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STABILITY OF WATER-IN-OIL-IN-WATER MULTIPLE EMULSIONS: INFLUENCE OF THE INTERFACIAL PROPERTIES OF MILK FAT GLOBULE MEMBRANE

ESTABILIDAD DE EMULSIONES AGUA-EN-ACEITE-EN-AGUA: INFLUENCIA DE LAS PROPIEDADES INTERFACIALES DE LA MEMBRANA DEL GLÓBULO DE GRASA LÁCTEA

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Abstract

The interfacial shear viscosity (η^{int}) and the creep compliance-time (J(t)) behavior of milk fat globule membrane (MFGM) films (4, 5 and 6% w/w) formed at the water-oil interface were evaluated. Films with higher MFGM concentration displayed higher η^{int} and interfacial viscoelastic properties. When esters of polyglycerol and polyriciniolate fatty acids (PGPR) were added to the oil phase, a competitive adsorption at the interface took place between PGPR and MFGM which caused a decrease in the interfacial viscoelastic properties of the films. The change in the rheological behavior of the films suggests that their interfacial structure was determined by complex interactions between the MFGM and PGPR molecules. Water-in-oil-in-water multiple emulsions (ME) with smaller surface-volume droplet size ($d_{3,2}$), greater stability, and higher storage (G') and loss (G") moduli were obtained when higher MFGM concentrations were used in the outer aqueous phase.

Keywords : milk fat globule membrane, esters of polyglycerol polyriciniolate fatty acids, multiple emulsions stability, bulk and interfacial rheological properties.

Resumen

La viscosidad de corte interfacial (η^{int}) y el comportamiento creep compliance-tiempo (J(t)) de películas de membrana de glóbulo de grasa láctea (MFGM) (4, 5 y 6% p/p) formadas en la interfase aceite-agua fueron evaluados. Las películas con mayor concentración de MFGM mostraron mayores η^{int} y propiedades viscoelásticas interfaciales. Cuando se adicionaron ésteres de ácidos grasos de poliglicerol y poliricinoleato (PGPR) a la fase oleosa, una adsorción competitiva tomó lugar en la interfase entre PGPR y MFGM, lo cual causó decremento en las propiedades viscoelásticas interfaciales de las películas. El cambio en el comportamiento reológico de las películas sugiere que su estructura interfacial fue determinada por interacciones complejas entre las moléculas de MFGM y PGPR. Emulsiones múltiples agua-en-aceite-en-agua con gotas de menor diámetro volumétrico superficial, mayor estabilidad y mas altos módulos de almacenamiento (G') y de pérdida (G"), se obtuvieron cuando se utilizaron concentraciones más altas de MGGL en la fase acuosa externa.

Palabras clave : membrana del glóbulo de grasa láctea, ésteres de ácidos grasos de poliglicerol y poliricinioleato, estabilidad de las emulsiones múltiples, propiedades reológicas interfaciales.

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1 Introduction

Milk fat exists as an emulsion of tiny, spherical oil globules dispersed in the whey (Jensen, 2002). The diameter of the milk fat globules ranges from (0.2-15 μm) and they are surrounded by a 4 to 10-nm multilayer membrane (MFGM) composed primarily of triglycerides, proteins, and phospholipids (Danthine *et al*., 2000; Singh, 2006). The MFGM ensures structural integrity, protection and stability of the milk fat in the aqueous phase (Ye *et al*., 2002; Bezelgues *et al*., 2009). Several studies have indicated that some of the components of MFGM have health-enhancing functions, in addition to MFGM having excellent emulsification properties (Kanno, 1989; Corredig and Dalgleish, 1998; Roesch *et al*., 2004; Thompson and Singh, 2006; Singh, 2006; Bezelgues *et al*., 2009). Both phospholipids and some proteins have shown to have inhibitory activity against breast cancer, and reducing the number of colon tumors (Spitsberg, 2005; Singh, 2006). Also phospholipids have shown beneficial effects against development of Alzheimer's disease (Parodi, 2001; Spitsberg, 2005), liver protection (Koopman *et al*., 1985), and memory improvement (Crook *et al*., 1991).

In milk, the phospholipid-protein layer acts as a surfactant to prevent coalescence and flocculation of the fat globules in their aqueous environment (Bezelgues *et al*., 2009). Mixtures of proteins and phospholipids are not only met in nature such as in biomembranes but also are widely used for the stabilization of emulsions and foams in food industry (Henry *et al*., 2010). Thus, the study of the adsorption behavior and interfacial rheology of mixed phospholipid/protein layers shed knowledge that allow to gain understanding regarding interfacial activity and emulsion stability (He *et al*., 2008).

The unique properties of MFGM have led to research into developing technologies for the isolation and separation of MFGM-enriched material from milk. MFGM-enriched material could potentially be incorporated into food emulsions, and could result in new functional foods and nutraceuticals. MFGM is released into the aqueous phase during cream churning (destabilization of milk fat globules) for obtaining butter, resulting in a by-product named buttermilk. Buttermilk is low cost and available in large quantities but has been considered for many years as a low value product. However, over the last two decades it has gained considerable attention due to its specific composition in proteins and polar lipids sourced from the MFGM (Vanderghem *et al*., 2010).

