

Accepted Manuscript

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PII: S0927-7765(18)30800-2
DOI: <https://doi.org/10.1016/j.colsurfb.2018.11.024>
Reference: COLSUB 9789

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 2 July 2018
Revised date: 25 October 2018
Accepted date: 9 November 2018

Please cite this article as: Naso JN, Bellesi FA, Pizones Ruiz-Henestrosa VM, Pilosof AMR, Studies on the interactions between bile salts and food emulsifiers under *in vitro* duodenal digestion conditions to evaluate their bile salt binding potential, *Colloids and Surfaces B: Biointerfaces* (2018), <https://doi.org/10.1016/j.colsurfb.2018.11.024>

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Studies on the interactions between bile salts and food emulsifiers under *in vitro* duodenal digestion conditions to evaluate their bile salt binding potential.

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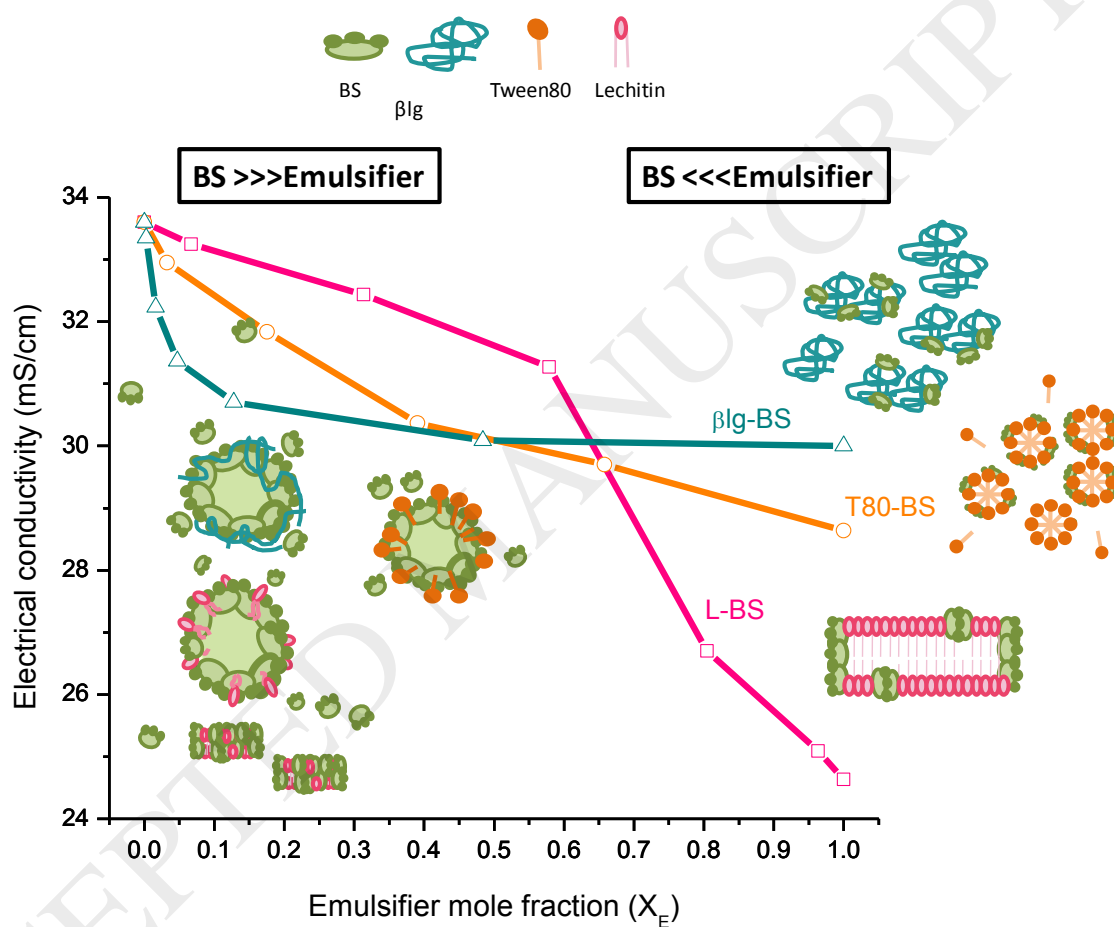
STATICAL SUMMARY

TOTAL NUMBER OF WORDS: 5427

TOTAL NUMBER OF FIGURES/TABLES: 8

Graphical Abstract

Graphical Abstract caption: Conductivity of emulsifier/BS solution, under simulated intestinal fluid



HIGHLIGHTS

- Interactions BS – emulsifiers represent a key factor to modulate lipolysis.
- The molar fraction where structural transitions occur were determined by conductivity
- Conductivity was an alternative method to compare emulsifier's ability to bind BS.

Abstract

During the last decade a special interest has been focused on studying the relationship between the composition and structure of emulsions and the extent of lipolysis, driven by the necessity of modulate lipid digestion to decrease or delay fats absorption or increase healthy fat nutrients bioavailability. Because bile salts (BS) play a crucial role in lipids metabolism, understanding how typical food emulsifiers affect the structures of BS under duodenal conditions, can aid to further understand how to control lipids digestion. In the present work the BS-binding capacity of three emulsifiers (Lecithin, Tween 80 and β -lactoglobulin) was studied under duodenal conditions. The combination of several techniques (DLS, TEM, ζ -potential and conductivity) allowed the characterization of molecular assemblies resulting from the interactions, as modulated by the relative amounts of BS and emulsifiers in solution.

KEY WORD: bile salt, emulsifiers, interactions, binding.

1. Introduction

During the last decade a special interest has been focused on studying the relationship between the composition and structure of emulsions and the extent of lipolysis, driven by the necessity of controlling the digestion of lipids to decrease or delay fat intake, to address the obesity crisis existing worldwide and the implications for long-term chronic diseases. The rate at which fatty acids (FA) are absorbed into the blood (i.e. postprandial triglyceride levels) is now considered to be important for human health; high postprandial triglyceride levels are associated with the activation of various inflammatory pathways and are recognized as risk factors for cardiovascular disease and diabetes[1]. On the other hand, the necessity of controlling the digestion of lipids is of great importance to assure maximum release of FA of nutritional importance, such as omega 3.

As lipolysis is an interfacial reaction, most of those studies have been done under the hypothesis that strengthening the barrier properties (mostly mechanical or sterical) of interfacial films to lipase action could control the rate and extent of lipolysis. However, many results have shown that this kind of barriers cannot significantly inhibit the action of lipase. Moreover, increasing amount of works suggest that there is an indirect impact of components of the interfacial film (or even unadsorbed components) on lipolysis, that rely on their ability to interact with biosurfactants and calcium, that are constituents of duodenal fluids [2].

