The dynamics of the biological membrane surrounding the buffalo milk fat globule investigated as a function of temperature

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ABSTRACT

The biological membrane surrounding fat globules in milk (the MFGM) is poorly understood, despite its importance in digestion and in determining the properties of fat globules. In this study, *in-situ* structural investigations of buffalo MFGM were performed as a function of temperature (4 – 60°C) using confocal microscopy. We demonstrate that temperature and the rate of temperature change affected the lipid domains formed in the MFGM with the lateral segregation i) of high Tm lipids and cholesterol in a Lo phase for both T < Tm and T > Tm and ii) of high Tm lipids in a gel phase for T < Tm. Rapid cooling favours nucleation, while slow cooling favours the growth, leading to the formation of small and large lipid domains respectively. Changes in the interfacial properties of the MFGM as a function of temperature could modulate the functions of fat globules during processing and digestion.

Keywords: milk fat globule membrane, confocal laser scanning microscopy, lipid phase separation, lipid domain, sphingomyelin, microstructure variations

1. Introduction

Milk is a natural oil-in-water emulsion in which the organization of lipids is complex and specific to this biological fluid (Lopez, 2011). Milk lipids are organized as colloidal assemblies called milk fat globules, the core of which is mainly composed of triacylglycerols (TAG, 98% of milk lipids) covered by a biological membrane that governs all the interfacial phenomena (e.g. hydrolysis of TAG by the digestive enzymes). These biological entities are secreted by all mammal females to deliver lipids and bioactive molecules to the gastrointestinal tract of newborns (Lopez, 2011). Milk fat globules are also functional elements within many dairy products consumed by infants and human adults (e.g. creams, cheeses). Despite the importance of the biological membrane surrounding fat globules in milk in determining the nutrition and in the properties of many dairy products this membrane is not well understood.

The milk fat globule membrane (MFGM) is thought to be comprised of three layers of polar lipids and proteins as a result of the mechanisms involved in milk fat globule secretion from the epithelial cells of the mammary gland (Heid & Keenan, 2005; Lopez, 2011). The first layer originates from the endoplasmic reticulum, while the outer bilayer results from the envelopment of milk fat globules in the apical plasma membrane of the epithelial cells during their secretion. The MFGM contains membrane-specific proteins and different classes of lipids such as the glycerophospholipids (i.e. phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; and phosphatidylserine, PS), the sphingolipids (mainly sphingomyelin, SM), and cholesterol (Heid & Keenan, 2005; Le, Van Camp & Dewettinck, 2014; Lopez, 2011; Ménard, Ahmad, Rousseau, Briard-Bion, Gaucheron & Lopez, 2010). Previous studies have reported that PE, PI and PS are mainly concentrated on the inner surface of the MFGM while SM, PC and the glycolipids are mainly

62 located in the outer bilayer of the MFGM (Deeth, 1997). The packing of the lipids and
63 proteins located in the outer bilayer of the MFGM has been recently further characterized
64 using confocal microscopy with fluorescent dyes. Structural analysis of the MFGM have
65 revealed heterogeneities i) in the lateral organization of the components (Evers, 2008), ii) in
66 the localization of membrane proteins (Lopez, Madec & Jimenez-Flores, 2010; Lopez &
67 Ménard, 2011; Nguyen et al., 2015) and iii) in the lateral packing of polar lipids with the
68 occurrence SM-rich domains at the surface of fat globules from bovine milks (Gallier,
69 Gragson, Jimenez-Flores & Everett, 2010; Lopez et al., 2010), breast milks (Lopez &
70 Ménard, 2011; Zou et al., 2012) and buffalo milks (Nguyen et al., 2015). These lipid domains
71 correspond to phase separation, where the non-fluorescent areas may be liquid ordered (Lo)
72 phase or gel phase domains while the fluid liquid disordered (Ld) phase form the surrounding
73 fluorescent matrix (Gallier et al., 2010; Lopez et al., 2010). The hypothesis is that the Lo
74 phase domains of the MFGM are rich in polar lipids with high phase transition temperature
75 (mainly SM that accounts for about 25% of polar lipids in the MFGM (Lopez, 2011), but also
76 saturated phospholipids and cholesterol), while the Ld phase is mainly comprised of
77 unsaturated glycerophospholipids (PC, PE, PI, PS) (Lopez et al., 2010; Lopez & Ménard,
78 2011). SM contains long chain saturated fatty acids (Sanchez-Juanes et al., 2009) responsible
79 for its high phase transition temperature (Tm = 34.3-35°C, Malmsten, Bergentahl, Nyberg &
80 Odham, 1994; Murthy, Guyomarc'h, Paboeuf, Vié & Lopez, 2015). Dipalmitoyl
81 phosphatidylcholine (DPPC) is also characterised by a high Tm (Tm = 41.1-41.7°C, Benesch
82 & McElhaney, 2014). Polar lipids containing one unsaturated fatty acid chain, such as DOPE,
83 also have a Tm above room temperature (Murthy et al., 2015). These high Tm lipids could
84 pack in the MFGM and segregate from the fluid Ld phase of unsaturated polar lipids to form
85 domains in the gel phase, as recently shown in model membrane with milk SM (Guyomarc'h
86 et al., 2014) and in monolayers of MFGM lipid extracts (Murthy et al., 2015). The domains

formed by milk SM in the absence of cholesterol (gel phase) have different shapes, different nanomechanical properties and protrude from the fluid phase of the membrane with a higher dimension than the domains formed in the presence of cholesterol (Lo phase) (Guyomarc'h et al., 2014).

The characteristics of the lipid domains (e.g. number, size, shape, lipid phase, nanomechanical properties) could be affected by temperature. Consequently changes induced by fluctuations in temperature could have consequences for the mechanisms involved in the absorption of lipids in the gastrointestinal tract (37°C) and in dairy processing where storage can be at 4°C and heat treatments can occur at 60°C or higher, potentially altering the mechanical properties of the fat globule. The SM-rich domains in the human MFGM have been found to be responsive to temperature, with a decrease in domain size observed when the temperature increased (Zou et al., 2012). The effect of temperature on the microstructure of the MFGM of other species, such as the temperature sensitivity of the lipid domains, needs to be further investigated to gain a better understanding of the factors affecting the organization of the MFGM and the species differences of these observations. Specifically, the change in lipid domains in the outer bilayer of the MFGM as a function of temperature will be useful not only to better understand the function of these globules in the gastrointestinal tract but also for understanding the potential impact of changes occurring during dairy processing.

