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The impact of HPMC structure in the modulation of in vitro lipolysis: The role of bile salts

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ABSTRACT

The behaviour of two hydroxypropylmethylcelluloses (HPMC), with different molecular structures: E5LV and E4M, has been analysed in the presence of the bile salts, both at the oil-water interface (emulsion behaviour, interfacial properties and ζ-potential) and in the aqueous medium (particle size distribution analysis, cloud point temperature and electrical conductivity).

HPMCs emulsions experienced different degrees and rate of lipolysis (E4M emulsions experienced a higher lipid digestion than emulsions stabilized by E5LV) that were not related to differences in the molecular weight/viscosity.

Differences in the kinetics of lipolysis can be attributed to the interaction with BS according to methyl/hydroxypropyl ratio of these HPMCs. The self-assembly of the E4M cellulose, being the more hydrophilic cellulose (with a lower methyl/hydroxypropyl ratio than E5LV) was more hindered by the bile salts adsorption, thus developing a higher untangling at the interface that would increase the available sites for the lipase.

These results allow a better understanding of the mechanisms that affect food emulsions digestion and it could allow to design polysaccharides stabilized emulsions with better functional properties.

1. Introduction

Fat digestion is a complex colloidal process that requires the adsorption of lipase to the o/w interface of the emulsified droplets, in the presence of colipase and bile salts (BS), in the small intestine (Scholten, Moschakis, & Biliaderis, 2014; Singh & Sarkar, 2011; Zhu, Ye, Verrier, & Singh, 2013), where the absorption of fat digestion products occur, although partial digestion also takes place in the stomach (Klinkesorn & McClements, 2010). Lipids are hydrolysed (lipolysis) resulting in the release of two free fatty acids and one 2-monoacylglycerol (Gallier, Ye, & Singh, 2012). The BS play a key role in the digestion of lipids by stabilizing oil droplets (BS adsorb at the o/w interface and prepare the interface for the enzymatic breakdown due to the action of the lipase-colipase complex) and by forming micelles which are the transport vehicles of the lipolysis products to the enterocytes (Abrahamse et al., 2012; Bellesi, Pizones Ruiz-Henestrosa, & Pilosof, 2014; Bläckberg, Hernell, & Olivecrona, 1981; Sarkar, Ye, & Singh, 2016b; Singh & Gallier, 2014; Torcello-Gómez & Foster, 2014). BS molecules, that are secreted from the liver and stored in the gall bladder, present a high surface activity that makes them very efficient in displacing the adsorbed emulsifiers (complete or partial displacement) from the o/w interface (Bellesi et al., 2014; Maldonado-Valderrama & Patino, 2010; Mun, Decker, Park, Weiss, & McClements, 2006; Sarkar, Horne, & Singh, 2010a; Singh & Ye, 2013; Torcello-Gómez & Foster, 2014; Torcello-Gómez, Maldonado-Valderrama, Jódar-Reyes, & Foster, 2013). The effect of the conformation that they adopt at the o/w interface is very important, as they tend to lay flat at the interface, by maximizing the interfacial area (Chu et al., 2010; Maldonado-Valderrama & Patino, 2010, 2008).

It is very interesting the possibility of manipulating the lipid digestion that could improve health by modifying serum lipid levels (Maldonado-Valderrama, Gunning, Ridout, Wilde & Morris, 2009), in order to produce novel foods that could permit the control of appetite, of the digestion and/or the control of nutrients delivery (Dickinson, 2008; Hur, Lee, Lee, Bahk, & Kim, 2015;

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Scholten et al., 2014; Zimet & Livney, 2009). This fact could allow to decrease the existing growing obesity crisis, associated to a negative impact in the health of the population (Bellesi et al., 2014; Marciani et al., 2009).

The characteristics of the interfacial layers surrounding the fat droplets have been reported to play a significant role in the extent of lipid digestion, using different surface active components, as well as the release rate of any entrapped components (Bellesi et al., 2014; Li & McClements, 2011; Malaki Nik, Wright, & Corredig, 2011; McClements & Li, 2010; Mun, Decker, & McClements, 2007; Sarkar et al., 2016a; Torcello-Gómez & Foster, 2014; Tzoumaki, Moschakis, Scholten, & Biliaderis, 2012; Ye, Cui, Zhu, & Singh, 2013).

Polysaccharides are natural biopolymers that are commonly employed in many food dispersed systems, such as emulsions and foams, and they are normally used to control the texture and stability of these products (Dickinson, 2003; Hanazawa & Murray, 2014). Most of these molecules do not tend to adsorb at fluid interfaces because of their high hydrophilic character (Camino, Pérez, & Pilosof, 2009a; Rodríguez Patino and Pilosof, 2011). However, there exist a group of surface active polysaccharides, such as cellulose derivatives, that have received a great interest recently because of their physico-chemical properties and many technological applications (Arboleya & Wilde, 2005; Pérez, Sánchez, Pilosof, & Rodríguez Patino, 2009; Rodríguez Patino and Pilosof, 2011). Methylcellulose (MC) and hydroxypropylmethylcellulose (HPMC) are considered the principal cellulose derivatives and they are used in a broad range of applications in pharmaceutical and food formulations (Pérez, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2015).

HPMCs are non-ionic cellulose derivatives with methyl (hydrophobic) and hydroxypropyl (hydrophilic) groups added to the anhydroglucose backbone and include a family of cellulose ethers that differ principally in molecular weight, viscosity, degree of substitution (DS). DS is the average number of hydroxyl groups per anhydroglucose unit where hydrogen is replaced by methyl and molar substitution (MS) is the average number of propylene oxide groups per anhydroglucose unit. The higher the degree of total substitution is, the higher the hydrophobicity of the polysaccharide will be, thus their surface/interfacial activity will also increase (Camino et al., 2009a; Chang & Gray, 1978; Daniels & Barta, 1994; Wollenweber, Makievski, Miller, & Daniels, 2000). Furthermore, it is also known that HPMCs provide health effects, such as the hypocholesterolemic effects (Kim et al., 2011; Maki et al., 2000, 2009; Reppas, Swidan, Tobey, Turowski, & Dressman, 2009; Torcello-Gómez & Foster, 2014; Yokoyama et al., 2011), as well as their promissory usage as vehicles for encapsulation of active food ingredients (Fathi, Martín, & McClements, 2014; Mun, Kim, Shin, & McClements, 2015) in order to get a controlled release in the gastrointestinal tract. Due to all these interesting properties and because of the necessary reduction in the fat consumed by the populations, their use in food systems could be a good strategy to diminish the effective caloric content of the foods, to control the lipid digestion or to control the satiety (Beysseriat, Decker, & McClements, 2006; Mudgil & Barak, 2013; Torcello-Gómez & Foster, 2014).