According to Golding and Wooster [3] the interfacial process of lipolysis involves essentially three key steps: (1) binding of the bile salts (BS) - lipase/colipase complex to the oil-water interface, (2) hydrolysis of the emulsified lipid to 2-monoacylglycerol and two free fatty acids (FFAs) and (3) desorption of these inhibitory lipolytic products by solubilization in mixed BS and phospholipids micelles. Diffusion of micelles then delivers solubilized components across the unstirred water layer covering the luminal side of the enterocytes, thus facilitating uptake of lipophilic components by the enterocytes. Once this role is fulfilled, the BS micelles transit the remainder of the small and large intestine where they are progressively reabsorbed. However, if this transport mechanism fails or is slow, the accumulation of lipolytic products at emulsion interfaces will result in self-regulation of fat digestion.

Recently Sarkar, Ye and Singh [4] demonstrated the role that BS play in the desorption of the inhibitory lipolytic products under simulated intestinal conditions. They showed that the presence of unadsorbed BS markedly enhanced the rate and the extent of lipid digestion. This could be attributed to considerable removal of lipolysis products (FFA, mono- and/or di-acylglycerols) in mixed micelles, which are known to inhibit lipid digestion, by the unadsorbed BS.

In this frame, the potential binding of BS by components forming an emulsion opens up a new area of research to understand the importance of this mechanism in

controlling lipolysis. BS can be partially bound by soy proteins [5], by soluble fibers as chitosan [6], glucan and arabinoxylan [7] or by cellulose ethers [8, 9].

A better understanding of the mechanisms involved in the modulation of the lipolysis is needed as it will help to rationally select the emulsifiers or stabilizers to formulate emulsions with controlled FA release.

In the present work the interactions, under *in vitro* duodenal conditions, between BS extract (BSE) and several typical emulsifiers used to formulate food emulsions are studied by means of several techniques: dynamic light scattering (DLS), conductivity, zeta potential and transmission electron microscopy (TEM). The following low molecular weight emulsifiers were studied in relation to their interaction with BSE: polysorbates (Tween 80) and phospholipids (lecithin). They are very surface active compounds with non ionic and zwitterionic character respectively. A typical milk protein used in the formulation of food emulsions (β -lactoglobulin) was also included in the present study. Many works in the literature have studied its performance during lipolysis [1, 10-13].

As said above, one of the essential function of BS on digestion is solubilization of cholesterol, lipids, FA, monoglycerides and fat soluble vitamins due to the formation of mixed micelles between BS and phospholipid, such as lecithin [4, 14-17]. This helps in effective digestion and absorption of lipids. However, the supra-molecular structures that BS form, may influence their solubilization capacity. Thus, understanding how typical food emulsifiers affect the structures that BS adopt under duodenal conditions of lipids digestion can aid to further understand how to control lipids digestion.

2. Materials and methods

2.1 Materials

Soy lecithin (L) Metarin P (de-oiled, powdered lecithin) was provided by Cargill (Germany). BioPURE β -lactoglobulin (β -lg) was obtained from DAVISCO Foods International, Inc. (Le Sueur, Minnesota) with a protein composition (dry basis) of

97.8%, being β -lactoglobulin 93.6% of total proteins. Tween 80 (T80) of analytical grade was purchased from Biopack. A bovine bile extract (BSE) obtained from Sigma-Aldrich (B3883), was used as the source of BS. This extract contains 50 wt % of bile acids and, according to the producer, the specific composition is: 30–40% taurocholic acid, 10–20% glycocholic acid, 5–10% glycodeoxycholic acid and 5–10% taurodeoxycholic acid, accounting for 60% cholic acid by mass of the total BS. By considering the composition of BS and their corresponding molecular mass, an average molecular mass of 442 g/mol was obtained; the latter was used to define the molar concentration of BS in the experiments.

2.1.1 Sample preparation

All the solutions were prepared in a simulated intestinal fluid (SIF) according to Bellesi, Pizones Ruiz-Henestrosa and Pilosof [18] (39mM K_2HPO_4 , 150mM NaCl and 30mM $CaCl_2$ and pH 7). The BSE concentrations used (Table 1) were all within the physiological concentration range [18-20]. Emulsifiers and BSE solutions were prepared at different concentration ratios for each determination, as detailed in Table 1 and 2. T80, β -Ig and BSE were dissolved in SIF with gently stirring. Lecithin solution has to be stirred overnight to ensure a proper dispersion, because of its low solubility into the aqueous media.

2.2 Methods

2.2.1 Particle size determination

A Zetasizer Nano-ZS analyzer with a He-Ne laser beam (633 nm) (Malvern Instruments, Worcestershire, United Kingdom) was used to determine the particle size distribution by dynamic light scattering, at a fixed scattering angle of 173° . The instrument's measurement range is from 0.6 to 6000 nm. Contin's algorithm was used to analyze the data for percentile distribution of particle/aggregate sizes. The size distribution obtained is a plot of the relative intensity of light scattered by particles in

various size classes (intensity particle size distribution). The intensity particle size distribution can be converted, using Mie theory, to volume particle size distribution [21].

2.2.2 ζ -potential measurements

ζ -potential was measured using a Zetasizer Nano-ZS analyzer. The ζ -potential was determined by measuring the direction and velocity of the particles moving in the applied electric field at 37 °C. The ζ -potential measurements are reported as the average and standard deviation of measurements made on two samples, with ten readings made per sample. The Zetasizer Nano series calculate the ζ -potential by determining the Electrophoretic Mobility and then applying the Henry equation.

2.2.3 Transmission electron microscopy (TEM)

TEM images were obtained by mean of a Zeiss EM 109T transmission electron microscope operating at 80kV. Grids with LRW membranes (300-400 mesh) were used. A drop of 10 μ L of the sample was deposited on the grid and 5 minutes later was removed with filter paper. After ten minutes of drying at room temperature, staining with a drop of a 2 % uranyl acetate solution was performed. This drop was again removed with filter paper and then washed with double distilled water following 5 minutes. The resulting stained film was dried for 15 minutes. Images were acquired with the Gatan ES1000W digital camera (11 Mpx).

2.2.4 Electrical conductivity

The electrical conductivity (mS/cm) of the solutions was determined at 37 °C using a conductimeter Thermo Orion, 125A Plus (USA).

2.2.5 Statistical analysis

The reproducibility of the results was assured from the standard deviation of at least two independent measurements. A one-way ANOVA analysis ($P < 0.05$ is significant) was performed.

