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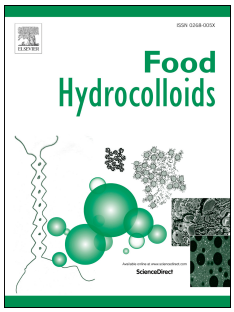

Nanoparticles assembled from mixtures of whey protein isolate and soluble soybean polysaccharides. Structure, interfacial behavior and application on emulsions subjected to freeze-thawing

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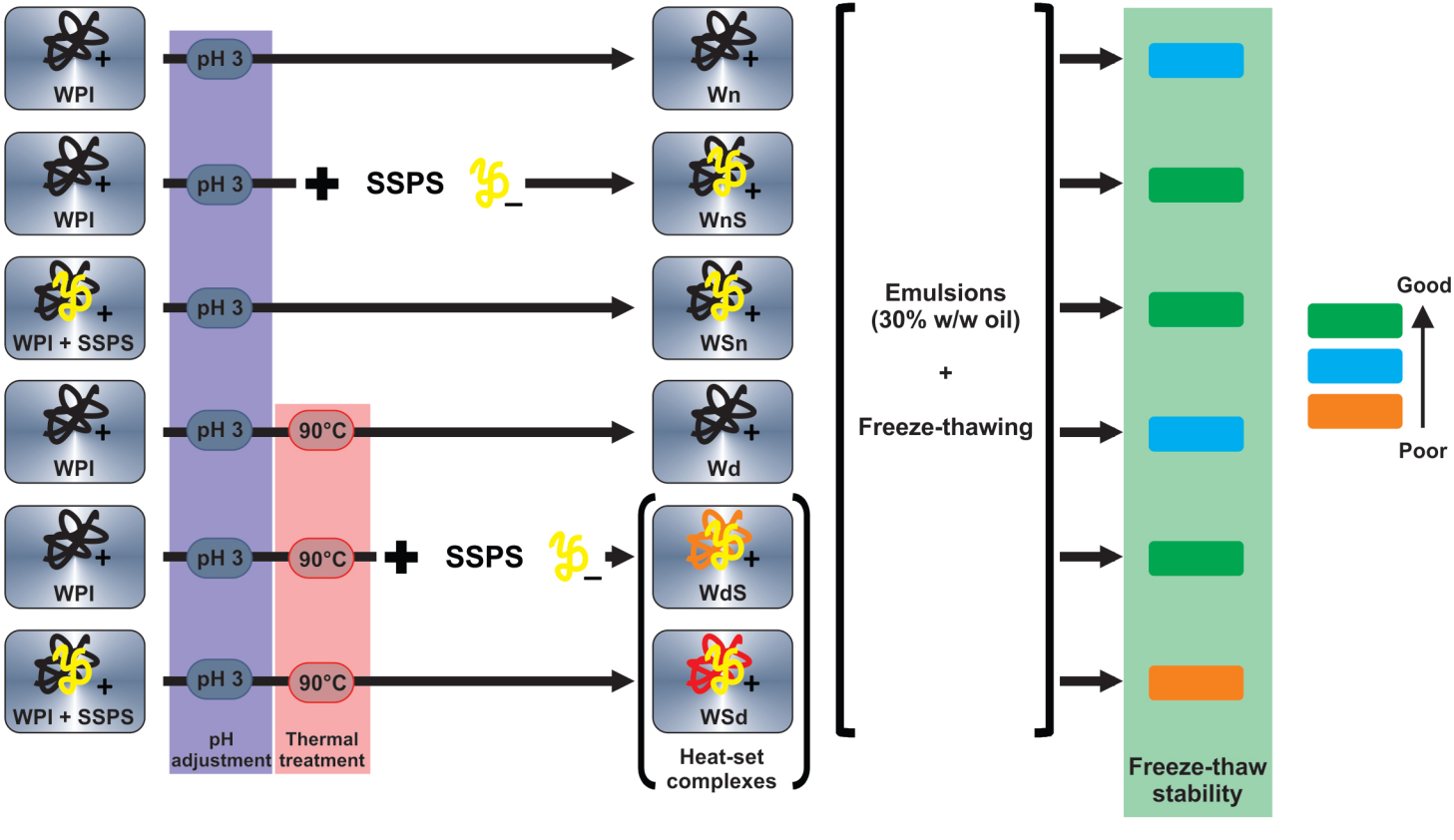
WPI
WPI
WPI + SSPS
WPI
WPI
WPI + SSPS
pH 3
pH 3
pH 3
pH 3
pH 3
pH 3
SSPS
90°C
90°C
90°C
SSPS
pH adjustment
Thermal treatment
Wn
WnS
WSn
Wd
WdS
WSd
Heat-set complexes
Emulsions (30% w/w oil)
+
Freeze-thawing
Freeze-thaw stability
Good
Poor

NANOPARTICLES ASSEMBLED FROM MIXTURES OF WHEY PROTEIN ISOLATE AND SOLUBLE SOYBEAN POLYSACCHARIDES. STRUCTURE, INTERFACIAL BEHAVIOR AND APPLICATION ON EMULSIONS SUBJECTED TO FREEZE-THAWING.

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Abstract

In this article, the freeze-thaw stability of emulsions prepared with nanoparticles assembled from mixtures of whey protein isolate (WPI, 2.0% w/w) and soluble soybean polysaccharides (SSPS, 0.5% w/w) was assessed. The assembly was performed by pH adjustment to 3.0 without and with heating (90 °C, 15 min). Moreover, the order of addition of SSPS to proteins, before or after heating, was also studied. The complexes were characterized by dynamic light scattering, turbidity, non-sedimentable protein content, aromatic surface hydrophobicity (H_0), interfacial tension and interfacial rheology measurements at the oil/water interface. In all cases, the dispersions evidenced slightly-positive ζ-potential values due to electrostatic associative interactions between proteins and SSPS. Moreover, the complexation increased the particle size, the interfacial activity and the non-sedimentable protein content. Oil-in-water emulsions (30% w/w sunflower oil) prepared with unheated WPI/SSPS mixtures were more stable to freeze-thawing (−18 ºC, 72 h; 20 ºC, 2 h) respect to those prepared with WPI alone. When SSPS was added to previously heated proteins, the resultant emulsions also evidenced a high freeze-thaw stability. The large sedimentable species, which contributed to form a film of high viscoelasticity, could stabilize the emulsions by a Pickering mechanism. However, when SSPS and WPI were heated together, the resultant emulsions exhibited a low freeze-thaw stability due to a combination of poor emulsification ability and limited interfacial adsorption of large particles. The results of this article might have important implications in the preparation of highly acidic emulsion-based products resistant to freeze-thaw treatments.

Keywords: emulsions, freeze-thawing, nanoparticles, soluble soybean polysaccharides, stability, whey protein isolate.

1. Introduction

Freezing is a widely used technology that preserves the sensory and nutritional properties of foods, as well as the microbial and chemical spoilage (Thiebaud, Dumay, & Cheftel, 2002). Nevertheless, for emulsion-based products freeze-thaw treatments constitute an important environmental stress causing sometimes their breakdown, due to rupture of membranes surrounding the droplets. In oil-in-water emulsions, this allows oil-to oil contact, leading to extensive coalescence process and oiling off after thawing. Broadly, the freeze-thaw stability of emulsions depends on two main aspects: the crystallization of water and/or lipid phases and the drastic changes in microenvironmental conditions surrounding the droplets (pH, ionic strength, osmotic pressure and viscosity) (Degner, Chung, Schlegel, Hutkins, & McClements, 2014; Ghosh & Coupland, 2008). The improvement of freeze-thaw stability of oil-in-water emulsions was assessed through different approaches including the control of ice crystal growth, vitrification of continuous phase, addition of cryoprotectants and modification of interfacial structure (Degner et al., 2014; Ghosh, Cramp, &Coupland, 2006; Ghosh & Coupland, 2008; Palazolo, Sobral, & Wagner, 2016).

