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Note

Stability and structure of protein–polysaccharide coacervates in the presence of protein aggregates

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Abstract

We have studied at pH 4.2 and three protein (Pr):polysaccharide (Pol) weight ratios (8:1, 2:1 and 1:1) the structure and stability of β-lactoglobulin/acacia gum/water dispersions containing protein aggregates (BLG/AG/W) or free from aggregates (AF-BLG/AG/W). Phase diagrams were characteristic of complex coacervation. BLG/AG/W dispersions displayed a larger biphasic area than AF-BLG/AG/W dispersions, that moved towards the protein axis. It was concluded that protein aggregates affected complex coacervation both by entropic (size and molecular masses of aggregates) and enthalpic (surface properties of aggregates) effects. Laser light scattering measurements revealed that the particles diameter (d_{43}) induced by demixing was controlled by protein aggregates in AF-BLG/AG/W dispersions. At 1 wt.% biopolymer concentration, particles were 15–20 times larger in AF-BLG/AG/W dispersions than in BLG/AG/W dispersions at (Pr:Pol) ratios of 2:1 or 1:1. Confocal scanning laser microscopy showed that AF-BLG/AG/W dispersions only contained spherical coacervates. BLG/AG/W dispersions contained both coacervates and aggregates coated with AG or/and BLG/AG coacervates. At a (Pr:Pol) ratio of 2:1 and 1:1, coacervates were vesicular or multivesicular. Coacervates were smaller in BLG/AG/W dispersions than in AF-BLG/AG/W dispersions. It was concluded that protein aggregates have the intrinsic ability to stabilize complex coacervates and could be used to design multifunctional delivery systems. This study showed that composite dispersions containing both protein aggregates embedded in protein–polysaccharide coacervates and free coacervates may be performed. In this respect, the design of protein aggregates with controlled size distribution and surface properties could open new possibilities both in the non-chemical control of coacervates stability and in the development of multifunctional delivery systems.

Keywords: Complex coacervation; β-lactoglobulin; Acacia gum; Microencapsulation; Light scattering; Confocal scanning laser microscopy

The area of multifunctional systems becomes increasingly important in the field of microencapsulation and controlled drug delivery. In addition, the use of biopolymers to create these multifunctional systems may be of great interest to encap-

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sulate hydrophilic molecules. In this respect, complex coacervation between biopolymers appear as an appropriate microencapsulation method (Bungenberg de Jong, 1949; Luzzi, 1970; Thies, 1982; Burgess, 1994; Schmitt et al., 1998). Complex coacervation refers to a liquid–liquid phase separation induced by electrostatic interactions between oppositely charged macromolecules. During demixing liquid droplets are produced, called coacervates, that are able to encapsulate molecules less polar than the solvent. Usually, complex coacervation is used to encapsulate lipid droplets containing active hydrophobic molecules. The major drawback of the technique is that complex coacervates are highly unstable and that toxic chemical agents, such as glutaraldehyde, are necessary to stabilize them. A major challenge to develop safe multifunctional delivery systems based on complex coacervation will be to find alternative routes to stabilize coacervates.

In the present study, we evaluated the ability of protein aggregates to generate potentially multifunctional dispersions by interactions with polysaccharides. More specifically, we compared in the conditions of complex coacervation at pH 4.2 the stability and structure of β-lactoglobulin/acacia gum/water dispersions free of β-lactoglobulin aggregates (AF-BLG/AG/W) with those containing aggregates (BLG/AG/W) using phase diagram determination, laser light scattering (LLS) and confocal scanning laser microscopy.

AG sample from *Acacia senegal* trees (lot 97J716) was a gift from CNI company (Rouen, France). Acid processed BLG (lot 818) was a gift from Lactalis Research Center (Retiers, France). The chemical composition of powders and physico-chemical properties of AG and BLG macromolecules were reported previously (Schmitt, 2000; Schmitt et al., 1999, 2000; Sanchez et al., in press). AG, BLG and AF-BLG aqueous stock dispersions at total biopolymer concentration comprised between 0.1 and 5 wt.% were prepared in deionized water (MilliQ, MilliPore, USA) by gentle mixing. AF-BLG refers to protein dispersions free of aggregates while BLG refers to dispersions containing process-induced aggregates (Schmitt et al., 2000). Stock dispersions were left overnight at 4 ± 1 °C to ensure good hydration of biopolymers. The pH of the resulting dispersions was adjusted to 4.2 using HCl or NaOH. BLG/AG or AF-BLG/AG dispersions at protein to polysaccharide weight ratios (Pr:Pol) of 1:1, 2:1 or 8:1 were obtained by gently mixing stock dispersions.