Information about buffalo milk and the microstructure of the buffalo MFGM is scarce compared to other milks from cows, goats and humans. Yet buffalo milk comprises approximately 13% of total world milk production (about 97 million tons per year) (IDF, 2009). Previous studies have demonstrated that the physicochemical properties of buffalo fat globules (e.g. size, zeta potential, composition of the MFGM) are different to bovine fat globules (Ménard et al., 2010). The amount of buffalo polar lipids, 2.6 mg/g fat and about

190 mg/L of buffalo milk, is 28% higher than the amount of bovine milk polar lipids (Ménard et al., 2010). The relative proportion of SM, which is assumed to be the major component of the lipid domains found in the MFGM (Lopez et al., 2010; Lopez & Ménard, 2011; Zou et al., 2012), is lower in buffalo MFGM compared to bovine MFGM (24.8% vs. 26.9% of polar lipids) (Ménard et al., 2010). Also, the amount of cholesterol is lower in buffalo milk as compared to bovine milk (7.0-10.2 mg/100mL vs. 10.5-19.8 mg/100 mL) (Strzalkowska, Jozwik, Baghnicka, Krzyzewski & Horbanczuk, 2009; Talpur, Memom & Bhanger, 2007; Zotos & Bampidis, 2014). These differences in lipid composition may provide structural specificities to the buffalo MFGM that require further investigation. In a first paper (Nguyen et al., 2015), we performed *in situ* structural investigations of the buffalo milk fat globules, showed the presence of cytoplasmic remnants, and characterized the heterogeneous distribution of proteins and lipids in the outer bilayer of the MFGM. All these experiments have been conducted at room temperature.

The objective of this work was to investigate the microstructure of the buffalo MFGM as a function of temperature and rate of temperature change (slow vs. rapid temperature gradients). The MFGM was characterized using confocal laser scanning microscopy (CLSM) under well-controlled temperatures ranging from 60°C to 4°C. This range of temperatures is pertinent to the technological processes used in the dairy industry and for the consumption and digestion of milk and dairy products.

2. Materials and methods

2.1. Buffalo milk samples

The buffalo milks used in this study were provided by Coopérative de Bufflonnes (Maurs, Cantal region, France). These milks corresponded to a mixture of the individual milks produced by 30 buffaloes of the Mediterranean breed *Bubalus bubalis* and collected from evening and morning milking. The growth of bacteria was prevented by adding NaN_3 (0.02% w/v) to the milks after their collection. The milk samples were stored at ambient temperature before microstructural analysis.

2.2 Microstructural analysis

An inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France) was used for the confocal laser scanning microscopy (CLSM) experiments, with a He-Ne laser operating at 543 nm wavelength excitation and emission detected between 565 nm and 615 nm. The observations were performed using a ×100 (numerical aperture NA 1.4) oil immersion objective. The staining protocols followed previously described methods for the investigation of the lateral packing of lipids in the outer bilayer of the MFGM (Lopez et al., 2010; Lopez & Ménard, 2011). Briefly, N-(Lissamine rhodamine B sulfonyl) dioleoylphosphatidyl ethanolamine (Rh-DOPE, concentration of 1 mg/mL in chloroform; Avanti polar lipids Inc., Birmingham, England) was used to label the phospholipids in the MFGM, *in situ* in the buffalo milks. Wheat germ agglutinin Alexa fluor 488 (WGA488, Invitrogen, Cergy Pontoise, France) was prepared at a concentration of 1 mg/mL in phosphate saline buffer and used to label the glycosylated molecules in the membrane, i.e. carbohydrate moieties containing N-acetylglucosamine and N-acetyl neuraminic acid (sialic acid) residues. A volume of 40 µL of the Rh-DOPE solution was placed in a glass vial and the chloroform was evaporated under nitrogen, to avoid the possible artefacts caused by this organic solvent. For dual staining of the MFGM polar lipids and carbohydrate moieties, 10 µL of the WGA-488 solution was also added into the vial containing Rh-DOPE. Then 1 mL of buffalo milk

158 sample was introduced in the vial. The stained milk samples were kept at room temperature
159 for at least 1 h prior to observation by CLSM. Optical microscopy using differential
160 interference contrast (DIC, also called Nomarski, (Cogswell & Sheppard, 1992) was also used
161 to characterize buffalo milk fat globules. DIC images were superimposed on the fluorescent
162 emission recorded in the CLSM images. The two dimensional images had a resolution of
163 512×512 pixels and the pixel scale values were converted into micrometers using a scaling
164 factor.

165 The microstructural analyses were performed at room temperature (19 ± 1°C) or between 4°C
166 and 60°C using a temperature-regulated stage (Linkam Scientific Instruments Ltd., Tadworth
167 Surrey, England). The rate of temperature change was controlled during cooling at dT/dt =
168 2°C/min and 20°C/min and during heating from at dT/dt = 2°C/min. For rapid temperature
169 gradient on cooling, the buffalo milks were heated at 60°C in a water bath and the sample (10
170 µl) was applied on a glass slide precooled at 4°C in the temperature-regulated stage of the
171 microscope (estimated rate of temperature dT/dt > 100°C/min).

172

173 # 3. Results and discussion

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175 ## *3.1 Microstructure of the buffalo MFGM at room temperature*

176 The microstructure of the buffalo MFGM was investigated *in situ* in milk at room temperature
177 (19 ± 1°C). As expected, the TAG-rich core of milk fat globules was not labelled by the
178 exogenous phospholipid Rh-DOPE (Figure 1A). The fluorescence from Rh-DOPE was
179 heterogeneously distributed in the membrane (Figure 1A). Figure 1 E shows the
180 heterogeneous distribution of glycosylated molecules (i.e. glycoproteins that are the main

MFGM proteins and glycolipids) in the MFGM. The double fluorescent labelling, i.e. RhDOPE and the lectin WGA-488, shows that the non-fluorescent areas observed with Rh-DOPE do not correspond to the localisation of proteins in the MFGM. The recording of non-fluorescent areas around milk fat globules was interpreted as the lateral packing of some lipids leading to the formation of domains in the MFGM (Figure 1A). These non-fluorescent areas observed at the surface of milk fat globules exhibited different shapes, ranging from circular shape with a diameter of 0.5 to 3 μm (Figure 1C) to irregular shapes (Figure 1 D and E). Figure 1 E shows an example of large irregular domains spreading at the surface of a buffalo milk fat globule. These domains could result from the connection of several circular domains at the surface of fat globules or from the different physical states of the lipids in the domains. The number of the lipid domains ranged from one (Figure 1 E) to six (Figure 1 C) on one side of fat globule.