Interfacial engineering is one of most promising strategies for the stabilization of oil-in-water emulsions subjected to environmental stresses. The knowledge related with the interactions between proteins and polysaccharides is extremely important to assess the obtaining of emulsion-based foods resistant to freeze-thaw treatments. Indeed, the preparation of multilayered emulsions through layer-to-layer interfacial electrodeposition technique improved the stability freeze-thaw stability of emulsions, as was stated in previous papers

(Fioramonti, Arzeni, Pilosof, Rubiolo, & Santiago, 2015; Gu, Decker, &McClements, 2007). The improvement of stability was ascribed to the formation of a thick interfacial layer that resists the stress imposed by the expansion of ice during the freezing.Moreover, the papers concerning with the freeze-thaw stability of Pickering emulsions stabilized by food-grade particles is fast cumulating in recent years. Again, regardless the type of emulsifier, the formation of thick layer of aggregated particles at the oil/water interface contributes to the maintenance of stability after freeze-thawing. In this regard, Marefati, Rayner, Timgrem, Dejmek, & Sjöo (2013) confirmed that the starch granule-base Pickering emulsions exhibited good freeze-thaw stability, which is enhanced by *in situ* partial gelatinization at the oil/water interface and the concomitant formation of gel-like network due to the presence of free starch particles in the aqueous phase. In other series of studies, the enhanced freeze-thaw stability of emulsions prepared with heated protein-based particles was attributed to the protein aggregation at the oil/water interface and the subsequent steric stabilization of droplets (Zhu, Zhang, Lin &, Tang, 2017a; Zhu, Zheng, Liu, Qiu, Lin, & Tang, 2017b; Zhu, Zheng, Liu, Qiu, Lin, & Tang, 2018). This steric stabilization can be also achieved though the associative interactions between protein and polysaccharides at interfacial level, as was stated in a previous paper (Xu, Zhang, Cao, Wang, & Xiao, 2016).

On the other hand, milk proteins (caseins and whey proteins) are highly effective emulsifiers (Dickinson, 2016). Whey protein isolate (WPI) is an important by-product of the cheese industry and it is mainly composed by β-lactoglobulin, α-lactalbumin, immunoglobulins, protease peptone, lactoferrin, glycomacropeptide and other minor proteins (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). WPI was widely used as the sole emulsifier or combined with polysaccharides in emulsion systems (Jiang et al., 2018; Laplante, Turgeon, & Paquin, 2005; Wang et al., 2017). In addition, whey protein-coated droplets are less prone to aggregation in highly-acid medium (pH < 4.0) than soy protein

isolate or caseinate-coated droplets and may, therefore be more appropriate for use in acid emulsions (Degner et al., 2014).

Moreover, soluble soybean polysaccharides (SSPS)are a family of pectin-like acidic biopolymers used not only as a source of dietary fiber but also as a functional ingredient (Xu & Liu, 2016). This sample, extracted from soybean cotyledons, is composed mainly by of a main rhamnogalacturonan backbone branched by β-1,4–galactan and α–1,3– or α–1,5–arabinan chains (Nakamura, Yoshida, Maeda, & Corredig, 2006). This polysaccharide sample can be classified as a surface-active polysaccharide. Nakamura, Yoshida, Maeda, Furuta, & Corredig (2004) separated the SSPS in two fractions by gel-filtration (310 kDa and 20 kDa, respectively). The protein moieties associated to high-molecular weight fraction aid to adsorption of SSPS onto oil/water interface, explaining the good emulsifying properties of SSPS. SSPS have high water solubility, low bulk viscosity, low pH and high temperature stability (Xu & Liu, 2016). The interactions between proteins and SSPS in acid emulsions have been previously investigated. The presence of SSPS at high enough concentrations stabilized the sodium caseinate emulsions against acid-induced aggregation (Liu, Verespej, Alexander, & Corredig, 2007). In a relatively recent work, Ray & Rousseau (2013) reported that the complexes between SSPS and denatured soy-whey proteins improved the quiescent stability of emulsions in acidic medium. The stability would be ascribed to denser packing of complexes around the oil droplets, providing greater steric stabilization. Nevertheless, in the aforementioned studies, the freeze-thaw stability of the emulsions prepared with protein–SSPS complexes was not evaluated.

On the basis of above considerations, the aim of current work is to evaluate the impact of the complexation between whey proteins and SSPS on the freeze-thaw stabilization of acid o/w emulsions. The obtaining of nanoparticles assembled from mixtures of WPI and SSPS at pH 3.0 are proposed evaluating the impact of thermal treatments and the order of addition of

polysaccharides to protein in relation to the heating.The structural and interfacial behavior of nanoparticles at the oil/water interface was firstly evaluated; then, the freeze-thaw stability of oil-in-water emulsions prepared with the complexes was assessed.

2. Materials and methods

2.1 Materials

Whey protein isolate (WPI, Lacprodan® DI-9224) was donated by Arla Foods Ingredients Argentina, S.A (Martínez, Buenos Aires, Argentina). Its chemical composition (% w/w, as was given by the producer) was: crude protein (N×6.25), 86.5; lactose, 0.1; total fat, 0.1 and salts, 1.25. Soluble soybean polysaccharides (SSPS, Soyafibe-S-CA100) were donated by Fuji Oil Co. Ltd (Osaka, Japan). Its chemical composition (% w/w, as given by the producer was) was: total dietary fiber, 75.1; crude protein (N×6.25), 7.8; moisture, 5.8; crude ash, 7.8. Moreover, the SSPS saccharide composition (% w/w) was: rhamnose, 5.0; fucose, 3.2; arabinose, 22.6; xylose, 3.7; galactose, 46.1; glucose, 1.2; galacturonic acid, 18.2. WSP and SSPS powder samples were used with no further purification. Refined sunflower oil was purchased in a local supermarket. 1-anilino-8-naphtalene sulfonate, ammonium salt (ANS) and β-lactoglobulin (> 90.0%, from bovine milk) were purchased from Sigma Co (MO, USA). All other chemical were analytical grade reagents purchased from Anedra (Research AG S.A; Buenos Aires, Argentina).

2.2. Preparation of protein-polysaccharides dispersions

Firstly, to ascertain the heating conditions to obtaining the nanoparticles, preliminary differential scanning calorimetry (DSC) assays were performed at pH 3.0 on concentrated WPI dispersions (20.0% w/w) in the absence and presence of SSPS (5.0% w/w). The pH adjustment was made by addition of 1.0 M HCl solution. Thermograms were obtained by heating samples at 5 ºC/min from 20 to 120 ºC (Q200 calorimeter, TA Instruments, Waters L.L.C; New Castle, DE USA), using an empty pan as reference. According to these assays, at pH 3.0, WPI dispersions showed only one endotherm (peak temperature, Tp = 79.38 ± 0.05 ºC; offset temperature, T_{offset}= 88.10 ± 0.98 ºC; protein denaturation enthalpy, ΔH=5.87 ± 0.83 J/g dry matter) which can be assigned to denaturation of β-lactoglobulin (Bernal & Jelen, 1985). At the same pH, in the presence of SSPS, Tp, T_{offset} and ΔH were 77.32 ± 0.08 ºC, 86.10 ± 0.50 ºC and ΔH=5.30 ± 0.33 J/g dry matter, respectively. On this basis, to perform the heating of dispersions, the temperature was set at 90 ºC, where the WPI proteins were totally denatured.

