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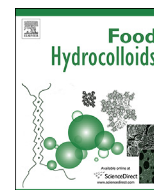
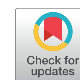


On the binding of folic acid to food proteins performing as vitamin micro/nanocarriers



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ABSTRACT

Folic acid (FA) encapsulation in protein matrices has been reported as a suitable method for preventing FA degradation upon storage or processing as well as to improve its bioavailability.

The ability of β-lactoglobulin (β-lg) and type A gelatin (G) to bind FA and form nano/microparticles under conditions of concentration (up to 5% w/w) and pH (3–7) that could have a technological application has been studied. The degree of folic acid binding to the proteins depended on their pH-dependent ζ-potential, indicating the occurrence of ionic bonds. Regardless of FA concentration, the percentage of bound FA to β-lg or G was 100% at pH 3.

At pH 3, the size of particles strongly increased by increasing the molar FA/protein ratio. Protein aggregation and further flocculation was observed at higher molar ratios. However, the size of particles could be modulated by high intensity ultrasound application.

FA/protein particles formed at pH 3 were totally reversible by shifting back the pH to 7.

This pH dependence is strongly favorable for the delivery of FA at the duodene (pH 7) and for the protection of FA at the pH prevailing in the stomach (pH 3).

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1. Introduction

Folates are a group of heterocyclic compounds based on the skeleton of 4 - (pteridin-6-methylamino) benzoic acid conjugated with one or more residues of L-glutamic acid. Folates are involved in fundamental biological processes, such as the synthesis of nucleic acids and proteins, thus an adequate intake can prevent congenital diseases, such as neural tube defects (Refsum, 2001). The role of FA in the prevention of cardiovascular diseases, megaloblastic anemia, cancer and Alzheimer's disease (Molloy & Scott, 2001) has been also reported.

Folic acid (4-[(pteridin-6-yl-methyl) amino] benzoic acid) is a synthetic and more stable form of folate and is commonly used for nutritional fortification and for formulation of pharmaceuticals. However, folic acid in its pure form is very sensitive to light, pH, temperature and oxygen exposure. FA has a marked sensitivity to ultraviolet light and heat, which degrades it in compounds without biological activity (Akhtar, Khan, & Ahmad, 1999; Off et al., 2005; Vora, Riga, Dollimore, & Alexander, 2002).

Folic acid is also susceptible to degradation under the acidic conditions prevalent in the stomach that can affect its bioavailability (Akhtar, Khan, & Ahmad, 2003; Alborzi, Lim, & Kakuda, 2013; Ball, 1998; Papastoyiannidis, Polychroniadou, Michaelidou, & Alichanidis, 2006). Thus delivery systems designed to protect FA from the acidic conditions of the stomach that can release the vitamin at the intestine (neutral pH) are needed. Food proteins, because of their particular physicochemical and physiological properties, as well as their biocompatibility and biodegradability, offer a great potential as a material for the preparation of nanoparticles and microparticles for oral delivery systems.

Folic acid encapsulation in protein matrices has been reported as a suitable method for preventing FA degradation upon storage or processing as well as to improve its bioavailability. Milk proteins can bind a variety of molecules and ions at different degrees of affinity and specificity, as they evolved to deliver essential nutrients from the mother to the newborn (Livney, 2010). The response of folic acid to UV irradiation in the presence of β-lactoglobulin (β-lg),

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bovine serum albumin (BSA) and α-lactalbumin (α-la) was investigated by Liang, Zhang, Zhou, and Subirade (2013). Photodecomposition of folic acid was delayed in the presence of the proteins, which ranked in the order β-lg > BSA > α-la in terms of effectiveness. The effectiveness was attributed to ligand-binding properties of proteins that can interact not only with folic acid but also with its photodecomposition products. Binding to β-casein appears to reduce the photodecomposition of folic acid more as it inhibited folic acid photodecomposition completely at 10 μM (Zhang et al., 2014).

Several protein nanoparticles have been found to protect folic acid in the acid environment of stomach. Casein nanoparticles stabilized by lysine (Peñalva, Esparza, Agüeros et al., 2015) and zein nanoparticles (Peñalva, Esparza, González-Navarro et al., 2015) were found to be resistant in gastric conditions. Whey protein concentrate matrix has been studied using electrospray and nanospray drying techniques (Pérez-Masiá et al., 2015) and proved to preserve folic acid from degradation.

All the reported protein/FA particles have been generally obtained at pH close to neutral. Interactions of folic acid at pH 7 with various proteins, including human and bovine serum albumin, β-lactoglobulin, α-lactalbumin and caseins, have been determined (Bourassa & Tajmir-Riahi, 2012; Bourassa, Hasni, & Tajmir-Riahi, 2011; Liang & Subirade, 2010; Liang et al., 2013; Pérez, David-Birman, Kesselman, Levi-Tal, & Lesmes, 2014; Zhang & Jia, 2006) by fluorescence quenching, isothermal titration calorimetry, circular dichroism and absorption spectroscopy.

Another protein widely used as an ingredient in foods and pharmacy is gelatin. One of the advantages of this protein as a carrier is the electrical nature of gelatin, which can be changed by the processing method of obtainment (Tabata & Ikada, 1998). Gelatin is derived from the fibrous protein collagen from animal skin, bone, and connective tissue. During the manufacturing of gelatin, raw animal material is treated with dilute acid or alkali, resulting in partial cleavage of the crosslinks: the structure is broken down to such an extent that "warm water-soluble collagen", i.e. gelatin, is formed (Schrieber & Gareis, 2007). Type A gelatin, that has a broad isoelectric range between pH 7 and 9, possesses a special interest; in contrast to most proteins it exhibits a positive charge in the pH range of most foods (2–7).

