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Cholesterol strongly affects the organization of lipid monolayers studied as models of the milk fat globule membrane: Condensing effect and change in the lipid domain morphology

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ABSTRACT

The biological membrane that surrounds the milk fat globules exhibits phase separation of polar lipids that is poorly known. The objective of this study was to investigate the role played by cholesterol in the organization of monolayers prepared as models of the milk fat globule membrane (MFGM). Differential scanning calorimetry and X-ray diffraction experiments allowed characterization of the gel to liquid crystalline phase transition temperature of lipids, Tm ~35 °C, in vesicles prepared with a MFGM lipid extract. For temperature below Tm, atomic force microscopy revealed phase separation of lipids at 30 $mN \cdot m^{-1}$ in Langmuir–Blodgett monolayers of the MFGM lipid extract. The high Tm lipids form liquid condensed (LC) domains that protrude by about 1.5 nm from the continuous liquid expanded (LE) phase. Cholesterol was added to the MFGM extract up to 30% of polar lipids (cholesterol/milk sphingomyelin (MSM) molar ratio of 50/50). Compression isotherms evidenced the condensing effect of the cholesterol onto the MFGM lipid monolayers. Topography of the monolayers showed a decrease in the area of the LC domains and in the height difference *H* between the LC domains and the continuous LE phase, as the cholesterol content increased in the MFGM lipid monolayers. These results were interpreted in terms of nucleation effects of cholesterol and decrease of the line tension between LC domains and LE phase in the MFGM lipid monolayers. This study revealed the major structural role of cholesterol in the MFGM that could be involved in biological functions of this interface (e.g. mechanisms of milk fat globule digestion).

1. Introduction

Lipids are secreted in milk in the form of colloidal biological assemblies, the milk fat globules (~4 μm diameter; [1]). The interface between the triacylglycerol core of milk fat globules and the aqueous phase is a biological membrane involved in the mechanisms of milk lipid digestion. Further studies are necessary to better understand the relation between the structure of the milk fat globule membrane (MFGM) and its functions.

The composition and structure of the MFGM depend on the mechanisms of milk fat globule secretion by the lactating mammary cells [2]. The MFGM is composed of glycerophospholipids (i.e. phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; and phosphatidylserine, PS), sphingolipids (mainly milk sphingomyelin, MSM), cholesterol and membrane-specific proteins [1,3]. The five main classes of milk polar lipids (PC, PE, PI, PS, MSM) are characterized by a wide diversity of saturated and unsaturated acyl moieties [4]. The MFGM components are distributed in a trilayer organization: a monolayer originating from the endoplasmic reticulum is in contact with the triacylglycerol core of fat globules and an outer bilayer resulting from the envelopment of fat globules in the apical plasma membrane of the mammalian epithelial cells [2]. As for other biological membranes (e.g. in human erythrocyte — [5]), authors reported an asymmetry in the localization of polar lipids. MSM, PC and cholesterol are preferably located in the outer bilayer of the MFGM while PE, PI and PS are mainly concentrated on the inner monolayer of the MFGM [6]. In recent years, structural investigations performed by confocal microscopy revealed lateral heterogeneities attributed to phase separation of polar lipids in the outer bilayer of the MFGM surrounding fat globules *in situ* in bovine milk [1,7,8], human milk [9,10] and buffalo milk [11]. In this approach, the continuous fluorescent phase was interpreted as a fluid liquid disordered matrix mainly comprised of unsaturated glycerophospholipids (PC, PE, PI, PS), while the non-fluorescent areas were thought to correspond to gel phase domains of polar lipids with high phase transition temperatures, such as MSM but also saturated PC, or to liquid ordered (l_o) phase domains in presence of cholesterol [1,8].

The functional role of cholesterol in the MFGM is poorly understood, despite the fact that cholesterol is known to play a crucial role in the

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lateral organization of lipids and formation of lipid rafts in mammalian cell membranes. Cholesterol strongly affects arrangements of membrane polar lipids, namely through decreasing the area occupied per phospholipid (condensing effect) and through blocking the lateral arrangement into l_o phase (fluidizing effect — [13]). Recently, atomic force microscopy (AFM) investigation of binary MSM/dioleylphosphatidylcholine (DOPC) and ternary MSM/DOPC/cholesterol model membranes of the MFGM showed that cholesterol modifies the morphology of the MSM domains and reduces the resistance of membrane bilayers to perforation [14].

The molecular organization of membrane phospholipids and cholesterol mixtures, models of biological membranes, is frequently studied in Langmuir films formed at the air/water interface. By using this technique, the condensing and ordering effect of cholesterol on membrane lipids has been widely investigated [15–17]. Moreover, the combination of Langmuir films with structural methods, e.g. microscopy, provides information on phase coexistence and domain morphology [16,17]. The interpretation of the results obtained on multicomponent monolayers is more complicated as compared to binary phospholipid/cholesterol films, however they better reflect the complex composition and properties of membranes. Various MFGM extracts were investigated using Langmuir films and Langmuir–Blodgett monolayers to study the phase behavior of lipids [12]. The role of cholesterol on MFGM lipid extracts remains to be investigated to better understand the lateral segregation of lipids in the MFGM.