On the other hand there is a need for edible delivery systems to encapsulate, protect and release bioactive and functional lipophilic and hydrophilic constituents within the food and pharmaceutical industries. In this sense multiple emulsions (ME) could be used for a number of purposes including targeting the delivery of bioactive components within the gastrointestinal tract, and designing food matrices that delay lipid digestion (McClements and Li, 2010). The droplets in water-in-oil-in-water ME used in the food and pharmaceutical industries may be stabilized by a variety of different emulsifiers, including small molecule surfactants, biopolymers and phospholipids. In particular, it is important that these delivery systems can be fabricated from food-grade ingredients using economically viable processing operations, and that they are robust enough to remain stable throughout their application in foods.

The objectives of this work were to: (a) determine the interfacial shear viscosity and interfacial viscoelastic properties of adsorbed films of MFGM; (b) evaluate the effect of the competitive adsorption between MFGM and esters of polyglycerol and polyriciniolate fatty acids (PGPR) on the viscoelastic properties of the films; and (c) evaluate the influence of the interfacial MFGM/PGPR films on the stability and bulk rheological properties of the water-in-oil-in-water ME.

2 Materials and methods

2.1 Materials

Canola oil (CO; Capullo®; Unilever de Mexico, S.A. de C.V.); carboxymethylcellulose (CMC; CMC® CEROL 50 000; Grupo Dermet, S.A. de C.V.); lipophilic emulsifier (PGPR; Grinsted® PGPR 90, esters of polyglycerol and polyriciniolate fatty acids; Danisco Mexico, S.A. de C.V.); hydrochloric acid, sodium hydroxide, chloroform, methanol, perchloric acid, ammonium molybdate, sodium bisulfite, sodium sulfite, and monopotassium phosphate (J.T. Baker, S.A de C.V.); 1-amino-2-naphtol-4-sulfonic acid (Alta Pureza Maquiladora S.A de C.V.) were all purchased in Mexico City, Mexico. Distilled deionized water (ddw) was used in all the experiments.

2.2 Isolation of MFGM

MFGM was isolated from fresh raw cream following the procedure described by Fong *et al*. (2007) with slight modifications. Ten L of fresh raw cream (40 g of

fat per 100 g; Planta Lechera, Universidad Autonoma Chapingo, Texcoco, State of Mexico, Mexico) was used in each of the experimental runs. The cream (30 ± 1 °C) was suspended in washing solution (ddw at 38 ± 1 °C) in a proportion of 1 L of cream per 3 L of ddw) applying gentle agitation (880 rpm, 15 min) with a mechanical mixer (CAFRAMO model RZR1, Cole-Parmer, Vernon Hills, IL, USA). The cream was subsequently separated with a bench-top cream separator. This washing step was repeated thrice. After being refrigerated overnight at 4 ± 1 °C, the washed cream was churned using a KitchenAid mixer (KSMC5OS KitchenAid Inc., St. Joseph, Michigan, USA) operated at 750 rpm at 10 °C until butter and buttermilk were obtained. Buttermilk was collected by filtration through 2 layers of cheesecloth, which was used to retain minute butter granules. The pH of the buttermilk was adjusted at 4.8 with hydrochloric acid (1M) for inducing MFGM precipitation. The resulting slurry was centrifuged in glass tubes with a laboratory centrifuge (Sorvall RC-5B; GMI Inc., Ramsey, MN, USA) at 10 000 rpm for 30 min, in order to separate the insoluble MFGM. The pH of the MFGM suspension was adjusted at 6.8 using sodium hydroxide (1M). The pooled MGFM suspension was freeze dried in a Lyph Lock® 4.5 freeze dryer (Labconco Corporation, MO, USA) and the resulting powder was stored at -20 °C until required for use.

2.3 *Determination of total protein, total lipid and phospholipids of MFGM*

The total protein content of the freeze-dried MFGM was determined by the Kjeldahl method (AOAC, 1995) using 6.38 as the conversion factor.

Lipids were extracted from MFGM following the procedure described by Sánchez-Juanes *et al*. (2009) with slight modifications. MFGM powder (1 g) was dissolved in 10 mL of chloroform/methanol (1:1 v/v), stirred overnight at 4 °C, and centrifuged at 1700 rpm for 10 min at 4 °C. The pellet was re-homogenized in 10 mL of chloroform/methanol (2:1 v/v), stirred for 45 min at 4 °C and centrifuged as described above. The new pellet was re-homogenized in 10 mL of chloroform/methanol (1:2 v/v) and stirred and centrifuged as above. The combined supernatants were reduced to one quarter of the original volume by drying at 40 °C in an oven (model HS-33 Rios Rocha, Mexico City, Mexico), kept overnight at -20 °C, and centrifuged at 1700 rpm for 10 min at 4 °C to remove insoluble material. The resulting extract was put into a Petri dish, dried in an oven at 40 °C for 24 h. Afterwards the Petri dish was put in a desiccator with $CaCl_2$ as desiccant for 24 h, and total lipids (TL) were determined gravimetrically.