The assembly of nanoparticles was performed by following the different strategies reported by Jones & McClements (2011) with some modifications: I) WPI and SSPS powders were jointly dispersed in distilled water by magnetic stirring for 2 h and the pH was adjusted to 3.0 by 1.0 M HCl addition. Aliquots of resultant dispersions (100 g) were heated at 90.0 (± 1.0) º C for 15 min with further cooling with running tap water to room temperature (25.0 ± 1.0 ºC); II) WPI powder was firstly dispersed in distilled water by magnetic stirring for 2 h and the pH was adjusted at 3.0 by 1.0 M HCl addition. The resultant dispersions (100 g) were heated at 90.0 (± 1.0) ºC for 15 min with further cooling with running tap water to room temperature (25.0 ± 1.0 ºC). Then, SSPS powder was dispersed by magnetic stirring for 2 h, readjusting the pH to 3.0. In both cases, the final concentrations of WPI and SSPS were 2.0 and 0.5% w/w, respectively (protein-to-polysaccharide mass ratio, R = 4.6). Unheated WPI/SSPS dispersions and individual WPI and SSPS dispersions were used as control

samples. Heating of dispersions was performed in sealed tall-glass beakers (length: 60 mm; internal diameter, 25 mm). The temperature was monitored using a Lufft Opus C-10 datalogger (Lufft, Meß und Regeltechnik, Germany); K thermocouples were located half way through dispersion. The warm up time was 5 min and the heating time (15 min) was registered once the dispersions reached the mentioned temperature at the center of the beaker.

Photographs at bulk scale of dispersions were performed using a digital camera (Kodak Easy Share, Eastman Kodak Company; Rochester, NY, USA). The nomenclature and visual appearance of all samples are summarized in Fig. 1.

2.3. pH dependence of turbidity of dispersions

WPI, SSPS and WPI/SSPS (R= 4.6) dispersions were prepared dispersing the powders with distilled water at 25 ºC by mild magnetic stirring for 2 h to ensure total dispersion and hydration. The final concentrations of WPI and SSPS were 2.0 and 0.5% w/w, respectively. To perform acid titration assays, the dispersions were previously diluted with distilled water (1:3 v/v). The pH values of WPI, SSPS and WPI/SSPS diluted dispersions were 6.90 ± 0.02, 5.95 ± 0.01 and 6.62 ± 0.02, respectively. Then, aliquots of these dispersions (20 ml) were titrated with repeated additions of 0.1 M HCl under mild magnetic stirring. The pH value was registered and the turbidity was expressed as the apparent optical density at 600 nm using a T60 UV-visible spectrophotometer (PG Instruments; Leicestershire, United Kingdom)

2.4. Determination of non-sedimentable protein content

196 The determination of non-sedimentable protein content, which is a measure of the
197 amount of protein incorporated into relatively small particles that cannot be removed by
198 centrifugation at high speed, was determined by following the procedure reported by Jones &
199 McClements (2010) with some modifications. Dispersions were placed in 50-ml screw-
200 capped tubes and subsequently centrifuged at 19,000×g for 30 min at 20 °C (Sigma 3-18KS
201 centrifuge, Sigma Laborzentrifugen GmbH; Osterode am Harz, Germany). Then, supernatants
202 were carefully separated in glass tubes. Appropriate dilutions with 2.0 % w/v Na_2CO_3
203 solution were made; then, the protein content was determined by the modified Lowry method
204 (Markwell, Hass, Bieber, & Tolbert, 1978), using β-lactoglobulin as standard protein. The
205 protein content was also measured on initial dispersions, without centrifugation. Thus, the
206 non-sedimentable protein (% w/w) was calculated as:
207
208 $$\text{Non-sedimentable protein (\% w/w)} = (sP/iP)\times 100 \quad (1)$$
209
210 sP is the protein content in the supernatant and iP is the protein content in the initial
211 dispersion, without centrifugation.
212
213 ***2.5. Particle size, ζ-potential and turbidity of nanoparticles***
214
215 The hydrodynamic diameter (or z-average diameter, D_Z) and ζ-potential of particles in
216 different dispersions were determined at 25 ºC by dynamic light scattering (DLS) using a
217 Horiba Scientific nanoparticle analyzer SZ-100 (Horiba Ltd.; Kyoto, Japan). All the
218 dispersions were diluted with double-distilled water, previously adjusted at pH 3.0 with HCl
219 1.0 M, to avoid multiple light scattering effects. The D_Z and ζ-potential were reported as the
220 mean and standard deviation of two separate assays, with ten readings made per assay.

Moreover, the intra-particle interactive forces of particles in dispersions were evaluated by dissociation tests according to the method reported by Zhu et al. (2017a), with some modifications. The dispersions were diluted with distilled water (1:3 v/v) adjusted at pH 3.0 with 1.0 M HCl in the absence and presence of 6.0 M urea, dithiothreitol (DTT, 30 mM), or their combinations. The final concentrations of urea and DTT in the diluted dispersions were 4.5 M and 22.5 mM, respectively. After being stored for 30 min, the turbidity of the diluted dispersions, expressed as the apparent optical density at 600 nm, was read using a T60 UV-visible spectrophotometer (PG Instruments; Leicestershire, United Kingdom).

2.6. Aromatic surface hydrophobicity

Aromatic surface hydrophobicity (H_0) was determined by fluorescence using the ANS probe, according to method of Mitidieri & Wagner (2002) with some modifications. Dispersions were serially diluted with double-distilled water previously adjusted to pH 3.0 with HCl solution to obtain protein concentrations ranging from $1.0 \cdot 10^{-2}$ to $1.0 \cdot 10^{-4}$ % w/v. Then, 40 µl of ANS (8.0 mM in double-distilled water) was added to 3.0 ml of dispersions. Fluorescence intensity was measured at 365 nm (excitation wavelength) and 484 nm (emission wavelength) using a Scinco FluoroMate FS-2 fluorescence spectrometer (Scinco Co, Ltd, Seoul, Korea) ($I_{F,\ ANS}$). Moreover, fluorescence intensity was measured in the same dispersions without ANS addition (I_F). From the plot of ΔI_F ($I_{F,\ ANS} - I_F$) as a function of protein concentration, H_0 was obtained as the initial slope. Finally, H_0 was expressed as a relative value, taking that of W_n sample as reference ($H_0 = 100$).

2.7. Interfacial behavior of nanoparticles

The interfacial behavior of particles was evaluated through both the interfacial tension and interfacial rheology measurements at the oil/water interface. Both assays were carried out on dispersions (2.0% w/w WPI; 0.5% w/w SSPS) without previous dilution. The equilibrium interfacial tension was measured at 25 ºC using an automated Lauda TD3 LMT 850 tensiometer (Lauda Königshofen, Germany) associated to a Peltier thermostating unit. The Du Noüy ring measurement method was selected in the device. The interfacial tension values of double-distilled water (adjusted to pH 3.0 with HCl 1.0 M) and aqueous dispersions at the same pH ($\gamma_{i,\,w}$ and $\gamma_{i,\,d}$, respectively) were obtained. Then, the equilibrium interfacial pressure (π_i) was calculated as:

$$\pi_i = \gamma_{i,\,w} - \gamma_{i,\,d} \quad (2)$$

In addition, the rheological properties of layers at oil/water interface were evaluated by the use of an oscillatory rheometer TA AR-G2 (TA Instruments, Waters, L.L.C; Newcastle, DE, USA) associated to a Du Noüy ring. Oscillatory shear measurements were performed at 25 ºC, and at constant frequency of 0.1 Hz. The strain was set to 5.0% within the lineal viscoelastic range. The complex viscosity (η^*) of layer, which includes both the elastic and the viscous responses, was calculated as:

$$\eta^* = [(G')^2 + (G'')^2]^{0.5}/\omega \quad (3)$$

G' is the interfacial elastic module, G'' the interfacial viscous module and ω the angular frequency. The overall evolvement of adsorption layer was monitored through the variation of η^* as a function of time (Baldusdottir, Fullerton, Nielsen, & Jorgensen, 2010).

2.8. Preparation of o/w emulsions

A two-step homogenization process was used to prepare the emulsions. Firstly, aqueous dispersions (2.0% w/w WPI; 0.5% w/w SSPS) were mixed with refined sunflower oil (30% w/w) using a high-speed rotor/stator device (Ultraturrax T-25 homogenizer, S25N–8G dispersing tool at 25,000 rpm for 2 min, IKA Labortechnik; Staufen, Germany). Then, the pre-emulsions were poured in a beaker and sonicated in an ultrasound homogenizer (Sonics Vibra Cell VCX750, Sonics & Materials, Inc.; Newtown, CT, USA) at 70% amplitude, with the standard tip immersed 1/3 in a glass beaker (28 mm diameter) for 2 min. The temperature increase during sonication was avoided putting the beaker in a water-ice bath.

