Electrostatic complexation of β -lactoglobulin aggregates with κ -carrageenan and the resulting emulsifying and foaming properties

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ABSTRACT

The electrostatic complexation of protein and polysaccharide and the functional properties of the complexes are significantly affected by the structure of protein aggregates and are important in the development of new food ingredients. In this work, natural globular β -lactoglobulin (NGBLG), β -lactoglobulin nanoparticles (BLGNP), and β -lactoglobulin fibrils (BLGF) were prepared and complexed with κ -carrageenan (κ -car). Phase diagrams of the NGBLG–, BLGNP–, and BLGF– κ -car systems were established and divided into 4 regions: mixed soluble polymers (I), intramolecular soluble complex (II), intermolecular soluble complex (III), and intermolecular insoluble complex (IV). Aggregation shifted the boundaries of regions III and IV of BLGF– or BLGNP– κ -car to lower pH and higher protein aggregates/ κ -car weight ratio (r), especially for BLGF– κ -car. The emulsifying and foaming properties of the 3 mixed systems were investigated in regions I and II. Complexes in region II had significantly better emulsifying properties than the corresponding mixtures in region I and the pure protein aggregates. Interestingly, phase separation resulted in different effects on the foaming properties of the 3 BLG– κ -car complexes, in which BLGF– κ -car complexation in region II decreased the foaming properties in region I but the complexation of NGBLG– κ -car and BLGNP– κ -car in region II increased the foaming properties. The BLGF– κ -car complex in regions I and II provided the best emulsifying and foaming properties. Interfacial data both on oil–water and air–water interfaces overall explained the emulsifying and foaming properties of the complexes.

Key words: electrostatic complexation, phase diagram, interfacial property, β -lactoglobulin aggregate

INTRODUCTION

Natural globular β -LG (NGBLG) represents about 60% of the proteins in bovine whey and is one of the most important food ingredients because of its functional properties of emulsification, gelation, foaming, and bioactive delivery (Livney, 2010; Nicolai et al., 2011). Natural globular β -LG in aqueous solution can form protein aggregates with specific sizes and shapes after heating under certain pH, including fibril, spherical particle, strand, fractal cluster, among others (Nicolai et al., 2011; Nicolai and Durand, 2013). Among these aggregates, fibrils and spherical particles have attracted much interest (Nicolai et al., 2011; Nicolai and Durand, 2013). β -Lactoglobulin fibrils (BLGF) are often prepared by heating BLG at 75 to 120°C and pH 2.0 to 3.5 (van der Linden and Venema, 2007; Adamcik and Mezzenga, 2012; Cao and Mezzenga, 2019). The fibrillation process of BLG is reported to include (1) partial unfolding and hydrolysis of the monomeric BLG, (2) protofilament formation via cross- β -sheet assembly of the hydrolyzed peptide, and (3) formation of mature fibrils via assembly and twisting of the multi-stranded protofilaments (Adamcik and Mezzenga, 2012; Cao and Mezzenga, 2019). β -Lactoglobulin nanoparticles (BLGNP) can be obtained by heating BLG of relatively low concentration (≤ 40 g/L) in a narrow pH range (4.6–4.8, 5.8–6.2) both above and below the isoelectric point (Schmitt et al., 2009; Nicolai and Durand, 2013).

Like other Pickering stabilizers, BLG aggregates can stabilize foams and emulsions via irreversible adsorption to the interface (Dickinson, 2012; Lam et al., 2014). β -Lactoglobulin fibrils with well-characterized structures are reported to be effective in the stabilization of emulsion and foam (Jung et al., 2010; Serfert et al., 2014; Gao et al., 2017; Peng et al., 2017; Hu et al., 2019), whereas the interfacial stabilization effect of pure BLGNP is not as successful as that of BLGF (Nicolai and Durand, 2013; Hu et al., 2019). Interfacial stabilization by BLGF is explained by the high storage

Received March 2, 2020.

Accepted May 19, 2020.

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modulus at the oil–water or air–water interface due to formation of the fibril network (Jung et al., 2010; Gao et al., 2017; Hu et al., 2019).

Protein and polysaccharide are 2 key food ingredients, and their electrostatic complexation has been widely used in the design and control of food texture (Krzeminski et al., 2014; Wu et al., 2014), the micro-encapsulation of active ingredients (Jain et al., 2015; Zhao et al., 2018), and the purification of protein or polysaccharide (Kong et al., 2015; Ren et al., 2019). Because of their superior amphiphilicity, protein–polysaccharide complexes exhibit excellent interfacial activity and are expected to be effective emulsifiers or foaming agents (Schmitt and Turgeon, 2011). Like proteins, protein aggregates can complex with polysaccharides. However, few studies have shown the complexation of protein aggregates with polysaccharide and the effects on functional properties (Jones et al., 2011; Khemissi et al., 2018; Wang et al., 2019). The structure of protein aggregates should significantly affect electrostatic complexation of protein–polysaccharide and the functional properties of the complex, which is important in the development of new food ingredients.

In previous works, an intramolecular soluble protein–polysaccharide complex was found to efficiently stabilize emulsions and protect PUFA (Li et al., 2012a, 2013; Yao et al., 2016). In a previous study, we systematically compared the air–water and oil–water interfacial properties of NGBLG, BLGF, and BLGNP, and found that their foaming and emulsifying properties were in the order of BLGF > NGBLG > BLGNP (Hu et al., 2019). In this work, we prepared NGBLG, BLGNP, and BLGF and complexed each with the polysaccharide κ -carrageenan (κ -car). To our knowledge, this is the first report to investigate the effect of BLG aggregation structure on the phase diagram of complexation of BLG aggregates with κ -car. Focusing on the phase regions of the mixed soluble polymers and the intramolecular soluble complex, we investigated the emulsifying and foaming properties of NGBLG–, BLGNP–, and BLGF– κ -car systems.