Ternary phase diagrams of AF-BLG/AG/Water and BLG/AG/Water systems were obtained by chemical analysis of the two separated phases obtained after two days. The binodal line was determined by the cloudy-point method. The point where demixing occurred was determined using spectrophotometry. Tie-lines of the different systems were determined on AF-BLG/AG/W and BLG/AG/W dispersions at 1:1 protein to polysaccharide weight ratio by analyzing water, protein and polysaccharide content in the equilibrium upper separated phase (Schmitt et al., 1999). The results were expressed in percentages and the ternary phase diagram was drawn using the Tcontour shareware (John Pilling, Michigan Technology University, MI). Ternary phase diagram of BLG/AG/W and AF-BLG/AG/W dispersions revealed both drop-shaped two-phase region located in the water rich corner and tie lines on each sides of the water corner bisector, which is the hallmark of complex coacervation or more generally associative phase separation (Fig. 1). The presence of aggregates induced the presence of a larger two-phase area that moved towards the protein axis. The BLG/AG/W dispersion was more unstable than the AF-BLG/AG/W dispersion. As a consequence, a higher total biopolymer concentration was needed to suppress coacervation in the former case (20 wt.%) than in the latter (4.5 wt.%). The decrease of the biphasic area in AF-BLG/AG/W could be ascribed to entropic effects since BLG aggregates are high molecular mass particles with polydisperse size distribution. However, the shift of the biphasic area in this case was caused by a difference in the strength of interactions between the protein and the polysaccharide (enthalpic effects). This hypothesis was based on the larger positive surface charge density of aggregated proteins demonstrated previously by electrophoretic mobility measurements (Schmitt et al., 1999, 2000). Another reason would be an increased interaction between the surface active AG and the hydrophobic protein aggregates.

The size distributions of BLG/AG/W and AF-BLG/AG/W particles were determined by LLS using a Mastersizer S granulometer equipped with a 5 mW He/Ne laser emitting at 632.8 nm (Malvern Instruments, Orsay, France). The optical system was composed by a 300 reverse Fourier lens covering a particle size ranging from 0.05 to 880 μm. The size distributions of particles were calculated on a volume basis after treatment of the scattered intensity data with a polydispersed model provided by the Malvern Mastersizer-S v2.17 software. The volume average diameter d_{43}, defined by $d_{43} = \Sigma_i^n n_i d_i^4 / \Sigma_i^n n_i d_i^3$, where n_i is the number of particles exhibiting a diameter d_i, was then calculated for each total biopolymer concentration. Since optical properties of the obtained particles were unknown, the determined diameters have to be considered as apparent diameters. All experiments were triplicated (5 assays per experiment). The average diameter (d_{43}) of particles was determined by LLS on 0.1–5 wt.% BLG/AG/W or AF-BLG/AG/W dispersions at three protein:polysaccharide (Pr:Pol) weight ratios (8:1, 2:1 and 1:1). Results revealed that d_{43} in BLG/AG/W dispersion was independent on the (Pr:Pol) weight ratio and total biopolymer concentration up to 1 wt.% concentration, with values around 10 μm (Fig. 2). Above 1 wt.% concentration, the d_{43} increased regularly as a function of concentration from 10 μm to 20–50 μm depending on the (Pr:Pol) ratio. Larger sizes were obtained for dispersions containing an excess of protein (Pr:Pol: 8:1). Knowing that higher protein concentrations induce the formation of larger aggregates, these results suggested that the particles size was controlled by the size of protein aggregates. A completely different picture was obtained for the AF-BLG/AG/W dispersions. The d_{43} sharply increased for the 2:1 and 1:1 ratios when the total biopolymer concentration was around 1 wt.%. The d_{43} values were 200 and 150 μm for the former and the latter, respectively, as compared to the 10 μm value found in BLG/AG/W dispersions. This could reveal an instability of the system caused by aggregation or coalescence of particles. For total biopolymer concentrations above 1 wt.%, the particles size was almost independent on the (Pr:Pol) ratio and total biopolymer concentration. The d_{43} values decreased and stabilised around 50 μm. The stabilisation of the size of particles by large biopolymer concentrations may be understood as the yield of coacervation suppression was approached. Besides the known effects of the (Pr:Pol) weight ratio and total biopolymer concentration on the stability of particles induced by complex coacervation, our results also suggested that stabilization of parti-