TL (0.15 g) were re-dissolved with chloroform/methanol (1:2 v/v) and diluted to 10 mL, and 1 mL sample was taken and dried by evaporation (40 °C). Total phospholipids content (TPC) was determined based on the procedure described by Nalto (1975), with modifications. The dried sample was added with 0.8 mL of perchloric acid (72 % v/v) in a 15 × 150 mm graduated glass tube, which was placed in a sand bath at 200 °C for 30 min, and cooled to room temperature (20 ± 2 °C). Approximately 6 mL of ddw + 0.5 mL of ammonium molybdate reagent (5 % w/v) were added and mixed, followed by the addition of 0.4 mL of reducing reagent (27.2 g of sodium bisulfite + 6 g of sodium sulfite + 0.5 g of 1-amino-2-naphtol-4-sulfonic acid, diluted to 250 mL). The content of the tube was diluted to 10 mL with ddw. Afterwards, the mixture was left to react for 20 min and the absorbance read in a spectrophotometer (Spectronics Genesys 5 UV/Vis, Spectronic Unicam, Rochester, NY, USA) at 660 nm. The spectrophotometer was zeroed with a reagent blank (0.8 mL perchloric acid). Calibration was done using 0.1 mL of a standard solution (KH_2PO_4, 0.5 mg mL^{-1} = 11.38 μg of P + 0.8 mL of perchloric acid). TPC was calculated using the equation 1.

$$TPC(mg/L) = (A_s/A_{std}) \times [P_{std} \times (V_{std}/V_s)] \times 25 \quad (1)$$

Where TPC = total phospholipids content ($mg.L^{-1}$); As = absorbance of sample; A_{std} = absorbance of standard solution; P_{std} = phosphorous in standard solution ($mg.L^{-1}$); V_{std} = volume of standard solution (mL); V_s = volume of sample (mL); and 25= factor for converting phosphorous (mg) to phospholipids (mg).

2.4 *Interfacial rheology*

The interfacial rheological measurements were done using a Physica MCR 301 Dynamic Shear Rheometer (Physica Meβtechnik GmbH, Stuttgart, Germany), with a stainless steel biconical disk (radius of disk, Rb, of 34.125 mm and disk double angle (2α) of 10°). A thermostated vessel (inner radius, Rc, 40 mm) was inserted in the measuring plate of the rheometer. Aqueous solutions (118 mL) of MFGM (4, 5, 6 % w/w) at pH 7.0 prepared 24 h before, were carefully spilled into the thermostated vessel. Afterwards the rheometer motor drive was lowered until the stainless steel biconical disk was placed at the MFGM solution surface. Then 118 mL of CO or CO + PGPR (were

carefully poured with help of a glass rod unto the vertical wall of the vessel until the oil formed a layer above the MFGM solution (Román-Guerrero *et al*., 2009). The age of the interface was taken from the moment the last droplet of oil was poured in.

2.4.1. Interfacial shear viscosity

The different MFGM interfacial films were submitted to a constant disk angular velocity (Ω) of 1.76×10^{-4} rad/s. The necessary torque (M) required for maintaining the steady rotational speed and angular displacement of the disk with time (θ_b) was monitored every 10 s for 15 min with the rheometer software. Plots of interfacial shear strain (γ^{int}) versus interfacial shear stress (σ^{int}) were obtained from the equipment software, and were used to determine the region in which the torque and/or interfacial stress at the interface attains steady-state behavior, which were used for calculating the interfacial shear viscosity (η^{int}) with Eq. (2) (Pérez-Orozco *et al*., 2004):

$$\eta^{\text{int}} = \frac{M}{4\pi\Omega}\left(\frac{1}{R_b^2} - \frac{1}{R_c^2}\right) \tag{2}$$

2.4.2. Interfacial creep compliance-time studies

The different MFGM and MFGM/PGPR interfacial films creep compliance-time behavior was carried out by subjecting them to a constant σ^{int} of 0.5658 mN/m during 60 min, after which σ^{int} was withdrawn, and the stress relaxation of the interfacial films was followed for further 7 min. The selected shear stress felt within the linear viscoelastic region of all of the interfacial films.

Plots of $J(t)$ versus t for the different interfacial films were obtained and the equipment software provided the values of the parameters of the following equation (Pérez-Orozco *et al*., 2011):

$$J(t) = J_0 + J_m\left(1 - e^{-(t/\lambda m)}\right) + J_N \tag{3}$$

where $J_0 = (1/E_0$; E_0 is the interfacial instantaneous elastic modulus) is the interfacial instantaneous elastic compliance in which bonds between the primary structural units are stretched elastically; $J_m = (1/E_R$; E_R is the interfacial retarded elastic modulus) is the interfacial mean retarded compliance of all the bonds involved; $\lambda_m = (J_m/\eta_m$; η_m is the interfacial mean viscosity associated with retarded elasticity) is the interfacial mean retardation time; and $J_N(= t/\eta_N)$ is the interfacial Newtonian compliance, which is characterized by an interfacial viscosity η_N. All measurements were carried out at 25 °C.