In this work, the ability of β-lactoglobulin and type A gelatin to bind FA and form nano/microparticles under conditions of concentration (up to 5% w/w) and pH (3–7) that could have a technological application has been studied. In addition, the performance under different pH conditions has been assessed.

2. Materials and methods

2.1. Materials

BioPURE β-lactoglobulin was supplied by DAVISCO Foods International, Inc. (Le Sueur, Minnesota). Its composition was: protein 97.8% (w/w dry basis) and β-lg making up 93.6% (w/w) of total proteins, 0.3% (w/w) fat, 1.8% (w/w) ash and 5.0% (w/w) moisture. Gelatin A sample was kindly provided by Rousselot Argentina S.A. (Hurlingham, Argentina). The isoelectric point of this acid gelatin sample is 7.5 and the pH value of 1% wt solution in Milli-Q water was 5.5. Folic acid was manufactured by DSM Nutritional Products Argentina S.A. and was gently donated by Laboratorios Bagó S.A. (La Plata, Argentina). Its purity was 98.8% (w/w, dry basis).

2.1.1. Single and mixed solutions

β-lg and FA powders were dissolved separately in double distilled water at room temperature, under agitation. Protein solutions (0.1–10% w/w) were kept 24 h at 4 °C to achieve complete hydration, while FA solutions (0.025–2.5% w/w) were prepared freshly on the same day of measurement. Gelatin powder was poured in double distilled water and was heated under agitation until 80 °C, where was kept for 10 min, to ensure complete dissolution (0.5–1% w/w). Then it was cooled to 35 °C, and held at this temperature until measurement. The pH was adjusted to 7.0 and 3.0 using 1 M NaOH or 1 M HCl (analytical grade).

Protein/FA systems were obtained by mixing the solutions at pH 7.0 under agitation to reach the required final concentration of the protein and vitamin in the bulk solution. In other set of samples, pH was further lowered to 3.0 using 1 M HCl.

2.2. Methods

2.2.1. Evaluation of FA binding

The percentage of FA bound to β-lg and gelatin was evaluated by difference between the amount of added and non-associated (free) FA, according to Arzeni, Pérez, LeBlanc et al. (2015), Arzeni, Pérez, & Pilosof, (2015). Free FA was determined by ultrafiltration using centrifugal filter devices with a molecular weight cut-off of 10 KDa (Amicon® Ultra-15, Millipore, Ireland). The concentration of FA in the filtrate was determined according to Zhang et al. (2011) with some modifications. Samples were diluted to 50% in a mixture of dimethyl sulphoxide (DMSO)/H2O 50/50 (v/v) and the absorbance was read spectrophotometrically at 365 nm. A calibration curve of FA was made for this purpose.

2.2.2. Particle size determination

The impact of FA in the proteins particle size distribution was determined by dynamic light scattering in a Zetasizer Nano-ZS, Malvern Instruments (Worcestershire, UK) provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. This instrument has a measurement range of 0.6 nm–6 μm, according to the manufacturer, and incorporates noninvasive backscattering (NIBS) optics. Samples were contained in disposable polystyrene cuvettes and measurements were carried out at a fixed scattering angle of 173°, at a temperature of 25 °C for β-lg and at 35 °C for gelatin. Data were collected over 10 sequential readings, and the average hydrodynamic diameter was obtained. The CONTIN method was used to analyze the data for percentile distribution of particle/aggregate sizes. The intensity size distribution obtained is a plot of the relative intensity of light scattered by particles in various size classes. Through Mie theory, with the use of the input parameter of sample refractive index, it was possible to convert the intensity size distribution to volume size distribution.

Larger particles/aggregates sizes were measured by static light scattering (SLS) using a Mastersizer 2000 device equipped with a Hydro 2000MU as dispersion unit, from Malvern Instruments Ltd (Worcestershire, UK). The pump speed was settled at 1300 rpm. Particle size is reported as $D_{3,2}$ diameter. $D_{3,2}$ is the volume–surface mean diameter or Sauter diameter ($D_{3,2}$ (μm) = $\Sigma\ n_i\ d_i^3/\Sigma\ n_i\ d_i^2$) where n_i is the number of particles of diameter d_i (Arzeni et al., 2012; Huang, Kakuda, & Cui, 2001; Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003).

Particle size is reported as the average of ten readings made on each sample.

2.2.3. ζ-Potential measurements

ζ-potential measurements were also performed in a Dynamic Laser Light Scattering instrument (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK). The ζ-potential was evaluated from the electrophoretic mobility of the particles. The conversion of the measured electrophoretic mobility data into ζ-potential was done using Henry's equation Eq. (1):

$$Ue = 2\varepsilon\zeta f(Ka)/3\eta \quad (1)$$

where Ue is the electrophoretic mobility, ε the dielectric constant, η the sample viscosity and $f(Ka)$ the Henry's function. The solutions were diluted 5 times with double distilled water at the same solution pH and placed into special folded capillary cells (DTS1061, Malvern Instruments, Worcestershire, UK). The reported values are the average of three measurements.

2.2.4. Confocal microscopy

Images of samples were recorded with a confocal laser scanning microscope (Model FV300, Olympus, Tokyo, Japan), provided with a He–Ne laser (543 nm). An objective PLAN APO 60x and a digital zoom of 2.5x were used. Non-covalent labelling of protein was performed with a few drops of 0.02% w/w rhodamine B solution (excitation wavelength 560 nm; emission maximum 625 nm).

In order to obtain simultaneous images of β-lg and FA, a spectral confocal laser scanning microscope (Model FV1000, Olympus, Tokyo, Japan) was employed. Measurements were performed by exciting the mixed system with two different sources, one multiline argon-ion laser (457 nm), for the FA, and a He-Ne laser (543 nm), for the rhodamine labelled protein. An Olympus UPlanSApo 60x oil immersion objective (Numerical Aperture = 1.35), was used, with a digital zoom of 2.5x. The emitted fluorescence was detected simultaneously by two channels, in the 475–530 nm range for the FA and the 555–655 nm range for the protein. Single FA was also observed by exciting it at 457 nm and detecting in the 475–530 nm range.