The behaviour of one of these HPMC emulsions has been recently evaluated (Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016) when exposing it to the complex simulated human gastrointestinal conditions to mimic their transit through the human digestive tract (exposition to the stomach juices and the pancreatic fluid (small intestine), containing enzymes such as trypsin, chymotrypsin, lipases, etc., and the biliary fluid, containing different bio-surfactants, such as the BS, phospholipids, cholesterol and the products obtained from the lipid digestion) (Mackie & Macierzanka, 2010; Nik, Corredig, & Wright, 2010). A much lower extent and rate of lipolysis was observed when using HPMC as emulsifier as compared to β-lactoglobulin. Thus HPMC seems to be a good emulsifier to reduce the extent of the lipolysis in the emulsions.

Torcello and Foster (Torcello-Gómez & Foster, 2014) have recently analysed the competitive adsorption of different modified celluloses and BS. They observed that the adsorption of the mixed systems (polysaccharides (10^{-3}% (w/w)) + BS) was controlled by the BS when using BS concentrations from 10^{-3} to 10^{-1} M. When analysing the behaviour of these systems in the aqueous phase (micro-DSC measurements) they observed the existence of interactions between these molecules that affected their adsorption to the o/w interface.

The objective of present work was to study the lipid digestion (free fatty acid release (FFA)) of the o/w HPMCs stabilized emulsions as affected by the molecular weight or hydrophobicity of HPMC and to understand the involved mechanism, mediated by the BS. Furthermore, the physico-chemical changes occurring in the emulsions and interfaces, when in contact with the BS (important physiological components for the lipase to develop the hydrolysis), as well as HPMCs-BS interactions in the bulk phase, have been assessed in order to explain the observed differences in the kinetics of the free fatty acid release.

2. Materials and methods

2.1. Materials

Commercial HPMCs: E5LV and E4M (food grade) from The Dow Chemical Company were kindly supplied by Colorcon (Argentina) and used without purification. Their properties (Table 1) have already been indicated by Camino and Pilosof (Camino, Sánchez, Rodríguez Patino, & Pilosof, 2011). HPMC solutions were prepared in hot water at 90 °C by dispersing the powder in Trizma [$(CH_2OH)_3CNH_2$/$(CH_2OH)_3CNH_3Cl$] buffer at pH 7. The solutions were prepared at 2% (w/w) and stored at 4 °C for 24 h in order to achieve their complete hydration (Camino et al., 2009a). Methocel E5LV was used due to its low molecular weight and high hydrophobicity (Camino & Pilosof, 2011), and Methocel E4M, was used because of its higher viscosity and molecular weight and lower hydrophobicity than E5LV (Table 1). The interfacial behaviour of HPMCs have been extensively studied in previous studies (Camino et al., 2009a, 2009b, 2011; Arboleya & Wilde, 2005; Camino & Pilosof, 2011; Wollenweber et al., 2000).

Commercial sunflower oil was purified, to eliminate the possible surface-active contaminants, with Florisil 60-100 Mesh (Fluka) as described by Bellesi et al. (Bellesi et al., 2014).

All the glassware was cleaned using ammonium persulfate-sulfuric acid to eliminate all the possible surface-active contaminants that could interfere in the measurements and rinsed with bi-distilled water.

Table 1
E4M and E5LV properties.

Properties	E4M	E5LV
% methyl	28.0	29.5
% hydroxypropyl	10.2	9.7
Methyl/hydroxypropyl ratio	2.3	3.0
Methyl substitution (DS)	1.90	1.90
Hydroxypropyl substitution (MS)	0.23	0.23
Total substitution (DS + MS)	2.13	2.13
Viscosity (cp), 2% wt solution (20 °C)	4965	5.4
Molecular weight (kDa)	90	2

2.2. Methods

The emulsions were prepared by mixing the HPMC solutions with the sunflower oil at a 10:90 ratio, using an ultrasonic processor Vibra Cell, model VCX 750 (Sonics & Materials, Inc., Newton, CT, USA) for 15 min at a frequency of 20 kHz and an amplitude of 20% at 0.5 °C (glycerine-jacketed) in order to dissipate the heat produced.

It was used as the subphase a simulated intestinal fluid (SIF) at pH 7.0, as indicated in the literature (Bellesi et al., 2014; Sarkar, Horne, & Singh, 2010b), simulating the ionic strength and the pH in the small intestine. Solutions of bovine bile extract (B3883, Sigma-Aldrich) were dissolved in the SIF at a final concentration of 5 mg/ml, a value that is within the physiological concentrations (Bellesi et al., 2014; Sarkar et al., 2010b; Ye et al., 2013).

Emulsions were mixed with the bile extract solution, at a 1:1 proportion (final concentration of BS at 5 mg/ml), both of them previously incubated at 37 °C, as well as it was also done with the mixture in order to simulate the physiological conditions.

2.2.1. In vitro digestion model

The in vitro gastrointestinal digestion of both HPMCs emulsions was carry out using the protocol reported by Bellesi et al. (2016). The in vitro digestion begins by mixing 15 ml of the emulsion with 15 ml of the simulated gastric fluid (SGF, pH 2.5, 100 mM NaCl, 3 mM $CaCl_2$, 5 mM NaH_2PO_4, and 22 mM KCl, T: 37 °C) under moderate agitation. Physiological concentrations of pepsin from porcine gastric mucosa (P700, powder ≥ 250 units/mg solid) and L-α-phosphatidylcholine from egg yolk (type XVI-E, P3556), both purchased from Sigma-Aldrich, were present in the SGF. After an hour, the gastric proteolysis was stopped by increasing the pH to 7 (1M $NaHCO_3$). The in vitro digestion continued by incorporating 7.5 ml of simulated intestinal fluid (SIF, pH 7.0, 39 mM K_2HPO_4, 150 mM NaCl and 30 mM $CaCl_2$), prepared as indicated by Sarkar et al. (2010a). Moreover, the SIF contained physiological concentrations of bovine bile extract (B3883), lipase from porcine pancreas (L3126, type II, 100–400 units/mg protein using olive oil − 30 min incubation), and the proteases: trypsin (type I, T8003) and L-α-chymotrypsin (type II, C4129), both from bovine pancreas. All the biochemical agents were purchased from Sigma-Aldrich. The mixture was stirred for 1 h at 37 °C and in the meanwhile, the pH was continuously monitored and controlled to maintain the pH at 7 by neutralizing the FFA released by the lipase activity using NaOH 0.5 M. Finally, the enzymes were inhibited using suitable concentrations of Orlistat (inhibitor of lipase activity) and trypsin and chymotrypsin inhibitor, both purchased from Sigma-Aldrich.

Three replicates were determined for each emulsion.

2.2.2. Particle size and ζ-potential

A Zetasizer Nano-ZS analyser with a He-Ne laser beam (633 nm) (Malvern Instruments, Worcestershire, United Kingdom) was used to determine the particle size by dynamic light scattering at a fixed scattering angle of 173° (Camino et al., 2009a; Martinez, Sánchez, Patino, & Pilosof, 2009; Pizones Ruiz-Henestrosa et al., 2014). The HPMCs solutions were measured at a 0.5% (w/w) concentration, while it was diluted the BS solution (5 mg/ml) at 0.05 mg/l. The aqueous solutions were used without any filtering step and they were measured at 37 °C.