2.9. Freeze-thaw protocol

Emulsion samples were transferred to vertical plastic containers (internal diameter = 30 mm with plastic lids), and the temperature was set at 20.0 ± 1.0 ºC. Then, they were isothermally stored in still air for 72 h at –18.0 ± 2.0 ºC. After storage at subzero temperature, frozen samples were thawed into a water bath at 20.0 ± 1.0 ºC for 2 h, and kept at this temperature before further characterization analyses.

2.10. Characterization of initial and freeze-thawed emulsions

The particle size distribution(PSD) was obtained by laser diffraction using a Malvern Mastersizer 2000E analyzer associated to a Hydro 2000MU wet dispersion unit (Malvern Instruments Ltd.; Worcestershire, United Kingdom). Emulsions were previously diluted with distilled water or 1.0% w/v sodium dodecyl sulfate solution adjusted at pH 3.0. Then,

emulsions were poured into the dispersion unit and stirred continuously (2,000 rpm) to ensure the sample homogeneity during PSD measurements. The optical parameters used to obtain the PSD were: refractive index of dispersant, 1.33, refractive and adsorption indices for the particles, 1.47 and 0.01, respectively. Particle size results were expressed as De Brouckere, volume-weighted mean diameters ($D_{4,3}$) and 90th percentile values ($D_{v,0.9}$), which are more sensitive to the presence of large particles.

The flocculation index (FI, %) of initial or freeze-thawed emulsions, was calculated as:

$$FI\ \% = [(D_{4,3} - D_{4,3\ SDS})/D_{4,3\ SDS}]\times 100 \quad (4)$$

$D_{4,3}$ and $D_{4,3\ SDS}$ are the De Brouckere mean diameters obtained from PSD measured in the absence and presence of SDS, respectively. This method allows evaluating only the flocs stable in the measurement conditions (Palazolo et al., 2011).

Moreover, the coalescence index (CI, %) of freeze-thawed emulsions was calculated as:

$$CI\ \% = [(D_{4,3\ f\text{-}t} - D_{4,3u})/D_{4,3u}]\times 100 \quad (5)$$

$D_{4,3u}$ and $D_{4,3f\text{-}t}$ are the De Brouckere mean diameters obtained from PSD of initial and freeze-thawed emulsions, respectively. PSD were measured in the presence of SDS (Palazolo et al., 2011).

2.11. Statistical analysis

All the characterization assays were conducted at least in triplicate and the results were expressed as mean ± standard deviation. The statistical analysis was performed by

analysis of variance (ANOVA) and Fisher test using the statistical program Statgraphics Centurion 18® (Statgraphics Technologies Inc., The Plains, VA, USA, 2017). Significance was considered at $p < 0.05$.

3. Results and discussion

3.1. pH dependence of aqueous dispersions of whey protein isolate and soluble soybean polysaccharides

In this section, the pH-dependence of the turbidity of unheated and diluted (1:3 v/v) aqueous dispersions containing WPI, SSPS and WPI/SSPS mixture was studied. The results are shown in Fig. 2a. WPI dispersions were clear in the pH range from 6.0 to 7.0 and at pH lower than 3.5. Nevertheless, the turbidity abruptly increased when pH was in the range 3.5-5.5 (maximum at pH ~ 4.4), indicating that the protein self-association had occurred due to weakening to electrostatic repulsion between protein molecules, as was well-established in the literature. (Alting et al., 2003; Bryant & McClements, 1999). Conversely, the turbidity of SSPS dispersions was close to zero across the entire pH range (pH 7.0 to 2.0).Although it is known that a protein fraction is associated to polysaccharidesin SSPS (Nakamura et al., 2004, 2006), the electrostatic and steric repulsion between the negatively charged and branched polysaccharides molecules avoided their self-association.

On the other hand, the pH dependence of the turbidity of WPI/SSPS mixture was considerably different from that of the WPI and SSPS dispersions (Fig 2a, b). In the pH range from 6.0 to 7.0, the turbidity of WPI/SSPS mixture was only slightly higher respect to that of individual WPI dispersion. These results would be consistent with a low degree of interaction between protein and polysaccharides in the pH range 6.0-7.0, presumably because both the

346 protein and polysaccharide molecules have a relatively high net negative charge (Kobori et
347 al., 2009). Indeed, according to Ibanoglu (2005), for proteins and carboxylated
348 polysaccharides, the complexation becomes very weak at neutral pH.However, the turbidity
349 increased with decreasing pH at pH below 5.3, had a maximum at pH ~ 4.3 and then slowly
350 decreased until pH 2.0. Even though the protein-to-polysaccharide mass ratiowas relatively
351 high (R = 4.6), the maximum value of turbidity of WPI noticeably decreased (~1.2 to ~0.6)
352 and it was shifted to lower pH values in the presence of SSPS. The WPI content ofall
353 dispersions was the same, so that the presence of polysaccharides inhibited the ability of
354 protein molecules to interact with each other and form large aggregates.
355 Theseobservationsindicate the occurrence of interactions between the protein and
356 polysaccharides present in the dispersions. Interestingly, the turbidity of WPI/SSPS mixture
357 was fairly high in the range of pH from 2.5 to 3.5 (Fig 2a,b). Under these pH conditions the
358 WPI and SSPS dispersions were completely clear (Fig 2b), so that the associative interactions
359 between protein and polysaccharides in the mixture are still relatively strong. Thus, the
360 assembly and characterization of nanoparticles was performed at pH 3.0.
361
362 ***3.2. Structural characterization of nanoparticles***
363
364 W_n and W_d dispersions exhibited a clear appearance (Fig. 1) and their ζ-potential were
365 close to + 20 mV, which are in agreement with those reported by other authors (Benichou,
366 Aserin, Lutz, & Garti, 2007) (Fig 3a). For SSPS clear dispersion (Fig. 1), the ζ-potential was
367 slightly negative; the magnitude of charge is low because the galacturonic acid has a pK_a near
368 to 3.0. Consequently, the opposite net charges of polysaccharides and proteins favored the
369 formation of complexes. Indeed, all mixed dispersions exhibited a turbid appearance,
370 regardless the preparation method, and their ζ-potential values were slightly positive (Figs. 1

and 3a). According to Benichou et al. (2007), WPI protein particles in the dispersions have a net charge equal to zero at pH 4.8. At pH 3.0, below the isoelectric point of the WPI, the numerous cationic groups on the protein molecules are partially neutralized by the anionic groups of SSPS, decreasing the ζ-potential of complexes (Fig 3a). At the same pH, the decrease of ζ-potential of soy proteins by SSPS complexation was also reported in previous papers (Ray & Rousseau, 2016; Xu & Liu, 2016).

Moreover, for all dispersions, the content of non-sedimentable protein (% w/w) is shown in Fig. 3b. This parameter was near to 100% for clear W_n, W_d and SSPS dispersions and a slight, but significant decrease, was evidenced when both WPI and SSPS were mixed without any thermal treatment (WS_n and W_nS), ($p<0.05$). In contrast, when heating was performed on dispersions, an additional reduction of non-sedimentable protein content was evidenced for WS_d. Thepositive net charge of all complexes was quite similar (Fig 3a) so that the particular behavior of WS_d complex can be influenced by other factors, such as the surface hydrophobicity and particle size. Indeed, the z-average values (D_Z) for species in the total and non-sedimentable fraction were also determined (Fig 3c). For the total fraction, all complexes exhibited larger particle sizes than those of W_n, $W_d$and SSPS, regardless the thermal treatment ($p<0.05$). In turn, WS_d and W_dS complexes evidenced larger particle size respect to those assembled from unheated proteins (WS_n and W_nS, $p<0.05$). In addition, the D_Z values measured on the supernatants were lower respect to those of the total fraction for all complexes (Fig 3c, $p<0.05$), which would reflect that the relatively large particles were removed by centrifugation.The D_Z values of WS_n and W_nS did not show significant differences ($p>0.05$). Conversely, noticeable differences were observed between the particle size of WS_d and W_dS complexes in the non-sedimentable fraction ($p<0.05$). These observations would support a different molecular organization of protein and polysaccharides for the complexes assembled in the heated dispersions.