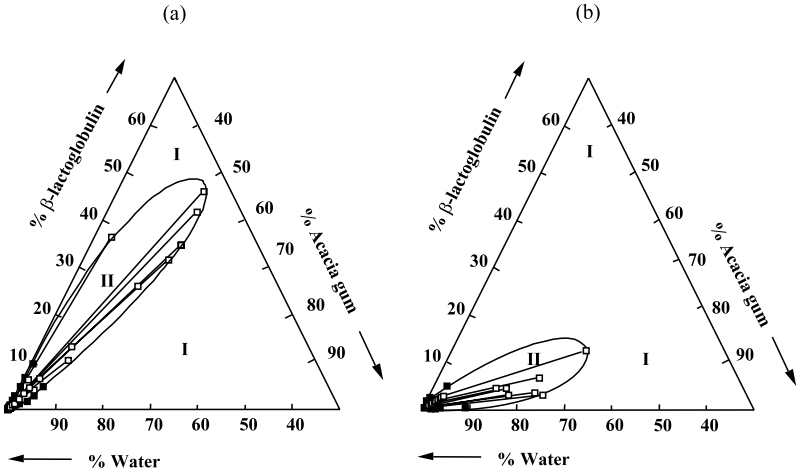

Fig. 1. Ternary phase diagrams of BLG/AG/W (a) and AF-BLG/AG/W (b) dispersions at pH 4.2. Numbers I and II refer to monophasic and biphasic regions, respectively, according to the cloudy-point method.

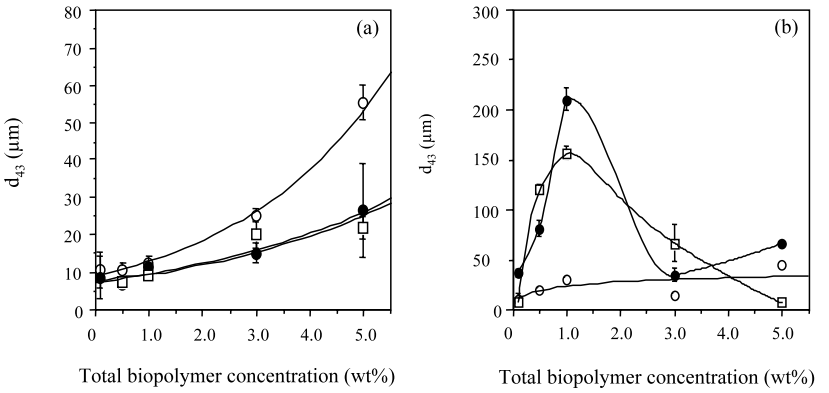

Fig. 2. Evolution of the particles diameter (d_{43}) determined by LLS as a function of total biopolymer concentration in BLG/AG/W (a) and AF-BLG/AG/W (b) dispersions at pH 4.2 and 8:1 (○), 2:1 (●) and 1:1 (□) protein to polysaccharide weight ratio.

cles could be achieved by use of protein aggregates of controlled size and surface properties.