MATERIALS AND METHODS

Materials

Natural globular β -LG of high purity was extracted from raw milk by heating at 40°C for 1 h to remove lipids, precipitating casein at pH 4.6, precipitating α -albumin at pH 3.7, dialyzing against water at 4°C for 72 h, and freeze-drying (Aschaffenburg and Drewry, 1957; Hu et al., 2019). κ -Carrageenan was supplied by FMC BioPolymer (Gelcarin GP-911NF, Philadelphia,

PA) and was converted into sodium type using ion exchange resin (Amberlite IR-120, Sigma Chemical Co., St. Louis, MO; Cao et al., 2015). All other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and were of analytical grade. Milli-Q water (Millipore, Billerica, MA) was used for sample preparation and experiments.

Preparation of β -LG Aggregates and Stock Solutions

β -Lactoglobulin nanoparticles and BLGF were prepared according to our previous work (Hu et al., 2019). Briefly, BLGNP was prepared by heating NGBLG solution at pH 5.8 and 85°C for 15 min, cooling in ice water for 20 min, dialyzing against pH 5.8 water at 4°C for 72 h [molecular weight cut-off (MWCO) = 50 kDa], and freeze-drying. β -Lactoglobulin fibrils were prepared by heating NGBLG at pH 2.0 and 80°C for 16 h, cooling in ice water for 20 min, dialyzing against pH 2.0 water at 4°C for 72 h (MWCO = 100 kDa), and freeze-drying. Transmission electron microscopy showed the particle size of NGBLG and BLGNP to be around 6 and 180 nm, respectively; the lengths of the BLGF were approximately 500 to 700 nm (Hu et al., 2019). Solutions of NGBLG, BLGNP, BLGF, and κ -car (0.2 wt%) were prepared by dispersing the respective powders in sterile Milli-Q water. After being fully dissolved, the solutions were passed through polyethersulfone filters (0.45- μ m pore size) to remove insoluble aggregates. The concentrations of the filtered solutions were determined by heating at 60°C with evacuation until a constant weight was achieved. The filtered solutions were diluted with Milli-Q water to obtain stock solutions of NGBLG, BLGNP, BLGF, and κ -car of 0.1% (wt/wt). Sodium azide (NaN_3 ; 0.005 wt%) was added to suppress micro-organism growth. The stock solutions were used for the following experiments except for emulsification.

Zeta-Potential Measurement of Protein Aggregate–Carrageenan Mixtures

The zeta-potentials of 3 pure protein aggregates (NGBLG, BLGNP, BLGF) and aggregate– κ -car mixtures as a function of pH were measured using a Zetasizer Nano-ZS apparatus (Malvern Instruments, Malvern, UK) with an MPT-2 pH autotitrator, as reported previously (Zhao et al., 2018). Protein aggregate– κ -car mixtures at varying ratios (r , wt/wt)—0.1, 0.5, 1.0, 2.0, 4.0, 10.0 and 20.0—with a total biopolymer concentration of 0.1% (wt/wt) were prepared. The pH of the above mixtures were decreased from 7.0 to 2.0 at a pH intervals of 0.4 units by automatic titration of NaOH (0.25 M) and HCl (0.25 and 2.5 M).

Construction of Phase Diagram of Protein Aggregate–Carrageenan Mixtures

The pH–composition phase diagrams of protein aggregate– κ -car mixtures were constructed as reported previously (Li et al., 2012b). Protein aggregate– κ -car mixtures at varying r values (0.1, 0.5, 1.0, 2.0, 4.0, 10.0, and 20.0, wt/wt) at a total concentration of 0.1 wt% were prepared and adjusted to pH 8.0 using NaOH (2.0 M) and HCl (2.0 M). Specifically, each aggregate– κ -car solution (10 g in triplicate) was mixed vigorously with glucono- δ -lactone powder (0.05–0.5 wt/wt) to initialize acidification and immediately subjected to in situ measurement of pH, light scattering, and turbidity. The change of pH over time during glucono- δ -lactone-induced acidification was monitored using an Orion 4 Star multifunctional pH meter (Thermo Electron Corp., Waltham, MA) at 25°C. Light scattering measurements (I_{173} and apparent R_h) at a refractive index of 1.41 were performed on a Zetasizer Nano-ZS apparatus (Malvern Instruments) equipped with a 4-mV laser at a scattering angle of 173° (I_{173}), where apparent R_h is the apparent hydrodynamic radius. Turbidity (τ) was measured at 500 nm using a UV-Vis spectrophotometer (TU-1900, Beijing Puyan General Instrument Co. Ltd., Beijing, China). The pH–time curve of each aggregate– κ -car solution was correlated with the time dependence of light scattering and turbidity measurements to obtain the structure transition information of the complexes with pH.

Preparation and Characterization of Protein Aggregate–Carrageenan Emulsions

Preparation. According to the phase diagram, pH and composition values in the region of intramolecular soluble complex were selected to prepare and characterize emulsions; that is, complex solutions with $r = 0.01, 0.1, 0.5$ (wt/wt) at pH = 7.0 and $r = 0.01, 0.05, 0.1, 0.2, 0.5$ (wt/wt) at pH = 4.0. Solutions of NGBLG, BLGNP, BLGF, and κ -car (1.875% wt/wt) were prepared by dispersing powders in sterile Milli-Q water. After dissolution, filtration, concentration determination, and dilution as described in the section Preparation of β -LG Aggregates and Stock Solutions, 1.5% (wt/wt) stock solutions of NGBLG, BLGNP, BLGF, and κ -car were prepared. Medium-chain triglyceride (MCT; 15% wt/wt) was used as the oil phase, and NaN_3 (0.005% wt/wt) was added to prevent microorganism growth. Emulsification was achieved in one simple homogenization step using a high-speed Polytron PT-MR2100 shear mixer (Kinematica Co., Lausanne, Switzerland) at 26,000 rpm for 2 min in an ice bath. The emulsions

were adjusted to pH 7.0 and 4.0 with HCl (1.0 M) and NaOH (1.0 M) and stored at $25 \pm 2^\circ\text{C}$ for 90 d.