ζ-potential measurements of the emulsions were also developed using this analyser at 37 °C. Samples were previously diluted at a 1:1000 proportion using the SIF to avoid multiple scattering effects. The ζ-potential of the different samples was determined by analysing the particles electrophoretic mobility. The values are reported as the average and standard deviation of duplicated measurements of ten readings per sample.

2.2.3. Emulsion droplet size analysis

The size distributions of the oil droplets were determined by a static laser light scattering technique (Malvern Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom).

A refractive index (RI) for the sunflower oil phase of 1.47 and its absorbance parameter of 0.001 were used to calculate the particle size distributions. Emulsions were analysed when the mixture with the BS was done and after 1 h by diluting the emulsions in an aqueous medium at the pH of the sample in order to avoid multiple scattering effects.

Measurements are reported as the surface weight mean diameter (Sauter diameter): $D_{3,2}$ (μm) = $\Sigma\, n_i\, d_i^3 / \Sigma\, n_i\, d_i^2$ (mean droplet size) and as the volume weight mean diameter (De Broucker diameter) that is more sensitive to fat droplet flocculation than Sauter diameter: $D_{4,3}$ (μm) = $\Sigma\, n_i\, d_i^4 / \Sigma\, n_i\, d_i^3$, where n_i is the number of oil droplets with a diameter d_i (Arzeni, Pérez, & Pilosof, 2012; Beysseriat et al., 2006; Camino & Pilosof, 2011; Mun et al., 2007; Ye et al., 2013). It was also reported the Specific Surface Area (SSA), that indicates the surface area per unit mass of the dispersed phase and is inversely proportional to the size of the oil drops (Carrera Sánchez & Rodríguez Patino, 2005; McClements, 1998).

The reported values are the average of measurements made in duplicate on two freshly prepared emulsions.

2.2.4. Interfacial properties

The surface pressure and the interfacial dilatational rheology were determined by using a Profile Analysis Tensiometer (PAT-1, SINTERFACE Technologies, Germany) with the pendant drop method, as indicated by Bellesi et al. (Bellesi et al., 2014), by recording the silhouette onto a CCD camera and then analysing the digital images fitted to the Young-Laplace equation with an accuracy of ±0.1 mN/m. Time-dependent interfacial pressure (π) of the different components adsorbed at the o/w interface was analysed during 11,100 s, until adsorption equilibrium was reached, by forming a droplet at the tip of a stainless steel capillary at a constant volume of 12 μl immersed in an optical glass cuvette containing purified sunflower oil, which was thermostated at a constant temperature of 37 °C. Regarding the subphase exchange experiments, it was used a coaxial capillary in order to inject the BS solution into the preadsorbed HPMC interfacial films, once it was formed a viscoelastic interfacial film (after 8000 s) (Bellesi et al., 2014; Ferri, Gorevski, Kotsmar, Leser, & Miller, 2008; Maldonado-Valderrama & Patino, 2015). The average standard accuracy of the surface pressure was roughly 0.1 mN/m.

The dilatational modulus of the interfacial films was obtained by applying a sinusoidal interfacial deformation (compression and expansion) at an angular frequency of 0.05 Hz and an amplitude of the oscillation at 3% of the initial drop volume. The system recorded the response of the interfacial tension to the deformation and it was then obtained the dilatational response of the interfacial film by applying a Fourier transform, obtaining the dilatational modulus (E) (Bellesi et al., 2014). The dilatational modulus is a complex quantity that is built up by a storage part (E′), representing the real part of the term, and a loss part (E″), describing the imaginary part of the modulus (Eq. (1)):

$$E = E' + i\,E'' = \varepsilon + i\,2\pi f\,\eta \tag{1}$$

where $E' = \varepsilon$ is the interfacial elasticity and $E''/\omega = \eta$ is the interfacial viscosity (Bellesi et al., 2014; Berthold, Schubert, Brandes, Kroh, & Miller, 2007; Pizones Ruiz-Henestrosa et al., 2014).

2.2.5. Cloud point temperature determinations

Five glass test tubes, containing the corresponding HPMC solutions in the presence and in absence of BS, were placed into a temperature-controlled bath. The samples were heated at 70 °C

and it was checked the inner temperature by introducing a thermometer in the tubes. The onset of clouding, that is the temperature where it appears an incipient turbidity, was determined as the average of at least two measurements (Pérez, Wargon, & Pilosof, 2006).

2.2.6. Electrical conductivity measurements

It was determined the electrical conductivity (mS/cm) of the HPMCs aqueous solutions, both in absence and in the presence of the BS, by using a conductometer (Thermo Orion, 125A Plus, (USA)). The conductivity values were obtained from the average of at least two measurements.

3. Results

3.1. Kinetics of lipolysis

The kinetics of lipolysis were determined by the neutralization of the free fatty acids (FFA) released during the simulated duodenal in vitro digestion. The consumed amount of NaOH during the digestion is related to the total amount of FFA released. Fig. 1 shows that E4M emulsions were more susceptible to lipolysis than E5LV emulsions (Table 2).

The amount of FFA released during the digestion process over time (Fig. 1) was fitted with the following empirical model (Eq. (2)) in order to describe the kinetics of the FFA release (Bellesi et al., 2016):

$$\%\ \mathrm{FFA}\ (t) = [(\%\ \mathrm{FFA})_{max}\ \mathrm{x}\ t] / (B + t) \quad (2)$$

where % FFA and $(\%\ \mathrm{FFA})_{max}$ are the % FFA released at the time t and at the "pseudo-equilibrium", respectively, and B is the time (min) needed to reach half the maximum % FFA [$(\%\ \mathrm{FFA})_{max}/2$], that is the lipolysis half-time. The initial rate of lipolysis (K_0), as well as the overall digestion rate constant (K), were also calculated (Eqs. (3) and (4)) (Bellesi et al., 2016; Pilosof, Boquet, & Bartholomai, 1985).

$$K_0^{FFA} = (\%\ \mathrm{FFA})_{max}/B \quad (3)$$

$$K^{FFA} = ((\%\ \mathrm{FFA})_{max} \times {}_B)^{-1} \quad (4)$$

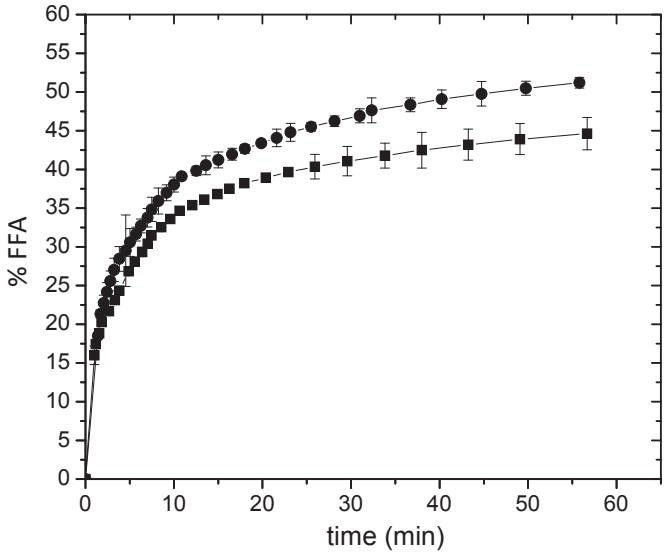

Fig. 1. % Free Fatty Acids (FFA) released from the E5LV emulsion (■) and E4M emulsion (●) under in vitro gastrointestinal conditions.