In fact, the aromatic surface hydrophobicity (H_0) was also determined (Fig 4a). As expected, SSPS evidenced very low H_0 due to the presence of sugar polar groups, which impair the interaction of the fluorescent probe with the non-polar aminoacid residues of the SSPS protein fraction. The increase of H_0 of W_n upon heating was consistent with the exposure of non-polar amino acids of WPI proteins, as was also previously reported (Zhu et al., 2017a). Neverthess, for W_d, the increase of the intensity of hydrophobic interactions was not sufficient to promote the protein self-association due to electrostatic repulsion between protein molecules (Fig. 3a,b). With regard to the WS_n and W_nS complexes, the addition of SSPS to the proteins, slightly increased the H_0 ($p<0.05$). In both cases, the complexation between both biopolymers would promote an exposure of non-polar amino acid residues, though to a limited extent. The impact of SSPS addition on H_0 was substantially different when the assembly was performed with denatured proteins. For W_dS complex, the significant decrease of H_0 with respect to that of W_d ($p<0.05$) would indicate that a shell of polysaccharides might be formed on the protein particles. This mechanism was also reported by Xu & Liu (2016) for nanoparticles assembled with soy protein isolates and SSPS. In contrast, when WPI and SSPS were heated together, an enhancing of H_0 was effectively observed ($p<0.05$). In this case, the globular proteins tend to stay associated with SSPS for a longer time during the thermal treatment (Jones & McClements, 2011). The increase of H_0 would be consistent with a higher degree of exposure of non-polar amino acid residues, indicating that the structural organization of protein and polysaccharides within the WS_d complex might be different respect to that of W_dS one.

In this context, the internal structure of complexes was also investigated by monitoring the turbidity of the total fraction at pH 3.0 in the absence and presence of protein-perturbing agents, such as urea and DTT (Fig 5b). Overall, in the absence of urea and DTT, all systems evidenced higher turbidity values for WS_n and WS_d dispersions and they were not

significantly affected by the DTT addition (data not shown). In the presence of urea, the turbidity values of W_nS and WS_n were negligible whatever the DTT addition. In contrast, the turbidity of WS_d dispersion was fairly higher, showing a slight, but significant decrease with DTT addition ($p<0.05$, Fig 5b). On the basis of these results, it can be hypothesized that hydrophobic effect as well as hydrogen and disulfide bonds would be also involved in the formation of nanoparticles, as was also stated in other systems (Liu & Xu, 2016, Zhu et al, 2017a). The urea directly interacts with proteins via strong Van der Waals dispersive interactions with protein side-chains and backbone compared to water, which promotes the intrusion of urea molecules in the hydrophobic core and to urea's preferential binding to all regions of proteins (Hua, Zhou, Thirumalai & Berne, 2008; Kamerzell, Esfandiary, Joshi, Middaugh, & Volkin, 2011). This interaction weakens the hydrophobic core and disrupts the intramolecular hydrogen bonds. The ζ-potential of W_dS and WS_d was similar ($p>0.05$); however, for the latter complex, significantly higher values of non-sedimentable protein content, particle size and H_0were observed ($p<0.05$, Figs 3b,c and 4a). Thus, the tighter interactions between protein and polysaccharides within the nanoparticles would increase their resistance to the perturbing effect of urea. Moreover, for WS_d complex, the formation of disulfide bonds via thiol-disulfide exchange would be promoted in a higher extent, although the extension of this reaction is limited in acidic medium (Monagan, Sherman, & Kinsella, 1995). This latter observation was consistent with the slight decrease of turbidity by DTT addition in the presence of urea (Fig 4b).

3.3. Interfacial behavior of nanoparticles

Firstly, the evolvement of the complex viscosity (η*) as a function of time is depicted in Fig. 5. ForW_n and W_d, a steep increase of η* was observed from the start of measurement

indicating a fast adsorption of protein molecules onto the oil/water interface (Baldursdottir et al, 2010, Fig 5a). Although W_d exhibited a high H_0, (Fig 4a) its interfacial pressure (π_i) was slight, but significantly lower than that of W_n (Table 1, $p<0.05$). The protein adsorption is a complex process, and depends on various factors such as hydrophobicity, net charge, charge distribution, structure and molecular weight (Haynes & Norde, 1994). In this context, the denatured protein particles, which have a higher particle size (Fig 3c), would be less prone to efficiently accommodate at the oil/water interface, promoting a lower increase of π_i. Nevertheless, the higher intensity of attractive interactions forces between denatured particles already adsorbed to the interface was consistent with the formation of a film of a higher viscoelasticity (Fig 5a,b). On the other hand, with the exception of WS_d, the time dependency of η^* for all the other complexes was substantially different to that of W_n and W_d dispersions; the complex viscosity progressively increased with increasing time and it leveled off after 30 min of adsorption, reaching higher η^* values at the end of assay (Fig 5a, b). At the same time, WS_n and W_nS complexes evidenced a higher interfacial activity, showing a slight, but significant higher π_i values than those of W_n. In analyzing comparatively the π_i of WS_d and W_dS complexes respect to that of W_d alone, a similar tendency was observed (Table 1, $p< 0.05$). The weakening of the electrostatic repulsion of protein molecules due to the complexation with SSPS (Fig 3a) would favor a more efficient adsorption at the oil/water interface of the complexes.

In order to elucidate if the large sedimentable particles can adsorb at the interface, the interfacial rheology tests were also performed on the non-sedimentable fraction (Fig 5c). For all complexes, a plateau at low η^* was reached from the start of measurement indicating a fast adsorption. Thus, the comparative analysis of the rheology profiles the total and non-sedimentable fractions revealed that the large sedimentable species play an important role in the stabilization of the interfacial film. These species slow down the adsorption rate, but at the

same time promote the formation of more viscoelastic films. This would be especially valid for WS_n, WS_d and W_dS dispersions. Conversely, for WS_d, although the species present in non-sedimentable fraction evidenced a fast adsorption, the large particles would only have a limited ability to adsorb at the oil/water interface.

3.4. Characterization of initial and freeze-thawed emulsions

The $D_{4,3}$ and $D_{v,\,0.9}$ values, obtained from PSD in the presence of SDS are shown in Fig 6. WS_n and $W_nS$complexes evidenced a better emulsifying behavior respect to that of W_n alone. According to Jafari, Assadpoor, He, & Bhandari (2008), the particle size of emulsions is the result of an equilibrium between droplet break-up and re-coalescence. In all cases, the concentration of emulsifier was high, so that there is enough emulsifier to cover the interface. Thus, the improved emulsifying behavior of WS_n and W_nS could be explained by a decrease of electrostatic repulsion, a concomitant increase of H_0, $\pi_{i.}$ (Figs. 3a and 4a; Table 1) and the ability of larger particles to reduce the re-coalescence during the emulsification.Conversely, for emulsions prepared with heated dispersions, $D_{4,3}$ decreased in the order: $WS_d>W_d>W_dS$ ($p<0.05$) and WS_d exhibited the highest $D_{v,\,0.9}$ value ($p<0.05$, Fig 6 a,b). As was seen above, the large sedimentable particles of WS_d are poorly adsorbed (Fig 5b,c). Unlike the interfacial tension and interfacial rheology assays, during the ultrasound emulsification, the emulsifier adsorption is forced because theinterfacial area is quicklycreated by cavitation(Jafari et al., 2008). In this condition, it is probable that the large sedimentable particles are also poorly adsorbed favoring the droplet re-coalescence and the resultant increase of particle size of WS_d emulsion (Fig. 6).

Moreover, the flocculation index (FI) of all initial emulsions was quite similar and lower than 5% (Table 2). For emulsions prepared with protein as emulsifiers, only the flocs

formed by a bridging mechanismare stable in the measurement conditions of PSD (high dilution and stirring) (Gu et al., 2007). The bridging flocculation is especially promoted when there is no enough emulsifier to cover the interface (Palazolo et al., 2011). Thus, the low FI values obtained in all cases are supported by the high concentration of emulsifier in the emulsions.

