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Biopolymer nanoparticles designed for polyunsaturated fatty acid vehiculization: Protein–polysaccharide ratio study

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ABSTRACT

Information about the design of biopolymer nanoparticles (BNPs) for polyunsaturated fatty acid (PUFA) vehiculization is provided. Linoleic acid (LA) was used as a model PUFA. The binding ability of LA to β-lactoglobulin (BLG) was applied for obtaining BLG–LA complexes. BLG–LA complex formation was monitored by fluorimetry and it was observed that a moderate heat treatment (60 °C, 10 min) enhanced BLG–LA complexation. Obtaining BNPs involved the electrostatic deposition of high methoxyl pectin (HMP) onto the BLG–LA complex surface. The phase behavior of biopolymer systems was discussed at different Prot:HMP ratio ($R_{Prot:HMP}$, wt.%) levels (1:1–6:1). Absorbance at 600 nm, particle size, and ζ potential were analyzed at pH 4.0. At 1:1–2:1 $R_{Prot:HMP}$, BNPs showed appreciable turbidity, a nanometric diameter (337–364 nm), and a negative ζ potential. Finally, intrinsic and extrinsic fluorimetry was used for examining the HMP protective role at the LA binding site. At 2:1 $R_{Prot:HMP}$, HMP cover could promote significant LA protection in BNPs.

1. Introduction

Currently, one of the main challenges in the development of new functional foods is the incorporation of bioactive compounds into food matrices. Because of recognized properties of bioactive compounds, the new generation of food products promotes functional and nutritional requirements, producing additional effects on health. Effective bioactive compound incorporation into food matrices depends on their physicochemical properties and the existence of favoured molecular interactions with other food ingredients. Unfortunately, the most interesting bioactive compounds to be incorporated are very sensitive to the environmental conditions of processing and storage of foods (oxygen, UV radiation and temperature). Hence, in order to reach the desired effects, a strategy for incorporating bioactive additives is needed, e.g. through encapsulation technologies. In this sense, several encapsulation techniques for sensitive bioactive molecules have been developed, according to their physicochemical properties and environmental susceptibility (Augustin & Hemar, 2009; Joye, Davidov-Pardo, & McClements, 2014; Matalanis, Jones, & McClements, 2011).

One of the encapsulation strategies uses biopolymer nanoparticles (BNPs) as matrices or vehicles for the transport, protection and controlled release of bioactive compounds (Jones & McClements, 2011; Joye et al., 2014; Zimet & Livney, 2009). These particles can be obtained under aqueous medium conditions in which protein–polysaccharide self assembly take place (Jones, Decker, & McClements, 2009; Jones, Lesmes, Dubin, & McClements, 2010). Moreover, the employment of these biopolymer materials is possible due to their high availability, biocompatibility and biodegradability. Moreover, the small size of BNPs could have a lot of advantages over conventional encapsulation systems, such as higher stability to aggregation and gravitational separation, higher optical clarity and improved bioavailability (Joye et al., 2014; Lesmes & McClements, 2009). Nevertheless, the design of BNPs for encapsulation purposes involves the systematic study of the functional properties of individual biopolymers, the molecular interactions between biopolymers and the bioactive compounds, and the process variables that govern such interactions. Another important aspect in the design of BNPs, for bioactive compounds vehiculization in foods, is the knowledge of factors involved in the phase behavior and colloidal stability, such as particle size and electrical properties. It is well established that a lower particle size and higher electrical potential are required for a good colloidal

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stability in aqueous mediums (Jones et al., 2009, 2010; Lesmes & McClements, 2009; Ryan et al., 2011).

In this context, the aim of the present paper was to provide experimental information about the design of BNPs for polyunsaturated fatty acid (PUFA) vehiculization in acidic, aqueous systems. β-Lactoglobulin was used due to its recognized ability for binding lipophilic compounds (Kontopidis, Holt, & Sawyer, 2004). Linoleic acid (LA) and high methoxyl pectin (HMP) were employed as models of PUFA and anionic polysaccharides, respectively. The applied strategy consisted of: (i) obtaining BLG–LA complexes via BLG ability for binding LA, and (2) BNP production via HMP electrostatic deposition (attractive electrostatic interaction) onto the surface of pre-formed BLG–LA complexes. LA encapsulation using BNPs is justified due to their high environmental susceptibility in food matrices (Perez, Andermatten, Rubiolo, & Santiago, 2014; Sponton, Perez, Carrara, & Santiago, 2014, 2015a, 2015b). This strategy was reported by Zimet and Livney (2009), supporting the idea that a cover of polysaccharide on the surface of the protein-PUFA complexes could favour the protection of PUFA molecules, mainly against oxidation. However, here we propose a systematic study about the process variables, such as pH and protein–polysaccharide concentration ratio ($R_{Prot:HMP}$), for obtaining BNPs for PUFA vehiculization with special emphasis on their phase behavior and colloidal stability.