These results are consistent with previously reported structural analysis of the buffalo MFGM performed at room temperature (Nguyen et al., 2015) and are in agreement with results obtained in the MFGM of various mammal species (Gallier et al., 2010; Lopez et al., 2010; Lopez & Ménard, 2011; Zou et al., 2012; Zou et al., 2015). The non-fluorescent areas characterised in the MFGM at room temperature are interpreted as the preferential tight packing of high gel to liquid crystalline phase transition temperature (high Tm) polar lipids that are composed by long-chain saturated fatty acids (i.e. SM and saturated phospholipids). From a physical point of view, these domains could be formed by lipids organized in the gel phase for $T < Tm$. Also, high Tm polar lipids can segregate together with cholesterol in the plane of biological membranes to form lipid domains in the Lo phase (Brown & London, 1998). A way to better understand the physical state of the lipid domains is to increase the temperature to reach $T > Tm$ of the saturated polar lipids located in the buffalo MFGM.

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206 ***3.2 Microstructure of the buffalo MFGM as a function of temperature and rate of***
207 ***temperature change***

208 The microstructure of the buffalo MFGM was investigated in various thermal conditions
209 applied between 60°C to 4°C by controlling the *in situ* temperature using a heating stage
210 directly under the microscope.

211 In a first set of experiments, the buffalo milks have been heated to 60°C and the impact of the
212 rate of cooling has been investigated. At 60°C, CLSM experiments showed the heterogeneous
213 distribution of both glycosylated molecules (mainly glycoproteins) and polar lipids and the
214 presence of non-fluorescent areas with circular shapes dispersed in the buffalo MFGM
215 (Figure 2). Interestingly, these experiments revealed the phase separation of polar lipids at
216 60°C and the occurrence of domains in the outer bilayer of the buffalo MFGM. The
217 temperature T = 60°C is higher than the Tm of milk SM and saturated PC molecular species
218 located in the membrane (Tm = 34.3 - 35°C for milk SM and Tm = 41.1 - 41.7°C for DPPC;
219 Benesch & McElhaney, 2014; Malmsten et al., 1994; Murthy et al., 2015). This temperature is
220 also higher than the final temperature of melting recorded with MFGM lipid extracts (Murthy
221 et al., 2015). Hence, the presence of domains formed by the high Tm lipids was not expected
222 in the MFGM heated at 60°C. Also, all TAG located in the core of fat globules are melted at
223 60°C (Lopez, Bourgaux, Lesieur & Ollivon, 2007). Then, crystals of solid TAG cannot be
224 responsible for the non-fluorescent areas observed at 60°C in the buffalo MFGM.

225 The impact of the rate of cooling milk from 60°C down to 4°C on the microstructure of the
226 MFGM and on the number and size of the lipid domains was investigated using Rh-DOPE.
227 On cooling of the milk samples from 60°C down to 20°C and then to 4°C at a rate dT/dt

228 ~2°C/min using the temperature-controlled stage under the microscope (with a plateau of
229 constant temperature for at least 10 min), we observed an increase in the size of the non-
230 fluorescent areas when the temperature decreased, which was interpreted as a growth of the
231 lipid domains (Figure 3). The independent non-fluorescent areas observed at 60°C tended to
232 connect and fuse at lower temperature. The increase in the area occupied by the lipid domains,
233 observed at the surface of milk fat globules when the temperature decreased, corresponds to
234 an increase in the proportion of the polar lipids able to integrate within the domains. This is
235 due to changes in the physical state of high Tm lipids with a transition from Ld phase to a
236 more ordered state for T < Tm forming packed domains in which the fluorescent dye Rh-
237 DOPE does not partition. On rapid cooling (dT/dt > 100°C/min) of the milk samples from
238 60°C down to 4°C, CLSM experiments revealed the formation of a high number of small non-
239 fluorescent areas with irregular shapes at the surface of the MFGM (Figure 4). By comparing
240 the microstructure of the MFGM at 4°C after cooling either at dT/dt ~ 2°C/min or at dT/dt ~
241 100°C/min, we observed that rapid cooling from 60°C favoured the nucleation mechanism of
242 the lipid domains instead of their growth. It is possible that the lipid domains formed in the
243 outer bilayer of the MFGM require long times to come into complete size equilibrium. The
244 differences in the shapes of the lipid domains induced by the rate of temperature change could
245 be interpreted as variations in the local composition of lipids (high Tm lipids vs. high Tm
246 lipids in interaction with cholesterol) and to the physical phase of the lipids (gel vs. Lo phase,
247 respectively). For the first time, these experiments showed that the rate of cooling milk from
248 60°C down to 4°C affected the number, the size and the shape of the lipid domains formed in
249 the biological membrane surrounding buffalo milk fat globules.

250 In a second set of experiments, the microstructure of the buffalo MFGM was investigated
251 after cooling at 4°C from room temperature. After storage of milk at room temperature (19 ±
252 1°C), milk samples were cooled rapidly (dT/dt ~20°C/min) down to 4°C directly in the

253 temperature-controlled stage inserted under the microscope. At room temperature, spherical
254 non-fluorescent domains with a size from 0.5 to 2 μm were formed in the outer bilayer of the
255 biomembrane surrounding milk fat globules (Figure 5 A). Rapid cooling down to 4°C
256 changed the morphology of the lipid domains. The surface of these fat globules showed an
257 increase in the connectivity between the lipid domains and a transition from circular shapes to
258 elongated and irregular shapes (Figure 5 B). These results showed that the initial temperature
259 (T=20°C < Tm vs. T=60°C > Tm of saturated polar lipids) and initial physical state of the
260 lipids affect the number, the size and the shape of the domains formed in the buffalo MFGM
261 at 4°C.

262 In a third set of experiments, we characterised changes in the microstructure of the MFGM as
263 a function of temperature on heating of milk from 4°C up to 60°C and returning again to 4°C
264 (Figure 6 A-C). The heating and cooling rates were dT/dt ~ 2°C/min, with plateaus at constant
265 temperature (e.g. 20°C, 40°C and 60°C) during 10 min. Although heterogeneities in the
266 number, shape and size of the domains existed between fat globules at 4°C, the main
267 observation for all fat globules was a decrease in the size of the domains with an increase in
268 temperature (Figure 6 A-C). This could correspond to a solubilisation of high Tm polar lipids
269 from the domain to the fluid phase, due to their phase transition from the gel phase to the Ld
270 phase. The fluid phase at the periphery of the domains allows integration of the exogenous
271 fluorescent phospholipid Rh-DOPE. The domains exhibited irregular shapes at 60°C, similar
272 to those observed at 4°C, possibly due to the additional time required to form spherical
273 domains. Interestingly, the changes in the morphological characteristics of the lipid domains
274 appear to be not completely reversible when the temperature decreases back to 4ºC (Figure 6
275 C-F). This may reflect the timeframes of the experiments and a longer period of incubation
276 may allow equilibrium to be established.