3. Results and discussion

3.1 Bile salts

BS are a family of soluble amphiphilic molecules with a chemical structure quite different from the classical amphiphilic molecule, which has a well defined hydrophilic head and hydrophobic tail [22]. BS possess a rigid steroid structure that exhibit planar polarity [23]. The concave side of the steroid skeleton is hydrophilic due to the presence of one, two or three hydroxyl groups and an acidic group that can be conjugated in general with taurine or glycine. The convex side is hydrophobic with an angular methyl group [22, 24]. The acidic group also provides charge, since it is dissociated under typical conditions, so BS are ionic amphiphiles [25]. Similar to classical amphiphiles, BS also form micelles above a certain concentration called critical micellar concentration (CMC), but they are generally smaller because of the rigidity of BS. The micellization is not only driven by the hydrophobic effect, but also hydrogen binding that results in a complex self-assembly behaviour as beyond the CMC, BS micelles self-assemble to form aggregates [25, 26]. A wide number of supra-molecular association modes are observed [27]. Their growth depends on parameters such as ionic strength, BS species and also the concentration range. A higher ionic strength implies a better screening of the electrostatic interactions and thus favors growth of the micelles. Several models have been proposed to describe the unusual self assembly of BS that is still under debate [25, 26, 28]. Micelle/aggregate growth in BS is documented by a lot of experimental evidences, and points out the existence of an almost continuous association process [27, 29].

The size of BSE aggregates formed in SIF (pH 7) at two concentrations relevant for duodenal digestion (0.5% and 1.5% wt, which means 5.7 and 17 mM of BS,

respectively) is shown in Figure 1. The concentration of BS in the duodenum in the postprandial state are ranged typically between 5 and 15 mM, depending on the time after ingestion of the meal, with peak values up to 40 mM [30].

As concentration increased, the size of particles shifted to larger sizes. At 0.5% most of BSE formed aggregates of 396 ± 53 nm with a small population at 5560 ± 237 nm, but at 1.5% three populations were apparent at 255 ± 48 , 712 ± 208 and 5560 ± 90 nm.

The aggregation number typically increases with BS concentration and then seems to saturate at about 30-50 mM for the different individual BS. There is a direct relationship between aggregation number, polydispersity and total BS concentration [25]. Moreover, the high ionic content of SIF may affect the degree of aggregation of BS. The aggregation number of BS micelles in water increases with increasing ionic strength [25, 28]. The influence of inorganic salt on the structure of BS in aqueous solution has been recently studied by means of dissipative particle dynamics simulations [31]. In the absence of inorganic salt, BS tend to form micelles with a low aggregation number, at about 5–6, in aqueous solution. Morphological changes were observed upon adding inorganic salts. Salt addition induced the growth of short oblate beltlike structures due to reducing solubility leading to reducing the surface of all micelles and due to the planar Janus-type structure of BS molecules. The quasi-two-dimensional beltlike structures grow because of parallel orientation of BS molecules, which place their hydrophobic groups in the micelle core.

TEM images in the background of Figure 1 show that the aggregates shape is consistent with a beltlike structure reported by Markina, Ivanov, Komarov, Khokhlov and Tung [31] by means of molecular simulation of BS self assembly in the presence of inorganic salts.

It is important to highlight that a crude bovine bile extract (BSE) is used in the present study, because it simulates the composition of duodenal fluids closely than by using a single BS. This adds complexity to the system as bile contains about 50% of

BS and variable concentrations of phospholipids, fatty acids and cholesterol [16, 32-34]. The ratio of bile salts/phospholipids in the BSE was 33/1.

The interactions of BSE at 0.5 and 1.5%, and the different emulsifiers in the simulated duodenal conditions (SIF) are analyzed in the following sections. Two mixed systems were studied for each emulsifier according to Table 1. The first one was a mixture with a concentration of emulsifier (1.5% wt) three times higher than the BSE (0.5% wt). Contrarily, the second system was a mixture with a concentration of BSE (1.5% wt) three times higher than the emulsifier (0.5 % wt). By considering that BS concentration in the BSE extract was 50%, different emulsifier to BS molar ratios (E/BS) were obtained according to the molecular weight of each emulsifier (Table 1).

3.2. Bile salts- β -lactoglobulin interactions

The volume size distributions of β -lg, BSE and the mixture β -lg/BS at a molar ratio 15/100 are shown in Figure 2A. At pH 7 in SIF only one peak was observed in the volume size distribution of β -lg (1.5% wt) with a maximum value at about 4.6 ± 0.1 nm which falls in between those corresponding to the monomeric (3.3 nm) and the dimeric form (6 nm) of β -lg. At neutral pH, β -lg exists in a dynamic equilibrium between its dimeric and monomeric form [35]. In conditions of higher ionic strength as occurs in SIF, the equilibrium is shifted towards the dimeric form. The volume size distribution of the mixture β -lg/BS at a 15/100 molar ratio, exhibited only one population that almost coincided with the peak of β -lg. The BS aggregates are not present anymore suggesting their disintegration and interaction with β -lg. This was further corroborated by TEM, where the beltlike structures observed in Figure 1 were not detected (data not shown).

The volume size distributions of β -lg, BSE and the mixture β -lg/BS at a molar ratio 1.6/100 in Figure 2B shows that in this case the peak corresponding to β -lg is not present anymore, as well as that corresponding to the BS aggregates at 255 ± 48 nm. Nevertheless, aggregates of 615 ± 190 and 5560 ± 94 nm were present. This result

indicates that β -lg would be bound to BS aggregates. The corresponding TEM image in Figure 3A shows a very dense image where particles cannot be clearly distinguished possibly because of the staining of the protein; anyway the image suggests the presence of big structures.

The ζ -potential values of single β -lg and BSE particles, as well as their mixtures at 15/100 or 1.6/100 β -lg/BS molar ratio, are shown in Figure 4. At the highest molar ratio, the ζ -potential of the mixture is similar to that of β -lg, even if BSE particles had a very low ζ -potential (-33 mV). This behavior suggests that in the associated particles, BS would accommodate in the interior of β -lg aggregates so that no extra negative charge is impaired. Small amounts of the BS may be strongly bound in the protein hydrophobic task [27].

On the other hand, when the molar ratio is 1.6/100, the binding of β -lg molecules on BSE aggregates (Figure 2B) partially screened the negative charge of BS aggregates (-36 mV), decreasing it to -20.8 mV, suggesting that β -lg would be bound to the surface of the big BSE particles observed in Figure 2B.