2. Materials and methods

2.1. Materials

β-Lactoglobulin (BLG) was provided by Davisco Food International (USA) and its chemical composition was (wt.%): 90.82% protein, 0.20% fat, 1.90% ash, 4.80% moisture and 2.28% other. Linoleic acid (LA, cis, cis-9, 12-octadecadienoic acid) was purchased from Sigma (USA). LA was kept under a N_2 atmosphere at −18 °C according to manufacturer advice. High-methoxyl pectin (HMP) was kindly supplied by Cargill (Argentina). HMP was obtained as a mixture extracted from citrus peels and apple pomace, and had the following characteristics (data supplied by Cargill): 68.0 ± 2.0% degree of esterification (DE) and composition (wt.%): 87.0% carbohydrate, 11.0% moisture and 2.0% ash (Na^+ 480 mg/100 g and K^+ 160 mg/100 g, Ca^{+2} 200 mg/100 g, Mg^{+2} 30 mg/100 g and Fe^{+2} 2 mg/100 g). Aqueous medium pH modifier, glucono-δ-lactone (GDL), was purchased in Sigma (USA). The fluorescence probe, 1-anilino-8-naphtalenesulphonic acid (ANS), was purchased in Fluka Chemie (Switzerland).

2.2. Obtaining of BLG–LA complexes

BLG dispersion was prepared at 0.16 wt.% in MilliQ ultrapure water, and the pH adjusted to 7.0 using 0.1 M HCl or NaOH. This stock dispersion was stirred for 1 h at room temperature and, subsequently, it was filtered through a glass microfiber pre-filter and cellulose ester filter of 0.22 and 0.45 μm pore size (Millipore, USA). Filtration was done in order to eliminate possible protein aggregates. The BLG stock dispersion was diluted at 0.08 wt.%. Additionally, a stock ethanolic solution of 4.0 M LA was prepared. BLG–LA complex production was carried out by the addition of 38 μl of the LA solution into 2 ml of the BLG diluted dispersion. This experimental condition (based on previous studies) was adequate for producing the binding site saturation on BLG (Perez et al., 2014). The ethanol concentration did not exceed 2.0%, therefore, it was assumed that no protein structural modification was produced (Sponton et al., 2014). After LA addition, tubes were vigorously stirred for 2 min in a vortex. It was assumed that ethanol dissipates in the aqueous medium, and the LA molecule binds to BLG producing BLG–LA complexes (Zimet & Livney, 2009). BLG–LA complex production was monitored by means of fluorimetry using a spectrofluorimeter (Hitachi 2000, Japan) equipped with a 1-cm pathlength quartz cell. Excitation and emission wavelengths were 295 and 332 nm, respectively. Fluorescence values were expressed in terms of relative fluorescence intensity (RFI), being RFI = F/F_0, where F is the fluorescence intensity of the BLG–LA complex and F_0 corresponds to the fluorescence intensity of pure BLG (Perez et al., 2014; Sponton et al., 2014). Usually, addition of LA to the BLG dispersion cause an increase in RFI, which would correspond to an increase in BLG–LA complex amount in aqueous solutions (Frapin, Dufour, & Haertle, 1993). Furthermore, the effect of temperature on RFI of BLG–LA complexes was evaluated in order to observe if heat treatment could modify the protein–ligand binding properties. For this, dispersions containing BLG–LA complexes were heated in a thermostatic bath (Dalvo instruments, BTMP model) in the range of 25 and 90 °C for 5 and 10 min. All experiments were done in triplicate.

2.3. Formation of biopolymer particles

2.3.1. Experimental

Biopolymer particles for LA vehiculization were obtained by HMP electrostatic deposition onto the BLG–LA complex surface in acidic pH. In this work, the effect of different levels of protein–HMP concentration ratio ($R_{Prot:HMP}$, wt.%) on the formation of biopolymer particles was evaluated. The $R_{Prot:HMP}$ levels comprised between 1:1 and 6:1 (wt.%) For this, BLG–LA complexes and HMP dispersions were prepared in MilliQ ultrapure water at pH 7.0 and appropriate volumes of these dispersions were mixed. The HMP dispersion was previously heated to 70 °C for 15 min, promoting adequate polysaccharide hydration. In all systems the BLG concentration was maintained constant at 0.08 wt.% (protein concentration used for BLG–LA complex production). Biopolymer particles formation at the different $R_{Prot:HMP}$ levels were evaluated by decreasing pH which promote the attractive electrostatic interaction between BLG–LA complex cationic groups and HMP anionic groups. Decrease in aqueous medium pH was conducted as will be mentioned below.

2.3.2. Phase behavior diagrams

The phase behavior of biopolymer systems was evaluated according the method described by Fioramonti, Perez, Aríngoli., Rubiolo., and Santiago (2014). In order to decrease the aqueous medium pH, GDL was added to mixed dispersions containing BLG–LA complexes and HMP. The GDL concentration used was 0.35 wt.%. From these mixed dispersions, temporal evolution of transmittance (T%) and pH values were determined. Transmittance measurements were performed in a Turbiscan MA 2000 (France). The variation of aqueous medium pH was automatically measured using a Sper Scientific pH meter (USA). Evolution of pH values were registered by means of instrument specific software. With the T% and pH dynamic profiles, curves of T% vs pH were constructed. From these curves, transition pH values (pH_c and pH_ϕ) were calculated as the intersections of the tangents between the inflection points of T% vs pH curves. In order to examine the phase behavior of the biopolymer mixed systems, pH vs $R_{Prot:HMP}$ phase diagrams were constructed from pH_c and pH_ϕ values (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003). All measurements were performed in triplicate at 25 °C.

2.3.3. Absorption and fluorescence spectroscopy

In order to evaluate the molecular characteristics of the biopolymer particles for LA vehiculization, absorption and fluorescence (intrinsic and extrinsic) spectroscopy was applied. In these experiments, biopolymer particles were obtained by decreasing

the pH slowly from 7.0 to an appropriate pH value (acidic) using HCl 0.1 M. This fixed acidic pH value was established according to phase behavior results.

