Effect of the Cluster Size on the Micro Phase Separation in Mixtures of β-Lactoglobulin Clusters and κ-Carrageenan

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Received September 9, 2005; Revised Manuscript Received November 3, 2005

The phase separation of globular protein clusters formed by heat-denatured β-lactoglobulin (β-lg) in mixtures with the polysaccharide κ-carrageenan (κ-car) has been studied at pH 7 and 20 °C. The effect of the protein cluster size on the phase separation was investigated by preparing clusters with radii between 20 nm and 1 μm. The formation of protein rich microdomains led to an increase of the turbidity starting at a minimum κ-car concentration that decreased with increasing cluster size, but was only weakly dependent on the protein concentration. The size and number of microdomains do not depend much on the cluster size, but their density decreases with increasing cluster size leading to a lower turbidity.

Introduction

In the past, the phase behavior of a wide range of globular proteins and polysaccharides has been studied.[1−5] If the net charge of the proteins and the polysaccharides is opposite, complexes are formed that may lead to associative phase separation with the formation of a phase rich in both polysaccharides and proteins. If the polysaccharides are neutral or oppositely charged, segregative phase separation was observed with separate protein rich and polysaccharide rich phases. However, mixtures of anionic polysaccharides and proteins far above the isoelectric point (p*I*) are usually compatible over a wide range of concentrations.

In the latter case, phase separation is nevertheless observed if the globular proteins are associated into large clusters.[3,4,6−8] Aggregation of globular proteins often occurs after heat-induced denaturation and leads to gelation above a critical protein concentration (C_g).[9−12] The presence of polysaccharides modifies the structure of the heat-set protein gels profoundly even if the mixtures are fully compatible before heating.[13−16] In the heated mixtures, the gels consist of connected and partly fused dense protein domains of a few microns. The overall structure and the mechanical properties of the gels depend not only on the concentration of the ingredients and the ionic strength but also on the heat treatment.

The origin of the coarse structure of heat-set globular protein gels in the presence of polysaccharides is micro-phase separation of protein clusters into protein rich microdomains.[14,17,18] The latter assemble and form the more or less homogeneous skeleton of the gel. The sensitivity of the gel structure to the heating temperature is explained by the different temperature dependence of the rate of protein aggregation and the rate of phase separation. The first is determined by the activation energy of denaturation and increases strongly with increasing temperature.[19] The temperature dependence of the latter is not well-known but is expected to be much weaker.

Obviously, it is difficult to study the process of phase separation in detail during the formation of the aggregates. Therefore, a few studies have been done on mixtures of preformed stable protein aggregates and polysaccharides at room temperature.[3,4,6−8] Stable protein aggregates can be formed either by heating for times shorter than the gelation time or for concentrations lower than C_g. Phase separation was observed in mixtures containing protein clusters, whereas the corresponding mixtures with native proteins were compatible. Attempts have been made to explain the phase separation in terms of depletion of polysaccharides between neighboring clusters.[3,4,6,20] Microscopy has shown that spherical protein rich domains are formed that have a tendency to associate but not to merge fully unless under strong centrifugal force.[7]

Here we present a detailed study of mixtures of β-lactoglobulin (β-lg) aggregates and κ-carrageenan (κ-car) at pH 7. Both materials are important ingredients for the food industry. β-lg is a globular protein with radius 2 nm and molar mass 18.4 kg/mol and is the main protein component of whey.[21−23] Its iso-electric pH is 5.2, and at pH 7, it has a net negative charge of 7. When heated at pH 7, β-lg forms well-defined so-called preaggregates that consist of about 100 monomers and have an elongated shape with length 50 nm and width 10 nm.[12,24,25] The preaggregates associate further to form a polydisperse distribution of self-similar clusters characterized by a fractal dimension $d_f = 2.0$.[12,26,27] The average size of the clusters formed after extensive heat treatment increases with increasing concentration and eventually a gel is formed above C_g. The aggregates are stable to cooling and dilution.

κ-car is a negatively charged polysaccharide issued from algae. The sample used for this study is rather polydisperse and has a weight average molar mass of 400 kg/mol.[28] We have chosen the conditions (temperature, type, and concentration of counterions) such that κ-car always has a random coil configuration.[29,30]

In earlier work,[13,14] it was shown that the denaturation and aggregation of native β-lg is not influenced by the presence of κ-car. In addition, the structure of the aggregates was found to be the same in the absence or presence of κ-car. However, the growth rate of the aggregates and the gelation rate increased rapidly with increasing κ-car concentration. In mixtures of aggregates and κ-car at room temperature, the formation of micron-size protein rich domains was observed which were similar to the domains found in the heated mixtures.[7] The microdomains slowly sediment under gravity, and the analysis of the supernatant showed that larger clusters separate prefer-

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10.1021/bm050662m CCC: $33.50
Published on Web 12/16/2005