The formation of mixed aggregates of β casein, and selected BS (sodium deoxycholate (NaDC) and sodium cholate (NaCh)) have been reported by Kuchlyan, Roy, Dutta, Sen and N.Sarkar [36]. The main driving forces for the mixed self-assembly formation are the hydrophobic interactions and hydrogen bonding between BS and β casein. β casein, because of its molecular structure, forms micelles; the β casein-BS complexes (26-31 nm) were slightly greater than the β casein micelles. By means of ionic conductivity measurements in dilute regime, at pH 6.5, Orioni, Roversi, Mesa, Asaro, G.Pellizer and D'Errico [37] demonstrated that a significant number of taurodeoxycholate (NaTDC) binds onto bovine serum albumin (BSA) [37]. Ghosh, Mondal and Mukherjee [38] performed a detailed characterization of the interaction of a series of BS, NaDC, NaCh, and sodium taurocholate (NaTC), with a model transport protein, human serum albumin (HSA). Their results highlight the fact that it is the hydrophobic character of the BS that governs the extent of interaction with the protein.

3.3 Bile salts- Tween 80 interactions

Polysorbate-80 or Tween-80 (polyoxyethylene-20-sorbitan monooleate) is a biocompatible nonionic surfactant and hence is widely used as an emulsifier, solubilizer and wetting agent in food, cosmetics and pharmaceutical applications [39, 40]. Like other surfactant molecules Tween 80, just above the limiting monomer solubility (the critical micelle concentration, CMC), begins to aggregate into micellar structures that are characterized by an inner core comprising the non-polar moieties and an outer surface of polar moieties in contact with the aqueous environment [41].

The volume size distributions of Tween 80 (T80), BSE and the mixture T80/BS at a 200/100 molar ratio are shown in Figure 2A. Only one peak was observed in the volume size distribution of T80 (1.5% wt) with a maximum value at 7.5 ± 0.17 nm.

Lafitte, Thuresson, Jarwoll and Nyden [42], reported a hydrodynamic diameter of 11.4 nm for T80 micelles in water, at a concentration of 5% and Bhattacharjee, Verma, Aswal, Date, Nagarsenker and Hassan [43] reported a hydrodynamic diameter of 12.2 nm for T80 micelles in 0.4 M NaCl. The lower hydrodynamic diameter in the present work is consistent with the higher ionic strength of SIF as addition of electrolytes may dehydrate hydrophilic groups of the nonionic surfactant.

Molecular simulation revealed that the self-assembly of a micelle of polysorbate 80 from randomly dispersed molecules is extremely fast, occurring within a nanosecond of simulation time. The micelle structure is compact with the surface being dominated by the polar terminal groups of the poly ethylene oxide (POE) chains, and exhibits a prolate ellipsoidal shape [41].

The volume size distribution of the mixture T80/BS at a 200/100 molar ratio, exhibited only one population that coincided with the peak of T80 (7.5 ± 0.17 nm). The BSE aggregates are not present anymore suggesting their disintegration and interaction with the T80 micelles. This was further corroborated by TEM, where the beltlike structures observed in Figure 1 were not detected (data not shown).

The volume size distributions of T80, BSE and the mixture T80/BS at a 23/100 molar ratio (Figure 2B), show that, in this case, the peak corresponding to T80 micelles is not present anymore, as well as those corresponding to BSE aggregates (255 ± 48 , 712 ± 208 nm). A new larger supra-molecular aggregate of 825 ± 182 nm is apparent, that would correspond to mixed T80-BSE aggregates. An increase of aggregates already present in BSE at 5560 ± 280 nm is also observed. TEM image from Figure 3B shows the morphology of big aggregates of variable size in accordance with the polydispersity shown by DLS (Figure 2B).

The ζ -potential values of single T80 and BSE particles, as well as their mixtures at 200/100 or 23/100 T80/BS molar ratio, are shown in Figure 4. At the highest molar ratio, the ζ -potential of the mixture is similar to that of T80, even if BSE particles had a very low Z-potential (-33 mV). This behavior suggests that in the associated T80/BS particles, BS would accommodate in the interior of the T80 micelle, so that no extra negative charge is impaired. However, when the molar ratio is 23/100, the binding of T80 molecules on BSE aggregates (Figure 2B) partially screened the negative charge of BSE aggregates (-36 mV), decreasing it to -13.9 mV, suggesting that T80 would be bound to the surface of the big particles as observed in Figure 3B.

Some works support the existence of interactions and the formation of mixed micelles between specific BS and polysorbates [43-47]. Conductivity and surface tension measurements were employed to study the mixed micellization behavior of some BS (NaCh and NaDC) with nonionic surfactants (Tween 20 and Tween 60). Synergism between the individual surfactants in the mixed micelles was determined. Binary mixtures of NaDC and Tween 80 have been studied by conductance, surface tension, and fluorescence techniques. An overall attractive force in the mixed micelle state was observed. The microstructure of mixed micelles composed of T80 and NaDC in a 0.4 M NaCl solution have been further characterized by Bhattacharjee, Verma, Aswal, Date, Nagarsenker and Hassan [43]

3.4 Bile salts- lecithin interactions

Lecithin creates vesicles (700-1000 nm) in water, where it self-organizes into liquid crystalline structures consisting of several bimolecular layers [14, 48-51]. In the present work, a main peak was observed in the volume size distribution of lecithin with a maximum value at 712 ± 116 nm (1.5% wt) (Figure 2A) and 615 ± 15 nm (0.5 % wt) (Figure 2B).

The volume size distribution of the mixture L/BS at a molar ratio 410/100, exhibited a bimodal distribution (Figure 2A). The original peak at 712 ± 72 nm greatly decreased and a new population at 38 ± 27 nm appeared. The BSE aggregates are not present anymore, suggesting their disintegration and interaction with lecithin. The structure of the particles present in the mixed system is shown in the TEM image of Figure 3C. A population of vesicular structures with size between 600 and 2000 nm can be seen with BS particles penetrating the lamellar structure of vesicles. The vesicles surfaces seems to be perturbed by the presence of the BS, because some of them appeared open, with pores or holes in the bilayer, and others have lost the normal integrity with rippled borders.

In dilute aqueous solutions, mixtures of lecithin and BS form spherical or elongated micelles and vesicles, depending on the sample concentration and composition [52]. The transition from micelles to vesicles is a smooth transformation involving a region where micelle and vesicles coexist [53]. During the last decades many authors have studied this phenomenon by different techniques [31, 49, 52-58]. However the comparison of mixed aggregates sizes measured with different techniques, is complicated by the sensitivity of the micelle size to temperature, the ratio of the components, their concentration, BS species, ionic strength and pH [54, 59]. Moreover, vesicles are non-equilibrium structure, so their size is kinetically controlled and thus also depends of the sample preparation procedure [52, 59].