In all cases, FluoView™ image acquisition software (Olympus) was used to acquire the images in multiple.tif format in 1024 × 1024 pixel resolution.

2.2.5. Thermal behavior

Samples were heated inside the DLS equipment (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) from 25/35 to 75 °C in steps of 10 °C and held at each temperature for 5 min to evaluate the variation of size with the increasing temperature. The autocorrelation function at each temperature was obtained and then the size of particles. Two approaches were used to obtain size information, Cumulants and Contin algorithm (Farías, Martinez, & Pilosof, 2010). The z-average is useful when citing a single average value for the purpose of comparison, but clearly inadequate for giving a complete description of the distribution results in polydisperse systems.

2.2.6. High intensity ultrasound (HIUS) treatment

β-lg solutions (5% w/w) with growing FA concentrations adjusted at pH 3, were sonicated for 20 min using an ultrasonic processor Vibra Cell Sonics, model VCX 750 (Newtown, Connecticut, USA) with a maximum net power output of 750 Watt, a frequency of 20 kHz and an amplitude of 20%. A 13 mm high grade titanium alloy probe threaded to a 3 mm tapered microtip was used to sonicate 5 ml of solution contained in a 15 ml glass tube. Samples were immersed into a glycerine-jacketed bath (Polystat, Cole-Parmer) with circulating water at a constant temperature of 0.5 °C, to dissipate the heat produced during sonication in order to evaluate the effect of HIUS alone. Each sonication was performed in duplicate.

3. Results and discussion

3.1. Folic acid binding

In this work the ability of β-lactoglobulin to bind FA under conditions that could have a technological application was analyzed. Thus, a protein concentration of 5% w/w was used, to which increasing amounts of FA (concentrations between 0.025 and 1.25% w/w) were added. This means FA/β-lg weight ratios between 0.005 and 0.25 and FA/β-lg molar ratios between 0.2 and 10. The degree of folic acid binding to the protein at pH 7 and 3 was evaluated by spectrophotometric determination of free FA in the permeate, after ultrafiltration of FA/protein mixtures. Regardless of FA concentration, the percentage of bound FA to β-lg was zero at pH 7 and 100% at pH 3.

The gelatin (G), because of its high viscosity, was used at 0.5% w/w and all the tests were performed at 35 °C (a temperature above the sol-gel transition of gelatin) at the highest FA/protein weight ratio (0.25). For this protein, at pH 3 the percentage of bound FA was 100%, as well as for β-lg. However, at pH 7 the percentage of FA bound to G was 38.6%.

At pH 3, both proteins have a strong positive charge (≈20–30 mV) and FA a strong negative charge (Fig. 1A and B). Regardless of FA concentration, the ζ-potential of the mixtures was similar to single proteins, suggesting that the observed binding of FA little altered the surface charge of proteins. Other authors have reported no differences in the ζ-potential of poly(DL-lactideco-glycolide) (PLGA) nanospheres after the addition of folic acid, independently from the ratio of PLGA and FA used (Stevanović, Radulović, Jordović, & Uskoković, 2008). This behavior may arise because only few positive charges of proteins would be neutralized by FA interaction or/and by the contribution of cations released from FA salt upon ionic interaction with the protein.

Among 162 amino acids in β-lactoglobulin, lysine (15), arginine (3) and histidine (2) could potentially be involved in ionic interactions with glutamic acid in FA at pH 3. Similarly, the main basic amino acids in gelatin that could be involved are arginine (8%) and lysine (4%).

The molecular structure of FA (pteroyl monoglutamic acid) consists of a pteridin ring linked to the para-amino benzoic acid (PABA) and a molecule of glutamic acid. It exhibits a pH dependent solubility. Under neutral/basic conditions FA is about 1000 times more soluble than in an acidic environment because it is in the form of salt. At pH below 5 due to protonation of the two carboxylic groups (acid) FA has very low solubility, leading to precipitates. The experimental solubility data of folic acid at pH values between 0 and 7 shows that minimum solubility is achieved at pH 3 (Wu, Li, Hou, & Qian, 2010). The particle size distribution for 0.5% FA solutions immediately after adjustment at pH 3 is shown in Fig. 2, where particles of 100 μm are predominant. When standing this solution, crystallization was observed (inset of Fig. 2).

As the FA/protein mixtures were prepared at pH 7 and further the pH was lowered to 3, it is possible that negatively charged FA would bind to positive charges arising on the proteins when decreasing the pH to 3.

At pH 7, β-lg is well above the pI (4.6–5.2, (Bromley, Krebs, & Donald, 2005; Das & Kinsella, 1989; Harnsilawat, Pongsawatmanit, & McClements, 2006)) and has a strong negative charge (−25 mV) as FA (−20 mV). The repulsive electrostatic forces could explain that FA could not be retained by the protein upon ultrafiltration. Nevertheless, the gelatin exhibited a low positive ζ-potential at pH 7 (3.5 mV) (Fig. 1 D) as the A gelatin type used has an isoelectric point of 7.5. This could account for by a significant degree of FA retention (38.6%) upon ultrafiltration the FA/G mixture at pH 7.

As for pH 3, the ζ-potential of the FA/G mixtures at pH 5 and 7 (Fig. 1C and D) was similar to single gelatin, as FA binding little altered the surface charge of protein.