Few authors have investigated the microstructure of the MFGM as a function of temperature. At the physiological temperature T = 37°C, non-fluorescent lipid domains have been observed in the human MFGM (Lopez & Ménard, 2011) and in the bovine MFGM (Zou et al., 2015). Authors reported a decrease in the number and size of the domains when the temperature is increased from 4°C to 37°C (Zou et al., 2012). In the bovine MFGM, authors reported a decrease in size and a more circular appearance of the Lo domains when the staining temperature increased from 22°C to 45°C and a reverse of this behaviour was also observed when the temperature decreased from room temperature to 4°C (Gallier, 2010). It should be noted that the experimental conditions applied could affect the microstructure of the MFGM. In some studies (Gallier, 2010; Zou et al., 2012; Zou et al.,2015), the staining of milk samples was performed at different temperatures but the temperature was not regulated during CLSM investigations. In this study, we characterised the microstructure of the buffalo MFGM and the effect of cooling rate (100°C/min < dT/dt ≤ 2°C/min) in the temperature range from 60°C to 4°C, directly under the microscope in well-controlled thermal conditions. The readers should note that the cooling and heating temperatures used in this study are reasonably fast compared to typical silo cooling conditions in dairy manufacturing.

3.3. Lateral separation of compositionally distinct polar lipid phases: impact of the temperature and rate of temperature change

In this study, we have shown that the MFGM is a non-random mixing of glycoproteins, glycolipids and polar lipids that is highly sensitive to temperature and to the rate of temperature change. We showed that the non-fluorescent areas observed at 60°C do not correspond to the location of membrane proteins (Figure 2) and that they result from the preferential partitioning of Rh-DOPE in the Ld phase of the buffalo MFGM, as previously

301 reported in simple lipid systems (Baumgart et al., 2007; Chen & Santore, 2014). Figure 7
302 proposes a schematic representation of the organisation of polar lipids in the buffalo MFGM,
303 as a function of temperature (i.e. for T < Tm and T > Tm of the saturated polar lipids, the
304 physical behaviour of which changes as a function of temperature). The non-fluorescent lipid
305 domains observed at 60°C cannot correspond to the lateral segregation of a gel phase formed
306 by high Tm polar lipids (milk SM and saturated phospholipids) since these lipids are in a fluid
307 state at this temperature (e.g. T=60°C > Tm). Authors previously showed by using differential
308 scanning calorimetry and X-ray diffraction that for concentration of cholesterol above 40%
309 mol, the gel to Ld phase transition of SM is abolished, that temperature-dependent changes in
310 the lamellar organization of SM do not occur and that the bilayers are in the Lo phase
311 (Chemin et al., 2008). Hence, we interpret that the lipid domains characterised by a circular
312 shape at 60°C correspond to the lateral segregation of high Tm lipids, mainly milk SM, with
313 cholesterol into a Lo phase (Veatch & Keller, 2005) (Figure 7). For T < Tm of milk SM and
314 other saturated phospholipids, the coexistence of two ordered phases (i.e. Lo phase domains
315 including high Tm lipids and cholesterol and gel phase domains composed only by high Tm
316 lipids) with one fluid phase composed by unsaturated polar lipids may occur (Figure 7), as
317 already discussed for ternary model systems (Veatch & Keller, 2005). It is likely that the gel
318 phase and the Lo phase formed at low temperatures do not represent true thermodynamic
319 phase separation but rather phase coexistence. The gel and Lo phases are immiscible with the
320 fluid phase and form micron-scale domains, permitting their observation by confocal
321 microscopy. The role played by cholesterol in the MFGM is important since a minimum
322 molar percentage of cholesterol is necessary to form the Lo phase with high Tm lipids, as
323 reported in phase diagrams built with controlled lipid compositions (Veatch & Keller, 2005).
324 The Ld phase is composed by unsaturated polar lipids, whatever the temperature and also by
325 high Tm lipids for T>Tm (Figure 7). Studies have shown that the diet affects the fatty acid

composition of the polar lipids located in the MFGM and can affect the Tm of the polar lipids (Lopez, Briard-Bion & Ménard, 2014). We can therefore hypothesize that the changes in the microstructure of the MFGM observed as a function of temperature could be affected, for example by the lowering Tm of the saturated phospholipids through dietary changes.

The organisation of lipids in the biological membrane surrounding fat globules in milk can impact the chemical and enzymatic reactions that occur at the TAG/water interface, affect the localisation of membrane-associated proteins and may be involved in the functional properties of fat globules. The temperature dependence of the phase behaviour of lipids located in the MFGM is of interest not only for the process-ability of fat globules but also for nutrition. The MFGM plays a key role in the physical stability of fat globules in milk (i.e. protection against coalescence and aggregation). Dairy processing involves the application of thermal treatments to milk (i.e. cooling down to 4°C during storage, heating during pasteurisation and processing) and such processes are used for a wide range of dairy products (e.g. cream, butter, cheese). Hence, the lipid domains formed in the MFGM and more particularly the different lipid phases formed as a function of temperature (gel, Lo, Ld phases) could impact on these processes and resulting dairy products. The main function of milk is to assure the survival of all mammal newborns. Since fat globules are secreted in milk to provide energy and bioactive molecules to newborns, their biological membrane is undoubtedly well-adapted for the mechanisms involved in lipid digestion and protection of the neonate. The role played by the lipid domains formed at the surface of milk fat globules at the physiological temperature of digestion (i.e. 37°C) needs to be further considered. The Lo phase domains are present at the surface of fat globules upon digestion in the gastro-intestinal tract and may play a role in the hydrolysis of fat globules by modulating the adsorption and activity of the digestive enzymes (bile salt-stimulated lipase, gastric lipase, pancreatic lipase), as already

discussed (Berton, Rouvellac, Robert, Rousseau, Lopez & Crenon, 2012; Gallier, Ye & Singh, 2012; Lopez, 2011; Lopez et al., 2010; Lopez & Ménard, 2011). Lipid phase separation would also influence the binding of molecules to the MFGM such as bacteria, viruses (Lopez, 2011). The MFGM is a highly interesting biomembrane that needs to be further studied to better understand its functions.

4. Conclusion

Despite the importance of the milk fat globule membrane (MFGM) in nutrition and dairy processing, this biological membrane is poorly understood. This study investigated the lipid phase behavior of the buffalo MFGM as a function of temperature. CLSM experiments performed in well-controlled conditions *in situ* in buffalo milks revealed that the organisation of the lipid components is sensitive to temperature and to the rate of temperature change. Polar lipids segregated in the outer bilayer of the MFGM into two or more liquid phases (e.g. gel, Lo and Ld phases) that can coexist and have different physical properties. The rate of temperature change affected the number, the size and the shape of the lipid domains. Rapid cooling favored the mechanisms of nucleation while slow cooling favored the growth of the domains. Whether or not changes in the morphology and physical properties of these lipid domains affect the bioavailability of milk lipids and technological processing remains to be elucidated.