Walter, Vinson, Kaplun and Talmon [56] studied the vesicle-micelle transition of egg PC and NaCh by cryo-TEM. They observed that the initial step in vesicle solubilization

by NaCh (PC/BS molar ratio 180/100) is associated with pore formation and reorganization of the lipid bilayer. If enough NaCh was added to a suspension of egg PC vesicles (122/100, $X = 0.45$), the dispersion became clear, indicating that the structures were small (about 4-5 nm). They also demonstrated that flexible cylindrical micelles are the micellar intermediate between vesicles and small micelles. Therefore, the population of 38 ± 27 nm in Figure 2A, may be attributed to the intermediate cylindrical mixed micelles described by Walter, Vinson, Kaplun and Talmon [56]. Maza and Parra [58] observed by DLS, at an equimolar PC to NaCh relation, a bimodal distribution that corresponds to the coexistence of vesicles (200-1000 nm) and mixed tubular micelle (30-100 nm, peak at 52 nm). Clear signs of bilayer disintegration with formation of open bilayer fragments were also observed. Kiselev, M.Janich, A.Hildebrand, Strunz, Neubert and D.Lombardo [57] observed by DLS and small angle neutron scattering, that with increasing amount of NaDC in a solution at a fixed PC concentration, the vesicles solubilization was characterized by the formation of a variety of supra-molecular structures ranging from ellipsoidal vesicles (160-200nm), ribbon-like structures (600-640nm), rod-like micelles (30nm) and ellipsoidal micelles (7-8nm) coexisting with big particles (900-1000nm). They concluded that the last particles were aggregates of the small ones.

The volume size distributions of lecithin, BSE and the mixture L/BS at a 46/100 molar ratio in Figure 2B show that the peaks corresponding to lecithin and BSE almost disappeared and a new larger supra-molecular aggregate at 825 ± 236 nm is apparent, that would consist of mixed aggregates of lecithin and BSE. An increase of aggregates already present in BSE at 5560 ± 263 nm is also observed. TEM image in Figure 3D confirms the disappearance of vesicles and resembles the morphology of BSE aggregates observed in Figure 1.

The ζ -potential values of single lecithin and BSE particles, as well as their mixtures at 410/100 or 46/100 L/BS molar ratio are shown in Figure 4. At the highest molar ratio, the ζ -potential of the mixture is lower than that of lecithin, suggesting that BSE

particles, that had a very low ζ -potential (-33 mV), were adsorbed onto lecithin particles. In fact, as shown in Figure 3C, at this molar ratio lecithin vesicles membranes are being penetrated by BS, and mixed micellar structures are present. However, when the molar ratio is 46/100, the charge of the BSE supra-molecular aggregates in Figure 2B (-36 mV), appears to be screened by lecithin, suggesting its presence at the surface of those particles.

3.5 Electrical Conductivity

In order to get a deeper understanding of the structural changes occurring as the molar fraction of the emulsifiers (E) increases in the mixed systems (Table 2), the conductivity of the E/BSE solutions in SIF was determined (Figure 5).

The interactions can be easily detected by analyzing the conductivity profiles of the E/BSE mixtures in the range of molar fractions between 0 (only BSE) and 1 (only emulsifier). Each one of the emulsifiers exhibited a very different profile (Figure 5A) that could be interpreted in terms of various molecular events. The initial conductivity of BSE ($X=0$) was 33.6 mS/cm.

The conductivity steeply decreased by increasing the molar fraction of β -lg in the mixture up to about 0.125 and then leveled off. The analysis of size and ζ -potential of the mixture β -lg/BS with molar ratio 15/100 discussed above corresponds to a molar fraction of $X_{\beta\text{-lg}} = 0.13$ (Table 1) that is above the critical value of 0.125. At this $X_{\beta\text{-lg}}$ the particles in solution consisted of β -lg monomers or dimers (Figure 2A), including the BS in the structure as suggested by size and ζ -potential values that did not change practically (Figure 2A and 4). The almost invariant conductivity at $0.125 \leq X_{\beta\text{-lg}} \leq 1$ can be related to increasing saturation of β -lg as BS increases in the mixture. In fact, if the conductivity profile is analyzed in the direction of descending molar fractions (starting from $X_{\beta\text{-lg}} = 1$), it is seen that by increasing BS the conductivity does not change up to the critical $X_{\beta\text{-lg}}$ (0.125) where β -lg would become saturated by BS and a structural change takes place. It has been reported that the number of deoxytaurocholate

(NaTDC) molecules moving as a whole kinetic entity with BSA, determined as the protein titration threshold by ionic conductivity is close to 20 at pH 6.5 [37]. This value corresponds to a BSA molar fraction of 0.048. The critical values for these proteins (β -lg and BSA) are very low in comparison with that for T80 and lecithin systems, as will be discussed below.

Well below the critical point, the particles in solution consisted of aggregated BSE particles that included β -lg mostly in the surface, as revealed by the size and ζ -potential values shown in Figure 2B and 4, corresponding to the mixture β -lg/BS with molar ratio 1.6/100 discussed above and a molar fraction of $X_{\beta\text{-lg}} = 0.016$ (Table 1). The steep decrease of conductivity in the range $0 \leq X_{\beta\text{-lg}} \leq 0.125$ may be mainly attributed to the decrease of surface charge (ζ -potential) of BS particles because of the interaction with β -lg (Figure 4).

The conductivity also decreased by increasing the molar fraction of T80 up to about 0.4, but more slowly than for β -lg. After the break point $X_{\text{T80}}=0.4$, the conductivity further decreased but with a decreased slope. At molar fractions well below the critical point, the particles in solution consisted of aggregated BS particles that included T80 mostly in the surface, as revealed by the size and ζ -potential values shown in Figure 2B and 4, corresponding to the mixture T80/BS with molar ratio 23/100 discussed above and a molar fraction of $X_{\text{T80}} = 0.18$ (Table 1). The decrease of conductivity in the range $0 \leq X_{\text{T80}} \leq 0.4$ may be mainly attributed to the decrease of surface charge (ζ -potential) of BSE particles because of the interaction with T80. Around the critical point a structural transition would take place to particles of smaller size and charge (close to T80 micelles) as shown above (Figure 2A and 4) for the mixture T80/BS of molar ratio 200/100 that corresponds to $X_{\text{T80}} = 0.66$ (Table 1).

The conductivity showed initially the lowest decrease in the presence of lecithin up to a break point $X_{\text{L}}=0.6$, where the conductivity experienced a steep decrease. A second break point can be observed at $X_{\text{L}}=0.8$, above which the conductivity further decreased but with a smaller slope.