Absorbance at 600 nm (A_{600}) was determinated in a Jenway 7305 spectrophotometer (UK). A_{600} was considered as a measure of system turbidity (Jones et al., 2009, 2010; Ryan et al., 2011; Fioramonti et al., 2014).

Fluorescence measurements were performed using a spectrofluorimeter (Hitachi 2000, Japan). Intrinsic fluorescence determination (due to Trp emission) was performed at 295 nm excitation wavelength, and the emission spectra were recorded at 300–450 nm. The maximum fluorescence intensity (FI^{M}_{Trp}) was identified from the spectra. Moreover, extrinsic fluorescence measurements were performed using ANS. For this, 100 μl 8.0 mM ANS were added to 2 ml of biopolymer mixed dispersion (Perez, Carrera-Sánchez, Rodríguez-Patino, Rubiolo, & Santiago, 2012). The excitation wavelength was 350 nm and the emission spectra were recorded between 400 and 600 nm. Maximum fluorescence intensity (FI^{M}_{ANS}) was identified from the spectra. All measurements were performed in triplicate at 25 °C.

2.3.4. Particle size distribution and ζ potential

Particle size distribution (PSD) and ζ potential determinations were performed in a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK). As was mentioned in the previous section, biopolymer particles were formed at an appropriate acidic pH, established by the phase behavior results. PSD was obtained by dynamic light scattering (DLS) at a set angle of 90°. The light source of the instrument operated at a wavelength of 632.8 nm. The particle diameter was obtained from the intensity (%) vs diameter (nm) curve. The PSD in volume (%), as well as polydispersity index (PdI) were also considered in particle diameter analysis. The PSD in volume (%) was generated from the PSD in intensity (%) by applying Mie theory. The PdI was calculated from correlation data of cumulants analysis. PdI values smaller than 0.05 indicate samples highly monodispersed, while values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the dynamic light scattering (DLS) technique (Malvern Instruments Limited, 2014). For ζ potential measurements, the instrument determined the electrophoretic mobility distribution of particles by means of the laser Doppler velocity technique. The ζ potential values were calculated according to Smoluchowski model using the instrument specific software. All determinations were performed in triplicate at 25 °C.

2.4. Statistical analysis

Statistical differences were determined through one way analysis of variance (ANOVA) using the StatGraphics Plus 3.0 software. For this, an LSD test at 95% confidence level was applied.

3. Results and discussion

3.1. Heat behavior of BLG–LA complexes

BLG has the ability to solubilize and transport lipophilic bioactive compounds, such as retinol, fatty acids and vitamin D, and it is believed that this property could be linked to possible biological functions (Kontopidis et al., 2004). Therefore, BLG binding properties can be applied for the development of PUFA vehiculization systems in order to protect them from deterioration factors, such as oxygen, UV radiation, and temperature (Sponton et al., 2014; Zimet & Livney, 2009). In a previous paper, we have reported that at pH 7.0, BLG binds to LA with an apparent dissociation constant of $K_d' = 1.3 \times 10^{-7}$ M, involving one binding site ($n = 0.93 \pm 0.05$) (Perez et al., 2014). Nevertheless, one interesting question considered in this paper was to evaluate if heat treatment could modify the LA binding properties of BLG. According to this, Fig. 1 shows the effect of temperature, in the range of 25–90 °C, on the relative fluorescence intensity (RFI) of BLG–LA complexes. Heat treatment was performed at two different times: 5 and 10 min. In the first place, it was observed that at 25 °C, RFI values were greater than 1.0, suggesting BLG–LA complex formation (Frapin et al., 1993). Subsequently, RFI values increased slightly with temperatures from 25 °C to around 60 °C, especially when BLG–LA complexes were heated for 10 min. A further increase in temperature promoted a gradual decrease in RFI values for the two times analyzed. The first behavior observed can be explained considering that a gradual increase in temperature promotes the progressive exposition of buried hydrophobic residues into the protein, and consequently, could have an optimal temperature range for promoting better LA binding properties. It is well known that around 60–65 °C BLG exists in a particular conformation, called a "*molten globule*" in which the protein exposes a greater amount of hydrophobic residues conserving a considerable compactation level (Iametti, De Gregori, Vecchio, & Bonomi, 1996; Nicolai, Britten, & Schmitt, 2011). According to this, we have hypothesized that in its *molten globule* state, BLG could better link LA at the binding site via increased hydrophobic interactions. This hypothesis is consistent with previous works and it highlights the idea that a moderate heat treatment (around 60 °C) could improve BLG–LA complex stabilization (Le Maux, Giblin, Croguennec, Bouhallab, & Brodkorb, 2012; Lisková et al., 2011).

On the other hand, as can be also observed in Fig. 1, from 65 °C, RFI values decreased abruptly with the increase in temperature. With respect to this behavior, it is well known that at temperatures above 60–70 °C, BLG denaturation and aggregation phenomenon would take place (Iametti et al., 1996; Nicolai et al., 2011). These phenomena could promote a decrease in RFI values (due to a lower amount of LA in the BLG–LA complexes) probably due to the displacement of the LA molecule from its binding site. In relation with our previous work, we think that preservation of the spatial domain around binding site could be relevant for maintaining LA linked to BLG (Perez et al., 2014). According to these results, it was interesting to consider BLG–LA complex treated at 60 °C for 10 min (BLGt–LA complex) for biopolymer particles obtention.