Table 2
Parameters obtained for the modulation of %FFA with time using an empirical model (Pilosof et al., 1985).

HPMC	$(FFA)_{max}$	K_0^{FFA} (1/min)	K^{FFA} (*10^3) (1/min)	R^2
E4M	51.3 ± 0.6	16.3 ± 0.9	6.2 ± 0.4	0.9892
E5LV	44.4 ± 0.7	16.4 ± 1.4	8.3 ± 0.6	0.9742

The kinetics parameters in Table 2 indicate that both emulsions were digested at the same initial rate (K_0) but the overall digestion rate constant (K) for E4M emulsions was lower than that for E5LV emulsions.

E4M, because of its higher molecular weight, has a higher viscosity than E5LV (Table 1). As the viscosity of the continuous medium retards the adsorption rate of the physiological components (enzymes, bile salts, etc.) (McClements, 2014), as well as the desorption of the lipolytic products, it may be expected a lower lipolysis for the E4M emulsion. However, it was the E5LV emulsion which experienced the lowest lipolysis. The extent and rate of lipid digestion between HPMCs emulsions may be related to the size of the oil droplets, to their molecular structure or to their interaction with BS, that have a key role in the lipolysis. Therefore, the effect of the size of the oil droplets and the impact of BS on the emulsions behaviour was first characterized.

3.2. Effect of BS on the stability of HPMC emulsions

The droplet size distributions for the fresh o/w emulsions are shown in Fig. 2 (A and B). E5LV emulsions showed a main population at 1 μm (Fig. 2A). E4M emulsions (Fig. 2B) presented a bimodal distribution, with a size of the droplets between 0.12 and 5.8 μm and a main peak population at 1.4 μm. These results are in accordance with that cited in the literature for these polysaccharides at pH 6 (Camino & Pilosof, 2011).

The influence of mixing these emulsions with the BS under physiological conditions on the emulsion stability was analysed. It was first checked the effect of mixing the HPMCs emulsions with the SIF (in the absence of the BS) in order to analyse the possible effect of this fluid. It was corroborated that SIF did not affect the particle size distribution of the emulsions. Fig. 2 and Table 3 shows that, when mixing the original emulsions with the BS solutions (filled symbols) just in the moment of the mixture (t = 0 min) or after 1 h (t = 60 min), there were no apparent changes in the droplet size distributions or in the mean diameters. These results indicate that both HPMC emulsions are quite stable under duodenal conditions.

It has been concluded in a recent work (Bellesi et al., 2016) that, irrespective of the emulsifier used, the higher the initial interfacial area of the emulsions the higher the initial rate of lipolysis. When analysing the initial interfacial areas of both HPMC emulsions, before and after addition of BS (Table 3), it may be concluded that only small differences exist among them, thus supporting the similar K_0 values exhibited by both emulsions (Table 2).

Therefore, the sequential adsorption of BS at HPMC covered films was then analysed in order to explain the higher extent of lipid digestion observed for E4M emulsions. It was demonstrated by this approach, in a previous work (Bellesi et al., 2014), that the susceptibility of protein interfacial films to the displacement by the BS would depend on the molecular feature of each protein as well as on the interfacial protein coverage.

3.3. BS adsorption onto HPMC films

The properties of the interfacial films formed by the HPMCs at pH 7.0 and T = 37 °C (Figs. 3 and 4) were determined. It was used a

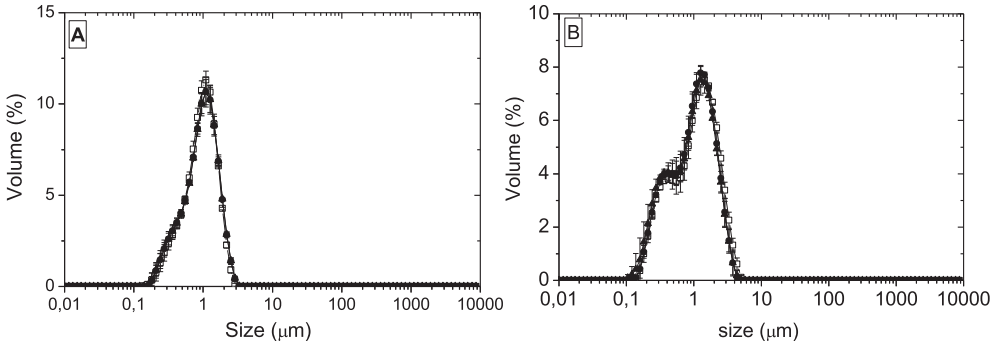

Fig. 2. Droplet size distributions of fresh HPMCs emulsions (E5LV (A) and E4M (B)) (open square) and emulsions after the mixture with BS (at the moment of the mixture (t = 0 min, filled circle) and 1 h after the mixture (t = 60 min, filled triangle)) under physiological conditions.

Table 3
ζ-Potential, average diameters $D_{3,2}$, $D_{4,3}$ and specific surface area (SSA) from fresh HPMCs emulsions (E5LV and E4M) and emulsions after the mixture with BS (at the moment of the mixture (t = 0 min) and 1 h after the mixture (t = 60 min) under physiological conditions.

Emulsion			ζ-Pot (mV)	$D_{4.3}$	$D_{3.2}$	SSA
E5LV	Original		−1.23 ± 0.07	1.083 ± 0.012	0.805 ± 0.048	8.62 ± 0.94
	BS	0 min	−7.13 ± 0.8	1.106 ± 0.017	0.801 ± 0.039	7.49 ± 0.37
		60 min	–	1.108 ± 0.025	0.801 ± 0.054	7.5 ± 0.5
E4M	Original		−0.54 ± 0.24	1.365 ± 0.046	0.771 ± 0.016	7.03 ± 0.82
	BS	0 min	−4.01 ± 0.8	1.25 ± 0.05	0.735 ± 0.008	8.31 ± 0.12
		60 min	–	1.221 ± 0.028	0.684 ± 0.071	8.81 ± 0.91

concentration of 0.5% (w/w) that allows the interface to be saturated. It is important to point out that we wanted to analyse the effect of BS upon a saturated interfacial film.

Fig. 3 shows the time evolution of π for both HPMCs. The HPMCs reached very fast a steady-state value of π (π ≈ 16 mN/m) that remained almost constant and very small differences were observed between these HPMCs.