2.5 Multiple emulsions preparation

Three multiple emulsions (ME) were prepared at 20 ± 2 °C using a two-stage emulsification procedure (Lobato-Calleros *et al*., 2009). In the first stage, a W_1/O emulsion was made with a 0.3 dispersed mass fraction (ϕ_1). The aqueous phase (W_1) (29.9 g of distilled water + 0.1 g of CMC) was poured drop-wise with continuous agitation into the oil phase (O) (66.8 g of CO + 3.2 g of PGPR) with the help of a high shear Ultra-Turrax T50 basic homogenizer (IKA Works, Inc. Wilmington, USA) operated at 6 400 rpm during 5 min. In the second stage the requisite amount of W_1/O primary emulsion was re-emulsified (6400 rpm for 4 min) into 4, 5, and 6 % w/w MFGM solutions adjusted at pH 7.0, yielding the ME with a 0.3 dispersed mass fraction (ϕ_2) that were coded as: $ME_{4\%}$, $ME_{5\%}$, and $ME_{6\%}$, respectively. During the emulsification process an ice bath was used to avoid temperature rising above 20 ± 2 °C.

2.6 Morphology and size of the ME droplets

The morphology of the ME droplets of the multiple emulsions was determined using an optical microscope (Olympus BX45, Olympus Optical Co., Tokyo, Japan) coupled to an image analyzer system (digital Olympus camera C3030, Olympus America Inc., USA).

The initial mean surface-volume droplet size ($d_{3,2}$) and its evolution with storage (4 ± 1 °C) time (3, 7, 10, 15 and 21 d) of the ME was determined with a Malvern particle size analyzer series 2600 (Malvern Instruments, Malvern, Worcestershire, UK).

2.7 Oscillatory rheological properties of the ME

The oscillatory rheological behavior of the ME was determined after 1 day of preparation with the Physica MCR 301 rheometer coupled to double gap concentric cylinders measuring system. ME were loaded into the measuring system and left to rest for 15 min for structure recovery and for temperature equilibration (25 °C). Dynamic amplitude sweeps (0.01-10 % strain) were performed under a constant frequency (1 Hz), in order to determine the linear viscoelastic range, where rheological properties are not strain or stress dependent. From the linear viscoelastic range a strain level of 0.2 % was chosen to perform frequencies sweeps at 0.01-10 Hz. The storage modulus (G') and

the loss modulus (G") as a function of frequency were obtained from the equipment software.

2.8 Data analysis

All of the experiments were done in triplicate using a randomized experimental design. Rheological properties of MFGM interfacial films were analyzed using simple classification variance analysis and whenever it was appropriate, Tukey's test was used in order to determine differences between the means. The significance was determined at $P \leq 0.05$. Data analysis was performed using Statgraphics Plus software (Statistical Graphics Corp., Manugistics, Inc., Cambridge, MA, USA).

3 Results and discussion

3.1 Yield and chemical composition of the MFGM

The yield of MFGM was 3.2 ± 0.3 g/L of cream. This result is close to that (3.6 ± 0.3 g/L) reported by Fong *et al*. (2007). The protein, lipids and total phospholipids contents of the MFGM were of 42.38 ± 1.70, 45.7 ± 1.4 and 30.05 ± 1.2 %, respectively. Literature data on the composition of the MFGM are highly variable due to differences in isolation method, type of raw material, pre-treatment of buttermilk or cream, purification and analysis techniques. Singh (2006) informed that protein accounts for 25-60 % of the mass of the MFGM, depending on the isolation method chosen and the sample history. Fong *et al*. (2007) reported a protein and lipid contents of 22.3 ± 1.5 % and 71.8 ± 1.7 %, respectively, in the pellet fraction of MFGM. Kanno and Kim (1990) obtained a MFGM containing 28 % of protein and 64 % lipid. Bezelgues *et al*. (2009) reported 55 % of protein, 37.9 % of lipids and 25 % of phospholipids contents for MFGM.

The MFGM phospholipids are primarily phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and sphingomyelin (SM), with small amounts of phosphatidyl serine and phosphatidyl inositol (PI) (Thompson and Singh, 2006). All of these phospholipids possess surface active properties. Interfacial rheology is an important method to obtain knowledge and understanding of interfacial activity and system stability (He *et al*., 2008).

3.2 Interfacial rheological properties of the MFGM films

3.2.1. Interfacial shear viscosity

When two droplets in a liquid environment approach one another, the behavior of the system is governed by the interplay of hydrodynamic forces and surface forces. The hydrodynamic forces lead to viscous flow of the liquid medium from between the droplets and to distortion of the form of the droplets because of the pressure developed between them. The radial flow of fluid from between them the droplets exerts a shearing force on them, tending to generate a circulation of liquid within each droplet. The distortion of the droplets is opposed by surface (interfacial) tension, because any departure from spherical form involves an increase of surface area (Kitchener and Musselwhite, 1969). While interfacial viscosity cannot cause static metastability, it might assist in dynamic stabilization, by slowing up the extrusion of liquid from between approaching surfaces between emulsion droplets.