3.2. Phase behavior of biopolymer particles

Molecular interactions between BLG and pectins have been researched by different authors (Jones et al., 2009, 2010; Sperber,

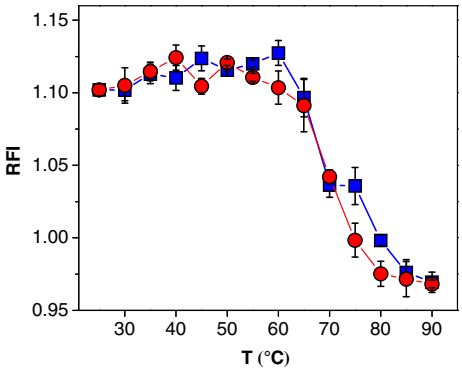

Fig. 1. Effect of temperature, in the range 25–90 °C, on the relative fluorescent intensity (RFI) of BLG–LA complexes. Symbols: (●) 5 min, (■) 10 min. RFI = F/F_0, where F is the fluorescence intensity of BLG–LA complexes and F_0 is the fluorescence intensity of pure BLG. Measurement conditions: pH 7.0, 0.08 wt.% protein.

Schols, Cohen Stuart, Norde, & Voragen, 2009). When the pH of BLG and pectin mixed dispersions is adjusted to a value below BLG isoelectric point (pI: 4.8–5.2), soluble complexes, coacervates and precipitates are formed depending on the aqueous medium conditions (pH, ionic strength, biopolymer relative concentration, biopolymer charge density, etc.). These hybrid biopolymer structures are characterized by attractive electrostatic interactions between BLG cationic groups and pectin anionic groups, as well as by hydrophobic interactions between the pectin methoxyl groups and the BLG non polar residues (Jones et al., 2009, 2010; Sperber et al., 2009). As was mentioned previously, the strategy employed in this work for obtaining BNPs for LA vehiculization in acidic aqueous systems was: (1) BLG–LA complex formation, and (2) BNPs production via HMP electrostatic deposition onto the surface of the pre-formed BLG–LA complexes. The term 'polysaccharide electrostatic deposition' has been proposed by some researchers, suggesting the idea that the formation of a polysaccharide cover onto the surface of protein–ligand structure could promote the ligand protection against environment factors (Santipanichwong, Suphantharika, Weiss, & McClements, 2008). Additionally, this term could be interpreted as the formation of a protein–polysaccharide complex via attractive electrostatic interactions.

The most important process variables in BNPs production are pH and protein–polysaccharide concentration ratio, which could determine the phase behavior (regions of soluble protein–polysaccharide complexes and complex coacervation) of the acidic aqueous systems, and consequently, possible applications in food technology (Fioramonti et al., 2014; Weinbreck et al., 2003). In this work, we evaluate the phase behavior of biopolymer mixed dispersions, considering the evolution of T% with aqueous medium pH. The effect of $R_{Prot:HMP}$ (Prot:HMP ratio, wt.%) on T% vs pH profiles of biopolymer mixed systems is provided in the Supplementary Material section (Fig. SM.1). From these profiles, transition pH values (pH_c and pH_ϕ) were calculated using the method described by Fioramonti et al. (2014). The pH_c corresponds to the pH value that delimits a region in which biopolymers coexist individually (without interactions) from a region of soluble protein–polysaccharide complexes. On the other hand, the pH_ϕ corresponds to the pH value that delimits a region of soluble protein–polysaccharide complexes from a region governed by complex coacervation, or associative phase separation in which the protein–polysaccharide complexes are insoluble as a consequence of their greater size and/or lower surface charge (Sperber et al., 2009). With the data on the transition pHs, it was possible to build pH vs $R_{Prot:HMP}$ phase diagrams. Phase diagrams for biopolymer dispersions containing HMP and native LA complexes (BLGn–LA) and LA complexes treated at 60 °C for 10 min (BLGt–LA) are shown in Fig. 2B and D, respectively. Phase diagrams for systems composed of HMP and native BLG (BLGn), and BLG treated at 60 °C for 10 min (BLGt) were included as controls without LA, in Fig. 2A and C, respectively. In general, it was noted that the increase in $R_{Prot:HMP}$ caused an increase in the magnitude of both transition pHs. This behavior can be explained considering the gradual charge neutralization of HMP anionic groups when the amount of cationic groups on the proteins (BLGn and BLGt) or LA complexes (BLGn–LA and BLGt–LA) increases in solution (increase in $R_{Prot:HMP}$) (Fioramonti et al., 2014; Jones et al., 2009, 2010; Weinbreck et al., 2003). From the same figure, it can be deduced that:

(i) The phase behavior of the control systems (BLGn:HMP and BLGt:HMP) was quite similar indicating that a moderate heat treatment (60 °C for 10 min) did not significantly affect the mode in which HMP interacts with BLG.
(ii) In general, the phase behavior of systems containing LA complexes (BLGn–LA:HMP and BLGt–LA:HMP) was similar to the phase behavior of the controls (BLGn:HMP and BLGt:HMP) suggesting that LA linked to BLGn or BLGt did not considerably change the mode in which HMP interacts with the proteins.