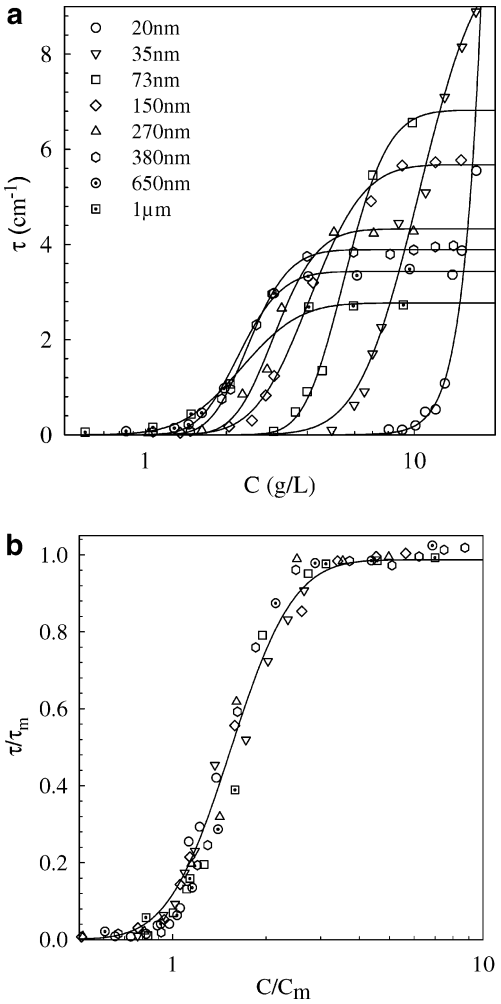

Figure 1. (a) Dependence of the turbidity on the κ-car concentration in mixtures with 2 g/L β-lg clusters of different sizes. The *z*-average radii of gyration of the clusters are indicated in the figure. The solid lines are guides to the eye. (b) Same data as in Figure 1a after normalization of the turbidity by its maximum value and the κ-car concentration by C_m. The solid line is a guide to the eye.

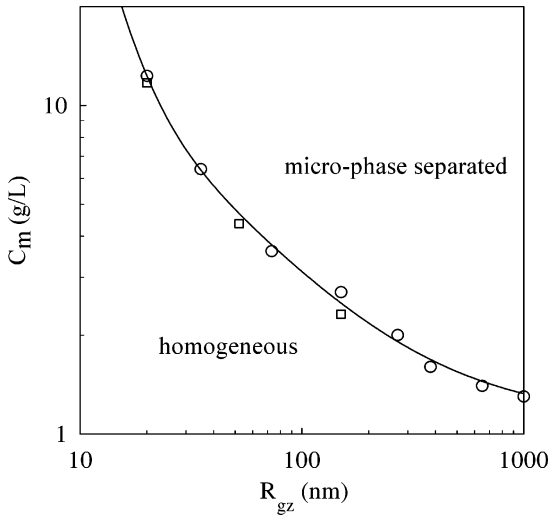

Figure 2. Dependence of the minimum κ-car concentration needed to induce micro-phase separation on the β-lg cluster size in mixtures with 2 g/L β-lg. The circles represent the results obtained for 0.1 M NaCl, whereas the squares represent the results in the absence of added NaCl.

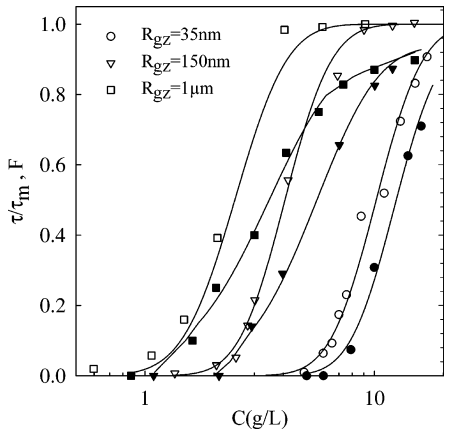

Figure 3. Comparison of the dependence on the κ-car concentration of the normalized turbidity (open symbols) and the fraction of phase separated protein clusters (closed symbols) in mixtures with 2 g/L β-lg clusters of different sizes indicated in the figure. The solid lines are guides to the eye.

entially. The objective of the present work was to study in detail the effect of the protein cluster size on the phase separation.

Materials and Methods

Materials. The β-lg used in this study was a gift from Lactalis (Laval, France) and contained about equal fractions of the variants A and B. We showed elsewhere that the aggregation rate is equal for the two variants in the mixture.[31] Solutions were extensively dialyzed against salt free Mili-Q water at pH 7 with 200 ppm NaN_3 added to avoid bacterial growth. The samples were filtered through 0.2 μm pore size Anotope filters, and the concentration was measured after filtration by UV absorption at 278 nm using extinction coefficient 0.96 L g^{-1} cm^{-1}.[32] β-lg clusters of different sizes were prepared by the heating of aqueous protein solutions at pH 7 at 80 °C for 24 h. After this heat treatment, almost all proteins were converted to clusters. Different sizes were obtained by varying the protein concentration and/or the ionic strength. The *z*-average radius of gyration of the clusters was determined using light scattering as described elsewhere.[33] The size of the largest clusters used for this study was estimated to be about 1 μm using convocal scanning laser microscopy.