Characterization. Emulsions were characterized by particle size, zeta-potential, and microscopic morphology (Li et al., 2012b; Gao et al., 2017; Zhao et al., 2017). The particle size of emulsions was measured on a Mastersizer 2000 laser particle size analyzer (Malvern Instruments) with a refractive index of 1.52 and represented by volume-weighted diameter (d_{43}). Zeta-potentials of emulsions were measured by a Zetasizer Nano-ZS apparatus (Malvern Instruments). Emulsion morphology was observed using an optical microscope (BT-1600, Bettersize Instruments Ltd., Dandong, China). Particle size and zeta-potential were measured in triplicate at $25 \pm 1^\circ\text{C}$.

Foaming Properties of Protein Aggregate–Carrageenan Mixtures

Using the 0.1% stock solutions, protein aggregate– κ -car solutions with a total biopolymer concentration of 0.1% and $r = 0.1, 0.2, 0.3, 0.4$, and 0.5 (wt/wt) were prepared and adjusted to pH 7.0 and pH 4.0 using 1.0 M HCl and NaOH. The foaming properties of the aggregate– κ -car solutions were investigated using the Krüss dynamic foam analyzer DFA100 (Krüss GmbH, Hamburg, Germany) with a porous glass frit (FL 4504, pores of 16–40 μm , Duran/DWK Life Sciences, Milville, NJ), which measures foam capacity, foam stability, and foam structure (Hu et al., 2019). The experiments were carried out at $25 \pm 2^\circ\text{C}$, and repeated 3 times to obtain average values.

Interfacial Adsorption Measurements of Protein Aggregate–Carrageenan Mixtures

The interfacial adsorption behavior of protein aggregate– κ -car mixture was measured on an automatic pendant drop tensiometer (Tracker, Teclis, Longessaigne, France), and changes in surface pressure (π) and surface dilatational modulus (E) under sinusoidal oscillation mode were analyzed during the adsorption of aggregate– κ -car onto the air–water or oil–water interface (Gao et al., 2017; Hu et al., 2019). To measure the adsorption behavior on the air–water and oil–water interfaces, one U-shaped sample needle was immersed in 5 mL of protein aggregate– κ -car solution in the sample cell and the volume of the air or oil droplet was set at about 10 μL . The deformation amplitude and oscillation frequency were 10% (within the linear range) and 0.05 Hz, respectively. Protein aggregate– κ -car mixtures were diluted to 0.1% (wt/wt) for air–water interface and 0.15% (wt/wt) for oil–water

interface, respectively, and adjusted to pH 7.0 and pH 4.0 for interface analysis. At $25 \pm 0.1^\circ\text{C}$, the density of air, MCT, and samples were 0.001185 g/cm^3 , 0.945064 g/cm^3 , and $\sim 1.001 \text{ g/cm}^3$, respectively. The interfacial tensions of air–water and MCT–water were 72.0 ± 0.1 and $24.7 \pm 0.1 \text{ mN/m}$, respectively.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft Corp., Redmond, WA) were used for statistical analysis. The significance of difference was determined using one-way ANOVA followed by the least significant difference (LSD) test ($n = 3$).

RESULTS AND DISCUSSION

Electrostatic Complexation and Phase Separation of BLG Aggregates with κ -Car

The zeta-potential and isoelectric point (pI) of NGBLG-, BLGNP-, and BLGF- κ -car mixtures are shown in Figure 1. The polysaccharide (κ -car) carried a negative charge throughout the pH range and its zeta-potential value was around -50 mV . The pI values of NGBLG, BLGNP, and BLGF were 4.6, 4.7, and 4.9, respectively (Hu et al., 2019), and the zeta-potential values of these protein aggregates were closely related to the pH of the aqueous solution. When the pH is greater than the protein pI, protein molecules appear negatively charged; when the pH is less than the pI, the protein is positively charged. The zeta-potential value of each protein aggregate- κ -car mixture was between that of the pure protein aggregate and that of κ -car and increased with increasing r (i.e., higher protein content). The maximum stoichiometry of NGBLG-, BLGNP-, and BLGF- κ -car could not be calculated because of the unavailable pI of NGBLG-, BLGNP-, or BLGF- κ -car mixtures at $r = 2, 1, 0.5$, and 0.1 .

During complexation induced by acidification, first the intramolecular soluble complex is formed, followed by the intermolecular soluble complex and intermolecular insoluble complex by aggregation (Weinbreck et al., 2003; Li et al., 2012b). The size of the intramolecular complex, intermolecular soluble complex, and intermolecular insoluble complex increased with decreasing pH, which was monitored by scattered light intensity I_{173} and turbidity τ . The scattered light intensity I_{173} measures the Rayleigh scattering of molecules or particles that are much smaller than the wavelength of the incident light, so I_{173} can be used to indicate the size change of macromolecules, intramolecular soluble complexes, and intermolecular soluble complexes during acidification (Mekhloufi et al., 2005; Li et al., 2012b). The I_{173} value

Figure 1. Zeta-potential (ζ) as a function of pH for mixtures with varying ratios (r). (A) Natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), (B) β -LG nanoparticles (BLGNP)- κ -car, (C) β -LG fibrils (BLGF)- κ -car. The variable r (0.1 to 20) represents the mass ratio of protein aggregate to κ -car (wt/wt).

of an intramolecular soluble complex is almost constant during acidification, whereas that of an intermolecular soluble complex increases during further acidification, indicating an increase in size. Turbidity is caused by the loss of transmitted light due to light scattering of particles of any diameter; it is more sensitive to larger

particles and can be used to represent the size change from an intermolecular soluble complex to an intermolecular insoluble complex (Mekhloufi et al., 2005; Li et al., 2012b).