The structure of BLG/AG/W mixtures at 1 wt.% total biopolymer concentration was determined by CSLM. The procedure for BLG and AG labelling, respectively by FITC and RITC, and the technical set-up were described previously (Schmitt et al., 2001). The focal plane of observation was about 30 μm from the inverted objective of the microscope. Pictures were processed ~ 160 s after mixing stock dispersions using the Laser Sharp MRC-1024 software version 3.2 (Bio-Rad, Germany). All experiments were duplicated using freshly prepared dispersions. CSLM was used in order to visualize microstructure of 1 wt.% dispersions at 8:1, 2:1 and 1:1 (Pr:Pol) weight ratios. Micrographs showed that AF-BLG/AG/W dispersions were made of spherical particles, the so-called coacervates, whose size depended on the (Pr:Pol) ratio. Apparent diameters of coacervates were below 5 μm for the dispersions at 8:1 ratio and between 1–25 μm for the dispersions at 2:1 and 1:1 ratios. These values were smaller than those determined from LLS, which may be caused by the necessary dilution of the dispersions at 1 wt.% concentration for LLS measurements. It is worth noting that a myriad of minute coacervates were present in all dispersions. Interestingly, vesicular and multivesicular coacervates were formed at 2:1 and 1:1 ratios by self-assembly between BLG and AG. Giant vesicles and microcompartmentation of coacervates were also observed by optical microscopy in the gelatin/acacia gum (Newton et al., 1977 Jégat and Taverdet, 2000), gelatin/pectin (McMullen et al., 1982), gelatin/gellan gum (Chilvers and Morris, 1987), gelatin/carboxymethylcellulose (Lévy and Andry, 1991), or gelatin/chitosan (Remuñan-López and Bodmeier, 1996) cases. The mechanism of vesicle formation is not known actually. However, considering that coacervates at 8:1 ratios were not vesicular, it may be supposed that charge neutralization of complexes could play a role in the formation of vesicles. More efficient charge neutralization can also be evoked to explain the strong instability of dispersions at a 2:1 ratio, instability that leads to extensive coalescence of coacervates. The microstructure of BLG/AG/W dispersions was characterized by the simultaneous presence of coacervates and heterogeneous aggregates. With an increasing protein content (8:1 ratio), aggregates became predominant. It was interesting to notice that exactly similar micrographs were obtained illuminating either the red or green channel, which was a proof that AG was also present at the aggregates surface. The size of coacervates in AF-BLG/AG/W dispersions at 2:1 and 1:1 (Pr:Pol) ratios were larger than in BLG/AG/W dispersions, demonstrating the stabilizing effect of protein aggregates. This stabilization of the particle size by 'insoluble' aggregates was not solely due to a decrease of the soluble protein content. The comparison of the particle diameters obtained for the 1 wt.% dispersion to those obtained for the 0.5 wt.% dispersion, and taking into

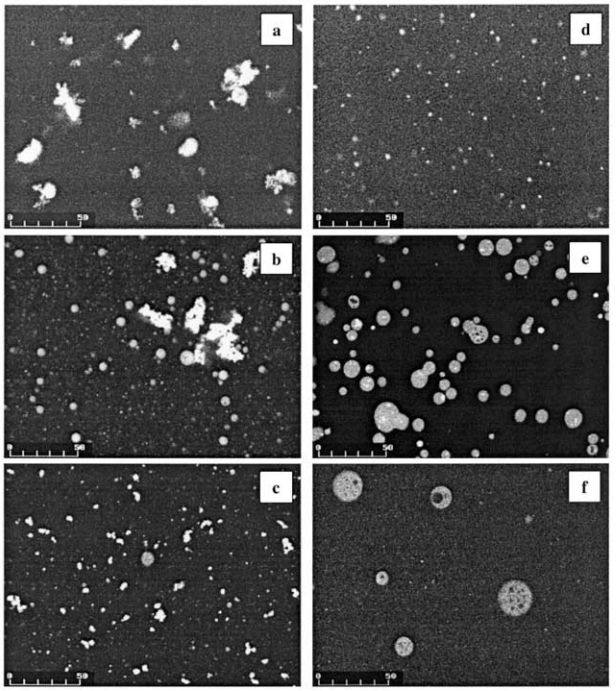

Fig. 3. Microstructures obtained by confocal scanning laser microscopy of BLG/AG/W (a–c) and AF-BLG/AG/W (d–f) dispersions at pH 4.2 and 1 wt.% total biopolymer concentration at 8:1 (a, d), 2:1 (b, e) and 1:1 (c, f) protein to polysaccharide weight ratio.

account the ~50% BLG insolubility at pH 4.2, showed that the values did not coincide with the (Pr:Pol) ratio (Fig. 3). Non-specific electrostatic or hydrophobic interactions between the surface active AG and BLG/AG coacervates (or hydrophobic BLG aggregates) could also play a role in the coacervate stabilisation (Torza and Mason, 1970; Snowden et al., 1987; Buchhammer et al., 1993; Gao and Dubin, 1999), either by modifying the effective (Pr:Pol) ratio in coacervates or by decreasing the number of coacervates available for coalescence.

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