In order to assess if the binding of FA is dependent on the protein surface charge, bound FA was related to the pH modulated ζ-

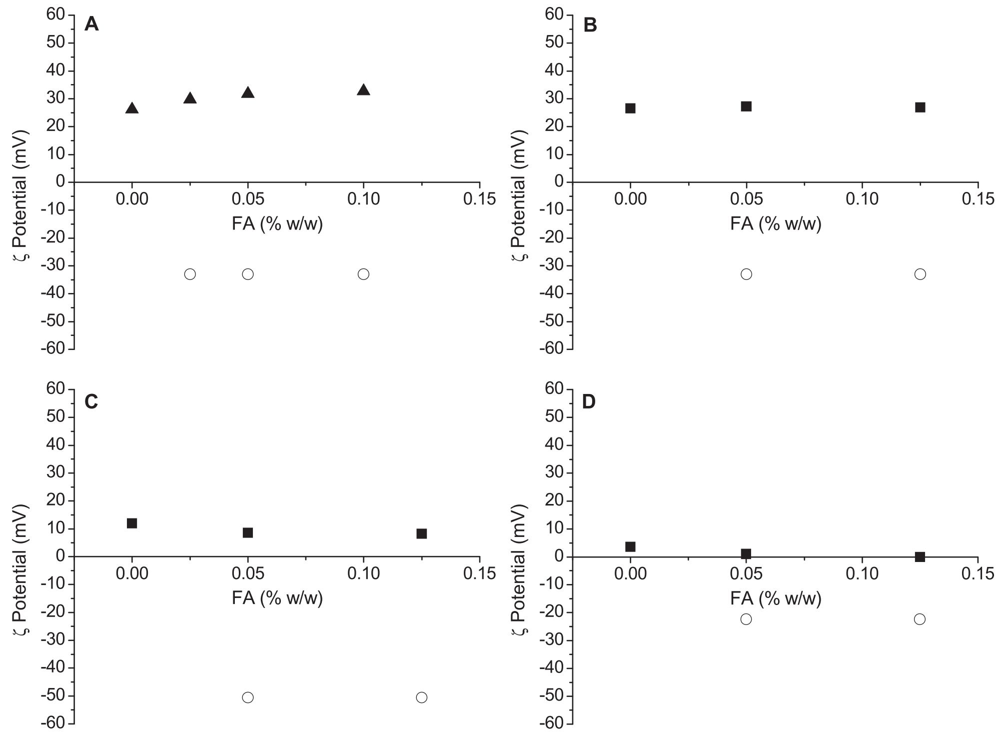

Fig. 1. ζ-potential of protein particles as affected by FA binding at pH 3: A,B at pH 3, C: pH 5 and D: pH 7 for β-lg 5% (▲) and Gelatin 0.5% (■). The ζ-potential of Folic Acid (○) is also shown at the corresponding pH.

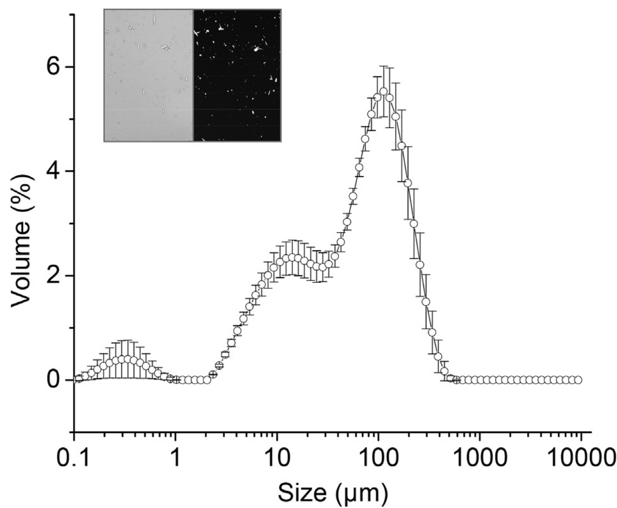

Fig. 2. Volume particle size distribution of Folic Acid (0.5%) at pH 3. Inset: Optical microscopy image of folic acid crystals at pH 3.

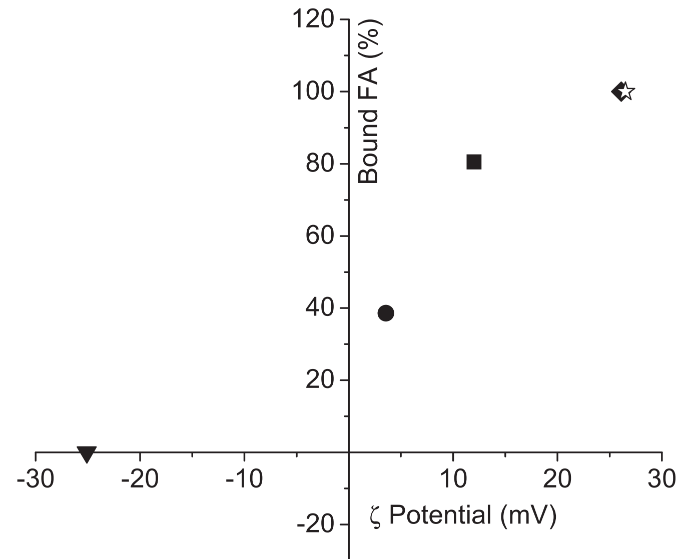

Fig. 3. FA bound to proteins as related to ζ-potential of single proteins. β-lg 5% pH 7 (▼), β-lg 5% pH 3 (◆), Gelatin 0.5% pH 7 (●), Gelatin 0.5% pH 5 (■), Gelatin 0.5% pH 3 (✰).