Three characteristic pH values (pI , pH_c , and pH_ϕ) are used to characterize protein-polysaccharide complexation induced by acidification. The pI s of protein aggregates are closely related to the formation of intramolecular soluble complexes based on the positive charge of protein aggregates and the negative charge of anionic polysaccharide (Li et al., 2012b). The critical pH value (pH_c) is traditionally defined as the onset of the increase in scattered light intensity and is generally considered to be associated with formation of intermolecular soluble complexes (Weinbreck et al., 2003; Mekhloufi et al., 2005; Li et al., 2012b). The pH_ϕ is defined as the maximum of scattered light intensity, which sometimes coincides with the onset of the increase in turbidity, which is associated with the formation of insoluble complexes, causing phase separation (Kaibara et al., 2000; Li et al., 2012b). For example, in our study, $pH_c = 5.2$ and $pH_\phi = 4.5$ for the NGBLG- κ -car complex at $r = 4$ (Supplemental Figure S1; <https://doi.org/10.3168/jds.2020-18457>). It should be noted that the values of I_{173} , τ , and apparent R_h were affected by the rate of decrease in pH. Thus, strictly speaking, these values observed at different rates of decreasing pH should be extrapolated to a decreasing rate of zero, but it was difficult to change this rate systematically. Therefore, some uncertainties among different research groups should be taken into account when these data are compared.

Supplemental Figures S1 to S3 (<https://doi.org/10.3168/jds.2020-18457>) show changes in I_{173} , τ , and

apparent R_h as a function of pH for aggregate- κ -car mixtures at different ratios. Based on pI , pH_c , and pH_ϕ derived from Supplemental Figures S1 to S3, the phase diagrams of aggregate- κ -car systems were built in a pH-composition ordinate and each phase diagram was divided into 4 regions (Figure 2A). These represent (I) regions of mixed soluble polymers ($pH > pI$ at small r value and $pH > pH_c$ at large r value), (II) intramolecular soluble complex ($pH < pI$ at small r value), (III) intermolecular soluble complex ($pH_c > pH > pH_\phi$), and (IV) intermolecular insoluble complex ($pH < pH_\phi$), which agreed with our previous work (Li et al., 2012b). Figure 2B shows a proposed model of the structural transition of NGBLG- κ -car during complexation. The models for BLGNP- κ -car and BLGF- κ -car are not provided in this work, because the particle sizes of BLGNP and BLGF were larger than the chain length of κ -car. More work will be done to characterize the aggregate- κ -car complexes in the future.

Comparing the boundary of the phase diagrams of the 3 mixed systems, it can be seen that the protein aggregation state significantly influenced the formation of intermolecular soluble complexes (III) and intermolecular insoluble complexes (IV), although it had little effect on the phase region of intramolecular soluble complexes (II). Compared with NGBLG- κ -car, the phase boundaries of the regions III and IV of BLGF- κ -car and BLGNP- κ -car shifted to lower pH and higher r values, and were most pronounced in BLGF- κ -car. For example, when $r = 2$, pH_c of BLGF- κ -car, BLGNP- κ -car, and NGBLG- κ -car were 5.4, 5.2, and 5.0, respectively, whereas the pH_ϕ were 4.9, 4.3, and 4.2, respectively. There are two possible explanations for this. (1) To form complexes of the same size with

Figure 2. (A) Phase diagrams of natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP)- κ -car, and β -LG fibrils (BLGF)- κ -car systems in a pH-composition ordinate. pI is the isoelectric point of NGBLG, BLGNP, or BLGF; pH_c (solid symbols) is the starting pH for the formation of soluble complexes during acidification, and pH_ϕ (open symbols) is the starting pH for the formation of insoluble complexes during acidification. Lines $\cdots$, $---$, and $- \cdot - \cdot -$ represent the border lines of NGBLG, BLGNP, and BLGF, respectively. (B) Proposed model of the structural transition of NGBLG- κ -car during complexation. The black coils stand for κ -car and the blue spheres for NGBLG. pI = the isoelectric point of NGBLG (or BLGNP or BLGF); pH_c = the starting pH for the formation of soluble complexes during acidification, and pH_ϕ = the starting pH for the formation of insoluble complexes during acidification.

κ -car via electrostatic complexation, protein aggregates with a larger particle size require fewer charge neutralizations and fewer molecules of κ -car. Also, it is easiest for BLGF- κ -car complexes to form a network structure because of the large length:diameter ratio and specific surface area of BLGF. That is why the pH values (pH_c and pH_p) for BLGF- κ -car to generate the intermolecular soluble complexes and intermolecular insoluble complexes were much higher. (2) The order of hydrophobicity is BLGF > BLGNP > NGBLG (Li et al., 2012b); thus, the hydrophobic interaction involved in complex formation was BLGF- κ -car > BLGNP- κ -car > NGBLG- κ -car.

Emulsifying Properties of Complex of BLG Aggregates with κ -Car

As the intermolecular soluble complex in region III was unstable and the intermolecular insoluble complex in region IV was always in the state of precipitation (Li et al., 2012b), this work focused on the mixtures in region I (the mixed soluble polymers, pH 7.0, $r = 0.01$ –0.5) and region II (the intramolecular soluble complex, pH 4.0, $r = 0.01$ –0.5) based on the phase diagram (Figure 2). At pH 7.0, the emulsification activity and emulsion stability of the NGBLG-, BLGNP-, and BLGF- κ -car systems increased with increasing r

Figure 3. Changes in emulsion particle size (volume-weighted diameter; d_{43}) of different ratios of natural globular β -LG (NGBLG; panels A and D) to κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP; panels B and E) to κ -car, and β -LG fibrils (BLGF; panels C and F) to κ -car during ambient storage at pH = 7.0 (A, B, C) and pH 4.0 (D, E, and F). Emulsion particle sizes were expressed as mean $\pm$ SD.

values (i.e., protein content; Figure 3A-C). The reason for this might be that protein and polysaccharide did not complex in region I and protein played a role in emulsification. In addition, emulsification activity and the emulsion stability of BLGF- κ -car were significantly higher than those of BLGNP- κ -car and NGBLG- κ -car at $r = 0.1$ and 0.5 . This is consistent with the previous results (Li et al., 2012b).