If the conductivity profile is analyzed in the direction of descending emulsifier molar fractions (starting from $X_L=1$), different types of interactions occurring in BS with phospholipid bilayers may be identified in accordance to the abundant existing literature mentioned before. At $X_L=1$ lecithin in SIF at pH 7 creates vesicles (600-700 nm) (Figure 2A). The BS at low concentration are inserted into the phospholipid vesicles without disrupting the membranes [49, 53] increasing the surface charge of liposomes and thus the conductivity.

At $X_L=0.8$, the system corresponds to that previously analyzed by DLS, TEM and ζ -potential, corresponding to a L/BS at a 410/100 molar ratio (Table 1). The structure of the particles present in the mixed system showed in the TEM image of Figure 3C revealed vesicular structures with sizes between 600 and 2000 nm, with BS particles penetrating their lamellar structure. In addition, a population at 38 nm, attributed to the intermediate cylindrical mixed micelles, as described above, was also present (Figure 2A).

Over the range $0.6 \leq X_L \leq 0.8$ vesicles would become solubilized when increasing the concentration of BS. Mixed vesicle–mixed micelle systems would predominate in this range. According to Almgren [60], surfactants added into an aqueous solution containing lecithin vesicles promoted disintegration of the lamellar structure, resulting in the formation of mixed micelles. Nevertheless this is a smooth transition that commences when a saturation of lamellae with the adsorbed surfactant is achieved. Below $X_L=0.6$ the existence of small mixed micelles (about 4-5 nm) has been reported for L/BS [52, 53, 56, 57, 61]. Nevertheless in this molar fraction range we have observed only large supra-molecular aggregates of 825 ± 236 nm (Figure 2B). Probably in the SIF medium, the small mixed micelles could be aggregated in those supra-molecular structures, because of the high ionic strength.

4. Conclusions

Besides their different nature, zwitterionic or non-ionic low molecular weight surfactants (lecithin and Tween 80) or macromolecular surfactant as a protein, all of them interacted with BS. The combination of several techniques (DLS, TEM, ζ -potential and conductivity) allowed the characterization of molecular assemblies resulting from the interactions, as modulated by the relative amounts of BSE and emulsifiers in solution. The conductivity technique further allowed to determine specifically the molar fraction where structural transitions occur. The molecular assemblies formed by interaction of BS and the studied emulsifiers could affect the ability of BS to remove fatty acids from the surface of oil droplets during lipolysis, thus affecting the rate or extent of fatty acids release. Whether these assemblies improve or decrease the ability of BS to solubilize the free fatty acids will be reported in the following work.

Acknowledgements

This work has been supported by Universidad de Buenos Aires (Grant Number 20020130100524), Consejo Nacional de Investigaciones Científicas y Técnicas, Agencia Nacional de Promoción Científica y Tecnológica (Grant Number: PICT-2014-3668).

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ACCEPTED MANUSCRIPT

FIGURE 1

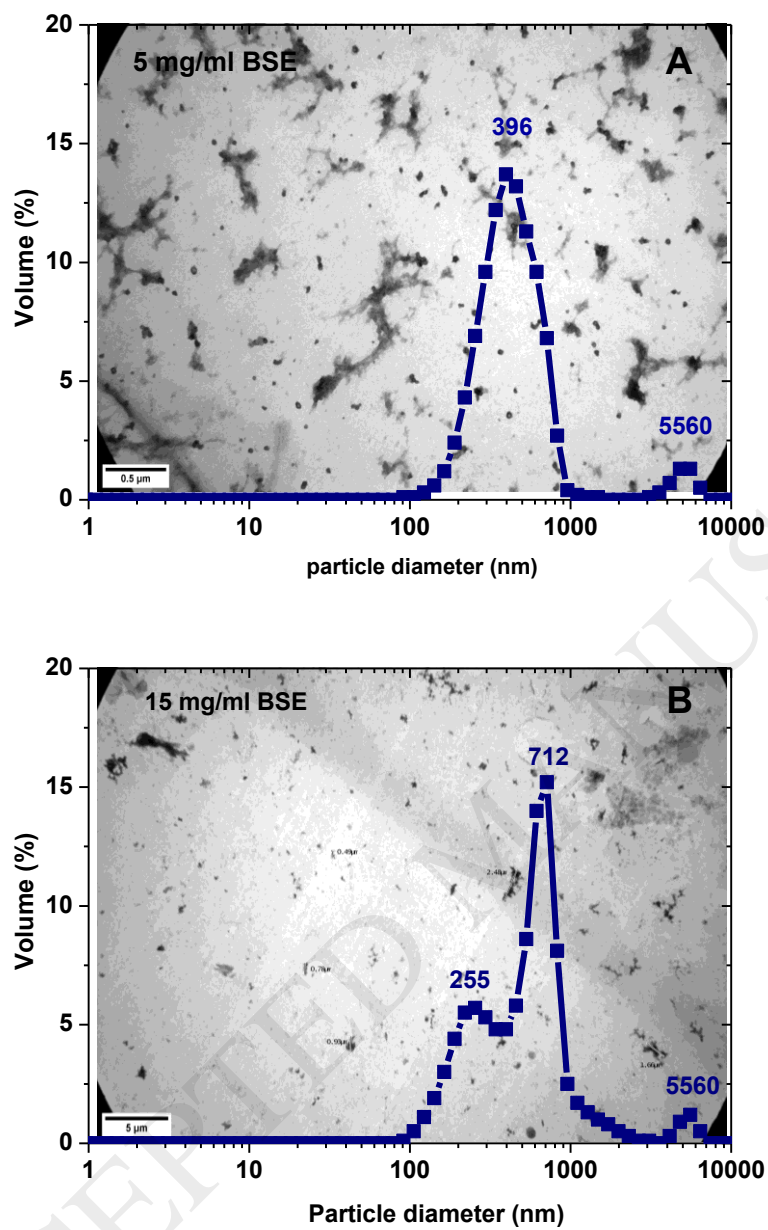


FIGURE 1 Volume size distribution and TEM micrographs of BSE at 0.5% wt (A) and 1.5 % wt (B). Magnification: 30000x and 3000x, respectively.