It is known that the freeze-thawing is a highly destabilizing treatment for emulsions, (Ghosh et al., 2006, Palazolo et al., 2011, 2016, Thiebaud et al., 2002). After freeze-thawing, all emulsions were destabilized by coalescence and flocculation, though to a different extent (Table 2). It worth noting that the emulsions were stored at subzero temperature during a relatively short time period and a low-melting-point oil phase was used. Hence, the mentioned differences could be mainly attributed to the processes that occur in the aqueous phase at interfacial level (Palazolo et al., 2011). Emulsions prepared with W_d dispersion showed slightly higher destabilization parameters (FI and CI, %) respect to that of W_n one ($p< 0.05$). This result was similar respect to that obtained by Zhu et al (2017a) for emulsions prepared with unheated and heated WPI after one cycle of freeze-thawing. For W_nS, W_dS and WS_n emulsions, an improvement of stability to coalescence and flocculation upon freeze-thawing was effectively observed, showing CI and FI values lower than 17 and 20%, respectively. Conversely, WS_d emulsion exhibited the highest destabilization degree to coalescence and flocculation; the CI and FI values were higher than those of W_d emulsions, without SSPS addition (Table 2).

According to the Zhu et al (2017 a,b), the freeze-thaw stability of emulsions prepared with proteins as the sole emulsifier can be enhanced by a Pickering mechanism, due to the adsorption to protein aggregates onto the oil/water interface. In this paper, the role of large sedimentable particles could be critical to improve the freeze-thaw stability of the emulsions. The Pickering steric stabilization would be supported by the low magnitude of surface charge

of complexes (Fig 3a). The species present in the sedimentable fraction, which contribute with the formation of interfacial film of high viscoelasticity (Fig. 5b,c), could also adsorb during the emulsification, improving the freeze-thaw stability of the WS_n, W_nS and W_dS emulsions respect to those prepared with W_n and W_d alone. In contrast, for WS_demulsions, the limited adsorption of large particles could have a negative impact on their freeze-thaw stability. Thus, the improvement of stability of WS_n, W_nS and W_dS emulsions to freeze-thawing would be directly associated to a combination of enhanced emulsification ability of complexes and their structures at the oil/water interface.

4. Conclusions

The present study has demonstrated that the improvement of the freeze-thaw stability of acid emulsions prepared with complexes assembled from whey protein isolate and soluble soybean polysaccharides would be ascribed to the adsorption of large particles during the emulsification, in agreement with the formation of an interfacial film of high viscoelasticity. The steric Pickering stabilization would be supported by the low net charge of complexes at pH 3.0. The behavior was clearly evident for the complexes obtained by assembly of unheated proteins and by the addition of polysaccharides to previously heated whey proteins. Conversely, the nanoparticles obtained when both biopolymers were heated together evidenced a low stability to freeze-thawing due to a limited adsorption of large particles at the oil/water interface. The internal organization of both biopolymers within the complexes, which was evidenced by measurements of surface hydrophobicity and turbidity in the presence of protein-perturbing agents, would play a key role to explain the differences in the adsorption behavior of both complexes obtained from heated dispersions. Although the complexation between protein and polysaccharides is a valid strategy to improve the stability

of emulsions against environmental stresses, such as the freeze-thawing, the impact of the assembly procedure of the nanoparticles cannot be dismissed. The results of this article might have important implications in the preparation of highly acidic emulsion-based products (such as sauces and beverages) resistant to environmental stresses, such as the freeze-thaw treatments.

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Figure captions

Figure 1: Schematic representation of obtaining of aqueous dispersions prepared from whey protein isolate (WPI, 2.0% w/w), soluble soybean polysaccharides (SSPS, 0.5% w/w) and their mixtures. The assembly of complexes was performed by pH adjustment to 3.0 without or with heating.The sample nomenclature was defined according to the order of SSPS addition respect to pH adjustment and heating. The visual appearance of dispersions is also showed.

Figure 2: a) Turbidity profiles during acid titration (1.0 M HCl solution) of whey protein isolate (WPI), soluble soybean polysaccharides (SSPS) and WPI/SSPS mixture; b) Visual appearances of WPI, SSPS and WPI/SSPS dispersions after acid titration at pH 3.0. Biopolymer dispersions (2.0% and 0.5% w/w for WPI and SSPS, respectively) were previously diluted 1:3 v/v with distilled water before HCl addition.

Figure 3: a) ζ-potential, b) non-sedimentable protein content (% w/w) and c) hydrodynamic, z-average diameters (D_Z) of unheated and heated (90 ºC, 15 min) WPI (2.0%) or WPI/SSPS (2.0/0.5 % w/w) dispersions (pH 3.0). The non-sedimentable nanoparticles were obtained by centrifugation (Section 2.4, Eq. 1). The sample nomenclature was defined in Fig. 1. Values are means of three replicates (n=3) and error bars indicate standard deviation. For ζ-potential and non-sedimentable protein content, the mean values with different lowercase letters at the top of bars indicate significant difference ($p < 0.05$). For D_Z values, the mean values (n=3) with different lowercase letters at the top of bars, indicate significant differences between different samples ($p< 0.05$). The mean values with different uppercase letters indicate significant differences between D_Z values in the total and non-sedimentable fraction ($p < 0.05$).

Figure 4: a) Aromatic surface hydrophobicity values (H_0) and b) Effect of various perturbing agents on the turbidity of aqueous dispersions (pH 3.0) prepared with WPI (2.0% w/w) or WPI/SSPS (2.0/0.5% w/w). For turbidity assays, the dispersions were diluted (1:3 v/v) with distilled water, urea 6.0 M solution or urea 6.0 M/dithiothreitol (DTT) 30 mM solution. The sample nomenclature

was defined in Fig. 1. Values are means of three replicates (n=3) and error bars indicate standard deviation. For H_0, the mean values with different lowercase letters at the top of bars indicate significant differences ($p < 0.05$). For turbidity, the mean values with different lowercase letters at the top of bars, indicate significant differences between different samples ($p < 0.05$). The mean values with different uppercase letters indicate significant differences between turbidity values measured with different perturbing agents ($p < 0.05$).

Figure 5: Evolvement of interfacial complex viscosity (η^*) at the oil/water interface against time for the total (a, b) and non-sedimentable fraction (c) of unheated and heated WPI (2.0% w/w) or WPI/SSPS dispersions (pH 3.0). The non-sedimentable nanoparticles were obtained by centrifugation (Section 2.4, Eq. 1). The sample nomenclature was defined in Fig. 1.

Figure 6: De Brouckere volume-weighted mean diameter ($D_{4,3}$) and 90th volume percentile ($D_{v,0.9}$) of initial and freeze-thawed emulsions (-18 ± 2 °C) prepared with different aqueous dispersions (pH 3.0) of WPI (2.0% w/w) and WPI/SSPS mixtures (2.0/0.5% w/w). Both parameters were obtained from particle size distributions measured in the presence 1.0% w/v SDS solution at pH 3.0. The sample nomenclature was defined in Fig.1. Values are means of three replicates (n=3) and error bars indicate standard deviation. The mean values with different lowercase letters at the top of bars indicate significant differences ($p < 0.05$) between different emulsions. The mean values with different uppercase letters indicate significant differences between $D_{4,3}$ and $D_{v0,9}$ values before and after freeze-thawing.

Table 1. Interfacial pressure (π_i) values at the oil/water interface for dispersions prepared with mixtures of whey protein isolates (WPI) and soluble soybean polysaccharides (SSPS). The sample nomenclature was defined in Fig 1.