At pH 4.0, emulsions of NGBLG-, BLGNP-, and BLGF- κ -car systems were stable at most r values, but their emulsification activities were greatly affected by r values (Figure 3D-F). The best emulsification activities (about $4\ \mu\text{m}$ of particle size of fresh emulsions) for NGBLG-, BLGNP-, and BLGF- κ -car were at $r = 0.1$, 0.2 , and 0.05 , respectively, indicating that the electrostatic complexation of a small amount of BLGF and κ -car could provide the best synergistic effect. The reason for this might be that the interaction between BLGF and κ -car was strongest due to the largest values of the length:diameter ratio, zeta-potential, and surface hydrophobicity of BLGF at pH 4.0 (Li et al., 2012b), and the BLGF- κ -car complex could form a firm adsorption layer on the MCT-water interface.

The fresh emulsion particle sizes of NGBLG, BLGNP, and BLGF at pH 4.0 are reported to be about 10, 40, and $10\ \mu\text{m}$, respectively (Li et al., 2012b). Compared with the mixed soluble polymers (pH 7.0) and the pure protein aggregates, the intramolecular soluble complexes (pH 4.0) had better emulsification activity and emulsion stability, indicating that intramolecular soluble complexation greatly improved the emulsification properties of pure protein aggregates. This agreed with previous reports (Li et al., 2012a; Xiang et al., 2015).

We also characterized the appearance, microstructure, and zeta-potential of the emulsions. Supplemental Figures S4 to S9 (<https://doi.org/10.3168/jds.2020-18457>) show the emulsion appearance and microstructure of the NGBLG-, BLGNP-, and BLGF- κ -car systems during ambient storage at pH 4.0 and pH 7.0 for 90 d; the results are consistent with Figure 3. Supplemental Figure S10 (<https://doi.org/10.3168/jds.2020-18457>) characterizes changes in zeta-potential values at pH 4.0 and pH 7.0. For the 3 protein- κ -car systems with different r values, we observed no significant differences in zeta-potential changes at pH 7.0 or 4.0, indicating

Figure 4. Time evolution of surface pressure (π) (A: pH 7.0; C: pH 4.0) and dilatational modulus (E) as a function of surface pressure (π) (B: pH 7.0; D: pH 4.0) for natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP)- κ -car, and β -LG fibrils (BLGF)- κ -car mixtures during their adsorption to the air-water interface. The total concentration of protein/polysaccharide mixture was 0.15% by weight, with optimal ratio r at the corresponding pH; that is, $r = 0.5$ for all 3 mixtures at pH 7.0, $r = 0.1$, 0.2 , and 0.05 for NGBLG (or BLGNP or BLGF)/ κ -car at pH 4.0, respectively.

Figure 5. The foam half-life (A: pH 7.0; B: pH 4.0) and foam capacity (C: pH 7.0; D: pH 4.0) of natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP)- κ -car, and β -LG fibrils (BLGF)- κ -car mixtures with different ratios (r). Bars within each group with different letters (a, b) are significantly different at $P < 0.05$. Values of foam half-life and foam capacity were expressed as mean $\pm$ SD.

that zeta-potential is not the main factor for emulsion stability in this work.

The adsorption property and dilatational rheology of NGBLG-, BLGNP-, and BLGF- κ -car systems at the oil-water interface are shown in Figure 4. The orders of surface pressure (π) and dilatational modulus (E) of the 3 systems at pH 7.0 and pH 4.0 were BLGF- κ -car > NGBLG- κ -car $\geq$ BLGNP- κ -car in both cases, indicating that BLGF- κ -car is easier to adsorb and stabilize the oil-water interface, which agreed with the best emulsification activity and emulsion stability of BLGF- κ -car (Figures 2 and 3). Comparing the interfacial properties of the 3 mixed systems at pH 7.0 and pH 4.0, we found that the complexing state significantly affected the interfacial viscoelasticity of BLGF- κ -car ($E = 40$ mN/m at pH 7.0 and $E = 55$ mN/m at pH 4.0), whereas intramolecular soluble complexation almost had no effect on the interfacial viscoelasticity of NGBLG- κ -car and BLGNP- κ -car (~ 22 mN/m at pH 7.0 and pH 4.0). This indicates that BLGF- κ -car in

the intramolecular soluble complex state co-adsorbed to the interface and improved the interfacial properties, whereas the intramolecular soluble complexation of the other 2 mixed systems had no such effect. This difference may be related to the high length:diameter ratio and high hydrophobicity of BLGF.

Foaming Properties of Complex of BLG Aggregates with κ -Car

Similar to the emulsifying properties, we focused on foaming properties of mixtures in region I (pH 7.0, $r = 0.1$ –0.5) and region II (pH 4.0, $r = 0.1$ –0.5). The foam half-life and foam capacity of NGBLG-, BLGNP-, and BLGF- κ -car mixtures are shown in Figure 5. With an increase in r value, the foam half-life of the 3 mixtures at pH 4.0 and pH 7.0 increased, indicating that protein content determined foam stability to a considerable extent. The overall order of foam half-life of NGBLG-, BLGNP-, and BLGF- κ -car mixtures at both pH values

was $\text{BLGF-}\kappa\text{-car} > \text{NGBLG-}\kappa\text{-car} > \text{BLGNP-}\kappa\text{-car}$, indicating that foam stability of $\text{BLGF-}\kappa\text{-car}$ was better because of the anisotropy of BLGF and the interfacial network of $\text{BLGF-}\kappa\text{-car}$ mixture. Overall, the foam stability of $\text{BLGF-}\kappa\text{-car}$ was greater at pH 7.0 than at pH 4.0, whereas the foam stability of $\text{NGLGB-}\kappa\text{-}$

car and $\text{BLGNP-}\kappa\text{-car}$ was greater at pH 4.0 than at pH 7.0. This suggests that the complex state caused by acidification was not a key factor in foam stability, which is different from the emulsion stability of the protein-polysaccharide complex as discussed previously. The foam capacities of the 3 mixtures at both

