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Young modulus of supported lipid membranes containing milk sphingomyelin in the gel, fluid or liquid-ordered phase, determined using AFM force spectroscopy

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ABSTRACT

The biological membrane surrounding milk fat globules (MFGM) exhibits lateral phase separation of lipids, interpreted as gel or liquid-ordered phase sphingomyelin-rich (milk SM) domains dispersed in a fluid continuous lipid phase. The objective of this study was to investigate whether changes in the phase state of milk SM-rich domains induced by temperature (T < Tm or T > Tm) or cholesterol affected the Young modulus of the lipid membrane. Supported lipid bilayers composed of MFGM polar lipids, milk SM or milk SM/cholesterol (50:50 mol) were investigated at 20 °C and 50 °C using atomic force microscopy (AFM) and force spectroscopy. At 20 °C, gel-phase SM-rich domains and the surrounding fluid phase of the MFGM polar lipids exhibited Young modulus values of 10–20 MPa and 4–6 MPa, respectively. Upon heating at 50 °C, the milk SM-rich domains in MFGM bilayers as well as pure milk SM bilayers melted, leading to the formation of a homogeneous membrane with similar Young modulus values to that of a fluid phase (0–5 MPa). Upon addition of cholesterol to the milk SM to reach 50:50 mol%, membranes in the liquid-ordered phase exhibited Young modulus values of a few MPa, at either 20 or 50 °C. This indicated that the presence of cholesterol fluidized milk SM membranes and that the Young modulus was weakly affected by the temperature. These results open perspectives for the development of milk polar lipid based vesicles with modulated mechanical properties.

1. Introduction

Biological membranes are complex systems composed of a wide spectrum of lipid molecules (e.g. glycerophospholipids, sphingolipids, cholesterol) and proteins. Depending on their chemical structure, polar lipids can be organized in different phase states as a function of temperature. Moreover, lipid-lipid driven interactions occurring in the plane of the membrane can lead to phase separation and the formation of domains [1–4]. The main lipids involved in the formation of these domains exhibit higher phase transition temperature Tm than ambient temperatures, e.g. the saturated dipalmitoylphosphatidylcholine (DPPC; Tm = 41 °C, [5]) and natural sphingomyelins (Tm close to physiological temperature; [6]). Cholesterol is also known to be involved in the formation of liquid-ordered (Lo) phase domains with the high Tm lipids [7].

The biological membrane surrounding fat globules in milk, called the milk fat globule membrane (MFGM), is of special interest since it represents 160 m^2 per liter of milk and is involved in unique functional and biological properties, especially towards the newborn [8]. The MFGM is the interface between the triacylglycerol core of the milk fat globules and the aqueous environment of milk. It is therefore involved in many mechanisms occurring at the interface, e.g. presentation of signaling proteins or adsorption of enzymes, bacteria, viruses, that are important for the digestion of milk lipids and protection of the gastrointestinal tract. This membrane is also essential to ensure the physical stability of milk fat globules. The structure of the MFGM results from the multi-step secretion of fat globules from the epithelial cells of the mammary cells and corresponds to a trilayer of polar lipids and proteins, i.e., an inner monolayer, and an outer bilayer originating from the apical plasma membrane [9,10]. Due to its cellular origin, the MFGM contains mainly glycerophospholipids (PE, PC, PI, PS), sphingolipids, proteins, and cholesterol [11,12]. The milk sphingomyelin (milk SM) is the main sphingolipid found in the MFGM. It represents 20 to 35 wt% of the total polar lipids and exhibits specific physical properties with a gel to fluid phase transition temperature Tm = 34.3 °C [13,14]. MFGM polar lipids exhibit a gel to fluid phase transition temperature Tm = 36.4 °C [13], due to the presence of saturated polar lipids, mainly milk SM. Meanwhile, the cholesterol represents

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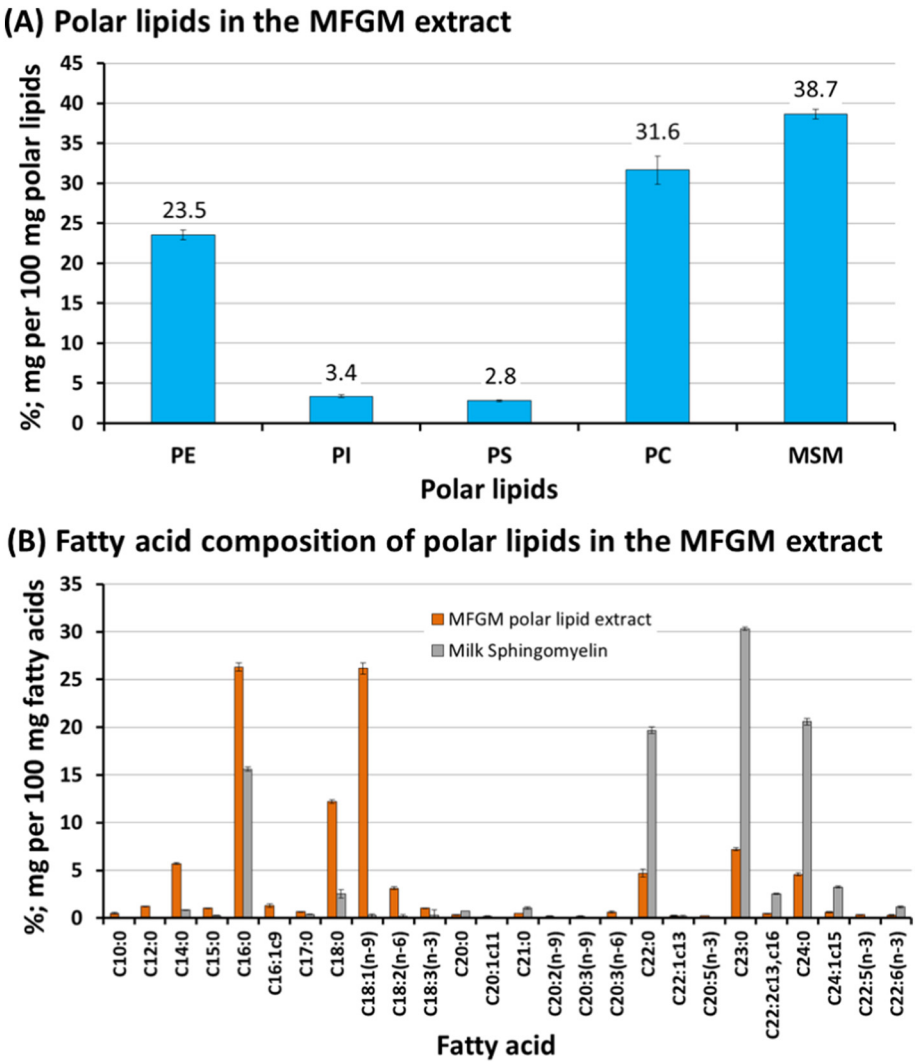