Regarding the interfacial dilatational rheology, it can be observed in Fig. 4 the evolution of E and η with the time of adsorption. A continuous increment in the value of E was observed (Fig. 4A), as the polysaccharides were adsorbed at the o/w interface (Camino et al., 2011). E5LV formed the most elastic interfacial film (higher values of E observed in Fig. 4A), which is a result of its higher methyl substitution (stronger hydrophobic interactions) (Camino et al., 2011; Rodríguez Patino and Pilosof, 2011). The

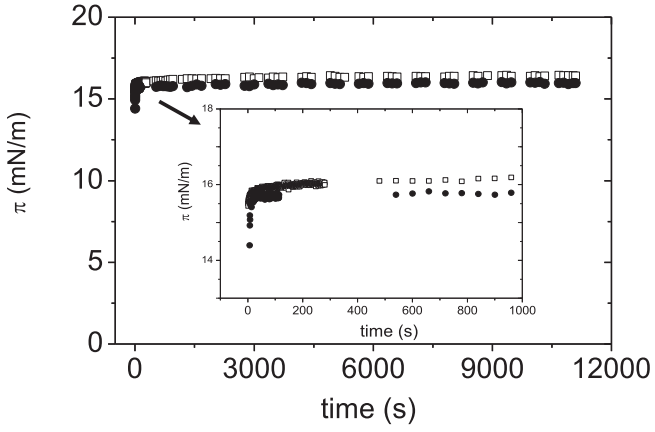

Fig. 3. Time-dependent surface pressure (π) for E4M (●) and E5LV (□) adsorbed films at the oil-water interface at a concentration of HPMC of 0.5% (w/w) in the bulk phase at pH 7. Temperature: 37 °C.

increment of E with time, even if the interfacial pressure attained constant values, has been attributed to increasing interactions between the adsorbed macromolecules during a multilayer formation beyond the collapse of the monolayer (Pérez, Sánchez, Pilosof, & Rodríguez Patino, 2008).

Regarding the interfacial viscosity of the films, E4M presented lower values than E5LV (Fig. 4B), so a more flexible structure of the interfacial film may be assumed for E4M compared to E5LV (Berthold et al., 2007). Nevertheless, both HPMCs formed viscoelastic films involving the association of the hydrophobic groups.

The effect of the adsorption of the BS in the previously formed interfacial film under simulated duodenal conditions was determined. However, it was first checked the behaviour of the HPMC interfacial film when introducing SIF without the BS. It was corroborated the irreversible HPMC adsorption at the o/w interface as no changes in the interfacial parameters were observed (data not shown).

The interfacial behaviour of the BS molecules alone has been studied previously (Bellesi et al., 2014) and it was observed that these molecules reached almost instantly a constant value of π at around 20 mN/m and very low values of E, forming a highly mobile interface.

It can be deduced from Fig. 5 that the adsorbed HPMC film may be penetrated by the BS, as the values of π experienced a fast increase, reaching the values obtained by the BS alone (π ≈ 20 mN/m). This fact reflects that BS dominates the surface pressure, as it was also observed by other authors when using different emulsifiers (proteins (milk proteins, soy protein, lactoferrin, …), polysaccharides, lipids and copolymers) (Bellesi et al., 2014; Chu et al., 2010; Gallier et al., 2014; Lesmes, Baudot, & McClements, 2010; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Maldonado-Valderrama & Patino, 2010; Sarkar et al., 2010b, 2010a; Singh & Sarkar, 2011; Torcello-Gómez & Foster, 2014; Torcello-Gómez et al., 2011a). It has been reported for some emulsifiers a displacement from the o/w interface that is driven via an orogenic mechanism (Bellesi et al., 2014; Euston, Baird, Campbell, & Kuhns, 2013;

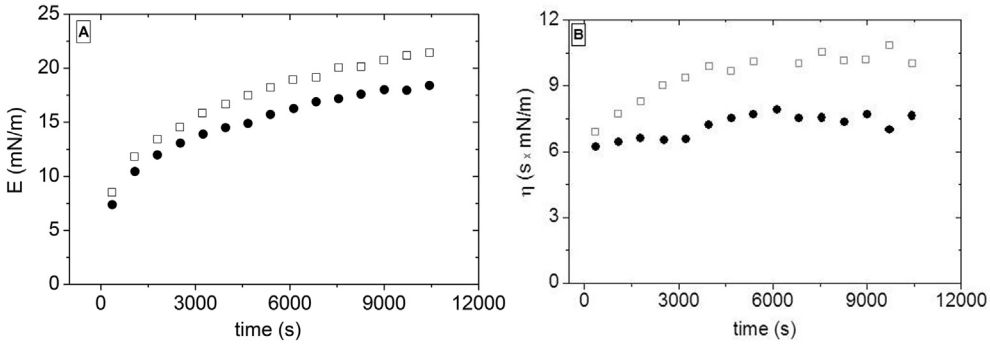

Fig. 4. Time-dependent dilatational modulus (A) and interfacial viscosity (B) for E4M (●) and E5LV (□) adsorbed films at the oil-water interface at a concentration of HPMC of 0.5% (w/w) in the bulk phase at pH 7. Temperature: 37 °C.

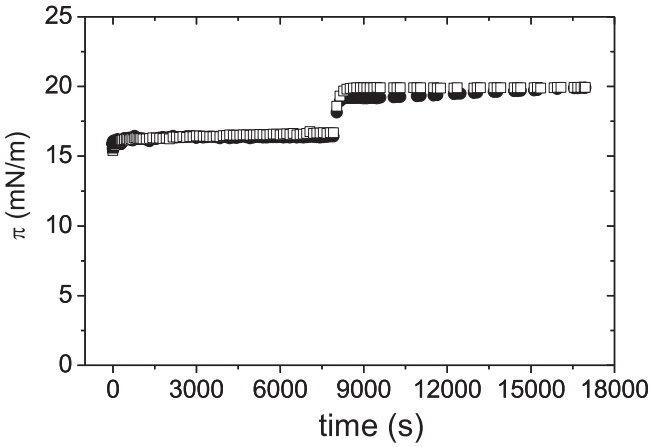

Fig. 5. Time-dependent surface pressure (π) for E4M (●) and E5LV (□) adsorbed films at the oil-water interface, at a concentration of HPMC of 0.5% (w/w) in the bulk phase at pH 7, upon exchanging the subphase by the BS solution at 8000 s. Temperature: 37 °C.

Mackie, Gunning, Wilde, & Morris, 1999; Maldonado-Valderrama et al., 2009).

Fig. 6 shows the effect of introducing the BS into the pre-adsorbed HPMC interfacial films in the time evolution of the dilatational modulus (E), as well as in the interfacial viscosity (η) (Fig. 6 (A and B)). The analysis of these parameters is very important as they can inform about the intermolecular interactions that take place in the interface. Furthermore, it is known that these properties depend on the structure and composition of the interfacial films and it is known that they influence the properties of the emulsion (formation and stability) (Maldonado-Valderrama and Patino, 2010).