Surface shear stress was measured as a function of strain for the MFGM at different concentrations and at aging times (Fig. 1). All of the MFGM concentrations showed similar shear-strain profiles. Independently of the MFGM concentration used, a finite aging time (12 h for 4 % of MFGM, and 8 h for 5 and 6 % of MFGM) had to elapse before the interfacial film produced an elastic response, *i.e.*, the increase in σ^{int} is proportional to increase in γ^{int}. The initial interfacial film forming induction period could be influenced by the diffusion rate of the surface-active MFGM components (proteins and phospholipids) and its interfacial affinity (Beverung *et al*., 1999). It is typical of macromolecular surfactants that their adsorption is very slow to approach equilibrium and is practically irreversible (Kitchener and Musselwhite, 1969). As continued interfacial adsorption and rearrangements of the surface-active components occurred greater number of molecular interfacial contacts caused an elastic film forming, which suffered reversible structural changes due to bond stretching between molecules without rupture. As aging time increased, the magnitude reached by σ^{int} in the elastic region increased, and was higher as the MFGM concentration was higher. The curves exhibiting an elastic response showed a maximum in σ^{int}, related with the amount of stress that the films can withstand before their structure yields, followed by a decrease in σ^{int}, indicating that the elastic limit of the adsorbed layer has been superseded, and that

a viscous deformation mechanism begins to occur. As γ^{int} continues increasing, σ^{int} continues dropping until a steady-state shear-stress value is reached (Ganzevles *et al*., 2006). The maximum steady-state shear-stress value was obtained after approximately 36 h, independently of MFGM concentration used. At this aging time the maximum σ^{int} values obtained varied as follows: 1.9 mN/m for 4 % MFGM film < 2.2 mN/m for 5 % MFGM film < 2.3 mN/m for 6 % MFGM film (Fig. 1); while the steady-state σ^{int} values varied as follows: 1.31 mN/m for 4 % MFGM < 1.64 mN/m for 5 % MFGM film < 1.71 mN/m for 6 % MFGM film. It can be seen that interfacial films at higher MFGM concentration required a larger σ^{int} for achieving a given γ^{int}.

The interfacial stress (torque) at the interface where the steady-state behavior was attained was used for calculating the η^{int}. Figure 2 shows the η^{int} versus time plots for the films using different MFGM concentrations. A marked increase in the η^{int} occurred with aging time, indicating the interactions among the proteins and phospholipids molecules at the interface depended on their total concentration. The η^{int} value reached after 36 h was higher as the MFGM concentration increased as follows: 1029.49 mNs/m (4 %), 1292.01 mNs/m (5 %) and 1352.26 mNs/m (6 %). Pérez-Orozco *et al*. (2004) reported increasing η^{int} values for mesquite gum-chitosan interfacial films with aging time. Waninge *et al*. (2005) reported that interfacial films made from a mixture of proteins and phospholipids resembling MFGM tended to form more complex structures than an adsorbed monolayer.

3.2.2. Interfacial creep compliance-time studies

The mechanical properties of macromolecular stabilized interfaces have been correlated with droplet stability. The mechanics of the approach of two drops in a fluid medium can give rise to several phenomena, most leading to droplets surface deformation, which on turn lead to the thinning out of interfacial films, to their collapse and to droplets coalescence. It has been proposed that interfacial films exhibiting high elasticity possess a better capability of "healing" disturbances at the interfacial region, so that time of coalescence of the droplets is greatly extended (Kitchener and Musselwhite, 1969). The interfacial creep compliance-time study is a non-destructive test permitting the determination of rheological parameters under conditions that approach the state of the sample at rest; this allows elucidating a more precise picture of the actual film (Román-Guerrero

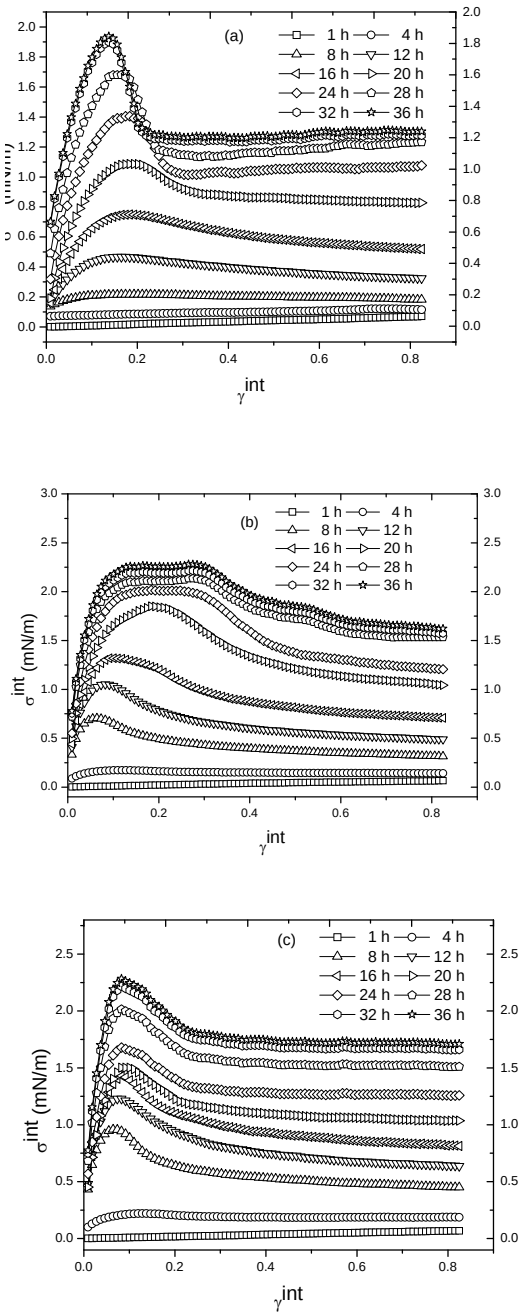

Fig. 1. Shear stress-strain relationship with aging time for MFGM films at different concentrations: (a) 4%, (b) 5%, and (c) 6%.

et al., 2009). All of the MFGM and MFGM/PGPR interfacial films exhibited typical interfacial creep compliance-time curves (Fig. 3), indicating that they exhibited viscoelastic behavior.