Fig. 1. (A) Composition, in % weight of the total polar lipids, of the milk fat globule membrane (MFGM) extract in polar lipids PE (phosphatidylethanolamine), PI (phosphatidylinositol), PS (phosphatidylserine), PC (phosphatidylcholine) and MSM (milk sphingomyelin); (B) Composition, in % weight, of the fatty acids present in the polar lipids of the MFGM extract (in orange) and in the milk sphingomyelin as a reference (in grey).

3–6 g kg^{-1} of the total fat in milk (among the mono-, di-, triglycerides, free fatty acids, polar lipids etc. – [15,16]). It is mainly located in the MFGM [17] with a cholesterol:polar lipid mass ratio of ~1:3. In the last decade, lipid domains of micron size have been observed *in situ* in the MFGM surrounding fat globules in milk using confocal microscopy. In analogy with cellular "rafts" [18], these microdomains were interpreted as the lateral segregation of high Tm polar lipids, notably milk SM, dispersed in a continuous fluid phase matrix of unsaturated PE, PS, PI, and PC [19–21]. Individual milk SM-rich domains are expected to be in the gel or in the liquid-ordered phase in the absence or presence of cholesterol, respectively [15]. Therefore, the gel, liquid-ordered and fluid phases are expected to coexist in the MFGM, making it a most complex colloidal system in native milk. Studies showed that the MFGM is indeed a dynamic system in which the organization of polar lipids [22–24] and the lipid domains are highly sensitive to temperature variations and time [15,25]. Increasing knowledge about the structure of the MFGM and its physical properties is therefore of primary interest to better control storage of human milk or to design bio-inspired lipid droplets e.g. in infant milk formulas. Among other physical properties, the resistance of MFGM to deformation is a key parameter to evaluate the stability of the milk fat globules towards mechanical stress, such as during dairy processes. In particular, the coupled influence of shear and heat is encountered in all stabilization operations, e.g. pasteurization or homogenization.

To do this, the calculation of the Young modulus out of force-indentation atomic force microscopy (AFM) spectroscopy experiments has proven an interesting approach to deal with as soft samples as biomembranes. Less invasive than the measurement of the yield force (or breakthrough force) of membranes, which successfully discriminate the gel, liquid-ordered and fluid phases of model supported lipid bilayers [26,27], calculation of the Young modulus also has the advantage to allow comparison with other techniques or with non-supported membrane objects [28]. In recent years, AFM determination of the Young modulus has for instance yielded values in the tens to hundreds of kPa range for the heterogeneous structure of fibroblasts [29] or other eukaryote cell membranes [30], for the decreased stiffness of cancer cells [31,32] or for the elasticity of bacterial cell wall [33]. Meanwhile, model supported lipid bilayers yield Young modulus values in the tens to hundreds MPa range [27,28,34,35]. In milk, only Balasuriya et al. [36] have reported that the Young modulus of the MFGM of entire milk fat globules varied with the various fractionation or homogenization treatment applied. Unfortunately, the complexity of the milk fat globule surface did not allow direct recognition of the lipid phases within the MFGM.

Therefore, the objective of the present work was to evaluate the Young modulus values of the different lipid phases found in bilayers

made of MFGM polar lipids, as a protein-free model for the native MFGM [37], using AFM imaging and force spectroscopy. The behavior of the gel, fluid and liquid-ordered phases have been scrutinized with respect to temperature variation from 20 to 50 °C, and in presence of cholesterol in order to unravel its possible role in stabilizing structures against environmental change. These temperatures were chosen as to stand respectively below and above the melting temperatures of MFGM polar lipids and of the milk SM. Hence, saturated polar lipids of the MFGM, and namely the milk SM, were in the gel phase at 20 °C and in the fluid liquid disordered (Ld) phase at 50 °C [13]. Meanwhile, a milk SM/chol ratio of 50:50 mol% has been shown to turn milk SM bilayers into liquid ordered phase [36], a phase state that does not show any transition as a function of temperature [14,38,39].

2. Experimental methods

2.1. Materials

Sphingomyelin from bovine milk (milk SM; purity > 99 wt%) and cholesterol (chol; from ovine wool, purity > 98 wt%) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The MFGM polar lipid extract used in this study was the same as in Murthy et al. [13,37]. It contained polar lipids with the following relative weight percentages: 38.7% milk SM, 31.6% PC, 23.5% PE, 3.4% PI and 2.8% PS, as determined by high-performance liquid chromatography coupled with evaporative light scattering detection [40]. Most of the long-chain saturated fatty acids were due to the high amount of milk SM, which contains ~20 wt% of C16:0, C22:0 and C24:0; as well as ~30 wt% of C23:0 and minor amounts of other chains, especially the unsaturated C24:1 (Fig. 1). Meanwhile, a majority of the long chain base of milk SM is the C18:1 sphingosine.

PIPES buffer (1,4-piperazinediethanesulfonic acid) was prepared as follows: PIPES 10 mM (purity ≥ 99 wt%; Sigma-Aldrich, Milwaukee, WI, USA), NaCl 50 mM (purity ≥ 99 wt%; Sigma-Aldrich), and $CaCl_2$ 0–5 mM as indicated in the text (purity ≥ 99 wt%; Sigma-Aldrich) were dissolved in Milli-Q water and adjusted to pH 6.7 using NaOH 5 M.