It can be observed in Fig. 6A that E values strongly decreased after the addition of the BS, reaching very low values, which indicates that the HPMCs interfacial layers become less elastic in the presence of the BS, with a less structured interface in such a way that the E values were practically close to that corresponding for single BS film (2 mN/m). With respect to the interfacial viscosity, more flexible interfacial films were formed after introducing the BS (Fig. 6B) similar to the BS interfacial films (η ≈ 1.5 s x mN/m).

These results indicate that BS penetrate the HPMCs films as also reported for other emulsions (Beysseriat et al., 2006; Chu et al., 2009; Lesmes et al., 2010; Li & McClements, 2011; Sarkar et al., 2010a; Torcello-Gómez et al., 2011b), hindering the hydrophobic interactions between the HPMC molecules at the o/w interface, thus destroying the gel-like behaviour of films. Nevertheless, both celluloses exhibited a similar interfacial response to the adsorption of BS.

3.4. Impact of BS adsorption onto HPMC films on ζ-potential

ζ-potential is an efficient way to assess changes of the surface composition or surface interactions of HPMCs and BS. Although this technique only provides qualitative information it is interesting to correlate it with results obtained with other experimental techniques (Gu, Decker, & McClements, 2007; Hur, Decker, & McClements, 2009).

The ζ-potential of the freshly prepared HPMC emulsions (2%, w/w) (Table 3) was measured in the absence of the BS and a practically neutral ζ-potential with a value around −1 mV was obtained, which

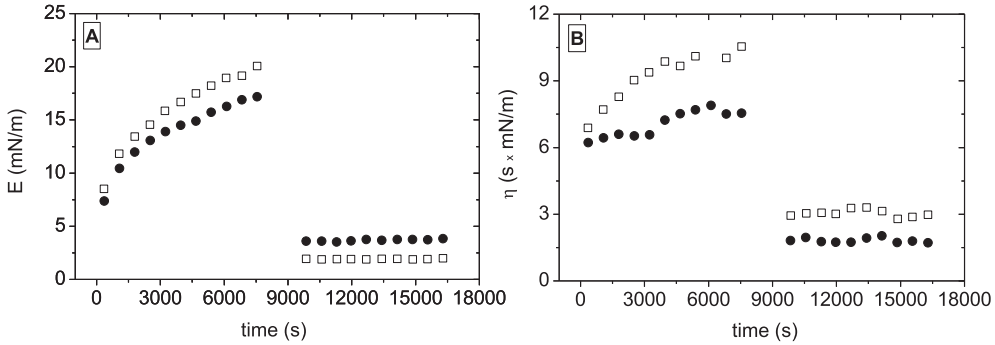

Fig. 6. Time-dependent dilatational modulus (A) and interfacial viscosity (B) for E4M (●) and E5LV (□) adsorbed films at the oil-water interface, at a concentration of HPMC of 0.5% (w/w) in the bulk phase at pH 7, upon exchanging the subphase by the BS solution at 8000 s. Temperature: 37 °C.

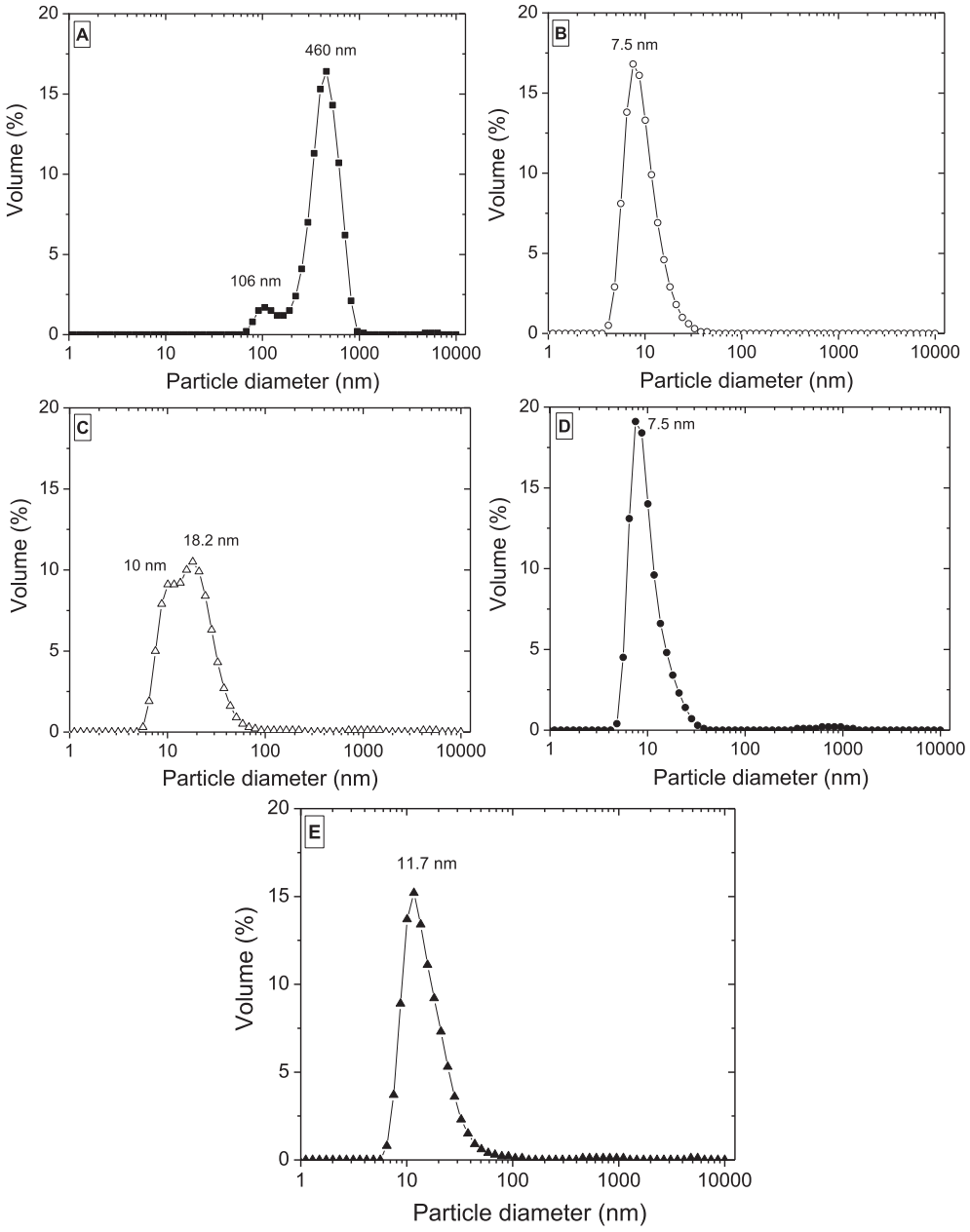

Fig. 7. Volume size distribution (%) for BS solutions (A), E5LV (B), E4M (C), and the mixed E5LV + BS (D) and E4M + BS (E) systems. Temperature: 37 °C.

was an expected result as these HPMCs are non-ionic polysaccharides (Camino et al., 2011).