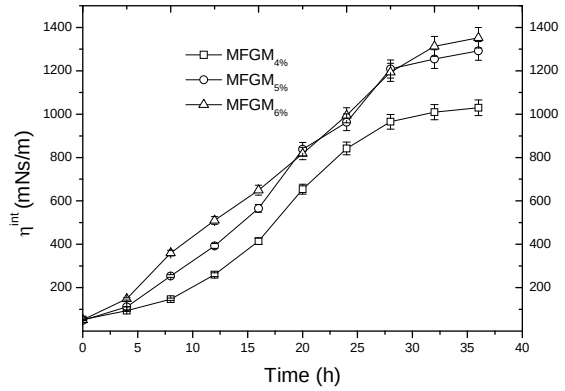

Fig. 2. Development of interfacial shear viscosity of MFGM films at different concentrations with aging time.

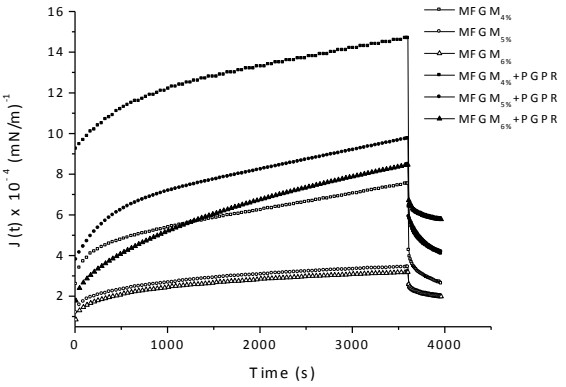

Fig. 3. Creep Compliance-time curves of the MFGM and MFGM + PGPR films aged 36 h.

The viscoelastic parameters characterizing each film are given in Table 1. Analysis of instantaneous interfacial compliance modulus (J_0), which is related with the instantaneous reversible deformation suffered by the strain applied to the film, indicates that as MFGM concentration increased (whether alone or combined with PGPR) the $E_0 = (1/J_0)$ value of the films increased. Thus, E_0, which provides a measure of elastic strength on the bonds making up the interfacial network structure (Lobato-Calleros *et al*., 2000) was higher for $MFGM_{6\%}$ > $MFGM_{5\%}$ > $MFGM_{6\%}$ + PGPR > $MFGM_{4\%}$ > $MFGM_{5\%}$ + PGPR > $MFGM_{4\%}$ + PGPR. The retarded interfacial elastic compliance region parameters J_m and λ_m (J_m/η_m), where bonds break and reform but not at the same rate, can provide more detailed information regarding the nature of the interfacial films (Román-Guerrero *et al*., 2009). In particular λ_m, which is the time taken for the delayed strain to reach approximately 63.2% (1-1/e), may be considered a measure of the complexity of the type and diversity of bonds making up the interfacial films. The more complex the structure, longer retardation times are needed to characterize the types of bonding that takes place (Lobato-Calleros *et al*., 2000).

Significantly higher values for λ_m were displayed as the MFGM concentration increased, but were significantly lower for the MFGM + PGPR films than for their pure MFGM counterparts. Likewise, J_N (= t/η_N) indicates that steady state deformation is reached when the creep deformation is mainly governed by viscous flow which is seen as a linear increase of the creep curve (Van Bockstaele *et al*., 2011). Higher η_N values were exhibited as the MFGM concentration increased, but again, η_N values decreased for MFGM + PGPR films in comparison to the MFGM films. For proteins η_N and G' are usually much higher than for low molecular weight surfactants (Murray, 2011). It is evident that there is a displacement of the adsorbed MFGM by PGPR. Because PGPR has a strong attraction for oil, its molecules must be, at least to some extent, in a dissolved state in the oil phase. Most of PGPR hydrocarbon chain lies deeply within the oil phase, and a small portion of the hydrophilic part is located at the oil-water interface. On the other hand, the MFGM molecules are more attracted to the water phase with only the hydrophobic moieties anchoring at the oil-water interface and the bulk of the molecules are projected into the aqueous phase (Doxastakis and Sherman, 1983). The adsorbed PGPR molecules disrupt MFGM-MFGM molecules interaction at the interface due to a lubricating action, but do not completely displace the MFGM molecules from the interface, resulting in a decrease of the viscoelastic properties (Dickinson, 1991).