potential of proteins (Fig. 3). The observed relationship points out the main role of proteins surface charge, as increasing the positive surface charge, bound FA increases, confirming the involvement of electrostatic/ionic interactions. Previous studies have shown that even at pH 7 several proteins can interact with FA, including human and bovine serum albumin, β-lactoglobulin, α-lactalbumin, caseins, zein (Bourassa et al., 2011; Bourassa & Tajmir-Riahi, 2012; Liang & Subirade, 2010; Liang et al., 2013; Peñalva, Esparza, Agüeros, et al., 2015; Peñalva, Esparza, González-Navarro, et al., 2015; Pérez et al., 2014; Zhang & Jia, 2006). The interaction between folic acid and proteins at neutral pH has been investigated using fluorescence quenching, isothermal titration calorimetry, circular dichroism and absorption spectroscopy. BSA has been reported to interact with folic acid by hydrophilic contact with tryptophan residue at position 132 (Trp132) on the protein surface and by hydrophobic contact with Trp212 inside the molecule (Bourassa et al., 2011). Jha and Kishore (2011) suggested that the binding between BSA and FA is dominated by electrostatic interactions with contribution from hydrogen bonding. β-lg could bind folic acid at the protein surface, possibly in the groove between the α-helix and the β-barrel, which was proposed to be the reason for the delay in the

photodecomposition of folic acid (Liang & Subirade, 2010). Among the external portions of β-lg that have been suggested as ligand-binding sites are the outer surface near Trp19–Arg124, the surface hydrophobic pocket in the groove between the α-helix and the β-barrel, a site near the aperture of the β-barrel and a site at the monomer–monomer interface of the dimer (Liang et al., 2013).

Whatever the exact bonds involved in such interactions at neutral pH, they would be too weak to be determined in the ultrafiltration test. At pH 7, only weak electrostatic interactions can take place throughout the positive patches of proteins and negative carboxylic groups of FA. Nevertheless, at pH values where the proteins carry a net positive charge, strong electrostatic interactions among opposite charged groups would explain the strong binding observed for FA and the studied proteins. A similar behaviour has been previously reported for the binding of FA to white egg proteins (Arzeni, Pérez, & Pilosof, 2015; Arzeni, Pérez, LeBlanc et al., 2015).

3.2. Particle size and microstructure of folic acid/protein mixtures at pH 3

The average particle size of the FA/protein mixtures at pH 3 was evaluated in comparison to that of the pure proteins. In Fig. 4 A the average diameter of FA/β-lg particles formed at pH 3 is shown as a function of FA concentration. At low FA concentration or molar ratios, the particles formed are in the submicronic range (≤1 μm). The average particle diameter increased rapidly up to molar ratios of 1–1.5, then remained constant at a value of about 25–34 μm. As stated above, all added FA was bound to β-lg, enhancing protein aggregation and further flocculation at higher molar ratios (Fig. 4 B). FA/β-lg particles at pH 3 were also analyzed by confocal microscopy. Fig. 5 shows that single FA (Fig. 5A) and β-lg (Fig. 5B) have an homogeneous appearance, in the first case due solely to the presence of rhodamine, which has no affinity for the vitamin and in the case of the protein, because at the pH studied, it is in the monomeric form (Uhrínová et al., 2000) that cannot be revealed by the technique. In mixed systems (Fig. 5C and D), protein aggregates of increasing size are observed at increasing FA concentration. These observations corroborate the results obtained by light scattering (Fig. 4).

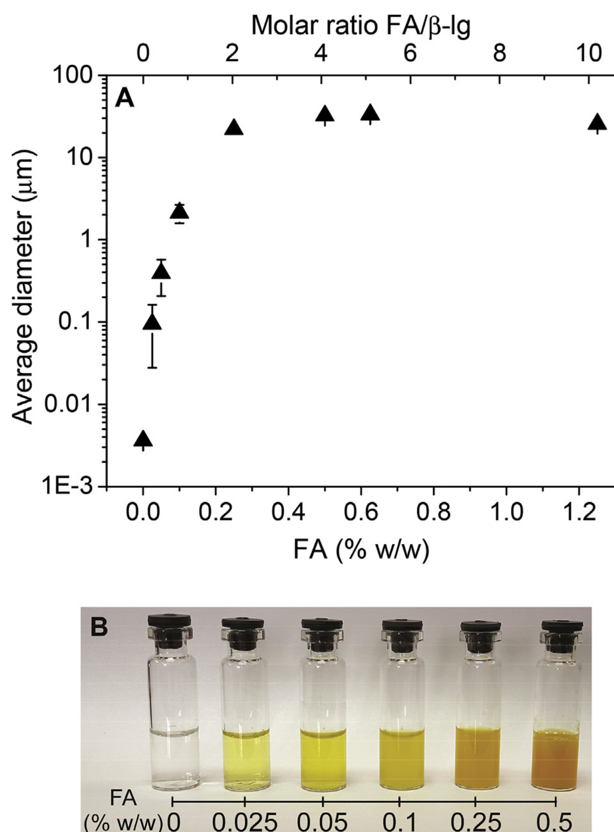

Fig. 4. A: Average particle size of β-lg (5% w/w) at pH 3 as FA concentration or molar ratio increases. B: Visual appearance of solutions.

In order to exclude the possible self-assembly/crystallization of FA at pH 3 in the mixtures, a spectral scanning microscopy was performed by exciting the mixed FA/β-lg system with two different sources and comparing to single FA at pH 3. As shown in Fig. 6 A, FA crystallization occurs at pH 3, however in the presence of β-lg these structures are not present anymore (Fig. 6 B-left) because of FA binding to protein.

A similar tendency was confirmed for FA/gelatin mixtures, as particle size increased with FA concentration or FA/G molar ratio (Table 1). The average diameter of particles achieved for FA/β-lg or FA/G were similar at FA/protein molar ratio of 0.57. At very high FA/protein molar ratios, FA/G particles were lower (9.86 μm) than those obtained for FA/β-lg particles (25–34 μm).