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FIGURE CAPTION

Figure 1: Microscopy images captured at room temperature (19 ± 1°C) showing the heterogeneous distribution of polar lipids within the buffalo milk fat globule membrane (MFGM). CLSM images after labelling of polar lipids with Rh-DOPE (red colour; A-B). Overlay of DIC optical microscopy images and CLSM images after labelling of polar lipids with Rh-DOPE (C-D) and glycosylated molecules (glycoproteins and glycolipids) with the lectin WGA-488 (green colour; E). The non-fluorescent areas (dark areas) observed in the buffalo MFGM correspond to the lateral segregation of high phase transition temperature polar lipids in domains (indicated by the white arrows). Images were captured using a 100x objective. TAG: triacylglycerols located in the core of fat globules. Thin arrows: lipid domains around fat globules; thick arrows: fat globules with lipid domains located at their surface. The scale bars are indicated in the figures.

Figure 2: Microscopy images the buffalo milk fat globule membrane (MFGM) captured at 60°C. (A, D) Overlay of DIC optical microscopy images and CLSM images after dual labelling of polar lipids with Rh-DOPE (red colour) and glycosylated molecules (glycoproteins and glycolipids) with the lectin WGA-488 (green colour). (B, E) CLSM images after labelling with the lectin WGA-488. (C, F) Overlay of DIC optical microscopy images and CLSM images after labelling of polar lipids with Rh-DOPE. The non-fluorescent areas (dark areas) observed in the buffalo MFGM correspond to the lateral segregation of lipids in domains. The scale bars are indicated in the figures.

Figure 3: Microstructural analysis of the milk fat globule membrane as a function of temperature, characterised on cooling from 60°C to 20°C and then to 4°C (dT/dt = 2°C/min with plateaus at constant temperature of 10 min). Overlay of DIC images and confocal scanning laser microscopy images with Rh-DOPE fluorescent dye. The scale bars are indicated in the figures.

Figure 4: Microscopy images showing the microstructure of the milk fat globule membrane (MFGM) after quenching from 60°C to 4°C (dT/dt > 100°C/min). The samples were stored at 60°C and introduced under the microscope in the temperature-regulated stage pre-cooled at 4°C. Overlay of DIC and confocal laser scanning microscopy images using the exogenous phospholipid Rh-DOPE to stain phospholipids within the MFGM (red colour). The scale bars are indicated in the figures.

Figure 5: CLSM images showing the changes in the microstructure of the milk fat globule membrane (MFGM) after rapid cooling from 20°C (A) down to 4°C (B) at dT/dt ~ 20°C/min. The exogenous phospholipid Rh-DOPE stains phospholipids within the MFGM and appears red. Images were captured using a 100x objective. The scale bars are indicated in the figures.

Figure 6: Microscopy images showing the changes in the lipid domains characterised in the buffalo MFGM at various temperatures. The temperature was increased from 4°C to 60°C (A, B, C) and then decreased from 60°C to 4°C (D, E, F). The heating and cooling rates were dT/dt ~ 2°C/min, with 10 min plateaus at constant temperature (e.g. 20°C, 40°C and 60°C). Phospholipids in the MFGM stained by Rh-DOPE appear red. The scale bars are 10 μm in length.

Figure 7: Organization of polar lipids in the milk fat globule membrane (MFGM) as a function of temperature proposed on the basis of confocal laser scanning microscopy experiments. For temperatures above the gel to liquid crystalline phase transition temperature (Tm) of saturated polar lipids (T>Tm), formation of lipid domains in the liquid-ordered (Lo) phase integrating cholesterol. For temperatures below the Tm of saturated polar lipids (T<Tm), the lipid domains form both the gel and Lo phases.

FIGURES

Figure 1

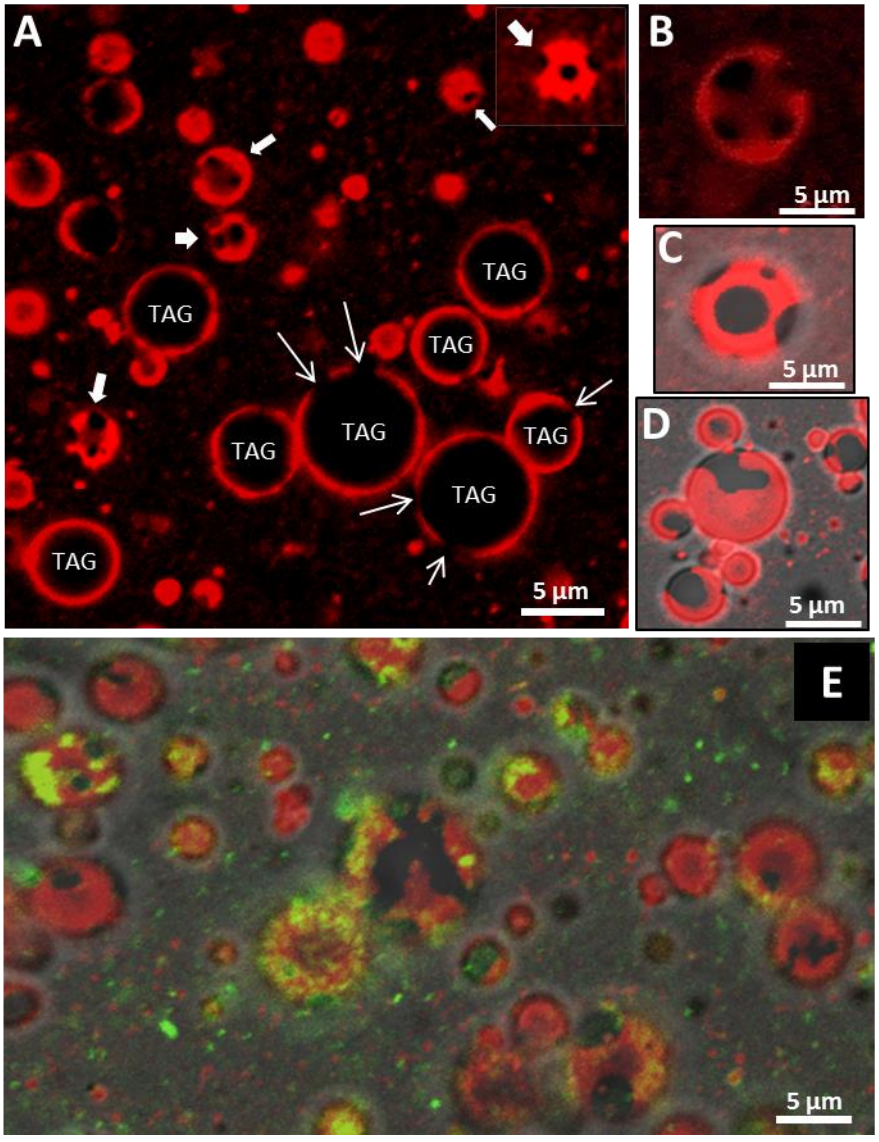
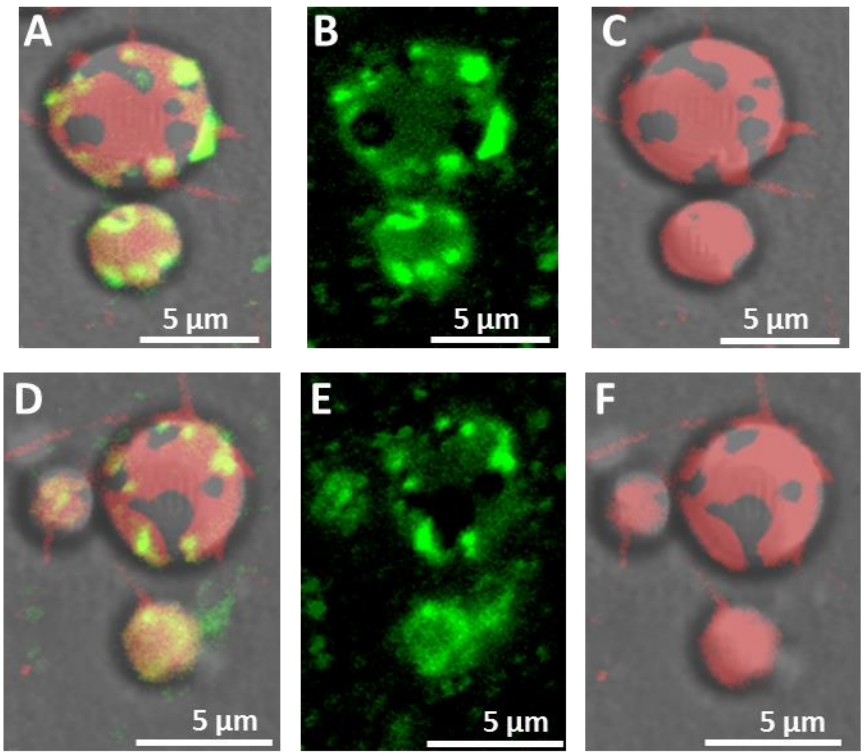


