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Thermotropic phase behavior of milk sphingomyelin and role of cholesterol in the formation of the liquid ordered phase examined using SR-XRD and DSC

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ABSTRACT

Sphingomyelin (SM) and cholesterol are major lipid components of biological membranes involved in the formation of ordered domains. In this study, we investigated the biophysical properties of milk-SM bilayers and determined the effect of cholesterol. The thermotropic phase behaviours of milk-SM and milk-SM/cholesterol mixtures were characterized using differential scanning calorimetry (DSC) and high flux synchrotron radiation X-ray diffraction (SR-XRD). The melting phase transition temperature determined for fully hydrated milk-SM bilayers was T_m = 34.3 ± 0.1 °C. The thermotropic phase behavior of milk-SM is complex, reflecting the mixture of different molecular species. Structural reorganisations successively occurring on heating of fully hydrated milk-SM bilayers were interpreted as follows: i) melting of C16:0-SM, ii) conversion of long and saturated SM species from fully interdigitated ($L_{\beta 1}$) to mixed interdigitated ($L_{\beta 2}$) lamellar structures evidencing gel phase polymorphism and then iii) transition to the fluid liquid-crystal L_α phase. We demonstrated that cholesterol modulates the physical properties of milk-SM bilayers and that building up of the lamellar liquid-ordered L_o phase is completed for 33 mol% of cholesterol. The ordering effect of cholesterol on milk-SM bilayers and the temperature-independent behavior of the L_o phase formed by milk-SM/cholesterol complexes were characterized. The findings of this work will contribute in a better understanding of the biological functions exerted by the milk-SM as a function of its phase state and interactions with cholesterol (e.g. hypocholesterolemic effect).

1. Introduction

The chemical composition and biophysical properties of lipids as well as their interactions govern the architecture of biological membranes and are involved in biological functions. Among all the lipids, sphingolipids (mainly sphingomyelin, SM) and cholesterol are important structural components of the plasma membranes of eukaryotic cells where they form tightly packed liquid-ordered (L_o) phase domains called "rafts" (Simons and Ikonen, 1997; Ramstedt and Slotte, 2006). Increasing knowledge about the biophysical properties of SM and SM/cholesterol complexes is a key step in a better understanding of their functions in biological membranes.

The biological membrane surrounding lipid droplets in milk, that is called the milk fat globule membrane (MFGM), is involved in important nutritional and health functions. The MFGM is composed of polar lipids i.e. glycerophospholipids and sphingolipids with mainly milk-SM that accounts for about 20–45 wt% of MFGM polar lipids (see review (Lopez, 2011)), cholesterol (30 wt% of cholesterol in the membrane lipid fraction, i.e. about 45 mol% (Et-Thakafy et al., 2017; Mesilati-Stahy and Argov-Argaman, 2014)) and membrane-specific proteins. The formation of ordered lipid domains of micronic size has been revealed in the MFGM using confocal microscopy (Et-Thakafy et al., 2017; Gallier et al., 2010; Lopez et al., 2010). These lipid domains have kept an ongoing interest in recent years since the MFGM is a biological interface involved in many mechanisms occurring in the gastrointestinal tract, i.e. milk fat globule digestion, immunity, maturity of the intestine. These MFGM microdomains have been observed *in situ* in milk over a wide range of temperatures (4–60 °C) and have been shown to be reactive to thermal kinetics (Et-Thakafy et al., 2017). The proposed hypothesis is that the MFGM microdomains are composed of high phase transition temperature (T_m) saturated polar lipids (mainly milk-SM; $T_m \sim 34$ °C; (Malmsten et al., 1994; Murthy et al., 2015) in the gel phase (for $T < T_m$) or correspond to milk-SM/cholesterol complexes in the L_o phase (Et-Thakafy et al., 2017; Lopez et al., 2010). Milk-SM

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contains a variety of molecular species with a heterogeneous composition, e.g. long-chain bases (mainly d18:1, d16:1, d17:1) and amide-linked acyl chains (mainly C16:0, C20:0, C22:0, C23:0, C24:0) (Byrdwell and Perry, 2007; Fong et al., 2007; Sanchez-Juanes et al., 2009). The complex and unique composition of milk-SM may lead to specific biophysical properties in membranes (e.g. packing properties, interaction with cholesterol and other membrane components) compared to other SM molecules (e.g. synthetic/semi-synthetic SM, egg-SM, brain-SM). However, information about the thermotropic phase behavior and biophysical properties of milk-SM remains scarce.