2.2. Methods

2.2.1. Preparation of small unilamellar vesicles

First, each lipid powder was weighted into glass vials and dissolved using chloroform/methanol (4:1 v/v) to make mother solutions of the MFGM polar lipids, milk SM or cholesterol (chol). Dried lipid films were then produced by placing or mixing the appropriate amounts of the mother solutions into glass vials, followed by evaporation of the solvent at 40 °C under a gentle stream of dry nitrogen. The dried lipid films were hydrated with PIPES buffer at 60 °C to reach a final concentration of 1 mg mL^{-1} for the MFGM polar lipids, or 0.1 mg mL^{-1} for the milk SM and milk SM/chol 50:50 mol%, then thoroughly vortexed. PIPES-NaCl buffer without calcium was used for the MFGM polar lipids, and PIPES-NaCl with 5 mM $CaCl_2$ was used for milk SM and milk SM/chol preparations [13,41]. Then, small unilamellar vesicles (SUV) were produced by sonication at 60 °C using a Q700 equipment (Q-sonica, Newtown, CT, USA) and a micro tip operating at 50% amplitude (~400 W) for 30–60 min. Noteworthy, the absence of calcium was required for the MFGM vesicles in order to ensure their stability. Those vesicles bear electronegative charge at pH 6.7 due to the presence of PI and PS, which is neutralized in presence of calcium, thus inducing aggregation [42,43]. Conversely, milk SM and milk SM/chol vesicles are electrically neutral in water due to the zwitterionic nature of the polar head group. The presence of calcium (and sodium, to a much lesser extent) shields the electronegative phosphate group, which results in increasing electropositivity with calcium addition, thus stabilizing the vesicle via electrostatic repulsion [43,44]. Furthermore, the presence of cholesterol favored fusion between vesicles. For that reason, increased dilution and sonication time were used to produce stable milk SM/chol vesicle suspensions.

2.2.2. Dynamic light scattering

The hydrodynamic diameter Dh of the SUVs was measured at 20 °C and in PIPES buffer using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) operating at a scattering angle of 173° and a wavelength of 633 nm. The mean Dh (± 5 nm) was calculated from the intensity distribution using the Stokes-Einstein relation and assuming that SUV had a spherical shape. The viscosity of the buffer was $\eta = 1.003$ mPa·s at 20 °C. The refractive index of the buffer was taken as 1.33. The mean diameter of SUV of MFGM polar lipids, measured in PIPES-NaCl without calcium, was 67 ± 22 nm. Meanwhile, the mean diameter of SUV of milk SM and milk SM/chol, measured in PIPES-NaCl with 5 mM of calcium, were 119 ± 58 nm and 111 ± 33 nm, respectively.

2.2.3. Atomic force microscopy (AFM) imaging and force spectroscopy

AFM imaging and force spectroscopy experiments were performed on supported lipid bilayers (SLB) in hydrated conditions. To form the planar SLB of MFGM polar lipids, 2.7 mL of PIPES-NaCl with 2 mM $CaCl_2$ buffer were pre-heated at 60 °C using a temperature-controlled Bioheater open liquid cell (Asylum Research, Oxford Instruments, Santa Barbara, CA, USA) mounted with 1 × 1 cm^2 freshly cleaved mica. Then, 300 µL of the hot SUV suspension were injected into the liquid cell, to reach 0.1 mg mL^{-1} of lipids. Introduction of calcium ions promoted vesicule fusion onto the mica, with no effect onto the suspension stability for the time of incubation [37]. The samples were then incubated at 60 °C for 1 h using an IPP programmed incubator (Memmert, Büchenbach, Germany) and saturated humidity chambers to prevent evaporation. The liquid cells were then slowly cooled down to 20 °C at a linear cooling rate of 18 °C per hour (~0.3 °C min^{-1}). For the saturated milk SM or the milk SM/chol samples, a small drop of the hot SUV suspension (~600 µL at 0.1 mg mL^{-1} lipids) was directly deposited onto a 7 mm-diameter disk of freshly cleaved mica glued on a stainless steel substrate. The smaller surface of bilayer prepared that way limited the formation of defects and cracks due to the reduction in molecular area upon the fluid/gel phase transition upon cooling milk SM samples. The sample was then incubated at 60 °C for 1 h and cooled down to 20 °C at a linear cooling rate of 35 °C per hour (~0.6 °C min^{-1}). Finally, the sample was carefully immobilized onto the temperature-controlled magnetic Peltier stage of a Cooler-Heater open liquid cell set at 20 °C (Asylum Research, Oxford Instruments, Santa Barbara, CA, USA).

Once equilibrated at 20 °C, the SLB composed of the MFGM polar lipids, milk SM or milk SM/cholesterol were carefully and extensively rinsed with the same PIPES buffer as during incubation onto mica, i.e. including 2 or 5 mM $CaCl_2$ for the MFGM or for the milk SM and milk SM/chol samples, respectively. The SLB were then characterized by AFM at 20 °C and at 50 °C after their heating using the temperature controlled cells, at a rate of 0.5 °C min^{-1}.

Imaging of the SLBs was performed in contact mode using an Asylum Research MFP-3D Bio AFM (Oxford Instrument, Santa Barbara, CA, USA) and MLCT probes (nominal spring constant $k \sim 0.03$ N·m^{-1}, Bruker Nano Surfaces, Santa Barbara, CA, USA). Individual probes were calibrated extemporaneously at 20 °C then 50 °C using the thermal noise method. The sensitivity was taken on the deflection curves beyond the breakthrough point of the SLB, i.e. when the tip was pressed against mica. The typical scan rate was 1 Hz for 512 × 512 pixels images. Up to 8 images with different scan sizes varying from 20 × 20 μm^2 to 2 × 2 μm^2 were recorded at various locations, on three different bilayers. The height difference between the lipid domains and the continuous phase was measured using cross sections of different images.