FIGURE 2

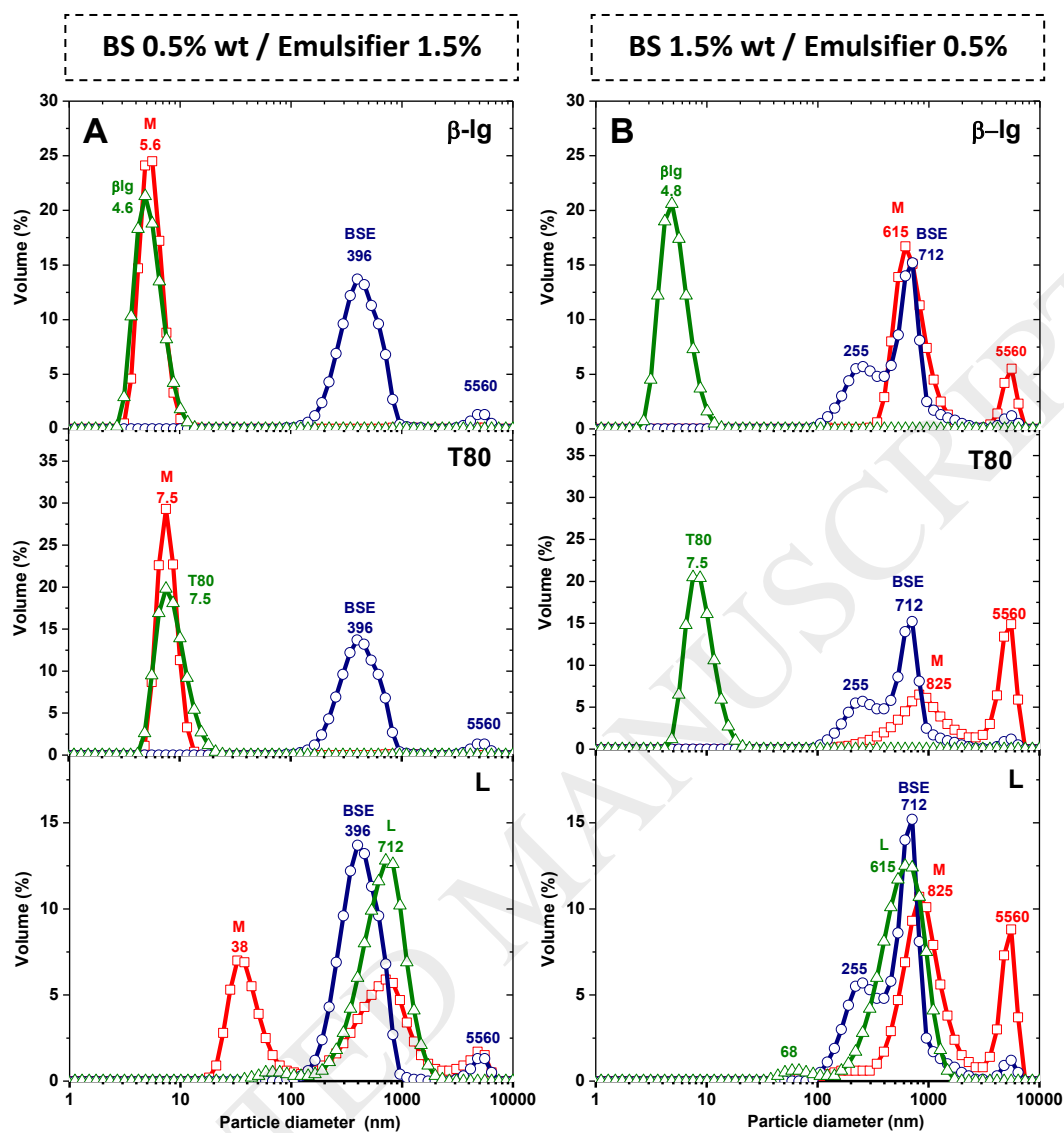


FIGURE 2 Volume size distributions of β -lactoglobulin, Tween 80 and lecithin in presence of BSE at 0.5% wt (A) and 1.5% wt (B). Emulsifier: (Δ), BSE (\circ) and Mixtures (\square).

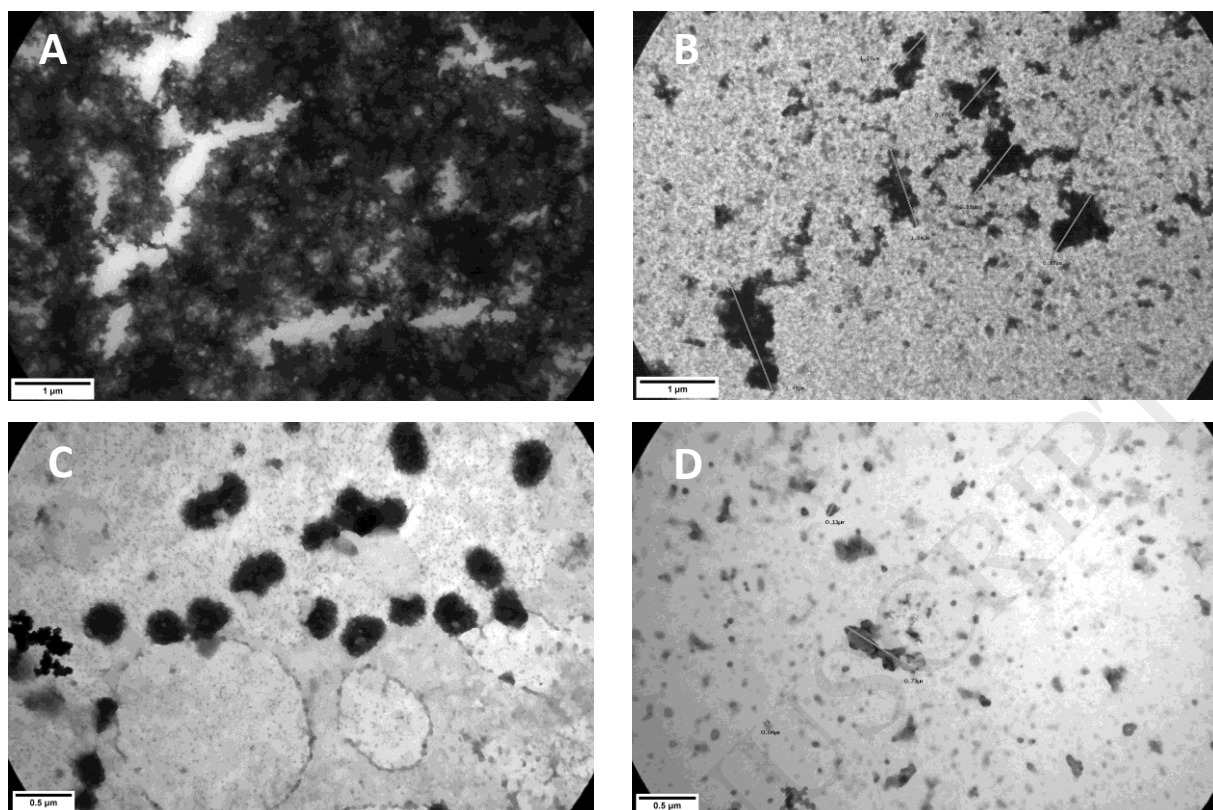


FIGURE 3 TEM micrographs of mixtures (%wt) formed by: β -Ig 0.5% and BSE 1.5% (A), T80 0.5% and BSE 1.5% (B), L 1.5% and BSE 0.5% (C) and L 0.5% and BSE 1.5% (D). Images magnification: 30000x