Figure 2



565 **Figure 3**

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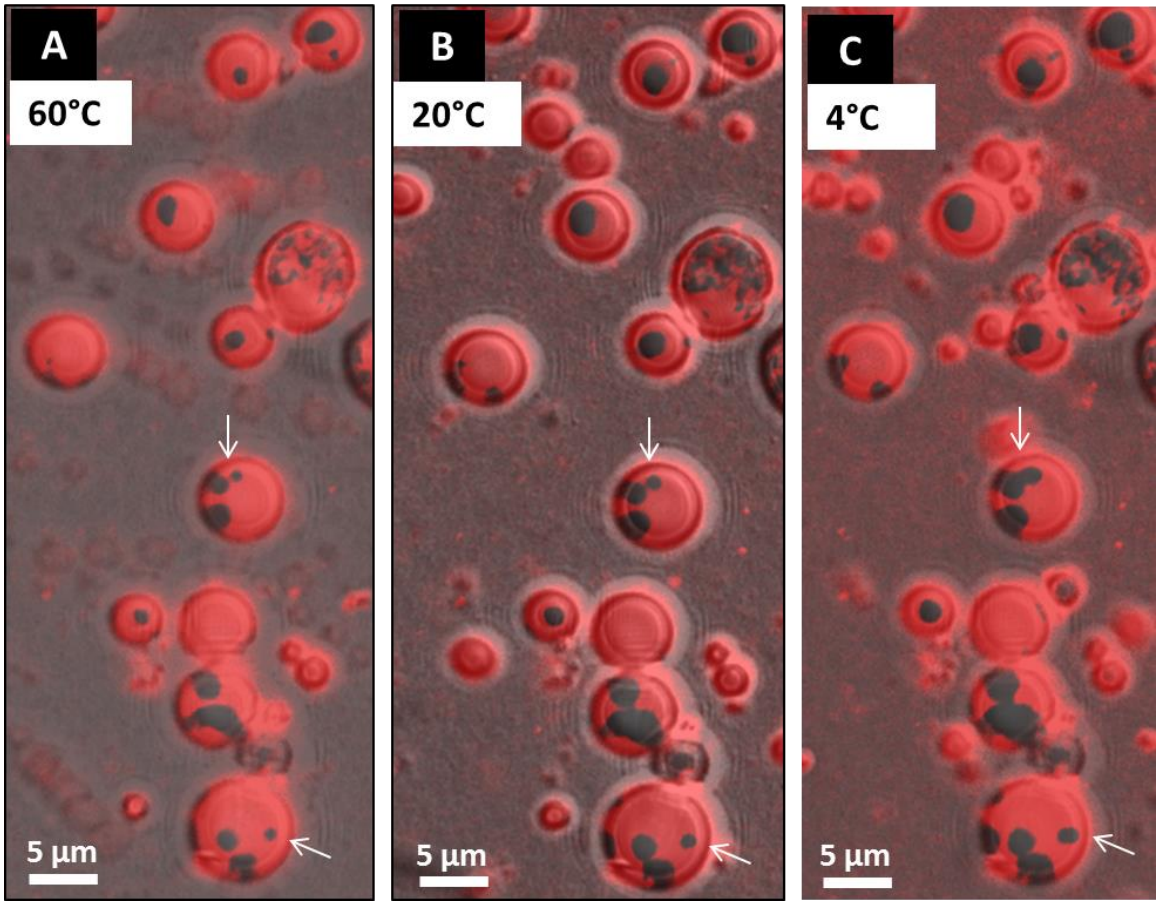