Force mapping was performed on the same imaged area and using the same probe as for AFM imaging. Typically, an applied load of up to 200 pN and a piezo speed of 2 µm·s^{-1} were used. Then, the bilayers Young modulus E was calculated by fitting the force curves using the classical Hertz model:

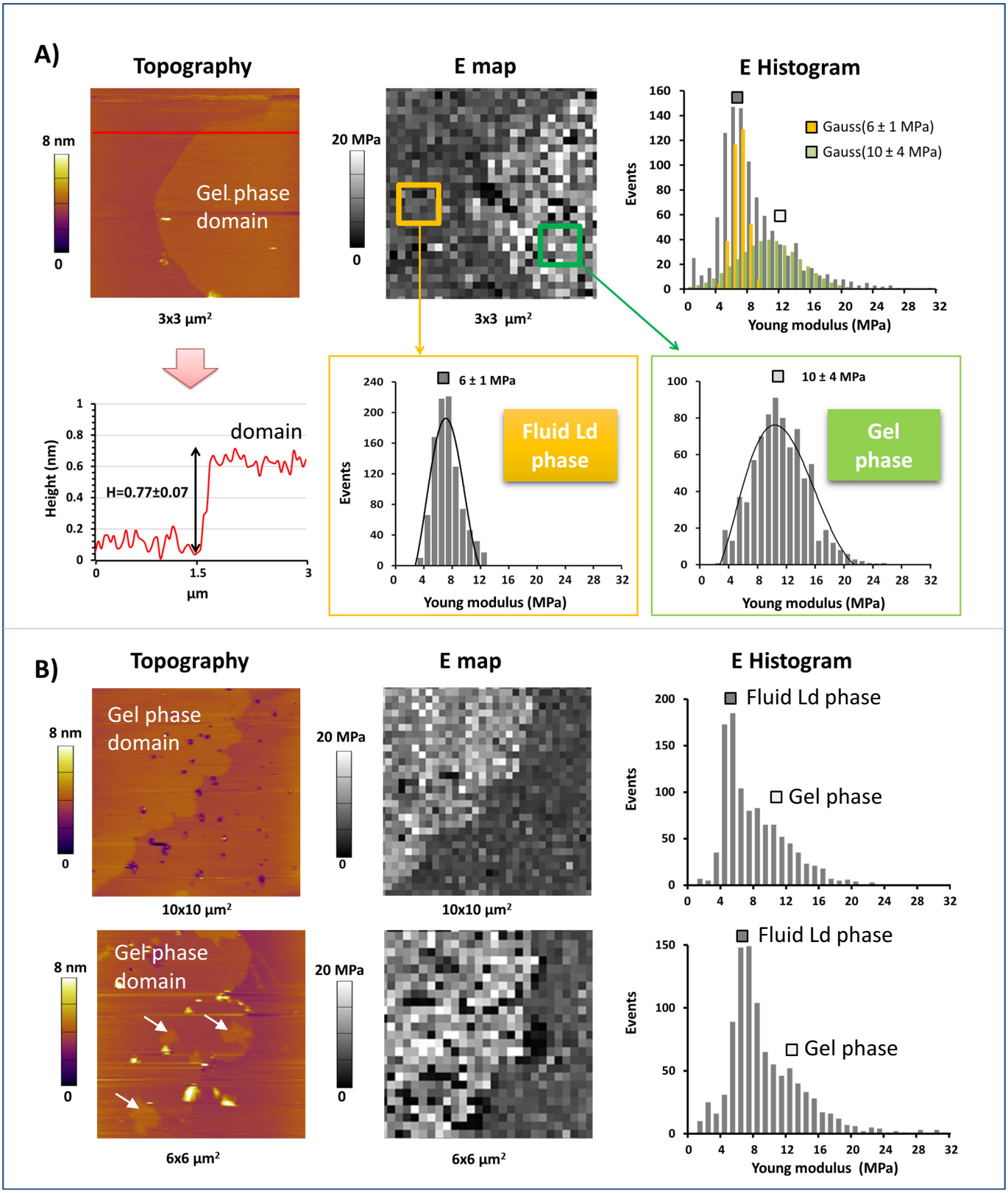

Fig. 2. (A) AFM topography images and the corresponding Young modulus (E) map and histogram of supported milk fat globule membrane (MFGM) polar lipid bilayers. In the orange and green inserts, we show the E histograms obtained after batch analysis of the individual force curves separately recorded upon separated mapping onto the fluid phase or gel phase, respectively. (B) Typical AFM topography images, with different scan sizes, of supported lipid bilayers of mixtures of MFGM polar lipids (left column), the corresponding Young modulus (E) mapping experiments (center column), and the resulting E histograms obtained after batch analysis of the individual force curves (right column). The white arrows indicate sub-domains. The white and dark grey colors of the square symbols refer to the maps' color scale, showing the existence of bimodal distributions. All images and force mapping experiments were performed in PIPES-NaCl-$CaCl_2$ buffer, pH 6.7, at 20 °C.

$$F = \frac{4}{3}\frac{E\delta^{3/2}\sqrt{R_{tip}}}{(1-\nu^2)}$$

where F: force, R_{tip}: tip radius (20 nm), δ: the indentation distance and ν the Poisson ratio, taken as 0.5 [45].

Analysis of variance was performed using the General Linear Model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp., Englewood Cliffs, NJ). Differences were regarded significant for $p < 0.05$.

3. Results & discussion

3.1. Topography and Young modulus of the MFGM polar lipid bilayers investigated as a function of temperature T

3.1.1. T = 20 °C < Tm of the MFGM polar lipids

Fig. 2 shows the topography of MFGM polar lipid bilayers and the corresponding Young modulus maps (E map) and histograms (E histogram). The topography images showed the coexistence of micrometer-wide domains (lighter zones) and a continuous phase (darker matrix). Cross section analysis of the AFM images showed that the height difference *H* between the domains (8 domains from 6 different images) and the surrounding matrix was $H \sim 0.77 \pm 0.07$ nm (Fig. 2A). These AFM images are in agreement with previous observations of hydrated SLB of MFGM polar lipids [13,46]. They are also in accordance with AFM images of binary systems composed of saturated SM in the gel phase and the unsaturated phospholipid dioleoylphosphatidylcholine (DOPC) as the fluid phase, i.e. milk SM/DOPC [41,46] or egg SM/DOPC [47].

The thick domains were interpreted as the lateral segregation of saturated polar lipids in the gel phase at 20 °C, i.e. mainly milk SM but also DPPC. The continuous phase is composed of unsaturated polar lipids that assume a thinner fluid phase organization at T > Tm. AFM force mapping experiment are delicate to handle on SLB, and there are many sources of experimental variation. Fig. 2B shows two other samples of AFM images of SLB composed of MFGM polar lipids to illustrate representativity. Subdomains were sometimes observed within the gel phase, exceeding the domains' surface by about 0.3 nm (Fig. 2B, second image). In MFGM polar lipid extract bilayers, subdomains have been interpreted as two laterally separated gel phases due to distinct forms of interdigitated packing of the milk SM molecules [46].