Authors reported for milk-SM a broad gel – L_α phase transition close to physiological temperature (Cheng et al., 2017; Filippov et al., 2006; Malmsten et al., 1994; Murthy et al., 2015; Shaw et al., 2012). Atomic force microscopy (AFM) investigations showed that in absence of cholesterol the lateral segregation of milk-SM in the plane of the bilayer matrix occurred for $T < 35\,^{\circ}C$ when milk-SM is in the gel phase, while no phase separation occurred above 35 °C when milk-SM is in the fluid state (Guyomarc'h et al., 2014, 2017; Murthy et al., 2016a). The gel phase domains formed by milk-SM below T_m induce structural and mechanical heterogeneities by having a higher thickness and a higher resistance to rupture compared to the surrounding fluid phase as revealed by AFM (Murthy et al., 2016a). Gel-gel phase separation in milk-SM domains has recently been reported and interpreted as lateral segregation of SM molecules with various chain lengths and to interdigitation (Guyomarc'h et al., 2014, 2017). A recent study highlighted the key role of the milk-SM acyl chain heterogeneities in the thermal and mechanical properties of bilayer membranes (Et-Thakafy et al., 2018).

The characterization of the biophysical properties of the L_o phase formed by SM molecules in presence of cholesterol has been the subject of a number of studies (Chachaty et al., 2005; Chemin et al., 2008; Quinn and Wolf, 2009). Much less attention has been devoted to the examination of the interactions between milk-SM and cholesterol in milks-SM/cholesterol mixtures (Cheng et al., 2017). Recent studies performed using Langmuir monolayers demonstrated the condensing effect of cholesterol on milk-SM below and above T_m of milk-SM (Cheng et al., 2017; Murthy et al., 2015). AFM experiments showed that cholesterol decreases the size of milk SM-rich domains and has a fluidizing effect in lipid bilayers studied as model of the MFGM (Murthy et al., 2016b). Further experiments are required to characterize the unique biophysical properties of milk-SM and the role of cholesterol in the formation of the L_o phase.

The objectives of this work were therefore to examine the biophysical properties of milk-SM and to increase the knowledge about the role of cholesterol on the properties of milk-SM bilayers. For this purpose, we analyzed the thermotropic phase behavior of milk-SM bilayers and milk-SM/cholesterol mixtures bilayers using differential scanning calorimetry (DSC) and synchrotron radiation X-ray diffraction (SR-XRD) at both small and wide angles.

2. Materials and methods

2.1. Materials

Sphingomyelin from bovine milk (milk-SM; > 99%) and cholesterol (chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Pipes buffer with an ionic strength and pH similar to milk was used to investigate the thermotropic phase behavior of milk-SM and milk-SM/chol bilayers. Pipes buffer was prepared as follows: Pipes 10 mM (1,4-piperazinediethane sulfonic acid; purity ≥ 99%; Sigma-Aldrich, Milwaukee, WI, USA) with NaCl 50 mM (purity ≥ 99%; Sigma-Aldrich) and $CaCl_2$ 5 mM (purity ≥ 99%; Sigma-Aldrich) were dissolved in Milli-Q water and adjusted to pH 6.7 using NaOH 5 M.

