



The miscibility of milk sphingomyelin and cholesterol is affected by temperature and surface pressure in mixed Langmuir monolayers



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ARTICLE INFO

Article history:

Received 28 July 2016

Received in revised form 12 November 2016

Accepted 12 December 2016

Available online 18 December 2016

Keywords:

Milk fat globule membrane

Lipid domain

Melting temperature

Langmuir film

ABSTRACT

The miscibility of milk sphingomyelin (milk-SM) and cholesterol was investigated in this study. The effect of different physical states of milk-SM on its interactions with cholesterol was determined by the recording of isotherms of compression of Langmuir films for temperatures above and below the gel to $L\alpha$ phase transition of milk-SM ($T_m \sim 34$ °C). For $T = 15$ °C $< T_m$, the liquid expanded (LE) to liquid condensed (LC) phase transition of milk-SM monolayers was observed at surface pressures of 10–15 mN/m. For $T = 43$ °C $> T_m$, the milk-SM molecules were in a LE phase regardless of the surface pressure applied. A phase diagram pressure – milk-SM/cholesterol composition was established. This study demonstrated that both temperature and surface pressure affected the miscibility between the milk-SM and cholesterol. The strongest attractive forces (i.e. condensing effect) were identified for 30 mol% cholesterol when the milk-SM was in the LE phase state.

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1. Introduction

The biological membrane surrounding milk fat globules is currently poorly understood despite its importance in the functional properties of milk lipids, in the mechanisms of milk lipid digestion and in nutritional and health benefits. Increasing the knowledge surrounding the packing and miscibility of lipid components (i.e. polar lipids, cholesterol) is of primary importance in order to better understand e.g. the functions of the milk fat globule membrane (MFGM) and the properties of emulsions containing lipid droplets coated with milk polar lipids.

The composition and architecture of the MFGM result from the mechanisms of fat globule secretion from the mammary epithelial cells (Keenan & Mather, 2006). The MFGM contains membrane-specific proteins and a highly complex assortment of polar lipids: sphingolipids (mainly sphingomyelin, milk-SM: 20–45% of polar lipids depending on mammal species), phosphatidylcholine (PC: 19–23.7%), phosphatidylethanolamine (19.3–32.7%), phosphatidylserine (1.9–19.7%) and phosphatidylinositol (6.1–13.6%) (Lopez, 2011; Rombaut, Dewettinck, & Van Camp, 2007). Apart from these lipids, the MFGM also contains about 30 wt% of cholesterol in the membrane lipid fraction (Mesilati-Stahy & Argov-

Argaman, 2014 – i.e. about 45 mol%). The MFGM is structured as a trilayer of polar lipids and proteins (Keenan & Mather, 2006; Lopez, 2011). The inner monolayer is in contact with the triacylglycerol core of the fat globules and originates from the endoplasmic reticulum of the epithelial cells. The outer bilayer envelops the fat globules during their secretion through the apical plasma membrane of the mammary cells. Previous authors have reported an asymmetry in the localization of polar lipids with milk-SM, PC and cholesterol being preferably located in the outer bilayer of the MFGM (Deeth, 1997). Studies performed *in situ* in milk by confocal microscopy have revealed a phase separation of polar lipids in the outer bilayer of the MFGM with the formation of lipid domains (Gallier, Gragson, Jiménez-Flores, & Everett, 2010; Lopez, Madec, & Jiménez-Flore, 2010; Lopez & Ménard, 2011; Nguyen et al., 2016; Zou et al., 2015). These lipid domains were assumed to be formed by the lateral segregation of saturated polar lipids with a high phase transition temperature, mainly milk-SM, and could also contain cholesterol (Gallier et al., 2010; Lopez, 2011; Lopez et al., 2010; Nguyen et al., 2016). The role played by cholesterol in the formation and biophysical properties of milk-SM rich domains in the outer bilayer of the MFGM is still poorly understood (Murthy, Guyomarc'h, & Lopez, 2016a; Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015). Recent studies performed by atomic force microscopy have shown that cholesterol affects the morphology of milk-SM domains (Guyomarc'h et al., 2014; Murthy et al., 2015, 2016a), and reduces the resistance to perforation of the membrane

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bilayers when studied as models of the MFGM (Guyomarc'h et al., 2014; Murthy et al., 2016a). Also, compression isotherms of the MFGM polar lipid monolayers revealed the condensing effect of the cholesterol, with concomitant changes in the topography of the Langmuir-Blodgett monolayers (Murthy et al., 2015). The structural and functional roles of cholesterol in the MFGM remain poorly understood (Guyomarc'h et al., 2014; Murthy et al., 2015, 2016a), despite cholesterol being known to play a fundamental role in the organization of cell membranes. Cholesterol has been reported to be involved in several mechanisms due to its close packing association with saturated lipids, inducing phase separation (McMullen, Lewis, & McElhane, 2004; Quinn & Wolf, 2009; Wolf, Koumanov, Tenchov, & Quinn, 2001) and forming tightly packed microdomains in membranes called "rafts", specifically with sphingolipids (Ramstedt & Slotte, 1999; Simons & Ikonen, 1997). The role played by the milk-SM in the activity of the gastric lipase at the surface of lipid droplets has been demonstrated (Favé et al., 2007) and the role of cholesterol and physical state of SM in the hydrolysis rate of SM by sphingomyelinase has been reported (Contreras, Sot, Ruiz-Argüello, Alonso, & Goñi, 2004; Jungner, Ohvo, & Slotte, 1997; Ruiz-Argüello, Veiga, Arrondo, Goni, & Alonso, 2002). The biophysical properties of the lipid domains present in the MFGM could therefore modulate the activity of lipolytic enzymes during milk fat globule digestion. Authors have recently shown that milk-SM improves lipid metabolism in high fat diet-fed mice (Norris, Jiang, Ryan, Porter, & Blesso, 2016) and that the milk-SM is involved in the reduction of cholesterol absorption in the intestine (Eckhardt, Wang, Donovan, & Carey, 2002; Noh & Koo, 2004; Nyberg, Duan, & Nilsson, 2000). Further studies are necessary to better understand the interactions between the milk-SM and cholesterol that could be involved in several functions, particularly in the gastrointestinal tract. These studies need to be performed under experimental conditions adapted to the biological conditions. Most of the biophysical experiments reported in the scientific literature were performed at room temperature (i.e. 20 °C). However, temperature governs the physical state of the milk-SM (temperature of phase transition $T_m \sim 34$ °C; Murthy et al., 2015) and MFGM polar lipids (Murthy, Guyomarc'h, & Lopez, 2016b) and milk lipids are digested at 37 °C in the gastrointestinal tract, i.e. at a temperature above the T_m of milk-SM.