Figure 6. Time evolution of the surface pressure (π) (A, B, C) and dilatational modulus (E) as a function of surface pressure (π) (D, E, F) for natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP)- κ -car, and β -LG fibrils (BLGF)- κ -car mixtures during their adsorption to the air-water interface at pH 7.0. The total concentration of protein/polysaccharide mixture was 0.1% by weight.

pH values were all greater than 1.0, except for samples with $r = 0.1$ at pH 4.0 (Figure 5C and D). The changes in foam microstructure of the 3 mixed systems at pH 7.0 and pH 4.0 (Figure S11; <https://doi.org/10.3168/jds.2020-18457>) were consistent overall with the results of foam stability (Figure 5A and B).

The foam stability of the protein-polysaccharide complexing system might be determined by several factors, such as protein content, protein hydrophobicity, and network structure of the complex, among others. The foam half-life values of 0.1% BLGF at pH 7.0 and 4.0 are reported to be about 7,000 and 9,000 s, respectively (Li et al., 2012b), which are much higher than the foam half-life values of BLGF- κ -car at either pH value in this work. This might be caused by the low

protein content and the addition of κ -car. The electrostatic complexation of BLGF with κ -car decreased the hydrophobicity and the foaming properties of BLGF because of the high hydrophilicity of κ -car. The phase separation induced by the electrostatic complexation of egg white and hydroxypropyl methylcellulose is reported to reduce foaming properties of egg white at pH 3 (van den Berg et al., 2015), whereas the network structure and aggregate particles formed at pH 3 and 5 by electrostatic complexation of lentil legumin-like protein and pectin are reported to improve foam stability (Jarpa-Parra et al., 2016). Therefore, existing research on the foam stability mechanism of the protein-polysaccharide complexes is limited and somewhat conflicting. To our knowledge, no research has

Figure 7. Time evolution of the surface pressure (π) (A, B, C) and dilatational modulus (E) as a function of surface pressure (π) (D, E, F) for natural globular β -LG (NGBLG)- κ -carrageenan (κ -car), β -LG nanoparticles (BLGNP)- κ -car, and β -LG fibrils (BLGF)- κ -car mixtures during their adsorption to the air-water interface at pH 4.0. The total concentration of protein/polysaccharide mixture was 0.1% by weight.

studied the effect of protein aggregate–polysaccharide complexation on foam properties. More work should be done to systematically study effects of particle size, morphology, and concentration of protein aggregates on the foaming properties of protein aggregate–polysaccharide systems.

The adsorption properties and dilatational rheology of NGBLG–, BLGNP–, and BLGF– κ -car at the air–water interface are shown in Figures 6 and 7. At pH 7.0, surface pressure (π) of the 3 mixed systems did not change but dilatational modulus (E) increased with increasing r values. The π and E values of BLGF– κ -car were significantly higher than those of NGBLG– κ -car and BLGNP– κ -car (Figure 6). Comparing data in Figure 5A and Figure 6, the interfacial properties of NGBLG–, BLGNP–, and BLGF– κ -car with different r values at pH 7.0 could be related to the foam stability results. At pH 4.0, changes in E as a function of π were complicated; in particular, BLGF– κ -car and BLGNP– κ -car at several r values showed a trend to first increase and then decrease (Figure 7). Hydroxypropyl methylcellulose and a complex of whey protein concentrate–sodium alginate on the air–water interface are reported to exhibit a similar trend in E and π measured with a pendant drop tensiometer, which might be due to collapse of the adsorbed interfacial monolayer when the surface pressure overcame the critical surface pressure (Pérez et al., 2008, 2009). Therefore, it is not suitable to use the largest E values of the mixed system to represent foam stability. This is why BLGF– κ -car at pH 4.0 had the largest E value but did not have better foam stability than NGBLG– κ -car. More research should be done to confirm the collapse of the interfacial monolayers.

CONCLUSIONS

The aim of this study was to investigate the effect of aggregation structures of BLG on the phase diagram of BLG aggregate– κ -car mixtures and the effect of phase separation on emulsifying and foaming properties. We demonstrated that aggregation had little effect on regions of mixed soluble polymers (I) and intramolecular soluble complex (II), but shifted the boundaries of regions of the intermolecular soluble complex (III) and the intermolecular insoluble complex (IV) of BLGF– and BLGNP– κ -car to lower pH and higher r values, especially for BLGF– κ -car. We compared the emulsifying and foaming properties of the 3 mixed systems in regions I and II. Three complexes in region II had significantly better emulsifying properties than the mixtures in region I, whereas phase separation had different effects on the foaming properties of the 3 BLG aggregate– κ -car complexes, in which complexation in

region II decreased the foaming properties of BLGF– κ -car in region I but increased that of NGBLG– and BLGNP– κ -car. Among the 3 complexes, BLGF– κ -car in regions I and II provided the best emulsifying and foaming properties.

ACKNOWLEDGMENTS

The research was supported by National Natural Science Foundation of China (31671811, 31972203) and International Cooperation Project of Hubei Provincial Department of Science and Technology (DWHZ2017000010). The authors have no conflicts of interest to declare.