The E maps recorded at 20 °C clearly showed a correlation between the structural heterogeneity of the bilayers (topography by AFM imaging) and their local Young modulus values (E maps – Fig. 2A and B, center column). The E histograms prepared from the E maps showed a broad range of Young modulus values, with bimodal distribution. To further investigate the Young modulus values of the polar lipids in relation to their phase state, E maps and the corresponding histograms have been recorded separately in the continuous fluid phase or in the gel phase domains. The Young modulus of the fluid phase, rich in unsaturated polar lipids, exhibited a mean value $E_{fluid} = 6 \pm 1$ MPa with a narrow distribution ranging from 4 to 16 MPa (Fig. 2A, in orange). In contrast, the mean Young modulus of the SM-rich gel phase domain was $E_{gel} = 10 \pm 4$ MPa with a distribution ranging from 4 to 32 MPa (Fig. 2A, in green). This allowed us to interpret the bimodal distribution of Young modulus values obtained over the biphasic system (Fig. 2A). The sum of events for each mode corresponds to the total number of pixels for each phase in a force map. Noteworthy, since the Young modulus values of the gel phase are more scattered and not enough higher than those of the fluid phase to form a separate peak, the E distribution of the gel phase is less visible than that of the fluid phase, even when gel phase is well represented. This is illustrated in Fig. 2A where the Gauss distributions of $E_{fluid} = 6 \pm 1$ MPa (in orange) and $E_{gel} = 10 \pm 4$ MPa (in green) are shown next to the E distribution of the biphasic system. Because the probe's geometry will affect calculation of the Young modulus, and because different probes were used to map individual bilayers, absolute values of E have to be taken with care. By comparing different experiments (Fig. 2A and B), it could be calculated that the average difference between the coupled Young modulus values of the gel and the fluid phases in *individual* images (i.e. the same probe is used) was $\Delta E = E_{gel} - E_{fluid} = 4.6 \pm 0.9$ MPa ($N = 5$). In conclusion, bilayers composed of the MFGM polar lipids exhibited significantly higher Young modulus values in the gel phase domains than in the fluid phase. These results are in agreement with Picas et al. [34] who reported Young modulus values of 19 and 28 MPa, respectively, for the fluid and gel phase of a DOPC/DPPC bilayer. As the Young modulus E and the breakthrough force of lipid membrane systems can be somewhat compared [28,35], the present results also agree with previous findings showing that the gel phase domains of MFGM polar lipids bilayers ruptured at higher force compared to the surrounding continuous fluid phase [48]. This is also in line with breakthrough force measurements on a large range of simple systems [49]. However, correspondence between the membrane's behavior in the linear regime (the Young modulus) and upon yield (the breakthrough force) also depends on other factors e.g. compressibility and possibility the internal crystalline gel structure [50]. For these reasons, Young modulus E and breakthrough force data may not always compare.

3.1.2. T = 50 °C > Tm of the MFGM polar lipids

In this set of experiments, changes in the topography and Young modulus of the MFGM polar lipid bilayers due to phase transition of the high-Tm polar lipids were investigated. Fig. 3A shows a typical example of AFM topography images and the corresponding Young modulus map (same area) of a MFGM polar lipid bilayer, recorded at 20 °C (T < Tm) then at 50 °C (T > Tm) after slow heating at 0.5 °C min^{-1}. The thick gel phase milk SM-rich domain, visible in the bilayer at 20 °C (Fig. 3A), completely disappeared after heating at 50 °C, leading to a homogeneous and flat membrane, in agreement with the melting of high Tm polar lipids [51–53]. Fig. 3B shows examples of the force curves recorded on each of the observed phase at 20 or 50 °C.

Furthermore, the bimodal and broad distribution of E values showing the contrast between the domain and the continuous phase at 20 °C evolved towards lower and homogeneous Young modulus values of $E_{50°C} = 4 \pm 2$ MPa upon heating (Fig. 3A), which were significantly lower to those of the continuous fluid phase at 20 °C ($E_{fluid} = 6 \pm 1$ MPa; Fig. 2A; $p < 0.05$). These results show that the physical state of the polar lipids, which evolves as a function of temperature, governs the lateral packing, phase separation and local stiffness of membranes. This is coherent with previous studies reporting low and homogeneous breakthrough force values in bilayers of MFGM polar lipids at 40 °C, compared to 26 °C [48]. Changes in the mechanical properties of the membrane with their phase state is a direct consequence of the order parameter of lateral lipid packing [54]. In gel phase, a tightly packed lateral organization results in an increase of the order parameter and to an enhancement of rigidity of the milk SM-rich domains within membranes of the milk polar lipids. An increase in temperature results in greater membrane fluidity as van der Waals interactions are disrupted and broken. Using the non-invasive measurement of the Young modulus of bilayers of a naturally complex mixture of polar lipids such as MFGM polar lipids, this confirmed previous reports obtained through the perforation approach on pure lipid mixtures. The breakthrough force of dilaureoyl-, dimyristoyl-, and dipalmitoylphosphastidylcholine (DLPC, DMPC, DPPC), but not that of palmitoyloleoylphosphatidylethanolamine (POPE; [55,56]), decreased sharply upon heating, as temperature crosses their respective Tm values [51,52]. The presented results were also in line with the sharp decrease of the bending modulus of phosphatidylcholine vesicles as they undertake gel ➔ fluid phase transition [57–59]. Furthermore, the decrease in Young modulus (or in breakthrough force - [48]) of the fluid phase with temperature is likely due to the increased molecular agitation and increased molecular distance between lipids upon heating of the bilayer [60]. Another reason could be that the melting of the high-Tm polar