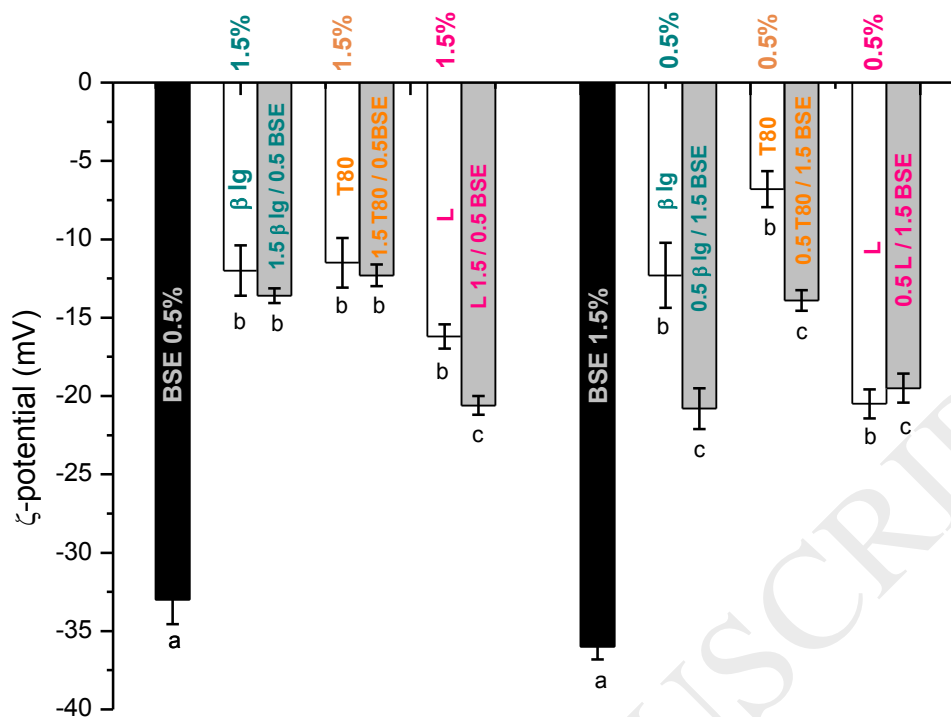


FIGURE 4 ζ -Potential of emulsifier (white bar), BSE (dark bar) and mixtures (grey bar). Error bar represent SD (n=3). Different letters indicate significant differences ($p < 0.05$) between BSE, emulsifier and mixtures.

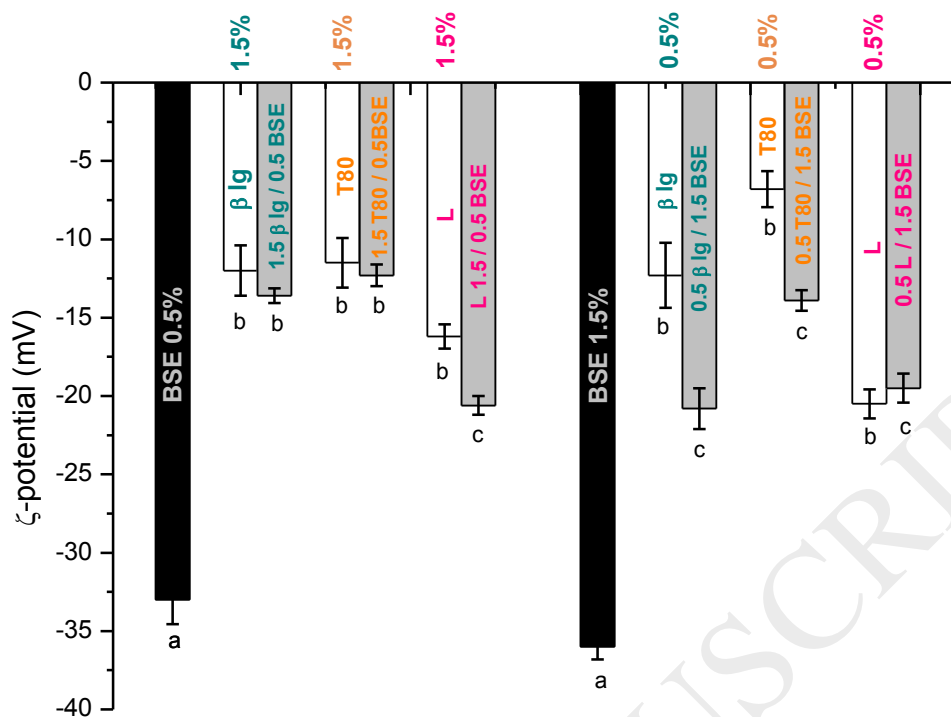


FIGURE 4 ζ -Potential of emulsifier (white bar), BSE (dark bar) and mixtures (grey bar). Error bar represent SD (n=3). Different letters indicate significant differences (p<0.05) between BSE, emulsifier and mixtures.

TABLE 1 Relations of emulsifier/BS used in TEM, DLS and ζ -Potential analysis

BSE (% wt)	Emulsifier (% wt)		Molar ratio (E/BS*)	X_E
0.5	β lg	1.5	15:100	0.13
	T80		200:100	0.66
	L		410:100	0.80
1.5	β lg	0.5	1.60:100	0.016
	T80		23:100	0.18
	L		46:100	0.31
*The molar concentration of BS was calculated as if represented the 50% wt of BSE				

TABLE 2 Relations of emulsifier/BS used in electrical conductivity analysis.

		<i>βlg</i>		<i>T80</i>		<i>L</i>	
BSE (% wt)	Emulsifier (% wt)	Molar ratio (E/BS*)	X_E	Molar ratio (E/BS*)	X_E	Molar ratio (E/BS*)	X_E
2	0	0:100	0	0:100	0	0:100	0
1.9	0.1	0.26:100	0.003	4:100	0.03	7.2:100	0.07
1.5	0.5	1.60:100	0.016	23:100	0.18	46:100	0.31
1	1	4.9:100	0.05	67:100	0.39	137:100	0.58
0.5	1.5	15:100	0.13	200:100	0.66	410:100	0.80
0.1	1.9	92:100	0.48	1283:100	0.92	2600:100	0.96
0	2	-	1	-	1	-	1

*The molar concentration of BS was calculated as if represented the 50% wt of BSE