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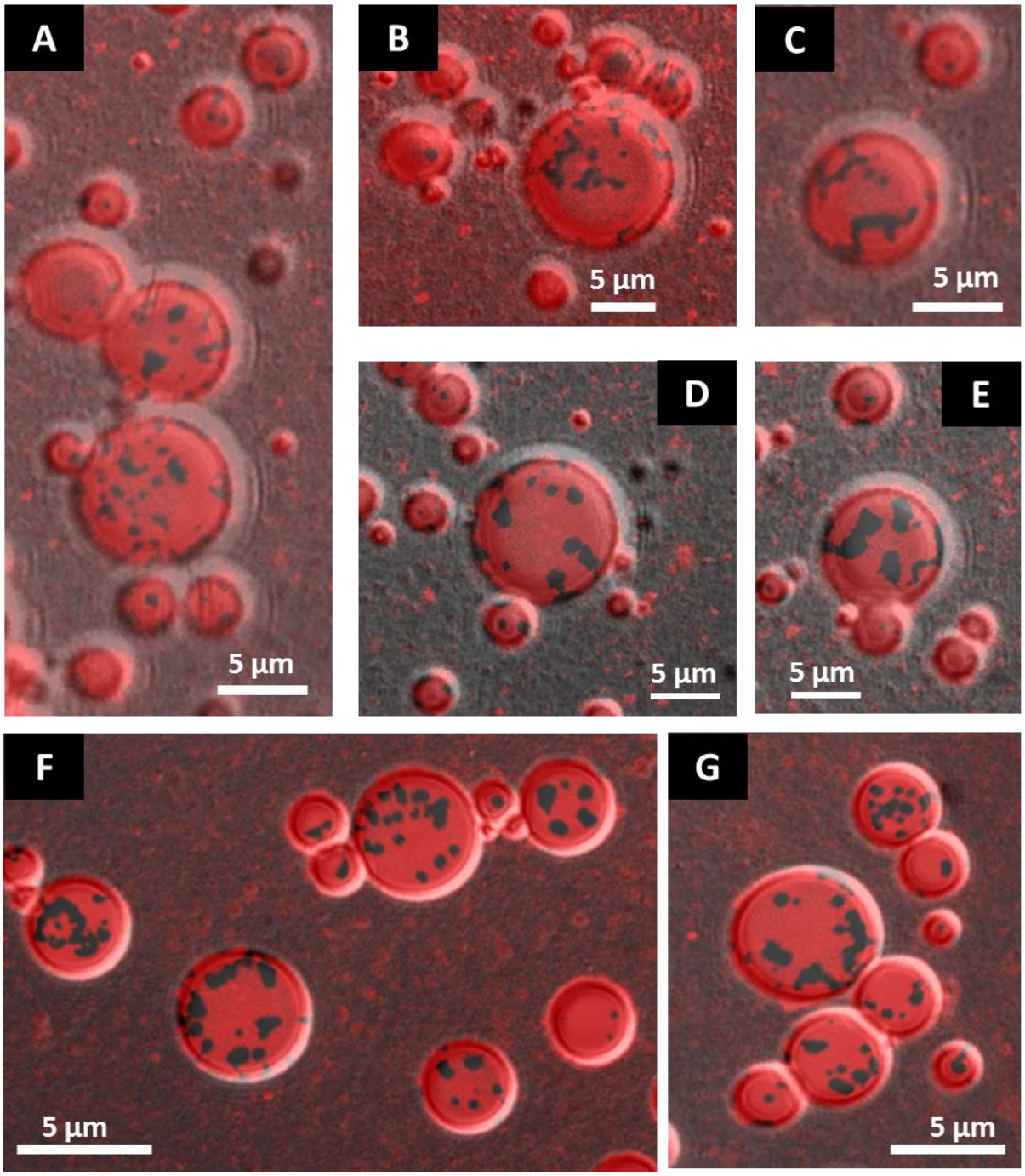
573 **Figure 4**



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577 **Figure 5**

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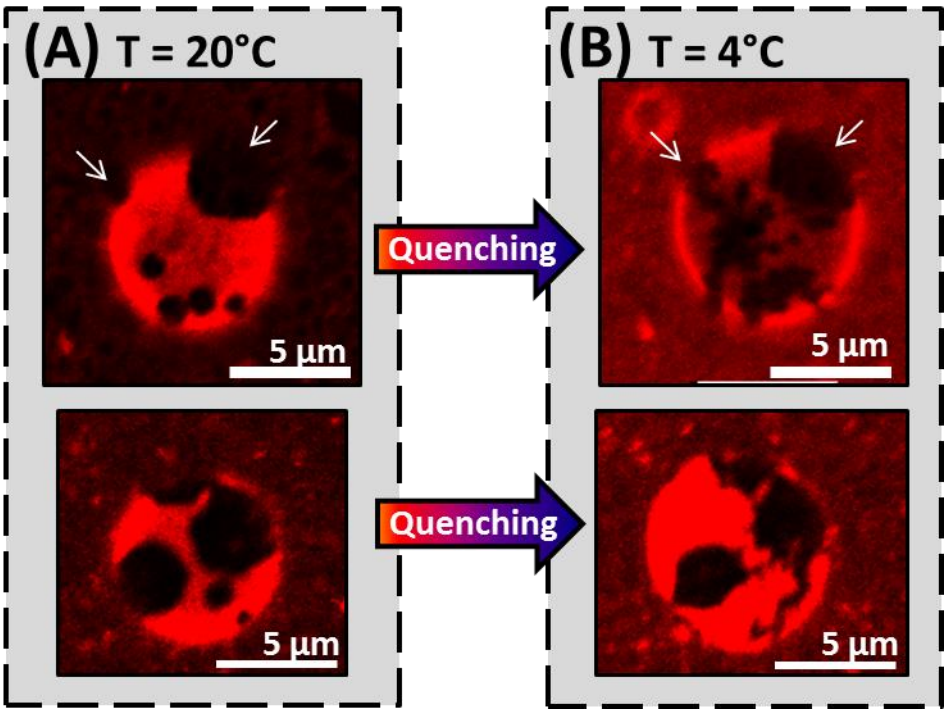