In this work, we determined the thermotropic phase behavior of lipids in a MFGM lipid extract and identified the lipid phases, using differential scanning calorimetry and X-ray diffraction respectively. These experiments showed that high phase transition temperature polar lipids contained in the MFGM lipid extract (i.e. MSM and saturated glycerophospholipids such as DPPC and POPE) are in an ordered phase for T < 25 °C. The properties of MFGM lipids in the presence of cholesterol at various concentrations were analyzed as a function of surface pressure using Langmuir films at 20 °C. The lateral segregation of lipids was characterized at the surface pressure $\pi = 30\ mN \cdot m^{-1}$, which is relevant for biological membranes such as the MFGM, and the role of cholesterol was investigated by probing Langmuir–Blodgett isotherms using AFM imaging.

2. Materials and methods

2.1. Materials

Sphingomyelin from bovine milk (MSM; >99%), cholesterol (Chol; from ovine wool, >98%), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. The MFGM lipid extract used in this study contained milk polar lipids with the following relative weight percentages: 38.7% MSM, 31.6% PC, 23.5% PE, 3.4% PI and 2.8% PS. These milk polar lipids were characterized by a large range of acyl moieties, as previously reported [4]. Knowing the individual molecular weights (MW) of milk polar lipids, the molar-averaged MW of the MFGM extract polar lipid molecules was calculated to be $757.4\ g \cdot mol^{-1}$. This value was used for calculations throughout the paper. The cholesterol content of the MFGM lipid extract was low, i.e. 1.34 wt.% of polar lipids, and was neglected in further calculation. PIPES 10 mM (1,4-piperazinediethane sulfonic acid; purity ≥ 99%; Sigma Aldrich, Milwaukee, WI, USA) buffer was prepared with NaCl 50 mM (Sigma) in Milli-Q water and adjusted to pH 6.7 using NaOH 5 M.

For differential scanning calorimetry (DSC) and X-ray diffraction (XRD) experiments, the MSM, DPPC, POPC, POPE and MFGM lipid extract powders were hydrated with PIPES buffer at 65 °C to reach a final concentration of 20 wt.% lipids for MSM, DPPC, POPC, POPE and 40 wt.% lipids for MFGM. The dispersions were thoroughly mixed in a vortex stirrer at 65 °C to form multilamellar vesicles.

For monolayer experiments, samples were prepared by dissolving appropriate stock solutions of MFGM lipid extract or cholesterol in chloroform/methanol (4/1 v/v) and mixing them to reach molar proportions of MSM/cholesterol from 100/0 to 50/50, corresponding to 0–26.8 mol.% cholesterol on a total MFGM polar lipids basis. The organic solvent was then evaporated under a stream of dry nitrogen at 50 °C. The samples were stored at −20 °C before use.

2.2. Differential scanning calorimetry (DSC)

The thermotropic properties of MSM, DPPC, POPC, POPE and MFGM multilamellar vesicles were determined by DSC using a DSC Q1000 apparatus (TA Instruments, Newcastle, DE). The calibration of the calorimeter was performed with indium standard (melting point = 156.66 °C, ΔH melting = $28.41\ J \cdot g^{-1}$). The samples were introduced in 20 μL aluminum pans hermetically sealed after sample introduction. An empty pan was used as a reference. The samples were heated at $2\ °C \cdot min^{-1}$ from −10 °C to 70 °C. For MFGM, cooling recordings were performed at $2\ °C \cdot min^{-1}$. All the thermal measurements were performed in triplicate. The temperature of phase transition was taken at the peak maximum (T_m) on heating and at the peak minimum on cooling (Tc), using TA Universal Analysis program (Universal Analysis 2000, v 4.1 D).

2.3. Temperature controlled synchrotron radiation X-ray diffraction (XRD)

X-ray scattering experiments were performed on the SWING beamline at synchrotron SOLEIL (Gif-sur-Yvette, France). A two-dimensional detector with sample to detector distance of 521 mm allowed the recording of XRD patterns in the range 0.08 $Å^{-1}$ to 1.8 $Å^{-1}$, thus covering both the small and wide-angles regions of interest to characterize the lamellar structures and to identify the packing of the fatty acid chains respectively. Diffraction patterns displayed a series of concentric rings as a function of the radial 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 silver behenate [18]. Small volumes (around 20 μL) of samples containing MSM or MFGM lipid multilamellar vesicles were loaded in thin quartz capillaries of 1.5 mm diameter (GLAS W. Muller, Berlin, Germany) and inserted in the set-up at a controlled temperature, i.e. T = 6 °C < Tm and T = 60 °C > Tm.

2.4. Pressure (π)–area (A) isotherms

The π–A measurements were carried out using a 10 × 90 cm Langmuir trough equipped with a Wilhelmy balance (Nima Technology, Cambridge, UK). About 1 mM lipid mixture in chloroform/methanol (2/1 v/v) were gently deposited onto PIPES buffer subphase with the Hamilton micro syringe, precise to 1.0 μL. After ~10 min spreading of the solution and evaporation of solvents, the lipid films were compressed continuously by closing barriers at a rate of $5\ cm^2 \cdot min^{-1}$ while monitoring both π and A until collapse of the lipid film (Nima Technology, Cambridge, UK). The temperature was kept constant at 20 ± 0.5 °C. The resistivity of ultrapure water was 18.2 MΩ·cm and the surface tension of ultrapure water was 72.3 ± 0.2 $mN \cdot m^{-1}$ (measurements performed using Wilhelmy plate method). Each experiment was repeated at least 2 times.

2.5. Langmuir–Blodgett (LB) films

For monolayer preparation, lipid films were prepared as described above, at 20 °C, on a 7 × 30 cm Langmuir trough (Nima Technology). After evaporation of the solvent, the lipid films were compressed at a rate of $5\ cm^2 \cdot min^{-1}$ to reach a constant surface pressure of