3.3 Morphology and stability of the ME

All of the fresh ME were characterized for exhibiting type C morphology (Garti, 1997), i.e. they were made up by spherical oil droplets containing within them a large number of water droplets (Fig. 4). The initial mean $d_{3,2}$ of the external droplets and its change with storage time was considered as a measure of the relative stability between the ME. According to the Fig. 5, we envisaged that the MFGM concentration in the external aqueous phase influenced the stability of the ME and the initial $d_{3,2}$ values. Lower concentrations of MFGM produced larger initial $d_{3,2}$ as follows: $ME_{4\%}$ (2.75 μm) > $ME_{5\%}$ (2.51 μm) > $ME_{6\%}$ (2.39 μm). Higher initial $d_{3,2}$ resulted in a droplet size polydispersity (noted in deviation bars in

Table 1. Viscoelastic parameters of the MFGM and MFGM + PGPR interfacial films

Film code	$J_0 \times 10^4$ $(mN/m)^{-1}$	$E_0 \times 10^{-4}$ (mNm)	$Jm \times 10^4$ $(mN/m)^{-1}$	λ_m (s)	$J_N \times 10^4$ $(mN/m)^{-1}$	$\eta_N \times 10^4$ $(mNs/m)^{-1}$
$MFGM_{4\%}$	2.6 ± 0.1^{bc}	0.4 ± 0.0^{b}	4.8 ± 0.2^{a}	950.2 ± 23.0^{c}	3.0 ± 0.1^{bc}	1197.0 ± 54.3^{a}
$MFGM_{5\%}$	1.2 ± 0.1^{a}	0.8 ± 0.0^{c}	2.2 ± 0.2^{ab}	1164.8 ± 19.0^{d}	0.8 ± 0.1^{a}	4456.3 ± 32.2^{b}
$MFGM_{6\%}$	0.9 ± 0.0^{a}	1.2 ± 0.1^{d}	1.8 ± 0.1^{ab}	1252.9 ± 15.3^{e}	0.7 ± 0.0^{a}	4633.2 ± 57.2^{b}
$MFGM_{4\%}$+PGPR	9.2 ± 0.7^{d}	0.1 ± 0.0^{a}	2.5 ± 0.2^{b}	322.9 ± 10.6^{a}	3.6 ± 0.3^{c}	1003.7 ± 70.1^{a}
$MFGM_{5\%}$+PGPR	3.7 ± 0.3^{c}	0.3 ± 0.0^{ab}	2.6 ± 0.2^{b}	472.4 ± 14.7^{b}	3.4 ± 0.1^{bc}	1052.1 ± 37.2^{a}
$MFGM_{6\%}$+PGPR	1.5 ± 0.6^{ab}	0.7 ± 0.2^{c}	1.4 ± 0.1^{a}	488.8 ± 15.2^{b}	3.0 ± 0.1^{b}	1213.1 ± 40.3^{a}

Means in a column followed by different letters are significantly different ($p \leq 0.05$). J_0: interfacial instantaneous compliance; E_0: interfacial instantaneous elastic modulus; J_m: interfacial mean compliance; λ_m: interfacial mean retardation time; J_N: interfacial Newtonian compliance η_N: interfacial viscosity.

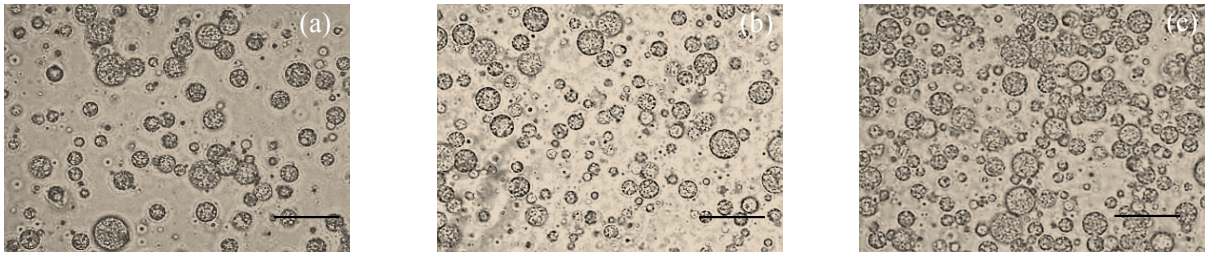

Fig. 4. Optical microscope images of ME droplets made up by spherical oil droplets containing within them a large number of water droplets: (a) $ME_{4\%}$, (b) $ME_{5\%}$, and (c) $ME_{6\%}$. Scale bar =10 μm.

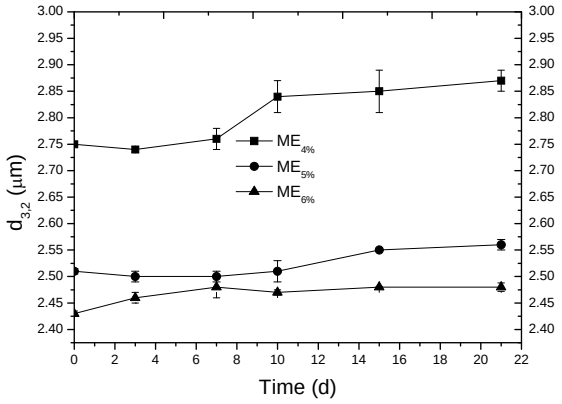

Fig. 5. Changes in the mean surface-volume droplet size ($d_{3,2}$) of the ME with aging time.