In order to confirm the phase behavior of the systems showed in Fig. 2, we selected pH 4.0, as a typical example, and the visual appearance of systems was examined. As can be seen in Fig. 3, the more turbid appearance of biopolymer mixed systems was observed at 2:1 $R_{Prot:HMP}$. It is important to highlight that these systems were temporally stable, suggesting the presence of soluble particles. At 4:1 and 6:1 $R_{Prot:HMP}$, systems became unstable after 30 min and complex coacervation or associative phase separation was observed. Another observation was that LA complexation and moderate heat treatment of BLG–LA complexes did not affect the appearance of biopolymer mixed systems (in comparison with their respective controls). So it can be concluded that the phase behavior of the biopolymer mixed systems observed at pH 4.0 (Fig. 3) was in agreement with the prediction of the phase diagrams (Fig. 2).

On the other hand, differences between the visual appearances of biopolymer mixed systems could be complemented, considering their absorbance at 600 nm (A_{600}) as a measure of the systems turbidity (Jones et al., 2009, 2010; Ryan et al., 2011). Fig. 4 shows the effect of $R_{Prot:HMP}$ (wt.%) on the A_{600} values. In this figure, proteins (BLGn, BLGt) and protein-LA complexes (BLGn–LA and BLGt–LA complexes) were included as controls. It was observed that at 1:1$R_{Prot:HMP}$, A_{600} values were quite similar to the controls, suggesting a decreased number of protein–polysaccharide interactions (as was also deduced from Fig. 2). Biopolymer mixed dispersions obtained at 2:1 $R_{Prot:HMP}$ showed intermediate A_{600} values. The increase in $R_{Prot:HMP}$ caused an increase in A_{600}, with maximum values at 4:1 and 6:1, indicating the presence of bigger or more numerous particles. It is important to note that A_{600} was measured as soon as the systems were prepared. Hence, it can be deduced that the complex coacervation phenomenon, with the concomitant phase separation, observed at 4:1 and 6:1 $R_{Prot:HMP}$ (Fig. 3) could be conducted by the progressive association of bigger and/or neutralized biopolymer particles. In this sense, the last systems would not be appropriate for LA vehiculization in acidic aqueous systems, because they did not remain soluble at this pH.

3.3. Size and ζ potential of biopolymer particles

The colloidal stability of biopolymer particle systems can be evaluated in terms of particle size and electrical properties (Jones et al., 2009, 2010; Lesmes & McClements, 2009). According to this, the effect of $R_{Prot:HMP}$ on the particle diameter (nm) and ζ potential (mV) of the biopolymer mixed systems was determined at pH 4.0, and is shown in Fig. 5A and B, respectively. Measurements of proteins (BLGn, BLGt) and LA complexes (BLGn–LA and BLGt–LA) were included as controls. It is important to note that all particle diameter values shown in Fig. 5A are derived from the analysis of PSD in intensity (%). However, we also report the relative percentages (not size) from the analysis of PSD in volume (%), because it provides information about the relative proportion of multiple components in the sample based on their mass or volume (Malvern Instruments Limited, 2014). This information is given in the Supplementary material section (Table SM.1). Hence, it is important to note that the size shown in Fig. 5A corresponds to particles with percentages comprised between 85 and 100% in mass or volume, and so could be considered monodispersed. This criterion is supported by the PdI values obtained which were lower than 0.26, suggesting samples with considerable monodispersity (Malvern Instruments Limited, 2014). From Fig. 5A it can be observed that:

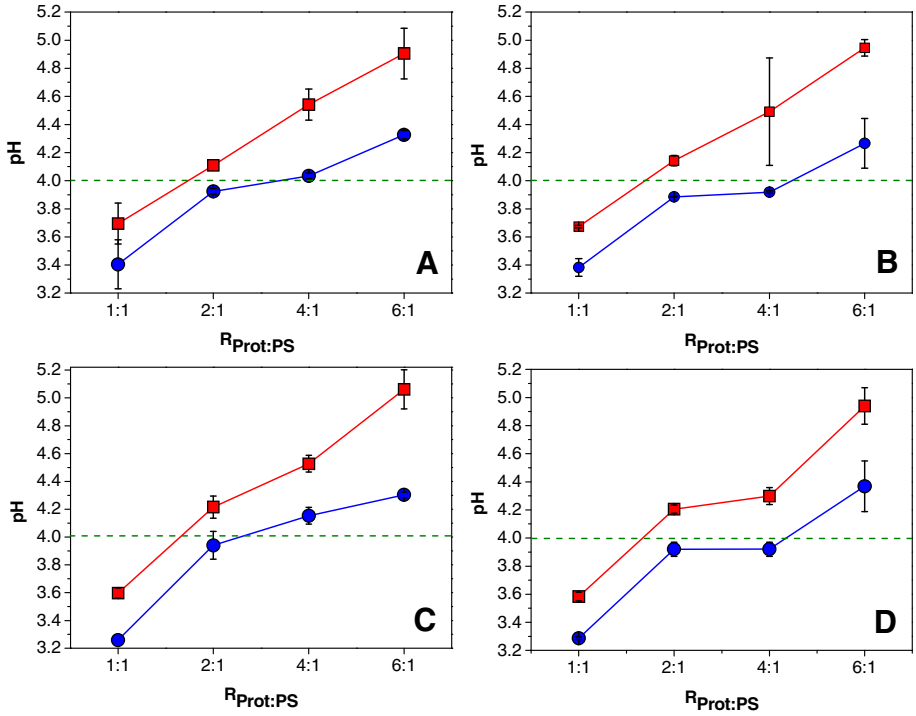

Fig. 2. Phase diagrams, pH vs $R_{Prot:HMP}$ (Protein–HMP ratio, wt.%), for BLGn (A), BLGn–LA (B), BLGt (C), y BLGt–LA (D). Symbols: (■) pH_c values, (●) pH_ϕ values. pH > pH_c corresponds to the biopolymer no interaction region, pH_c < pH < pH_ϕ corresponds to the soluble biopolymer particle region and pH < pH_ϕ corresponds to the insoluble biopolymer particle region (complex coacervation or associative phase separation). Dash line at pH 4 is marked as a reference. Temperature: 25 °C.