The objective of this work was to determine whether the milk-SM and cholesterol were miscible and how temperature influenced their miscibility. The original approach taken in this study was to investigate the effect of the phase state of milk-SM molecules, through changes in temperature (i.e. $T < T_m$ or $T > T_m$ of milk-SM), on the interactions between milk-SM and cholesterol. The thermotropic phase behaviour of the milk-SM was determined by differential scanning calorimetry and X-ray diffraction. The miscibility of cholesterol and the milk-SM was investigated by the isotherms of compression of Langmuir monolayers of several milk-SM/cholesterol mixtures. For the first time, this work demonstrated the condensing effect of cholesterol on milk-SM monolayers with a maximum area of condensation recorded for 30 mol% cholesterol when the milk-SM was in the LE phase state.

2. Materials and methods

2.1. Materials

Sphingomyelin from bovine milk (milk-SM; >99%) and cholesterol (chol; >99%) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and were used without further purification. Sphingomyelin has sphingosine as the hydrophobic backbone (mainly 18:1), together with an amide-linked acyl chain. The acyl chain composition of milk-SM is as follows: 19% C16:0, 3% C18:0,

1% C20:0, 19% C22:0, 33% C23:0, 20% C24:0, 3% C24:1 n-9 (Filippov, Orädd, & Lindblom, 2006; Guyomarc'h et al., 2014). PIPES buffer (1,4-piperazinediethanesulfonic acid) with an ionic strength and pH similar to milk was used to investigate the thermotropic phase behaviour of milk-SM bilayers and for Langmuir monolayers experiments. PIPES buffer 10 mM (1,4-piperazinediethane sulfonic acid; purity $\geq 99\%$; Sigma) with NaCl 50 mM (Panreac, Germany, purity > 99%) and CaCl₂ 5 mM (Panreac, Germany, purity > 98%) was adjusted to pH 6.7 using NaOH 5M. Purified water (Milli-Q waters) was used for buffer preparation.

2.2. Thermotropic phase behaviour of milk-SM bilayers determined by differential scanning calorimetry

Milk-SM bilayers were prepared by hydrating the milk-SM powder in PIPES buffer to obtain 20 wt%. The suspension was heated at 60 °C and thoroughly mixed in a vortex stirrer in order to ensure the formation of multilamellar vesicles. Before analysis, the samples were kept at 20 °C for at least 24 h. Differential scanning calorimetry (DSC) measurements were performed with a DSC Q1000 (TA Instruments, New Castle, DE). An aliquot of the milk-SM sample was loaded into DSC hermetically sealed aluminum pans (TA Instruments). An empty, 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 into the calorimeter at 20 °C, and then cooled down to 0 °C at 2 °C/min. Heating scans were run at a rate of 2 °C/min from 0 °C to 80 °C. Data analysis was performed using TA Universal Analysis program. The melting transition temperature (T_m) was taken at the peak maximum on heating.

2.3. Identification of lipid phases by synchrotron radiation X-ray diffraction

X-ray scattering experiments were performed on the SWING beamline at synchrotron SOLEIL (Gif-sur-Yvette, France). On the X-ray beamline operating at 15 keV, a two-dimensional detector allowed the recording of XRD patterns in the range 0.08–1.8 Å⁻¹, thus covering both the small and wide-angle regions of interest. XRD at small angles allows identification of the lamellar organization of milk-SM molecules while XRD at wide angles provides information on the lateral packing of their sphingoid base and acyl chains. Each diffraction pattern, displaying a series of concentric rings, was integrated circularly to yield the intensity as a function of the radial scattering vector q . The scattered 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. The channel to scattering vector q calibration of the detector was carried out with pure tristearin (β 2L form) and silver behenate as previously reported (Murthy et al., 2015). Small volumes (around 20 μ l) of samples containing fully hydrated milk-SM vesicles (20 wt%) were loaded into thin quartz capillaries of 1.5 mm diameter (GLAS W. Muller, Berlin, Germany) and inserted into the set-up. The samples were characterized at 15 °C (i.e. for $T < T_m$ of milk-SM) and at 45 °C (i.e. for $T > T_m$ of milk-SM).

2.4. Langmuir film balance experiments

The solutions of milk-SM and cholesterol (with concentrations of around 1 mg/ml) were prepared by dissolving the investigated components in chloroform (analytical grade, Carlo Erba reagents, Val de Reuil, France). Mixed solutions were prepared from the respective stock solutions to the required molar ratio $X_{\text{cholesterol}}/(1-X)_{\text{milk-SM}}$. Surface pressure (π) – area (A) isotherms were recorded with a KSV Langmuir trough (KSV 3000, KSV, Finland) with a total area = 70 cm² (70 × 10 cm) placed on an anti-

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