Fig. 5) throughout storage time. Smaller initial $d_{3,2}$ produced smaller increases in droplet size during the 21 days storage time. Thus, the relative increases of $d_{3,2}$ between t = 0 d and t = 21 d was as follows: $ME_{4\%}$ = 4.4 %, $ME_{5\%}$ = 2 %, and $ME_{6\%}$ = 1.6 %. These results indicate that although all of the ME emulsions were quite stable, those made using 6 % of MFGM suffered the smallest droplet size variation, so that they may be considered to be more stable. The destabilization processes that the ME may suffer are varied; for example, the outer droplet interface may coalesce with one or more multiple emulsion droplets; the individual internal aqueous droplets can be expelled sequentially from the multiple emulsions droplets; gradual shrinkage of the internal droplets is possible due to osmotic gradient between the inner and continuous aqueous phases, when net mass transport of water occurs from the inner phase to the outer continuous phase through the oil film acting as a "semi-permeable membrane"; and conversely, when the osmotic gradient acts in the opposite direction, water diffusion through the oil film from the continuous phase into the encapsulated aqueous droplets will produce swelling of these inner droplets (Hernández-Marín *et al.*, 2013). However, it has been established that adequate combinations of emulsifiers at the inner as well at the outer phase have beneficial effects on the stability of the ME (Garti, 1997). The optimum use of emulsifiers in ME formulation depends on their interfacial rheological properties (Maldonado-Valderrama and Rodríguez-Patino, 2010). A positive correlation between emulsion stability against droplet coalescence and surface shear viscosity has been found in experiments with oil-in-water emulsion-sized droplets introduced into the vicinity of a planar oil-water interface aged with a pure protein (Dickinson *et al.*, 1988).

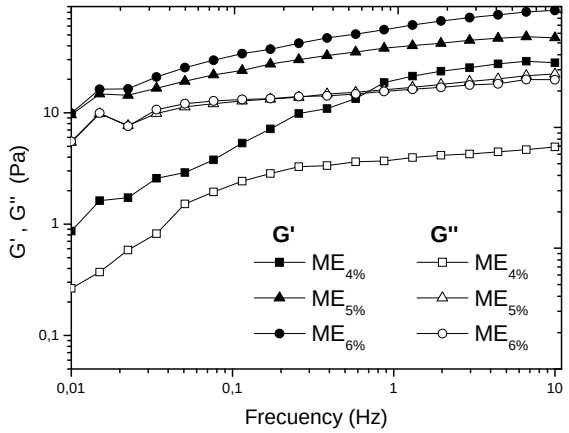

Fig. 6. Evolution of the storage (G') and loss (G") moduli of the ME with frequency sweep at 0.2% strain.

In addition, highly viscoelastic protein films tend to dampen down surface fluctuations, thus inhibiting the mechanism responsible for film rupture (Dickinson, 1992). The stability results of the ME tend to confirm that the stability of the ME was closely related to the interfacial rheological properties of the MFGM films.

3.4 Oscillatory rheological properties of the ME

Small-amplitude dynamic oscillatory shear tests are a powerful tool to obtain information about the microscopic structure of viscoelastic materials like emulsions, providing the values of the storage (G') and loss (G") moduli. G' is proportional to the extent of the elastic component of the system, and G" is proportional to the extent of the viscous component of the system (Wulff-Pérez *et al*., 2011). All of the ME made with different MFGM concentrations showed increases in G' and G" as a function of frequency, displaying higher G' than G" values over the whole frequency range studied (Fig. 6). Both, G' and G" were frequency dependent, although this dependency decreased as MFGM concentration increased. The kind of behavior depicted in figure 6 is typical of concentrated emulsions having a weak gel structure that arises from flocs held together rather strongly by polymer bridges, but that are nonetheless susceptible to reorganization on aging and in the presence of shear fields (Dickinson and Pawlowsky, 1996; Barnes, 2004). G" and G" values increased with increasing MFGM concentration and this behavior seems to be closely interconnected with the $d_{3,2}$ shown by the ME. Smaller $d_{3,2}$ imply higher numbers of ME droplets per unit volume and a more tightly interwoven floc structure.

Conclusions

Interfacial rheology results indicated that higher concentration of pure milk fat globule membrane (MFGM) produced films with higher interfacial instantaneous elastic modulus (E_0): 0.4 mN/m for 4 %; 0.8 mN/m for 5 %; and 1.2 mN/m for 6 %; and higher interfacial viscosity (η_N) 1197.0 mNs/m for 4 %; 4456.3 mNs/m for 5 % and 4633.2 mNs/m for 6%. When PGPR was added to the oil phase MFGM molecules were displaced in some extent from the interface resulting in a weakened interfacial film, but still mechanically strong. A close interrelationship was found to exist between the stability of the multiple emulsions and the interfacial rheological properties of the MFGM + PGPR films. Multiple emulsions stabilized by MFGM in different concentrations displayed frequency dependent viscoelastic properties where the storage modulus had higher values than the loss modulus over the whole frequency range studied. This behavior is characteristic of weak-gel structures arising from polymer bridging among flocs. Higher MFGM concentration produced higher viscoelastic moduli and lower frequency dependence, thus suggesting a close correspondence with multiple emulsion droplet size.

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