REFERENCES

- Adamcik, J., and R. Mezzenga. 2012. Proteins fibrils from a polymer physics perspective. *Macromolecules* 45:1137–1150. <https://doi.org/10.1021/ma202157h>.
- Aschaffenburg, R., and J. Drewry. 1957. Improved method for the preparation of crystalline β -lactoglobulin and α -lactalbumin from cow's milk. *Biochem. J.* 65:273–277. <https://doi.org/10.1042/bj0650273>.
- Cao, Y., and R. Mezzenga. 2019. Food protein amyloid fibrils: Origin, structure, formation, characterization, applications and health implications. *Adv. Colloid. Interface Sci.* 269:334–356. <https://doi.org/10.1016/j.cis.2019.05.002>.
- Cao, Y., L. Wang, K. Zhang, Y. Fang, K. Nishinari, and G. O. Phillips. 2015. Mapping the complex phase behaviors of aqueous mixtures of κ -carrageenan and type B gelatin. *J. Phys. Chem. B* 119:9982–9992. <https://doi.org/10.1021/acs.jpcc.5b05002>.
- Dickinson, E. 2012. Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends Food Sci. Technol.* 24:4–12. <https://doi.org/10.1016/j.tifs.2011.09.006>.
- Gao, Z., J. Zhao, Y. Huang, X. Yao, K. Zhang, Y. Fang, K. Nishinari, G. O. Phillips, F. Jiang, and H. Yang. 2017. Edible Pickering emulsion stabilized by protein fibrils. Part 1: Effects of pH and fibrils concentration. *Lebensm. Wiss. Technol.* 76:1–8. <https://doi.org/10.1016/j.lwt.2016.10.038>.
- Hu, J., J. Yang, Y. Xu, K. Zhang, K. Nishinari, G. O. Phillips, and Y. Fang. 2019. Comparative study on foaming and emulsifying properties of different beta-lactoglobulin aggregates. *Food Funct.* 10:5922–5930. <https://doi.org/10.1039/C9FO00940J>.
- Jain, A., D. Thakur, G. Ghoshal, O. P. Katar, and U. S. Shivhare. 2015. Microencapsulation by complex coacervation using whey protein isolates and gum acacia: an approach to preserve the functionality and controlled release of β -carotene. *Food Bioprocess Technol.* 8:1635–1644. <https://doi.org/10.1007/s11947-015-1521-0>.
- Jarpa-Parra, M., Z. Tian, F. Temelli, H. Zeng, and L. Chen. 2016. Understanding the stability mechanisms of lentil legumin-like protein and polysaccharide foams. *Food Hydrocoll.* 61:903–913. <https://doi.org/10.1016/j.foodhyd.2016.07.017>.
- Jones, O. G., S. Handschin, J. Adamcik, L. Harnau, S. Bolisetti, and R. Mezzenga. 2011. Complexation of β -lactoglobulin fibrils and sulfated polysaccharides. *Biomacromolecules* 12:3056–3065. <https://doi.org/10.1021/bm200686r>.
- Jung, J. M., D. Z. Gunes, and R. Mezzenga. 2010. Interfacial activity and interfacial shear rheology of native β -lactoglobulin monomers and their heat-induced fibers. *Langmuir* 26:15366–15375. <https://doi.org/10.1021/la102721m>.
- Kaibara, K., T. Okazaki, H. B. Bohidar, and P. L. Dubin. 2000. pH-Induced coacervation in complexes of bovine serum albumin and cationic polyelectrolytes. *Biomacromolecules* 1:100–107. <https://doi.org/10.1021/bm990006k>.