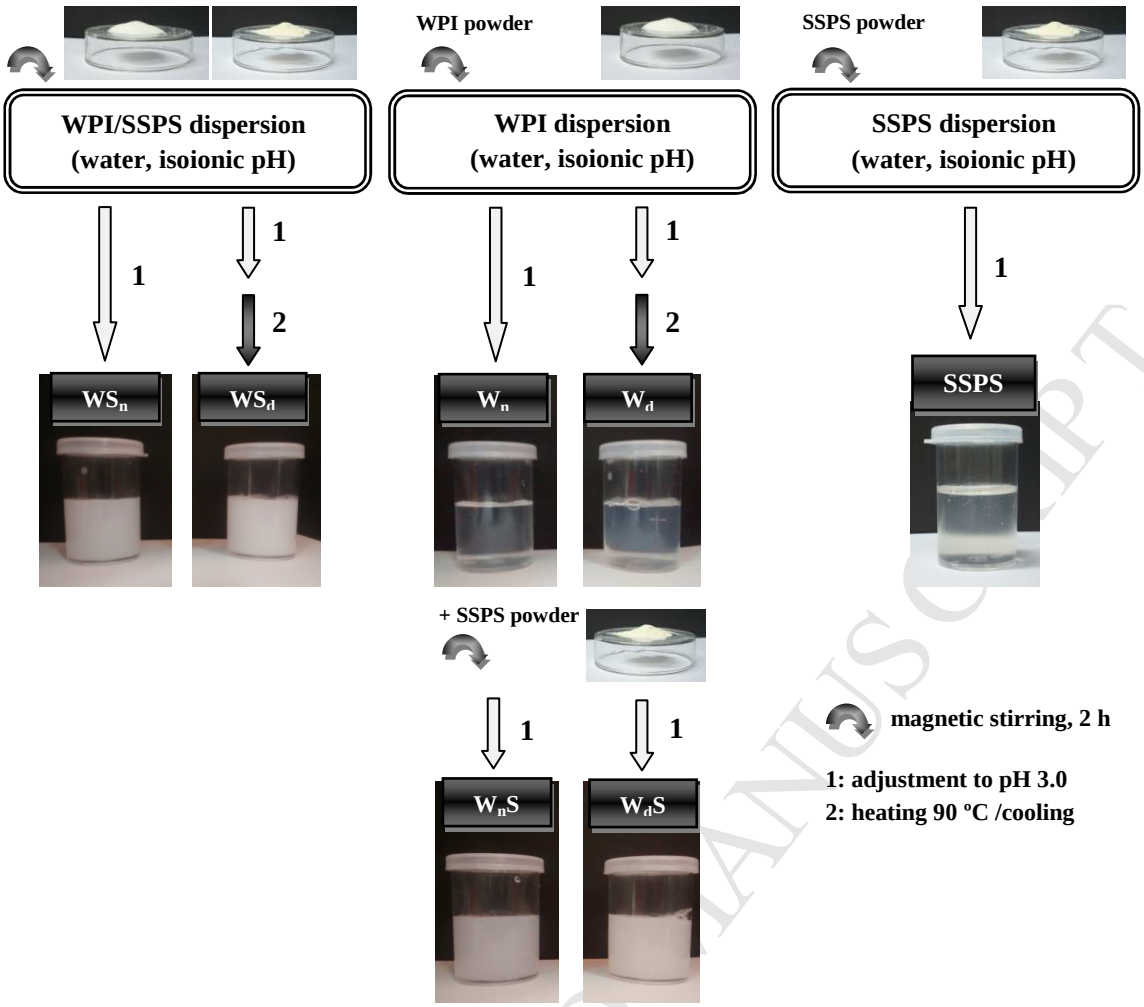
Aqueous dispersions	π_i (mN/m)
W_n	11.58 ± 0.06^{c}
WS_n	12.39 ± 0.06^{a}
W_nS	12.27 ± 0.07^{a}
W_d	10.88 ± 0.10^{d}
WS_d	11.76 ± 0.09^{b}
W_dS	11.84 ± 0.08^{b}

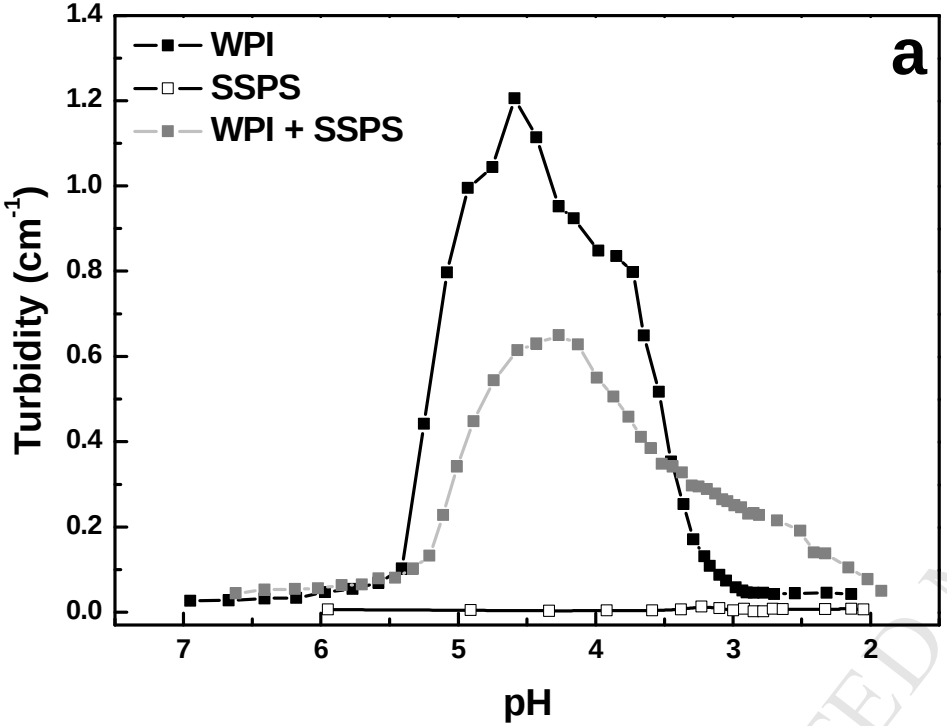
Mean values (*n*=3) with different lowercase letters indicate significant differences between different aqueous dispersions, as determined by Fisher's test ($p<0.05$)

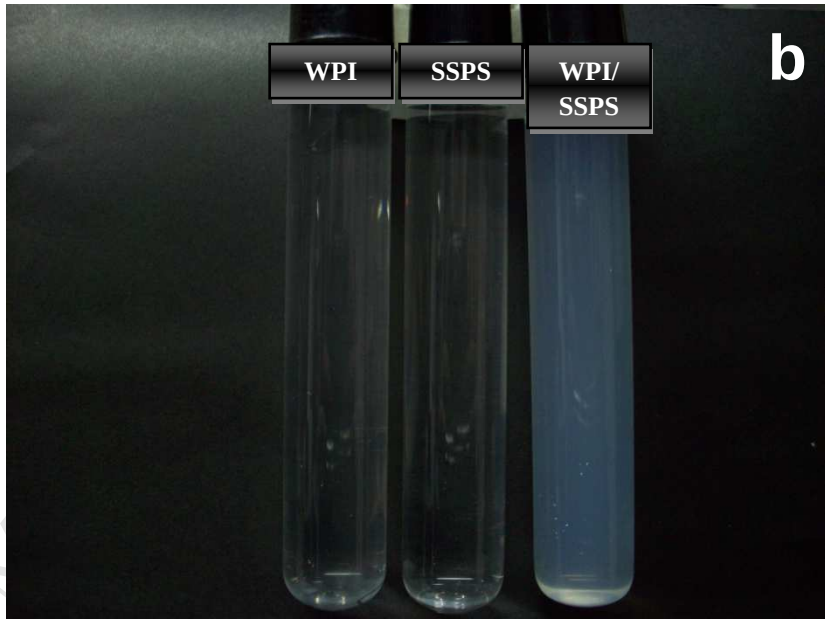
Table 2: Destabilization parameters (flocculation and coalescence indices, FI and CI, respectively) of initial and freeze-thawed o/w emulsions prepared with different aqueous dispersions of WPI (2.0% w/w) and WPI/SSPS mixtures (2.0/0.5% w/w). Sample nomenclature was defined in Fig.1.