3.3. Thermal behavior of folic acid/protein particles at pH 3

Fig. 7 shows the average size of particles upon heating at pH 3 single proteins and their mixtures with FA, systems with the smaller FA/protein weight ratio (0.005) were selected for both β-lg and G in order to get particle sizes in the measurement range of Zetasizer Nano-ZS. The particle size of single β-lg little varied by increasing temperature (Fig. 7 A) suggesting that at this pH the protein is not prone to aggregation. As reported by Martinez and Pilosof (2018) at pH 3, the native protein denatures upon heating at 85 °C and then the molecules aggregate by means of weak interactions like van der Waals forces and hydrogen bond. In this pH range the denaturation of β-lg molecules did not exhibit the thiol residues and the reactivity of the thiol groups is low. The initial average particle size of FA/β-lg mixture (β-lg 5% and FA 0.025%) was 1711 nm as shown in Fig. 7 A because of FA induced aggregation. The size was decreased by increasing temperature above 45 °C and leveled off at about 700 nm at 75 °C.

The initial average size of FA/G mixture (G 0.5% and FA 0.0025%) was 3684 nm and sharply decreased up to 60 °C where leveled off at about 1300 nm (Fig. 7B).

Therefore, for both FA/protein mixtures at pH 3 the increase of temperature reduced the particle size, which can be attributed to the decrease of electrostatic interactions on heating, due to the exothermic nature of this kind of non covalent bonds.

3.4. pH-reversibility of FA/protein particles

The FA/protein particles formed at pH 3 were tested for pH-reversibility. To this end FA/β-lg particles of 33 μm formed at pH 3 (β-lg 5% and FA 0.625%) were adjusted to pH 7. Fig. 8 shows the visual appearance of solutions that shifted from turbid at pH 3 to transparent at pH 7. Moreover, a particle size distribution similar to that exhibited initially by the system at pH 7 (before aggregation) may be observed in Fig. 8 A.

Similarly, FA/G particles of 10 μm formed at pH 3 (G 0.5% and FA 0.125%) were adjusted to pH 7 and the particle size distributions after the experiment did coincide with the original size distribution at pH 7, before being aggregated by FA (Fig. 8 B).

The pH reversibility of protein/FA aggregates verifies the ionic character of the interaction between proteins and FA. The interaction would be reversible due to a reversible change in the ionization

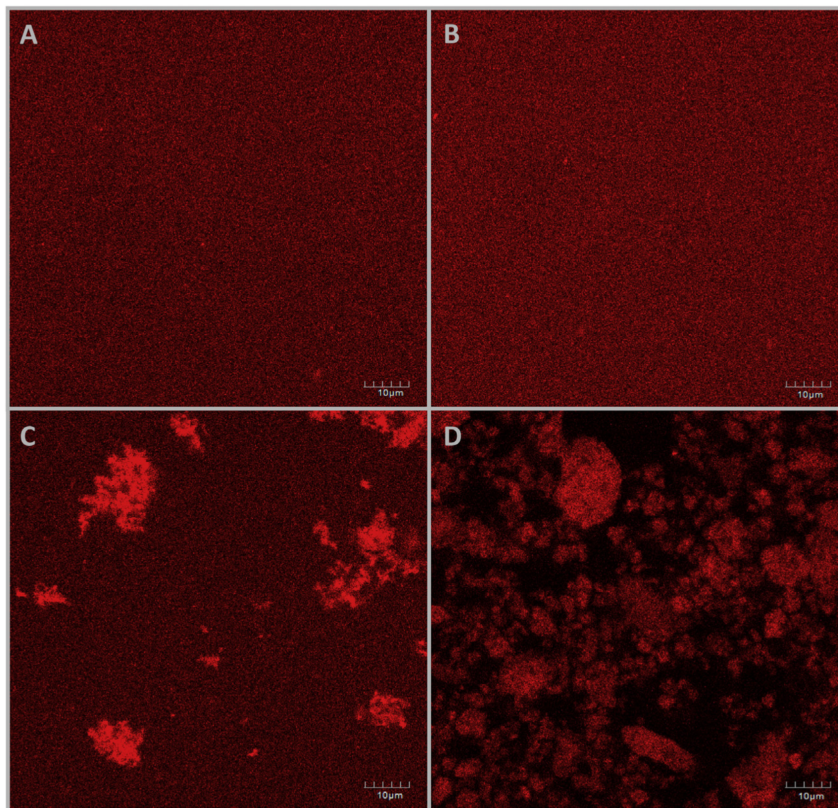

Fig. 5. Confocal microscopy images of A: FA 0.5% pH 3, B: β-lg 5% pH 3, C: β-lg 5% - FA 0.1% pH 3, D: β-lg 5% - FA 0.5% pH 3.