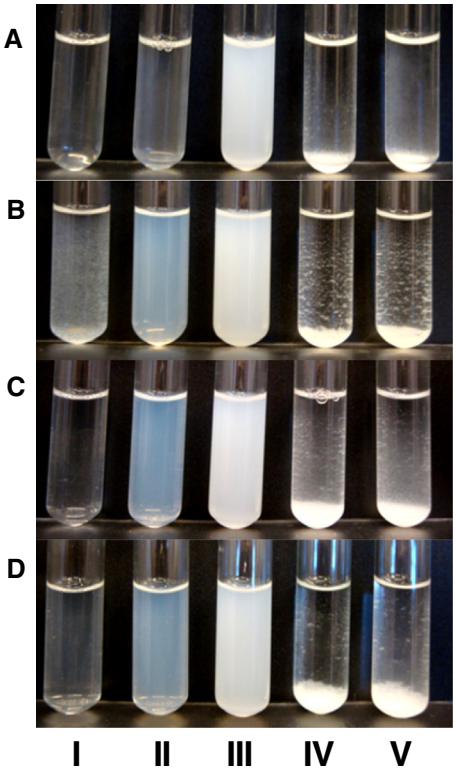

Fig. 3. Visual appearance of biopolymer particle systems for LA vehiculization. Nomenclature: (A) BLGn, (B) BLGn–LA, (C) BLGt, (D) BLGt–LA, (I) protein (BLGn, BLGt) or protein-LA controls, (II) 1:1 $R_{Prot:HMP}$ wt.%, (III) 2:1 $R_{Prot:HMP}$ wt.%, (IV) 4:1 $R_{Prot:HMP}$ wt.% and (V) 6:1 $R_{Prot:HMP}$ wt.%. Experimental conditions: pH 4.0, 25 °C, 0.08 wt.% protein, 30 min stabilization time.

(i) BLGn particle size was around 256 nm. It is well known that at pH 4.0, BLGn exists under the oligomer conformation and their particle size is highly dependent on the aqueous medium conditions, such as ionic strength, protein concentration, etc. (Harnsilawat, Pongsawatmanit, & McClements, 2006).

(ii) LA complexation and moderate heat treatment (60 °C, 10 min) caused an increase in BLGn size. With respect to the effect of LA complexation, it is important to note that the increase in BLGn size should be only interpreted in terms of LA binding behavior, as no significant differences ($p < 0.05$) were observed taking into account the size of the protein control in the presence of ethanol (BLGn-ol), as can be observed in Table SM.1 of the Supplementary material section. This control was included in the PSD analysis in order to confirm any BLGn structure modification, due to the ethanol used in binding experiments.

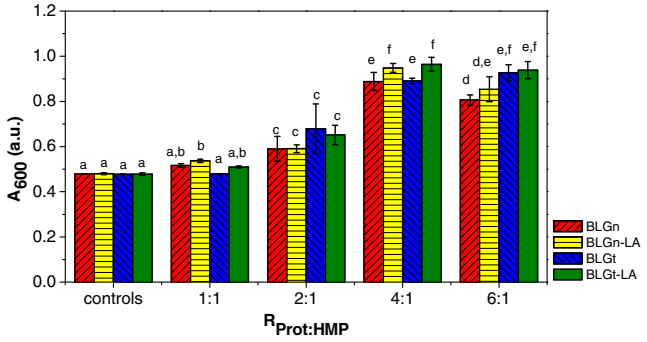

Fig. 4. Effect of $R_{Prot:HMP}$ (Prot:HMP ratio, wt.%) on the absorbance at 600 nm (A_{600}) of biopolymer particles for LA vehiculization. Protein (BLGn, BLGt) or protein-LA systems are included as controls. Measurement conditions: pH 4.0, 25 °C, 0.08 wt.% protein. Different letters indicate significant differences ($p < 0.05$).

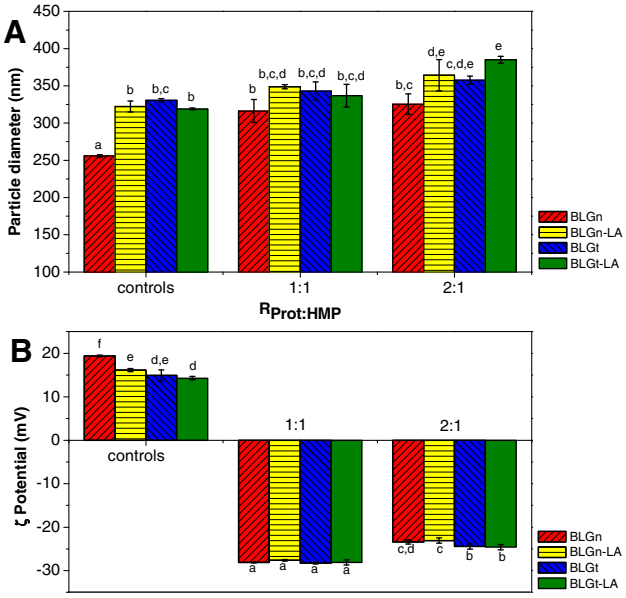

Fig. 5. Effect of $R_{Prot:HMP}$ (Prot:HMP ratio, wt.%) on the particle diameter (A) and ζ potential (B) of biopolymer particles for LA vehiculization. Protein (BLGn, BLGt) or protein-LA systems are included as controls. Measurement conditions: pH 4.0, 25 °C, 0.08 wt.% protein. Different letters indicate significant differences ($p < 0.05$).