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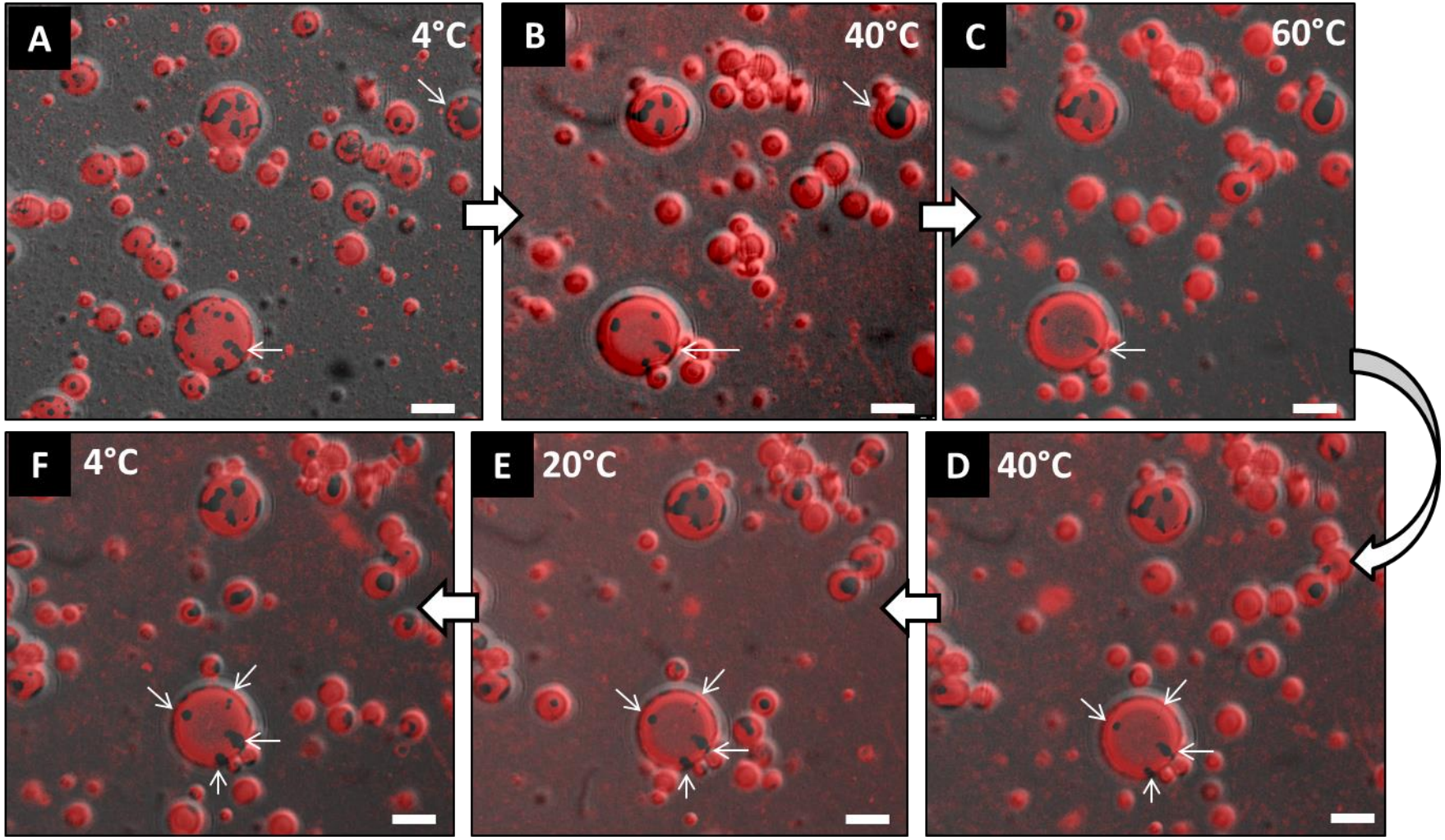

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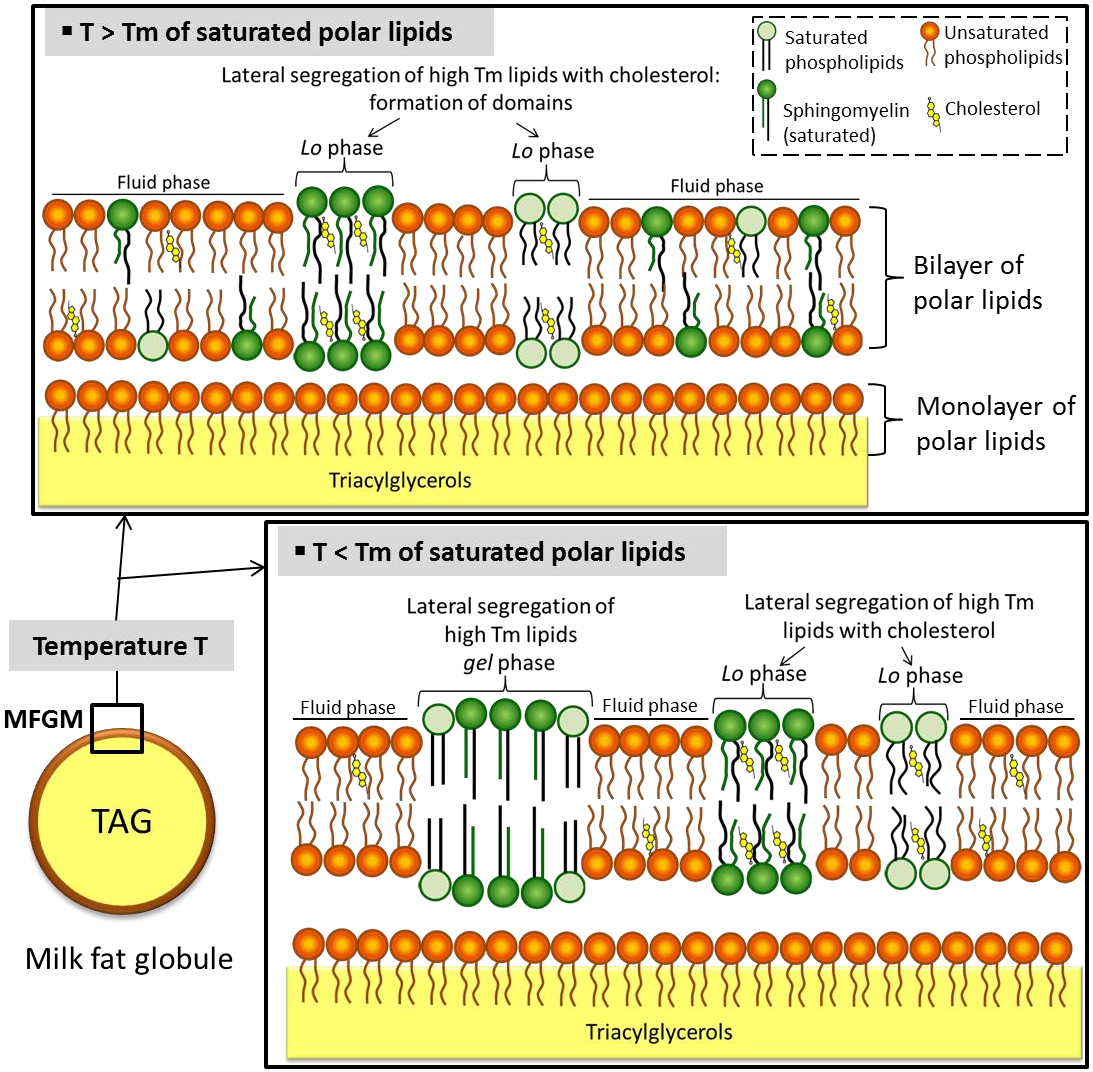
583 **Figure 7**



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