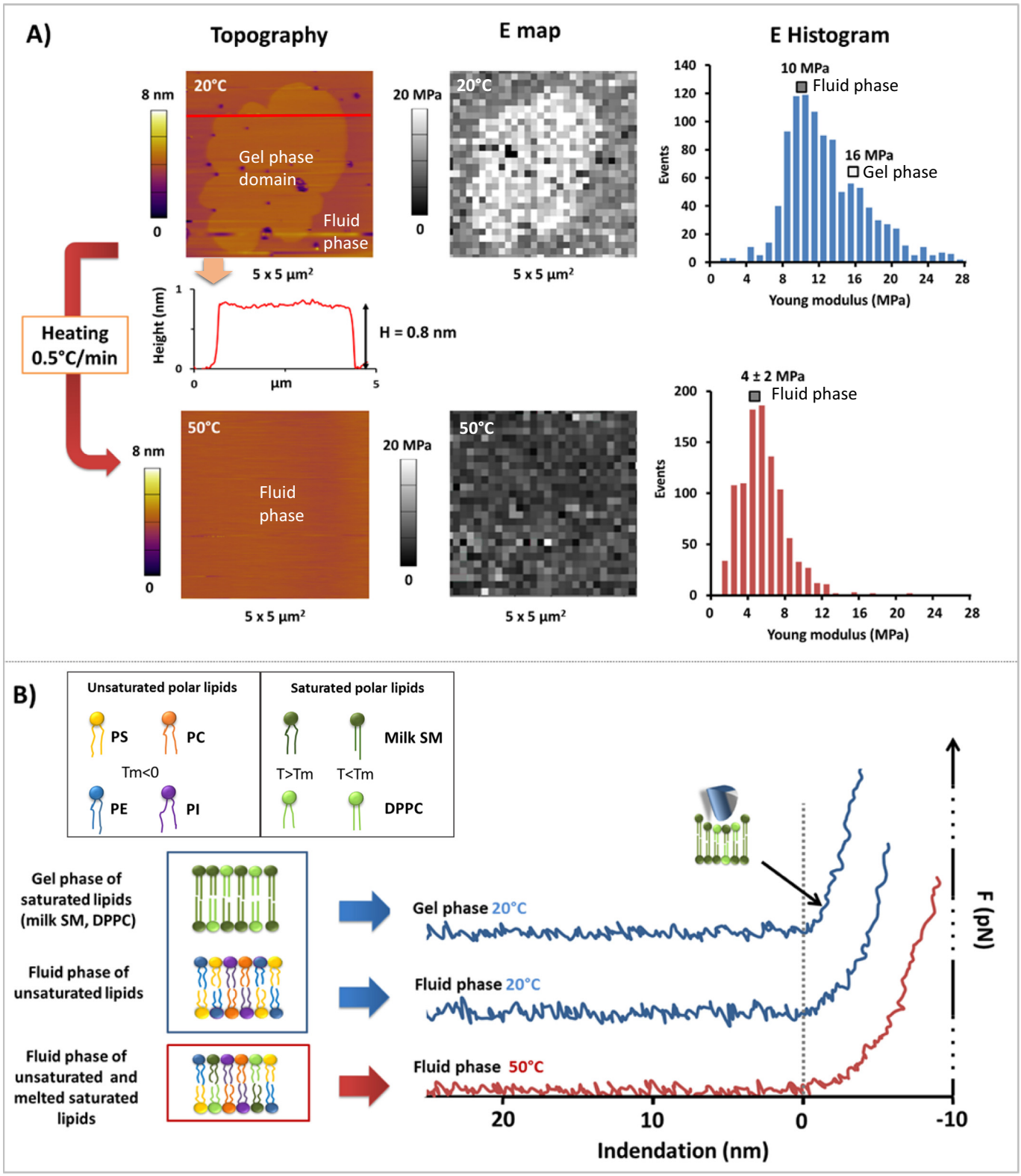

Fig. 3. (A) Effect of heating on the lateral organization and the mechanical properties of MFGM polar lipids supported bilayers investigated by atomic force microscopy (AFM). AFM images and the corresponding Young modulus mapping and histograms were recorded at 20 °C and 50 °C in PIPES-NaCl-$CaCl_2$ buffer, pH 6.7. (B) Typical force-distance curve recorded onto the gel or fluid phases at 20 °C or onto the fluid phase at 50 °C.

lipids modifies the composition of the fluid phase, thereby increasing the complexity for optimal lateral packing of the molecules.

3.2. Role of phase state on the Young modulus of milk-SM bilayers

In the native MFGM, cholesterol may be involved in the formation of liquid-ordered (Lo) phase domains that have been observed at as elevated temperature as 60 °C, e.g. above the Tm of the MFGM polar

lipids or of the milk SM [15]. The Young modulus values of these Lo phase domains, mainly composed of milk-SM and cholesterol, need further investigation to provide information useful in a biological context. Previous reports showed that the addition of 25–30 mol% cholesterol in binary 50:50 mol% SM/DOPC bilayers induced dispersion or inversion of the domain phase [47,61–63]. Specifically, a proportion of 33 mol% cholesterol is required to fully convert milk SM bilayers in the Lo phase [14]. Equally, the addition of cholesterol to the MFGM polar lipid extract bilayers, to reach a milk SM/chol ratio of 50:50 mol%, induced the almost complete dispersion of the Lo phase milk SM-rich domains into the fluid phase [13,37]. Therefore, resolution of the Young modulus mapping was expected to be too low to properly image such systems. For these reasons, it was decided to prepare milk SM and milk SM/cholesterol bilayers, as models of the ordered-phase domains of the MFGM. In this part of the study, we therefore examined the Young modulus values of milk SM bilayers as a function of their phase state, i.e. in the gel phase (T = 20 °C < Tm; [13,64]), in the fluid phase (T = 50 °C > Tm; [13,64]), and in the Lo phase (with 50 mol% of cholesterol in the bilayer, T = 20 and 50 °C; [37]).

Topography images at 20 and 50 °C (Fig. 4B, D, G, I) showed that both the milk SM and the milk SM/chol hydrated bilayers were homogeneously flat, thereby indicating that the composition and phase state of the milk SM/chol bilayers were evenly distributed. In particular, no gel/Lo phase coexistence could be suspected in the milk SM/chol bilayers at 20 °C. At that temperature, the Young modulus values of milk SM in the gel phase ranged from 2 to 50 MPa with a mean value of $E_{milkSM\text{-}gel}$ = 18 ± 6 MPa. These values are slightly higher than those

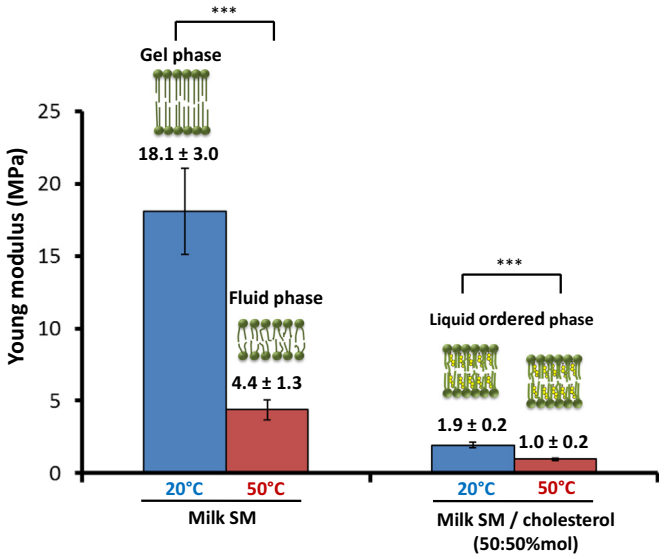

Fig. 5. Comparison of the average Young modulus values obtained at 20 °C or at 50 °C in PIPES-NaCl-$CaCl_2$ buffer, pH 6.7, on indenting milk SM or milk SM/chol (50:50 mol%; liquid ordered phase) supported bilayers. Symbol *** means significant difference ($p < 0.05$; N = 2-9 images for each category).