When mixing these emulsions with the BS under physiological conditions similar interfacial response to the adsorption of BS was observed for both celluloses (Table 3). As expected, the ζ-potential were more negative than those of the original emulsions; however, these values were significantly lower than those corresponding to the BS alone (−45.30 ± 2.97 mV). This points out that, despite BS penetrate the HPMCs films disrupting the original elastic interfacial film (as shown above), BS may be trapped within the HPMC structure, being such a "sequestration" from the surface the cause of a low surface charge of the oil droplets. Nevertheless, both HPMCs performed in a similar way.

As neither the differences between E5LV and E4M emulsions characteristics nor the response of their interfacial films to the BS adsorption could explain the observed differences in the rate and extent of lipolysis, the molecular interactions between both HPMCs and BS in the bulk phase were further studied.

3.5. Bile salts-HPMC interactions in the aqueous phase

BS interact with a large amount of compounds in solution that may affect their self-assembled structure. BS self-assemble in aqueous solution, similar to classical amphiphiles, being the micellization primary driven by hydrophobic interactions, but also by hydrogen bonds. Moreover, BS are rigid, almost flat molecules with weakly separated hydrophobic and hydrophilic faces that results in an incomplete separation of the hydrophilic and hydrophobic domains in the aggregates; some hydrophobic moieties may remain in contact with water and hydrophilic parts might be buried within micelles (Madenci & Egelhaaf, 2010). As a result of these particular structural characteristics BS exhibit a complex self-

Table 4
Cloud point temperature ($T_{cloudpoint}$) and time ($t_{cloudpoint}$) for HPMC/BS mixtures.

	$T_{Cloudpoint}$ (°C)	$t_{Cloudpoint}$ (min)
E5LV 1% (w/w)	59.5 ± 0.7	4.88 ± 0.17
E5LV 2% + SB 2% (1:1) (w/w)	56.2 ± 0.3	2.68 ± 0.31
E4M 1% (w/w)	54.1 ± 0.1	2.58 ± 0.26
E4M 2% + SB 2% (1:1) (w/w)	40.2 ± 2.1	1.27 ± 0.15

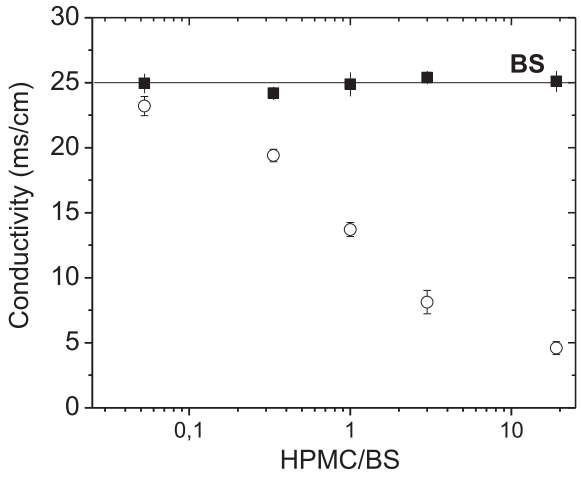

Fig. 8. Conductivity evolution with the mixed HPMC/BS ratio (○) at 37 °C. The values corresponding to the BS alone (■) are indicated as a reference.

assembly behaviour and the critical micellar concentration of BS is considered a concentration range rather than a critical value. There exist a stepwise association process leading to aggregates growing in size with concentration and ionic strength (Calabresi, Andreozzi, & La Mesa, 2007).

BS dissolved in SIF at a final concentration of 5 mg/ml, a value that is within the physiological concentrations (Bellesi et al., 2014; Maldonado-Valderrama & Patino, 2011), mainly self-assembled in micellar aggregates of 460 nm (Fig. 7A), but also formed aggregates of 106 nm.

Fig. 7 (B and C) shows the volume-size distribution of 0.5% (w/w) solutions of the HPMC studied. The size distribution was monomodal for E5LV (Fig. 7B), with a population at 8 nm, and it was bimodal with predominant peaks at 10 and 20 nm for E4M (Fig. 7C). Nevertheless, the peaks were broad, being the width indicative of the existence of polydispersity. All HPMCs form aggregates or clusters, being the size of the aggregates increased with the polysaccharide concentration, indicating that they are concentration-dependent (Camino et al., 2009a). The self-assembly of HPMC would be driven by hydrophobic interactions between the hydrophobic substituents (Kato, Yokoyama, & Takahashi, 1978).

The volume-size distributions of the particles in the mixed BS-HPMC solutions are presented in Fig. 7 (D and E). BS micellar aggregates are not any more apparent in the presence of both HPMCs, showing that BS had been bound by HPMC particles. The size of E5LV-HPMC particles was not affected by the interactions with BS (Fig. 7D) but E4M-HPMC clusters of 20 nm were mostly "dissolved" by BS (Fig. 7E). When BS are interacting with the hydrophobic groups in the cellulose backbone, they would impart a negative

