to PGalCer, resulted less favorable interaction with milk-SM compared to GalCer or GlcCer.

The gangliosides, due to their large oligosaccharide hydrophilic head groups, containing sialic acid moieties, could experience rearrangements, resulting in cooperative changes among metastable phases within a ganglioside-enriched bilayer (Cantu et al., 2009). This study included GM3 and GD3, the predominant gangliosides in human milk, which are solely found in the MFGM (Ma et al., 2015; Lee et al., 2018). Gangliosides, due to their considerably bigger head groups, induced less stable phases in POPC bilayers and affected their interaction with milk-SM. We noticed one notable observation while comparing the characteristics of gel phases between GM3 and GD3. Despite possessing a bigger headgroup, GD3 was capable of inducing gel phases that exhibit slightly greater thermostability compared to GM3. This may be attributed to the potential electrostatic interactions between the negatively charged GD3 and the zwitterionic head groups of POPC or milk-SM. The presence of additional -OH groups in the head group could also promote the intra- and intermolecular hydrogen bonding network throughout the head group region of GD3.

4.1. From molecular perspective to practical applications in food and nutrition

Our results provide important background information for understanding the characteristics of GSLs and how they potentially affect the formation of the milk-SM gel phase. Since both milk-SM and GSLs are in the outer bilayer of MFGM, their interaction is very important for maintaining the structure, stability, integrity, and biological activity of the MFGM. Their interaction might enhance the emulsifying characteristics of the MFGM, facilitating the bioavailability of fat-soluble vitamins and other nutrients. Previous reports indicate that the interfacial composition and phase behavior of MFGM greatly affect the digestion of milk fat globules (Garcia et al., 2014; Liang et al., 2017; Ye et al., 2017). Therefore, our findings demonstrating the stabilization of milk-sphingomyelin gel phases by GSLs might be highly relevant in regulating lipid digestion and metabolism. In MFGM, phospholipids simultaneously exhibit both liquid-ordered and liquid-disordered domains. In the liquid-disordered domain, it appears that the SM region interacts with bile salt, while the PC regions within these domains interacts with pancreatic lipase and colipase (Maldonado-Valderrama et al., 2011). The glycolipids in MFGM, which feature sugar moieties and complex branched structures, create steric hindrance that prevents lipase or bile salts from accessing the fat globule interface (O`Riordan et al., 2014).

Moreover, our findings indicating the role of GSLs in enhancing the thermostability of milk-SM are significant and will provide valuable insights for the future development of MFGM processing technologies, storage and stability in infant formulas and various dairy products. As observed, thermal processing stands out as the most predominant method used in the dairy industry for extending the shelf-life of the product and preventing the microorganisms responsible for pathogens and spoilage (Tunick et al., 2016). Thermodynamic parameters greatly affect the physical state of lipids, their interactions with other macromolecules, the morphology and characteristics of lipid domains in the MFGM (Lopez et al., 2018; Et-Thakafy et al., 2017; Murthy et al., 2016; Nguyen et al., 2016). Thermal treatment influences the formation of lipid domains and the presence of different lipid phases (gel, liquid ordered, and liquid disordered) in MFGM, and the quality of resulting products. According to recent studies, the coalescence of milk fat globules may be influenced by lipid rafts through phase transition following heat treatment (Wei et al., 2023). GSLs, together with cholesterol, facilitate the formation of microdomains (lipid rafts) in cell membranes, which are specialized regions rich in particular lipids and proteins (Simons and Ikonen, 1997; Lingwood and Simons, 2010). The existence of similar raft-like structures in MFGM and the role of GSLs in such events require in-depth exploration to enhance current knowledge of the organization and interaction of MFGM-associated proteins. It has been noticed that the selective interactions with milk proteins, such as casein, are controlled by the phase and charge of the polar lipids in membrane bilayers (Obeid et al., 2019). Considering that GSLs are charged lipids due to their sugar moieties, understanding their interactions with milk proteins is important for the future development of dairy products with adaptable protein adsorption properties, facilitated by alterations in the phase state and charge of the interfacial lipids. Therefore, the objective of future research should include more comprehensive investigations, particularly concerning the interaction of various GSLs with cholesterol and milk proteins.

5. Conclusion

This study demonstrates the impact of glycosphingolipids on the lateral phase segregation of milk-SM. To better understand the influence of glycosphingolipid head groups, PCer was also incorporated in this study, as they all possess an identical ceramide structure, with the primary distinction arising from the head group region. Our results clearly indicate that GSLs effectively stabilized the milk-SM gel phases in POPC bilayers, facilitated by favorable interactions between milk-SM and GSLs. Throughout this experiment, we observed the clear impact of sphingolipid head groups, wherein glycosphingolipids with smaller head groups exhibited better interaction with milk-SM and promoted the gel phase stabilization. This study established a solid foundation for understanding the structural role of GSL in their interaction with milk-SM, which might have a significant influence on the physicochemical characteristics and lateral phase separation of milk polar lipids in the MFGM, as well as the digestion and bioavailability of milk lipids. In contrast to the complex structure and composition of human MFGM, the findings of this study were derived from an *in-vitro* model membrane system. Consequently, our findings may provide a partial characterization of the lipid interactions of a more complex MFGM, where a variety of molecular species coexist and engage in complex interactions.

CRediT authorship contribution statement

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

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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.

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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.2025.105526.

Data availability

Data will be made available on request.

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