- Khemissi, H., H. Bassani, A. Aschi, I. Capron, L. Benyahia, and T. Nicolai. 2018. Exploiting complex formation between polysaccharides and protein microgels to influence particle stabilization of W/W emulsions. *Langmuir* 34:11806–11813. <https://doi.org/10.1021/acs.langmuir.8b02383>.
- Kong, X., L. Kong, Y. Ying, Y. Hua, and L. Wang. 2015. Recovering proteins from potato juice by complexation with natural polyelectrolytes. *Int. J. Food Sci. Technol.* 50:2160–2167. <https://doi.org/10.1111/ijfs.12851>.
- Krzeminski, A., K. A. Prell, M. Busch-Stockfisch, J. Weiss, and J. Hinrichs. 2014. Whey protein–pectin complexes as new texturising elements in fat-reduced yoghurt systems. *Int. Dairy J.* 36:118–127. <https://doi.org/10.1016/j.idairyj.2014.01.018>.
- Lam, S., K. P. Velikov, and O. D. Velev. 2014. Pickering stabilization of foams and emulsions with particles of biological origin. *Curr. Opin. Colloid Interface Sci.* 19:490–500. <https://doi.org/10.1016/j.cocis.2014.07.003>.
- Li, X., Y. Fang, S. Al-Assaf, G. O. Phillips, and F. Jiang. 2012a. Complexation of bovine serum albumin and sugar beet pectin: Stabilising oil-in-water emulsions. *J. Colloid Interface Sci.* 388:103–111. <https://doi.org/10.1016/j.jcis.2012.08.018>.
- Li, X., Y. Fang, S. Al-Assaf, G. O. Phillips, X. Yao, Y. Zhang, M. Zhao, K. Zhang, and F. Jiang. 2012b. Complexation of bovine serum albumin and sugar beet pectin: Structural transitions and phase diagram. *Langmuir* 28:10164–10176. <https://doi.org/10.1021/la302063u>.
- Li, X., Y. Fang, G. O. Phillips, and S. Al-Assaf. 2013. Improved sugar beet pectin-stabilized emulsions through complexation with sodium caseinate. *J. Agric. Food Chem.* 61:1388–1396. <https://doi.org/10.1021/jf304658q>.
- Livney, Y. D. 2010. Milk proteins as vehicles for bioactives. *Curr. Opin. Colloid Interface Sci.* 15:73–83. <https://doi.org/10.1016/j.cocis.2009.11.002>.
- Mekhloufi, G., C. Sanchez, D. Renard, S. Guillemin, and J. Hardy. 2005. pH-Induced structural transitions during complexation and coacervation of β -lactoglobulin and acacia gum. *Langmuir* 21:386–394. <https://doi.org/10.1021/la0486786>.
- Nicolai, T., M. Britten, and C. Schmitt. 2011. β -Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocoll.* 25:1945–1962. <https://doi.org/10.1016/j.foodhyd.2011.02.006>.
- Nicolai, T., and D. Durand. 2013. Controlled food protein aggregation for new functionality. *Curr. Opin. Colloid Interface Sci.* 18:249–256. <https://doi.org/10.1016/j.cocis.2013.03.001>.
- Peng, D., J. Yang, J. Li, C. Tang, and B. Li. 2017. Foams stabilized by β -lactoglobulin amyloid fibrils: Effect of pH. *J. Agric. Food Chem.* 65:10658–10665. <https://doi.org/10.1021/acs.jafc.7b03669>.
- Perez, A. A., C. R. Carrara, C. C. Sánchez, L. G. Santiago, and J. M. Rodríguez Patino. 2009. Interfacial dynamic properties of whey protein concentrate/polysaccharide mixtures at neutral pH. *Food Hydrocoll.* 23:1253–1262. <https://doi.org/10.1016/j.foodhyd.2008.08.013>.
- Pérez, O. E., C. C. Sánchez, A. M. R. Pilosof, and J. M. Rodríguez Patino. 2008. Dynamics of adsorption of hydroxypropyl methylcellulose at the air–water interface. *Food Hydrocoll.* 22:387–402. <https://doi.org/10.1016/j.foodhyd.2006.12.005>.
- Ren, J. N., Y. Hou, G. Fan, L. Zhang, X. Li, K. Yin, and S. Pan. 2019. Extraction of orange pectin based on the interaction between sodium caseinate and pectin. *Food Chem.* 283:265–274. <https://doi.org/10.1016/j.foodchem.2019.01.046>.
- Schmitt, C., C. Bovay, A.-M. Vuillomenet, M. Rouvet, L. Bovetto, R. Barbar, and C. Sanchez. 2009. Multiscale characterization of individualized β -lactoglobulin microgels formed upon heat treatment under narrow pH range conditions. *Langmuir* 25:7899–7909. <https://doi.org/10.1021/la900501n>.
- Schmitt, C., and S. L. Turgeon. 2011. Protein/polysaccharide complexes and coacervates in food systems. *Adv. Colloid Interface Sci.* 167:63–70. <https://doi.org/10.1016/j.cis.2010.10.001>.
- Serfert, Y., C. Lamprecht, C. P. Tan, J. K. Keppler, E. Appel, F. J. Rossiermiranda, K. Schroen, R. M. Boom, S. Gorb, C. Selhuberun- kel, S. Drusch, and K. Schwarz. 2014. Characterisation and use of β -lactoglobulin fibrils for microencapsulation of lipophilic ingredients and oxidative stability thereof. *J. Food Eng.* 143:53–61. <https://doi.org/10.1016/j.jfoodeng.2014.06.026>.
- van den Berg, M., F. L. Jara, and A. M. R. Pilosof. 2015. Performance of egg white and hydroxypropylmethylcellulose mixtures on gelation and foaming. *Food Hydrocoll.* 48:282–291. <https://doi.org/10.1016/j.foodhyd.2015.03.001>.
- van der Linden, E., and P. Venema. 2007. Self-assembly and aggregation of proteins. *Curr. Opin. Colloid Interface Sci.* 12:158–165. <https://doi.org/10.1016/j.cocis.2007.07.010>.
- Wang, X., Y. Nian, Z. Zhang, Q. Chen, X. Zeng, and B. Hu. 2019. High internal phase emulsions stabilized with amyloid fibrils and their polysaccharide complexes for encapsulation and protection of β -carotene. *Colloid. Surf. B Biointerfaces* 183:110459. <https://doi.org/10.1016/j.colsurfb.2019.110459>.
- Weinbreck, F., R. De Vries, P. Schrooyen, and C. G. De Kruif. 2003. Complex coacervation of whey proteins and gum arabic. *Biomacromolecules* 4:293–303. <https://doi.org/10.1021/bm025667n>.
- Wu, B. C., B. Degner, and D. J. McClements. 2014. Soft matter strategies for controlling food texture: formation of hydrogel particles by biopolymer complex coacervation. *J. Phys. Condens. Matter* 26:464104. <https://doi.org/10.1088/0953-8984/26/46/464104>.
- Xiang, J., F. Liu, R. Fan, and Y. Gao. 2015. Physicochemical stability of citral emulsions stabilized by milk proteins (lactoferrin, α -lactalbumin, β -lactoglobulin) and beet pectin. *Colloid. Surf. A* 487:104–112. <https://doi.org/10.1016/j.colsurfa.2015.09.033>.
- Yao, X., S. Xiang, K. Nie, Z. Gao, W. Zhang, Y. Fang, K. Nishinari, G. O. Phillips, and F. Jiang. 2016. Whey protein isolate/gum arabic intramolecular soluble complexes improving the physical and oxidative stabilities of conjugated linoleic acid emulsions. *RSC Advances* 6:14635–14642. <https://doi.org/10.1039/C5RA26040J>.
- Zhao, M., N. Cui, F. Qu, X. Huang, and Y. Yang. 2017. Novel nano-particulated exopolysaccharide produced by *Klebsiella* sp. PHRC1.001. *Carbohydr. Polym.* 171:252–258. <https://doi.org/10.1016/j.carbpol.2017.05.015>.
- Zhao, M., Y. Wang, X. Huang, M. Gaenzle, Z. Wu, K. Nishinari, N. Yang, and Y. Fang. 2018. Ambient storage of microencapsulated *Lactobacillus plantarum* ST-III by complex coacervation of type-A gelatin and gum arabic. *Food Funct.* 9:1000–1008. <https://doi.org/10.1039/C7FO01802A>.

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