2.2. Thermotropic phase behavior and structural properties of milk-SM and milk-SM/cholesterol bilayers

2.2.1. Sample preparation

2.2.1.1. Milk-SM bilayers. Samples were prepared by hydrating the milk-SM powder in Pipes buffer at 70 °C to obtain the desired weighted composition. The suspensions were heated at 70 °C, i.e. above the chain melting transition temperature of milk-SM, and thoroughly mixed in a vortex stirrer in order to ensure the formation of lipid bilayers in large multilamellar vesicles and to have good sample homogeneity. Before analysis, the samples were kept at 20 °C for 24 h.

2.2.1.2. Milk-SM/cholesterol bilayers. Samples were prepared by dissolving the appropriate amounts of milk-SM and cholesterol in chloroform/methanol (4/1 v/v) and mixing them from stock solutions in the desired proportions, denoted as milk-SM/chol molar ratios. The organic solvent was subsequently evaporated under a stream of oxygen-free dry nitrogen at 50 °C. The milk-SM and cholesterol dried mixtures were hydrated with Pipes buffer at 70 °C to reach the final concentration of 20 wt% lipids, for which milk-SM is fully hydrated. The dispersions were heated above the chain melting transition temperature of milk-SM and thoroughly mixed in a vortex stirrer in order to form large multilamellar vesicles and to ensure good sample homogeneity. Before DSC and coupled SR-XRD/DSC analysis, the samples were kept at 20 °C for equilibration during at least 24 h.

2.2.2. Differential scanning calorimetry (DSC) experiments

DSC measurements were performed with a DSC Q1000 (TA Instruments, Newcastle, DE). An aliquot of the milk-SM or milk-SM/chol samples was loaded into DSC hermetically sealed aluminum pans (TA Instruments). An empty and hermetically sealed aluminum pan was used as reference. The calorimeter was calibrated with indium ($\Delta H = 28.41$ J/g; Melting point = 156.66 °C). The DSC pans were introduced in the calorimeter at 20 °C, and then cooled down to 0 °C at 2 °C/min. To study the thermotropic phase behavior of milk-SM bilayers and milk-SM/chol samples, heating scans were run at a rate of 2 °C/min from 0 °C to 80 °C and then cooling scans were recorded at 2 °C/min from 80 to 0 °C. To study the thermotropic phase behavior of dry milk-SM, an aliquot of powder was successively heated at 2 °C/min from 0 to 150 °C. Data analysis was performed using TA Universal Analysis program. The melting transition temperature of milk-SM was taken at the peak maximum (T_m). Enthalpy changes of the transitions (ΔH_m) were obtained from the area under the peak and normalized by the milk-SM mass. To calculate the area under the peak a baseline connecting the linear segments of the heat capacity curve between the initial and endpoint of the transition was substracted.

2.2.3. Temperature-controlled SR-XRD experiments coupled with DSC

High-flux synchrotron radiation X-ray diffraction (SR-XRD) experiments were performed at SOLEIL synchrotron (Gif-sur-Yvette, France) on the SWING beam line. Data were collected by a two-dimension charge-coupled device detector. The diffracted intensity was reported as a function of the scattering vector $q = 4\pi\sin\theta/\lambda$ where 2θ is the scattering angle and λ the wavelength of the incident beam ($\lambda = 0.828$ Å; 15 keV). The sample-to-detector distance was set to 520 mm to allow recording of XRD patterns in the q-range from 0.03 $Å^{-1}$ to 1.8 $Å^{-1}$, thus covering the small- and wide-angle regions of interest for fully hydrated lipid bilayers. Calibration of the q-range was carried out with pure β-tristearin (Lavigne et al., 1993) for wide angles and silver behenate (Blanton et al., 2000) for small angles. Intensity values were normalized for beam intensity, acquisition time and sample transmission. Each XRD pattern recorded as a function of temperature displayed concentric signal rings which were integrated circularly to yield the intensity versus q. The samples of fully hydrated milk-SM and milk-SM/chol mixtures (around 20 µL; 20 wt% lipids in Pipes buffer) were introduced into thin quartz capillaries of 1.5 mm diameter (GLAS