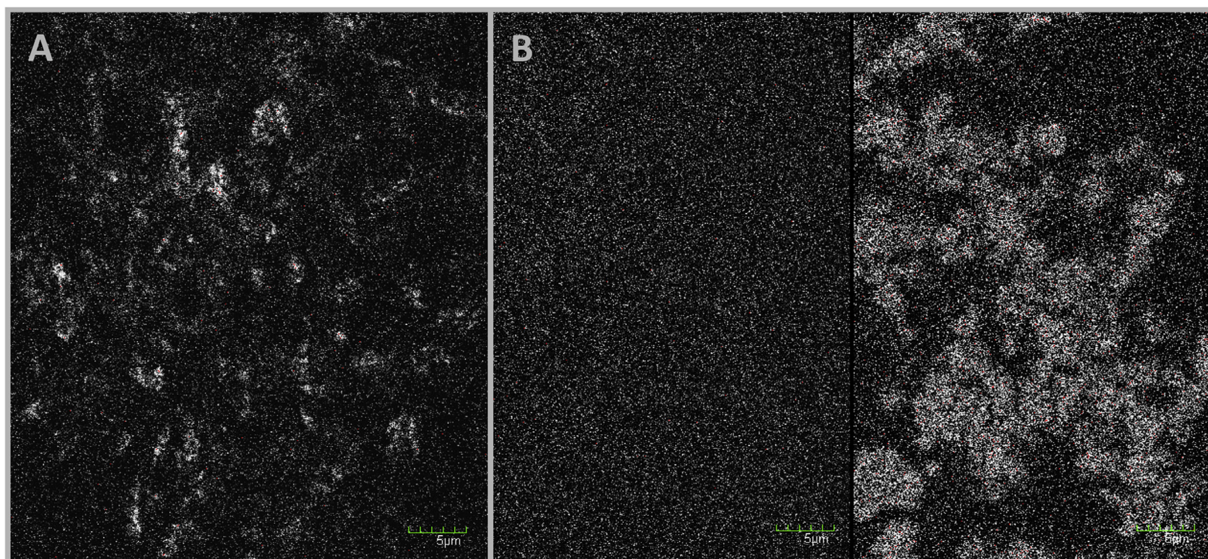

Fig. 6. Spectral confocal microscopy images of A: FA 0.5% pH 3, B: β-lg 5% - FA 0.5% (left: FA channel – right: Rhodamine channel).

Table 1
Average diameter of gelatin (0.5% w/w) particles at different FA concentrations, pH 3.

FA (% w/w)	FA/G Molar ratio	Average diameter (μm)[1]
0	0	0.13 ± 0.05^a
0.0025	0.57	3.68 ± 0.74^b
0.125	28.32	9.86 ± 0.23^c

[1] Mean values with different letters were significantly different ($P < .05$).

state of key amino acid residues (i.e., lysine, histidine, arginine).

3.5. *Modification of particle size of formed aggregates*

Particle size of FA/protein aggregates is very important for practical application of those particles as FA carriers in foods. Thus, we investigated whether lower total concentrations of the β-lg/AF mixture at pH 3, maintaining the maximum molar ratio studied

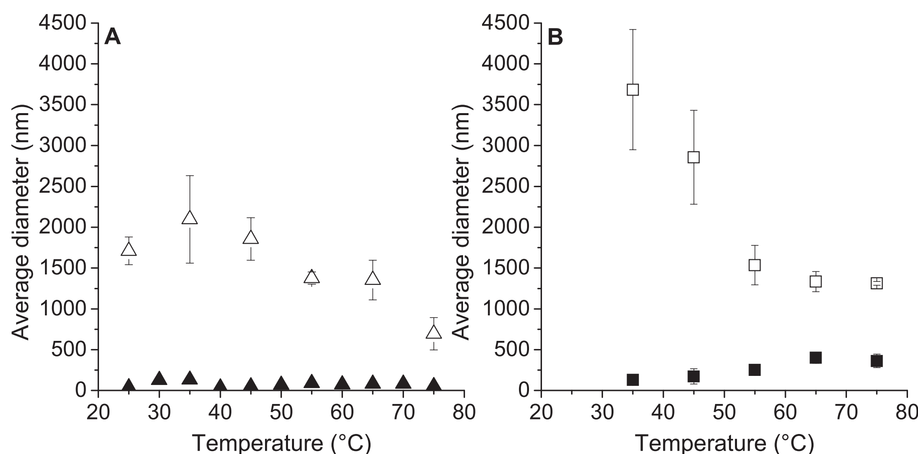

Fig. 7. Average particle size of FA/protein particles at pH 3 as affected by temperature. β-lg 5% - FA 0.025% (△) as compared to β-lg 5% (▲); B: Gelatin 0.5% - FA 0.0025% (□) as compared to Gelatin 0.5% (■).

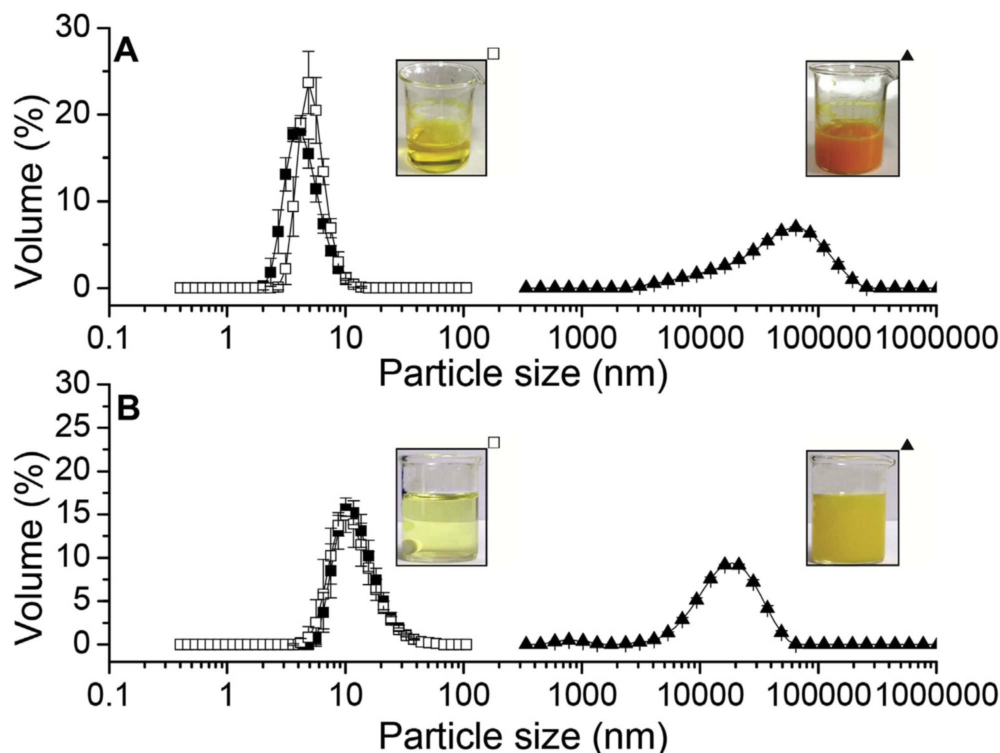

Fig. 8. Volume particle size distributions and visual appearance of the solutions for (A) β-lg 5% - FA 0.625%, (B) Gelatin 0.5% - FA 0.125%. With change of pH from 7(■) to 3(▲) and then returning to 7(□).