(iii) BLGn–LA and BLGt–LA complexes showed similar sizes confirming that moderate heat treatment of LA complexes did not produce significant changes in particle size and/or conformation.

(iv) Biopolymer particle diameters at 1:1 and 2:1 $R_{Prot:HMP}$ were comprised between 316 and 385 nm. In the food sector, the definition of nanometric materials is still not clear. Particles with both size strictly <100 nm and with few hundred nm could be defined as "nanoparticles" (Gutierrez et al., 2013; Joye et al., 2014). Hence, biopolymer particles formed at 1:1 and 2:1 $R_{Prot:HMP}$ could be considered as BNPs. It is relevant to highlight that particle size data should be interpreted considering the phenomenon of electrostatic deposition of HMP onto LA complex surfaces (i.e. protein–polysaccharide complexes formation) because BNPs showed intermediate sizes between the LA complexes (around 320 nm) and HMP (990 nm, as it was shown in Table SM.1 of the Supplementary material section).

(v) At 1:1 $R_{Prot:HMP}$ practically no significant differences in particle size were observed ($p > 0.05$). In general, at this condition, BNPs had the same sizes as their respective controls (with exception of BLGn) suggesting the existence of a decreased number of attractive electrostatic interactions between the HMP and LA complexes (BLGn–LA and BLGt–LA). These results are consistent with the prediction of the phase diagrams, visual appearance and A_{600} values, previously discussed.

(vi) At 2:1 $R_{Prot:HMP}$ the biggest particle sizes were found, especially for BNPs formed with BLGn–LA and BLGt–LA complexes. Hence, it can be deduced that LA complexation and moderate heat treatment (60 °C, 10 min) significantly affects the particle size ($p < 0.05$). These results were also compatible with the prediction of the phase diagrams, visual appearance and A_{600} values, and in general, would suggest that a slight increase in BNPs size (with the increase in $R_{Prot:HMP}$) could have notable repercussions on the visual appearance of the biopolymer mixed dispersions (as it can be seen in Fig. 3).

On the other hand, ζ potential measurements were also carried out. The electric potential at the shear plane between the bound layer of ions surrounding a particle and the bulk solution is known as ζ potential. The ζ potential can be modified by adsorption of a charged species, such as ions and ionic polymers onto the particle surface. From Fig. 5B it can be deduced that at pH 4.0:

(i) BLGn had a positive ζ potential confirming the positive net charge of the protein at pH below the pI (4.8–5.2) (Harnsilawat et al., 2006).

(ii) BLGt had a positive ζ potential but its magnitude was significantly lower than that of BLGn. This behavior can be explained considering the occlusion/exposition of some charged surface residues after the moderate heat treatment of BLGn (Fioramonti et al., 2014; Zhao, Li, Carvajal, & Harris, 2009).

(iii) BLGn–LA and BLGt–LA complexes had positive ζ potentials and their magnitudes were significantly lower than that of BLGn. The LA molecule has an apolar tail (aliphatic chain) and a polar head (carboxylic group) conferring an amphiphilic character. Hence, if LA was bound to BLG by means of hydrophobic interactions through the apolar tail, the ionized polar head would be oriented on the complex surface, so it is in contact with aqueous medium, decreasing the proteins (BLGn and BLGt) positive net charge.

(iv) BNPs had negative ζ potentials. According to these results, it can be deduced that the net charge of BNPs was governed by the negative net charge of HMP, which was −33 mV (result not shown). Therefore, BNPs ζ potential data should be interpreted considering the HMP electrostatic deposition onto the surface of the LA complexes (i.e. protein–polysaccharide complexes formation).

(v) At 1:1$R_{Prot:HMP}$, the magnitude of negative net charges of BNPs were greater than those obtained at 2:1 $R_{Prot:HMP}$. These results would suggest the gradual charge neutralization of HMP anionic groups when the amount of cationic groups in the proteins (BLGn and BLGt) and/or LA complexes (BLGn–LA and BLGt–LA) increases in solution (increase in $R_{Prot:HMP}$).

In general, the magnitude of BNPs ζ potential confirms the colloidal stability observed for systems at 1:1and 2:1$R_{Prot:HMP}$,(as it can be observed in Fig. 3). Thus, colloidal stability could be promoted by a high repulsion between the negative net charges of BNPs.

3.4. Fluorescence features of biopolymer particles

As was mentioned above, in a BNP designed for PUFA vehiculization, the cover of polysaccharide on protein–ligand complexes could favour ligand protection against environmental factors (Jones et al., 2009, 2010; Santipanichwong et al., 2008; Zimet & Livney, 2009). In this last section, we have evaluated if HMP electrostatic deposition onto the surface of LA complexes could favour protection of the LA binding site. For this, intrinsic and extrinsic fluorescence experiments were conducted.