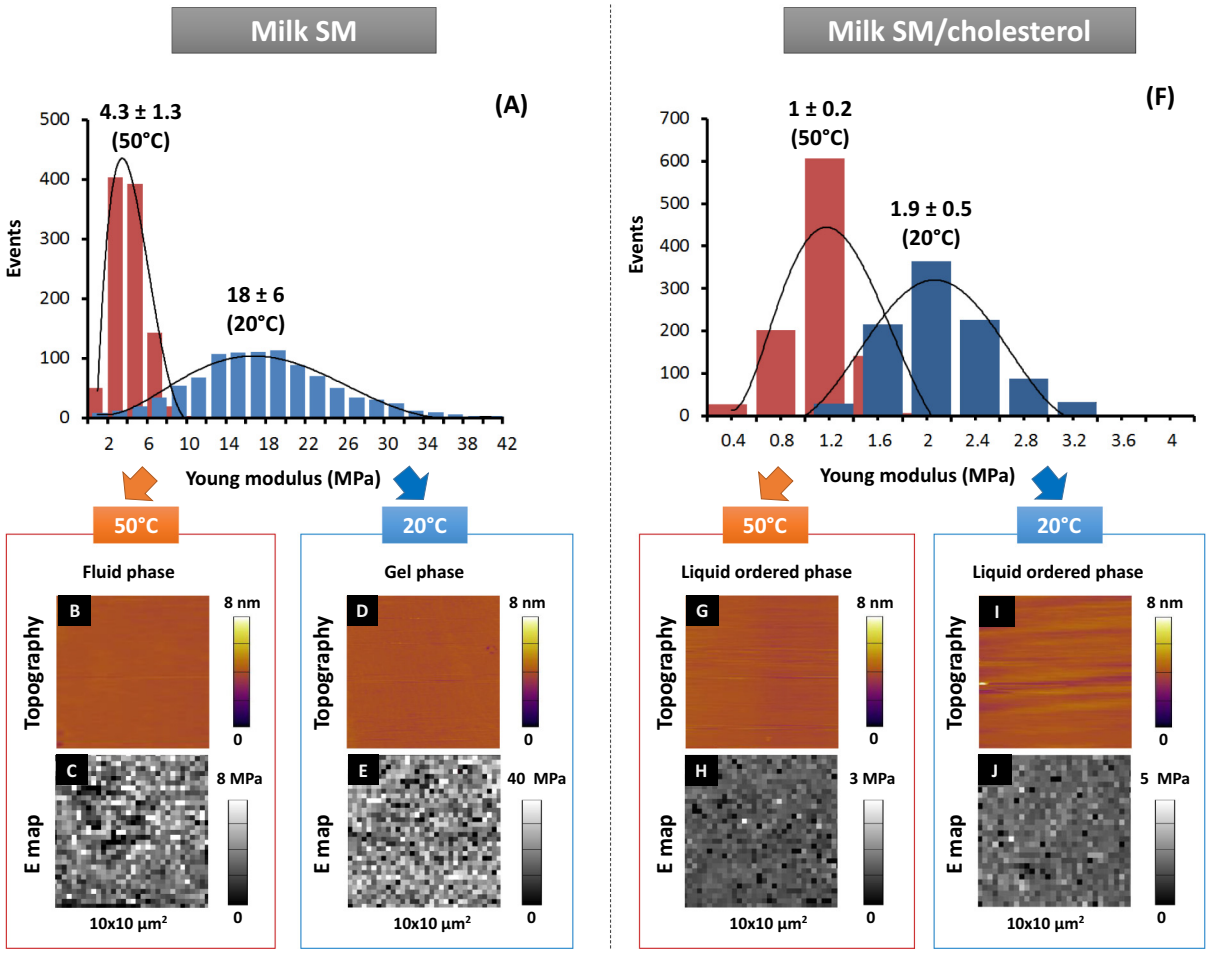

Fig. 4. Effect of the phase state of milk SM, as modified by temperature and cholesterol, on the topography and Young modulus values of supported milk SM bilayers. AFM topography images, Young modulus mapping and the corresponding histograms were recorded at 20 °C or 50 °C in PIPES-NaCl-$CaCl_2$ buffer, pH 6.7.

of the gel phase domains in MFGM polar lipids ($p < 0.05$). This may be explained by differences in the polar lipid compositions, where domains in the MFGM polar lipid extract may also contain other saturated molecules than the milk SM (e.g. DPPC), thereby increasing the complexity and decreasing order. Furthermore, the increased molarity in calcium in the buffer may increase molecular order and the membrane rigidity [44,65]. At 50 °C, all Young modulus values of milk SM membranes in the fluid phase were lower than 10 MPa, with a mean value of $E_{milkSM\text{-}fluid} = 4.3 \pm 1.3$ MPa (Figs. 4A and C; 5). This result showed that the melting of the gel phase of milk SM into the fluid phase, induced by heating the samples from 20 to 50 °C, significantly decreased the Young modulus values E of the milk SM membranes. This is in agreement with the results obtained with the MFGM polar lipids (Fig. 2), where the milk SM-rich domains are also expected to be in the gel phase at 20 °C.