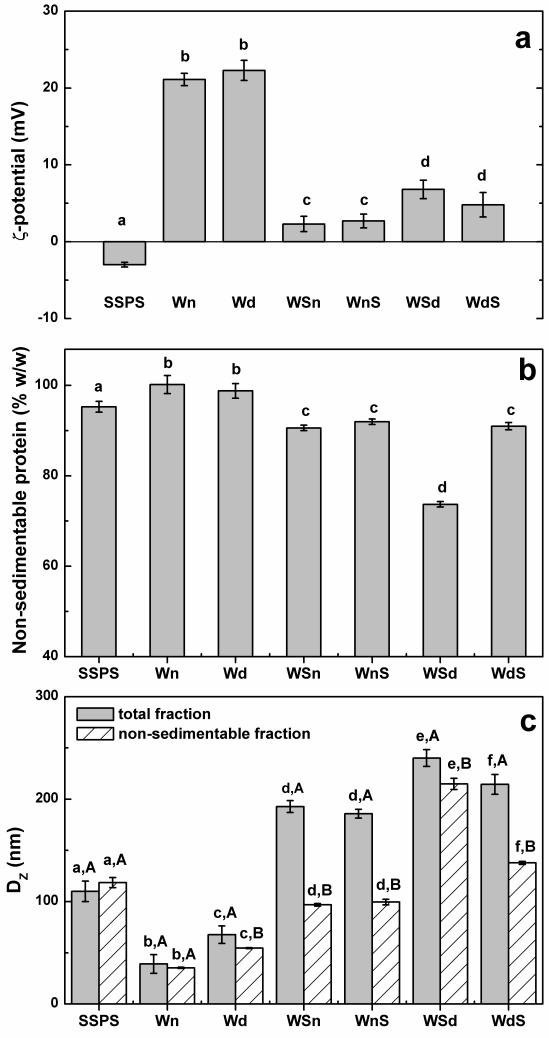
Emulsions	FI (%)		CI (%)
	Initial	After freeze-thawing	After freeze-thawing
W_n	0.8 ± 0.1^{b}	36.2 ± 4.0^{c}	34.9 ± 3.9^{c}
WS_n	2.5 ± 0.1^{a}	19.3 ± 2.0^{d}	13.5 ± 1.9^{d}
W_nS	0.5 ± 0.1^{b}	7.1 ± 0.9^{e}	16.2 ± 2.0^{d}
W_d	2.2 ± 0.2^{a}	51.0 ± 3.7^{b}	51.4 ± 6.0^{b}
WS_d	0.7 ± 0.1^{b}	154.2 ± 5.0^{a}	107.6 ± 3.5^{a}
W_dS	0.7 ± 0.1^{b}	15.2 ± 0.8^{d}	16.8 ± 0.3^{d}

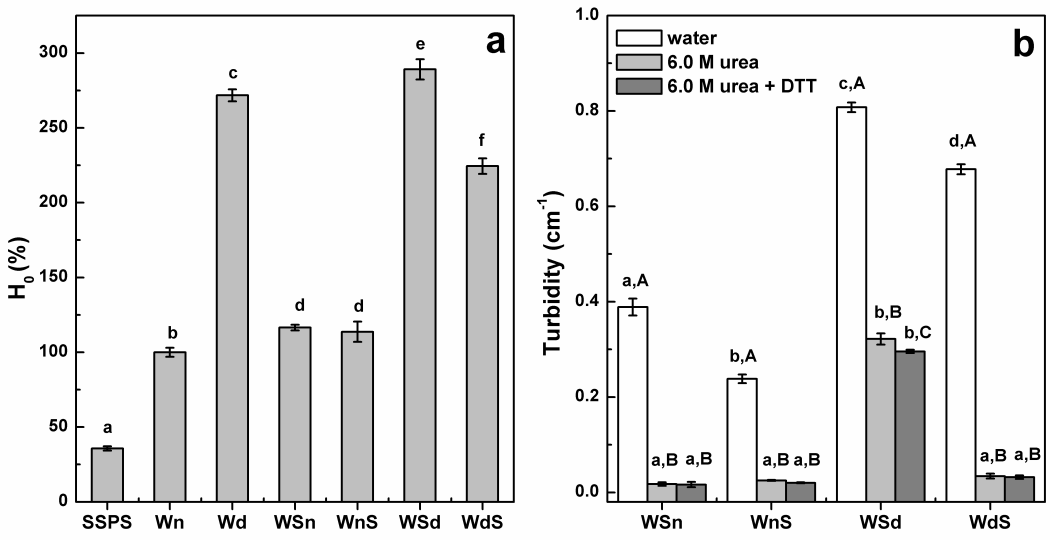
Mean values (n=3) with different lowercase letters within the same column indicate significant differences between different aqueous dispersions, as determined by Fisher's test ($p<0.05$).

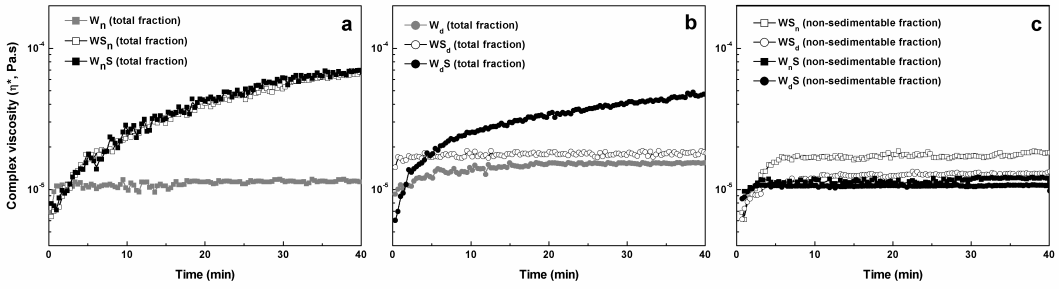


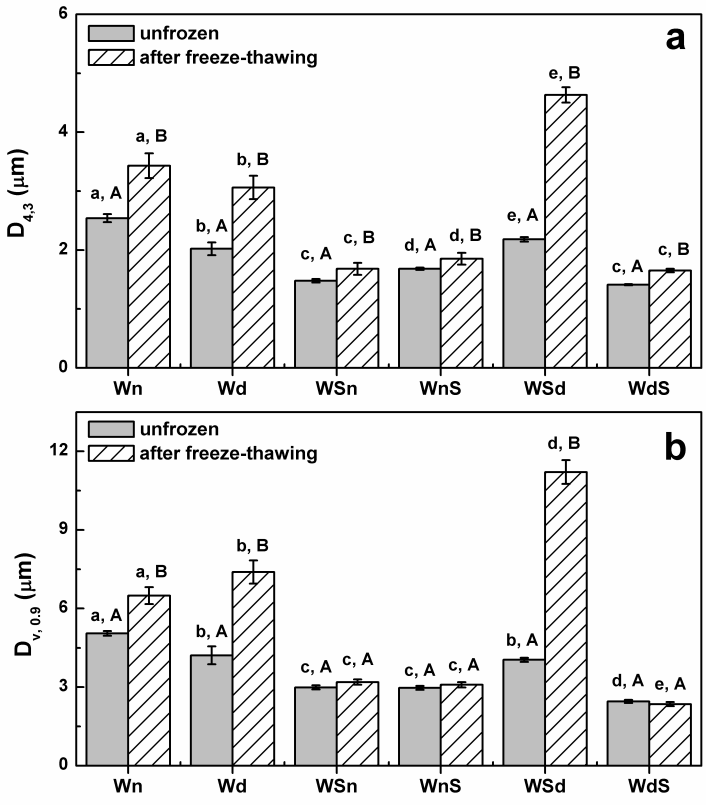












HIGHLIGHTS

WPI/SSPS nanoparticles were assembled at pH 3.0 using different strategies.

The structural and interfacial properties of complexes depended on their assembly procedure.

Freeze-thawing was evaluated on emulsions prepared with WPI/SSPS complexes.

The freeze-thaw stability of emulsions was affected by the assembly procedure of complexes.