The effect of $R_{Prot:HMP}$ on the intrinsic and extrinsic fluorescence of biopolymer mixed systems is shown in Fig. 6A and B, respectively. As controls, proteins (BLGn and BLGt) and LA complexes (BLGn–LA and BLGt–LA) were included. In general, it was observed that HMP electrostatic deposition caused a significant decrease in both FI^{M}_{Trp} (Fig. 6A) and FI^{M}_{ANS} (Fig. 6B). HMP electrostatic deposition onto the surface of proteins (BLGn and BLGt) and/or LA complexes (BLGn–LA and BLGt–LA) could decrease significantly the accessibility of fluorophores (Trp residues and ANS) for fluorescence emission, due to the formation of hydrid protein–polysaccharide

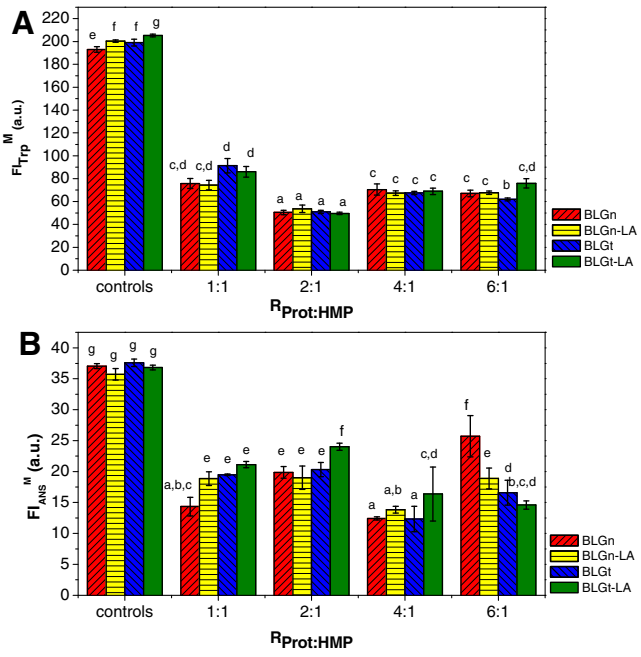

Fig. 6. Effect of $R_{Prot:HMP}$ (Prot:HMP ratio, wt.%) on the intrinsic (A) and extrinsic fluorescence (B) of biopolymer particles for LA vehiculization. Protein (BLGn, BLGt) or protein-LA systems are included as controls. Measurement conditions: pH 4.0, 25 °C, 0.08 wt.% protein. Different letters indicate significant differences ($p < 0.05$).

structures (Perez, Carrara, Carrera-Sánchez, Rodríguez-Patino, & Santiago, 2009). According to this, we consider the hypothesis that the proteins and/or LA complexes could be located at an internal domain of the biopolymer particles, so the external cover of polysaccharide could promote the protection of the LA binding site and, simultaneously, confer a lower hydrophobicity (higher particle hydrophilicity). As can be observed in Fig. 6A, a minimum in FI^{M}_{Trp} at 2:1$R_{Prot:HMP}$ was found, indicating a greater protection by HMP cover on the LA binding site in the BNPs (Fioramonti et al., 2014). Hence, in relation to the LA binding site protection and colloidal stability, a 2:1 $R_{Prot:HMP}$ could be appropriate for designing LA vehiculization systems based on BNPs, when intrinsic fluorescence is considered. Moreover, it can be deduced that a moderate heat treatment of LA complexes would seem to have no effect on the intrinsic fluorescence of BNPs. Nevertheless, it is important to remark that complementary studies about PUFA stability against different storage environmental factors (oxidation, UV radiation, temperature, etc.) would be needed in order to confirm these results. These aspects will be considered in a future contribution.

On the other hand, as was presented in Fig. 6B, HMP electrostatic deposition caused a complex behavior in the magnitude of FI^{M}_{ANS} of biopolymer particles. However, with respect to the controls, a pronounced decrease in FI^{M}_{ANS} was observed, suggesting a decrease in global surface hydrophobicity of the biopolymer particles (increased particle hydrophilicity). In the case of BNPs obtained at 1:1 and 2:1the $R_{Prot:HMP}$ the decrease in surface hydrophobicity could also contribute to the observed colloidal stability (as it can be observed in Fig. 3).

4. Conclusions

BPNs for LA vehiculization were successfully constructed by applying two functional properties: (i) LA binding ability of β-lactoglobulin (BLG) for obtaining BLG–LA complexes, and (ii) high methoxyl pectin (HMP) electrostatic deposition onto the surface of pre-formed BLG–LA complexes at acidic pH. Evaluation of the Prot:HMP concentration ratio ($R_{Prot:HMP}$, wt.%) in the range of 1:1 and 6:1 allowed defined conditions, in which BNPs would be adequate for LA vehiculization. At pH 4.0, stable BNPs were obtained at 1:1 and 2:1 $R_{Prot:HMP}$, exhibiting sizes around 337–364 nm and ζ potential comprised between −25 and −28 mV. In terms of the HMP protective role at the LA binding site, an 2:1 $R_{Prot:HMP}$ could favour greater LA protective effects in BNPs turning the most suitable condition for the design of LA vehiculization systems in acidic mediums.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.05.043.

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