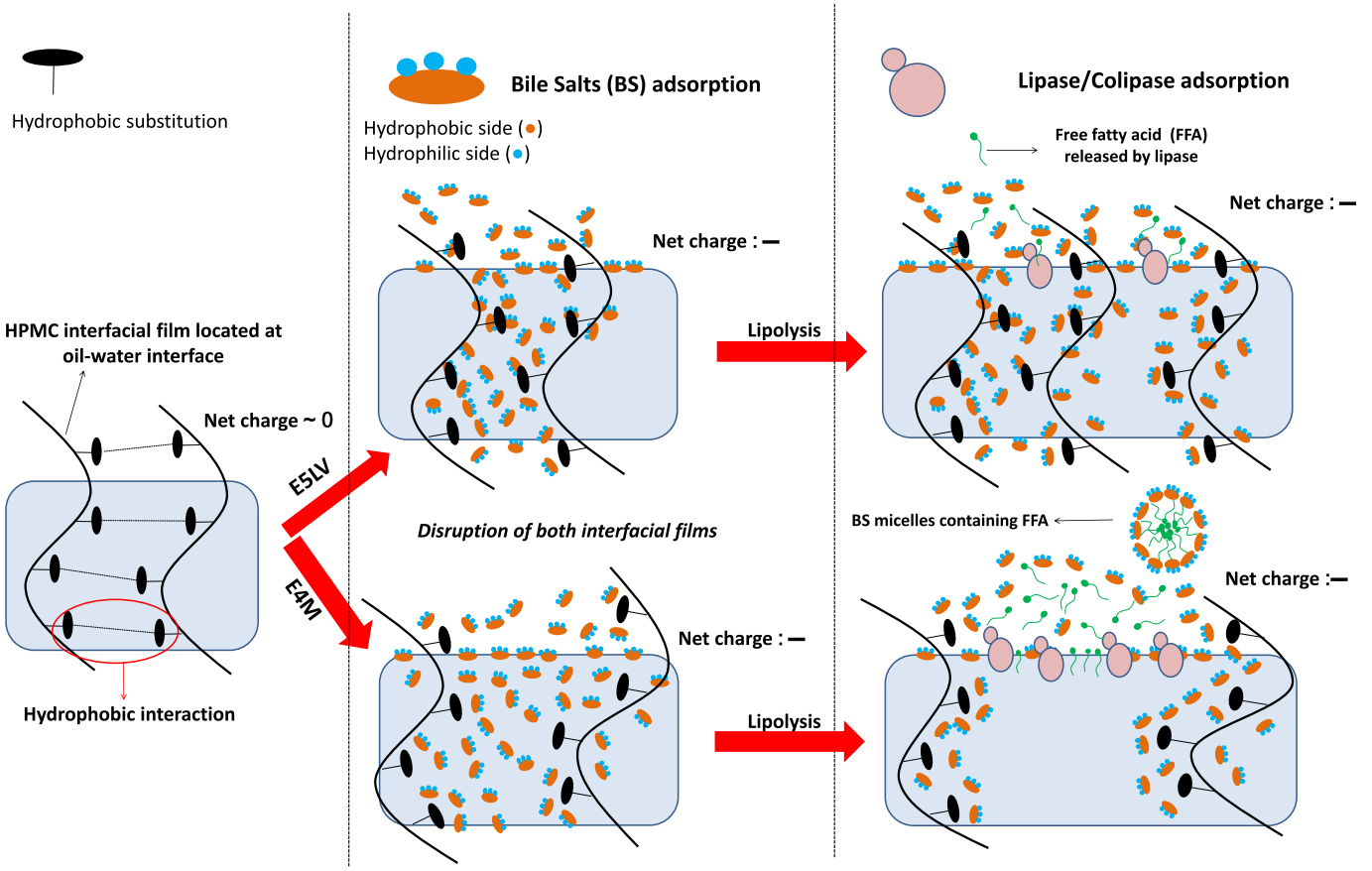

Fig. 9. Schematic representation of the proposed model: Adsorbed BS onto the E4M interface produce a higher disentanglement of the molecules allowing more available sites for the lipase adsorption than the interface formed by E5LV, resulting in a higher extent of lipolysis.

charge that increases the repulsion between the HPMC molecules, thus decreasing their tendency to the aggregation. This seems to be evident for E4M where the clusters of 20 nm are "dissolved" by BS. According to Torcello-Gómez et al. (2015), the cellulose ethers containing more hydroxypropyl groups seem more susceptible to the presence of BS. In fact, E5LV has a higher methyl/hydroxypropyl ratio (Table 1) and seemed less affected by BS.

Moreover, the cloud point of E4M was decreased at a larger extent than that of E5LV, as shown in Table 4. Hydrophobic interactions are also responsible for the gel formation of HPMC on heating. As the temperature is increased, the molecules absorb translational energy and they gradually lose their hydration, resulting first in the lowering of the viscosity. Eventually, a polymer-polymer association takes place due to hydrophobic interactions, causing cloudiness in solution and an infinite network structure which results in a sharp rise in viscosity and of turbidity as long as the concentration is relatively high (Sarkar & Walker, 1995). The negative charge that imparts the adsorbed BS onto HPMC increases the repulsion between HPMC molecules, thus facilitating the disentanglement process promoted by the heating before the cloud point occurs, thus leading to a decrease in the cloud point temperature (Table 4). The E4M cellulose resulted, as shown above, more susceptible to BS, as its self-assembly tendency was more decreased; it was also the cellulose that exhibited the highest reduction in the cloud point.

In order to confirm the binding of BS by HPMC, the conductivity of single HPMC and BS as well as of their mixtures at increasing HPMC/BS ratios was determined (Fig. 8). The conductivity of BS was almost constant ($\approx$25 mS/cm) in the range analysed, but it was strongly decreased by increasing the HPMC/BS ratio, despite the very low conductivity of the non-ionic HPMC (3 mS/cm) (both HPMCs showed similar conductivity values). This reflects the evidence that BS bind onto HPMC, but also that BS charges are screened by this interaction, suggesting that BS micelles are not interacting with water and may be trapped in the core of HPMC clusters. Nevertheless, no significant difference on BS binding ability was found between E5LV and E4M celluloses. This behaviour is similar to that occurring at the interface, as indicated previously.

The binding of BS onto cellulose ethers has recently been assessed by differential scanning calorimetry (DSC) and linear mechanical spectroscopy (Torcello-Gómez & Foster, 2014; Torcello-Gómez et al., 2015). Hydrophobic interactions were postulated to take place between cellulose ethers and the BS, which were reflected in the inhibition of the thermal structuring of cellulose ethers. The hydrophobic nature of the interaction of BS with other amphiphilic molecules has also been reported. In a recent work (Roy, Kundu, Banik, Kuchlyan, & Sarkar, 2015) it was confirmed that BS affect the core region of pluronic P123 micelles.

From a ^{13}C NMR study, it was deduced that soluble dietary fibres SDFs can interact with BS micelles either by forming dynamic complexes with the micelles (as indicated by systematic chemical shift changes in BS resonances) or by trapping BS micelles in a local aggregate structure resulting in reduced BS mobility and consequent broader, less intense BS resonances (Gunness, Flanagan, Mata, Gilbert, & Gidley, 2016).

The strong evidences from the different techniques used to assess the interactions of BS and HPMCs suggest that, in the aqueous phase, BS are bound or "sequestered" by HPMC, mainly by interactions with the hydrophobic core of HPMC, thus been partially screened their charge.

4. Conclusions

This study shows that HPMCs emulsions presented different degrees and rate of lipolysis that cannot be attributed to differences in the molecular weight/viscosity or to the size/surface area available to the action of lipase/colipase nor to differences in the interfacial film properties. The observed susceptibilities to lipolysis could rely on molecular events occurring at the interface upon BS adsorption. Besides both HPMC have shown similar abilities to adsorb and "sequester" BS in the bulk, as well as at the interface, some special features arise from their different methyl/hydroxypropyl ratio. Both methyl and hydroxypropyl groups can bound BS but the more hydrophilic E4M cellulose containing a lower methyl/hydroxypropyl ratio when adsorbed at the interface would be more affected by BS (Torcello-Gómez & Foster, 2014). In fact, as shown above, the self-assembly of E4M cellulose in the bulk was more hindered by BS adsorption. Thus in parallelism we propose that BS adsorbed onto the E4M interface would provoke a higher disentanglement of the molecules allowing more sites available for lipase adsorption (Fig. 9), resulting in a higher extent of lipolysis.

Although it was used a simple model where it was omitted the changes that may occur in the mouth and stomach, that are known to affect in the digestibility of the emulsions in the small intestine, these results will be very useful in order to design interfacial films to control fat digestion.

Acknowledgments

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This work is dedicated in ever loving memory of Nerina A. Camino.

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