(AF/β-lg = 10), could led to smaller particles. Fig. 9A shows the particle size distributions, which were monomodal and less polydisperse when using β-lg 0.1–1%. This particle size could be of interest for an application of these particles as FA carriers. Fig. 9B shows that by lowering β-lg concentration, the average particle size decreases, thus constituting a tool to control the particle size. Nevertheless from an industrial point of view, is not usual to work with such low total concentrations. Thus, the application of high intensity ultrasound (HIUS) was investigated as a way to modify particle size of FA/protein aggregates.

The application of HIUS has been effective in order to control particle size, producing in general its reduction in protein systems (Carolina Arzeni, Pérez & Pilosof, 2015; Gordon & Pilosof, 2010). In Table 2 it is shown that indeed the average diameter of the particles generated by the interaction of FA and β-lg is reduced by HIUS treatment. The initial average diameter of the particles (D_o) in the submicronic range was reduced by 3–10 times by application of HIUS, however for particles with diameters between 25 and 34 μm the maximum reduction was 50%. Generally larger particles are more likely to be attacked by cavitation energy, whereas small particles have lower relaxation times and thus can dispel the stress imposed by sonication more easily (Tsaih & Chen, 2003). However, in this case because of the high viscosity of the systems formed by the larger particles, the US treatment would be less effective. The higher the viscosity, the higher the US intensity threshold for the onset of cavitation (Behrend, Ax, & Schubert, 2000).

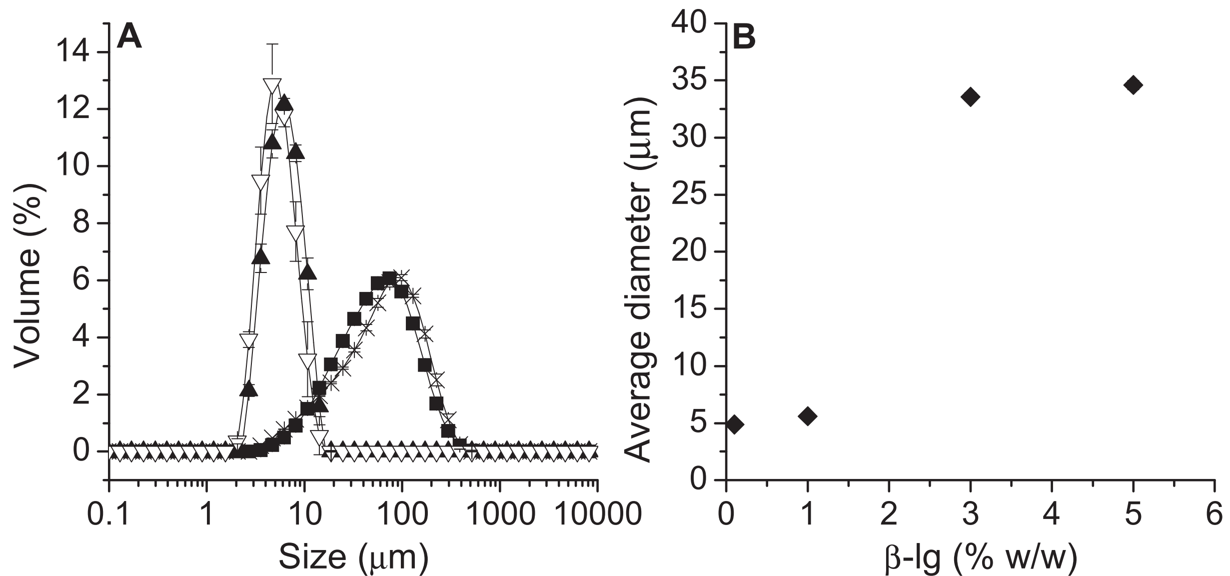

Fig. 9. A: Volume particle size distributions of FA/β-lg mixtures with constant molar ratio = 10. β-lg 5% - FA 1.25% (■), β-lg 3% - FA 0.75% (×), β-lg 1% - FA 0.25% (▲), β-lg 0.1% - FA 0.025% (▽). B: Average diameter of the same solutions.

Table 2
Average diameter of β-lg (5% w/w) particles at different FA concentrations, before (D_o) and after the HIUS application (D_{US}), pH 3.

FA (% w/w)	Do (μm)[1]	DUS (μm)[1]
0.025	0.095 ± 0.001^a	0.070 ± 0.001^a
0.050	0.393 ± 0.169^a	0.113 ± 0.021^a
0.100	2.370 ± 0.765^b	0.254 ± 0.067^a
0.500	33.054 ± 2.477^g	17.481 ± 2.955^c
0.625	33.553 ± 3.081^g	20.473 ± 5.032^d
1.25	28.644 ± 4.588^f	26.204 ± 1.568^e

[1] Mean values with different letters were significantly different ($P < .05$).

4. Conclusions

β-lg and gelatin were able to bind FA by ionic bonds at acidic pH, forming particles with growing sizes as the FA/protein molar concentration increased. Interestingly the FA/protein particles were pH-reversible, being this dependence strongly favorable for the delivery of FA at the duodene (pH 7) as well as for the protection of FA at the pH prevailing in the stomach (pH 3). Moreover, the sizes of FA/protein particles can be modified by changing total protein concentration or by HIUS application.

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