Fig. 5 furthermore shows a comparison between the Young modulus values recorded on the same milk SM and milk SM/chol (50:50 mol%) bilayers at 20 °C then 50 °C. In the presence of cholesterol, the Young modulus values of the milk SM bilayers in the Lo phase were $E_{milkSM\text{-}Lo\text{-}20°C} = 1.9 \pm 0.5$ MPa and $E_{milkSM\text{-}Lo\text{-}50°C} = 1 \pm 0.2$ MPa at 20 °C and 50 °C, respectively. Therefore, the Young modulus value of milk SM in the gel phase was higher than that of the same lipid in the Lo phase. This in accordance with the described fluidizing effect of cholesterol onto gel-phase polar lipid packing of sphingomyelins [3,4,7,66–69] or of DPPC [70]. By intercalating between lipid acyl tails, cholesterol prevents ordered packing of lipids, thus increasing their freedom of motion [66]. In the same line, cholesterol has also been shown to decrease the bending rigidity of gel-phase sphingomyelin in vesicle membranes [58,71]. The fluidizing effect of cholesterol was also indirectly observed in domains of MFGM polar lipids membranes, through the decrease of the height mismatch *H* and of the breakthrough force values measured onto the domains with cholesterol addition [13,37].

When comparing the different compositions (i.e. different bilayers), it appeared that the Young modulus values of the milk SM/chol were somewhat lower than those of the fluid phase of pure milk SM at 50 °C (Figs. 4C, H and J, 5). This was unexpected as order of the Lo phase is normally greater than that of the fluid phase [4], thanks to the condensing effect of cholesterol that reduces molecular area of the (fluidized) saturated polar lipids in the domains [72]. Tighter lipid packing is expected to increase interactions between adjacent molecules and hence, mechanical resistance. In melted DPPC bilayers ($T > Tm = 41$ °C) or in dilaureoylphosphatidylcholine bilayers, Redondo-Morata et al. [52] or Garcia-Manyes et al. [49] measured greater breakthrough force values in the presence (Lo phase) than in absence of cholesterol (fluid phase). Similarly, Liang et al. [73] showed that Egg PC bilayers ($T > Tm = -15$ °C) exhibited higher Young modulus when cholesterol was added (i.e., in Lo phase rather than fluid phase). From the analysis of liposomes of several compositions with both fluorescence and AFM force spectroscopy, Takechi-Haraya et al. [74] calculated increasing bending rigidities in the order fluid < Lo < gel phase. On the other hand, interfacial elasticity has been shown to be lowest for the Lo phase, when compared to that of liquid-condensed (~gel) or liquid-expansed (~fluid) phases of sphingomyelin monolayers [75]. In bilayers made of red blood cell lipid extract, García-Arribas et al. [76] measured as low breakthrough forces as those of a fluid phase, even though the bilayers were expected to be in Lo phase due to their cholesterol content.

Finally, it could be noticed that milk SM in the Lo phase at 20 °C exhibited lower Young modulus values (2 MPa) than the fluid phase of the MFGM at the same temperature (~6 MPa). Chiantia et al. [77] or Sullan et al. [63] however reported that the force needed to puncture Lo SM-rich domains were significantly higher than for a fluid DOPC-rich phase, but this essentially holds true for amounts of cholesterol < 25 mol%. For higher amounts, both fluid and Lo phases of a biphasic saturated SM/unsaturated polar lipids bilayers merge into similar breakthrough force values [37].

Overall, we conclude that milk SM bilayers exhibited dramatically higher Young modulus values (tens of MPa order or magnitude), hence stiffness, when in the gel phase, as compared with the fluid or Lo phases (MPa order of magnitude). The latter phases showed close Young modulus values of a few MPa, but the Lo phase was unexpectedly less stiff than the fluid phase [74].

These results show that cholesterol plays an important role in modulating the stiffness or softness of biological membranes, by fluidizing the gel phase state into Lo molecular packing. Moreover, cholesterol in equimolar amount to the milk SM counterpart is capable of maintaining the membrane phase into a stable Lo phase structure, with slightly decreasing Young modulus values with increasing temperature ($p < 0.05$). In cells, and most importantly in SM-rich cellular "rafts", cholesterol is likely to be implicated in the regulation of membrane curvature, which is important for many cellular events such as membrane fusion, budding, etc. [74,78–81].

In the MFGM, the cholesterol content is in the order of 1 mol of chol for ~2 mol of polar lipids, i.e. over 1 to 1 molecule with respect to the milk SM only. Meanwhile, the human erythrocyte membrane contains up to 45 mol% of cholesterol [82,83]. Even though the distribution and fluxes of cholesterol in cells are complex [84], this indicates that gel phase is probably inhibited in many biologically relevant situations, even though indirect observations pointed to some gel phase domains in the MFGM [15], not to mention the role of other compounds like ceramides [76]. The high amounts of cholesterol in plasmatic membranes, compared to those used in model vesicle or supported membranes, may account for the lower Young modulus values generally found when directly probing cells or milk fat globules. Phase diagrams [85] or nanomechanical data [76] point to the complex, and sometimes unexpected, behavior of polar lipid mixtures with biological composition. In the same line, milk SM vesicles are significantly less stiff that vesicles of the single sphingomyelins, probably as a result of disorder in the packing of milk SM [86]. Research is still necessary to more closely investigate structures of the lipid phases actually existing in biological membranes and their role in the modulation of some of the cells functional properties [82,87]. In the case of the milk fat globule, these research open perspectives for a better understanding of its reactivity towards mechanical or heat stresses, and hence for a better control of its maneuverability during dairy operations. Noteworthy, phase diagrams for detailed knowledge on the demixing behavior of milk polar lipids with composition and temperature is still missing, knowing that milk SM has a particularly complex composition. Finally, better knowledge of the MFGM and its physical properties will help the design of biomimetic milk fat globules or of milk-lipid based vesicles with designed mechanical properties.

4. Conclusion

Combination of AFM imaging and the non-invasive mapping of the Young modulus values of MFGM, milk SM and milk SM/chol bilayers has evidenced direct correlation between the lipid phases and their elastic property. Among all phases, the gel phase formed in milk SM rich domains at $T < Tm$ exhibited largest Young modulus values of the tens of MPa order of magnitude. Meanwhile, liquid-disordered and liquid-ordered phases exhibited Young moduli of a few MPa. The fact that the Young modulus value of the Lo phase was almost invariant with temperature adds to the multiple actions of cholesterol towards polar lipid bilayers [88]. This is new evidence that the finely tuned and complex sterol/polar lipid composition of biological membranes such as the MFGM is in the core of the membranes' biological functions.

Transparency document

The Transparency document associated with this article can be found, in online version.

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