The κ-carrageenan used for this study is an alkali treated extract from *Eucheuma cottonii* and was a gift from Degussa Texturant Systems (Baupte, France). Using NMR, it was found that the sample contained less than 5% ι-carrageenan.[34] A freeze-dried sample of κ-carrageenan in the sodium form was dissolved while stirring a few hours in hot Mili-Q water (70 °C) with 200 ppm sodium azide added as a bacteriostatic agent. The pH was adjusted to 9 to eliminate the risk of hydrolysis during preparation. The solution was extensively dialyzed against Mili-Q water at pH 7 containing 0.1 M NaCl and subsequently filtered through 0.45 μm pore size Anatope filters. The concentration was determined by measuring the refractive index using refractive index increment 0.145 g/ml.

Methods. Light scattering measurements were made using an ALV-5000 multi-bit multi-tau correlator and a Spectra Physics solid state laser operating with vertically polarized light with wavelength $\lambda = 532$ nm. The range of scattering wave vectors covered was $3.0 \times 10^{-3} < q < 3.5 \times 10^{-2}$ nm^{-1} ($q = 4\pi n_s \sin(\theta/2)/\lambda$), with θ the angle of observation, and n_s the refractive index of the solution). The temperature was controlled by a thermostat bath to within ± 0.1 °C.

The turbidity (τ) was determined at 685 nm using cells with path lengths between 1 and 10 mm depending on the turbidity. The temperature was controlled by a Peltier system and mineral oil was added to prevent water evaporation.

Confocal scanning laser microscopy (CSLM) was used in the fluorescent mode. Observations were made with a Leica TCS−SP2 (Leica Microsystems Heidelberg, Germany). As β-lg and κ-car do not exhibit intrinsic fluorescence at these wavelengths, only β-lg was labeled

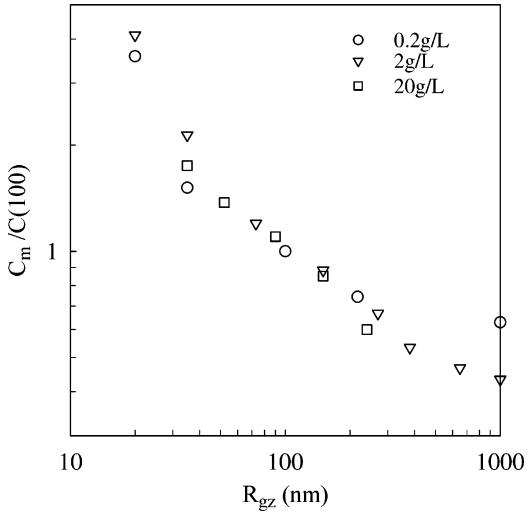

Figure 4. Dependence of the minimum κ-car concentration needed to induce micro-phase separation on the β-lg cluster size in mixtures with different β-lg concentrations indicated in the figure. C_m is normalized by the value at $R_{gz} = 100$ nm.

with fluorochrome, rhodamine B isothiocyanate (RITC). Thus, RITC (2.5 mg of RITC/g of β-lg) was added to the β-lg solution under magnetic stirring for 1 h. Samples were poured between a concave slide and a coverslip and then hermetically sealed. Observations of β-lg were made by excitation of RITC at 543 nm, the emission being recorded between 560 and 700 nm.

Results and Discussion

Phase Behavior. β-lg clusters with a wide range of sizes were mixed with κ-car at room temperature. The protein concentration was fixed at 2 g/L while the polysaccharide concentration was varied. The viscosity of κ-car solutions increased strongly at higher concentrations,[35] which limits the upper value that can be used in the mixtures to about 15 g/L. The pH was set at 7, and 0.1 M NaCl was added to partially screen electrostatic interactions. Above a minimum κ-car concentration (C_m), the mixtures became immediately turbid. The turbidity (τ) continued to increase weakly after mixing, but then it stabilized until after a few days sedimentation set in. The rate of sedimentation decreased with increasing polysaccharide concentration as was discussed in ref 7.

Figure 1a shows the dependence of the turbidity on the κ-car concentration for a range of protein cluster sizes. For each cluster size, the turbidity rises rapidly above C_m and levels off at about 3 times this value. The minimum κ-car concentration needed to induce phase separation and the maximum turbidity (τ_m) of the mixtures decrease with increasing protein cluster size. Figure 1b shows that the data obtained for different cluster sizes superimpose if C is normalized by C_m and if τ is normalized by τ_m. This enables an accurate estimation of these parameters even for the smaller clusters where τ_m is not reached even at the highest κ-car concentration used in the experiment.

The dependence of C_m on the z-average radius of gyration of the clusters (R_{gz}) is shown in Figure 2 in a double logarithmic representation. C_m decreases with increasing cluster size, but the dependence becomes weaker for larger clusters. An increase of the incompatibility with increasing cluster size is expected, because the loss of translational entropy of the proteins caused by phase separation decreases with increasing cluster size. In addition, depletion forces are expected to be larger for larger clusters.[36,37] However, it is difficult to even estimate the latter effect more quantitatively, because other interactions are involved such as van der Waals attraction and electrostatic

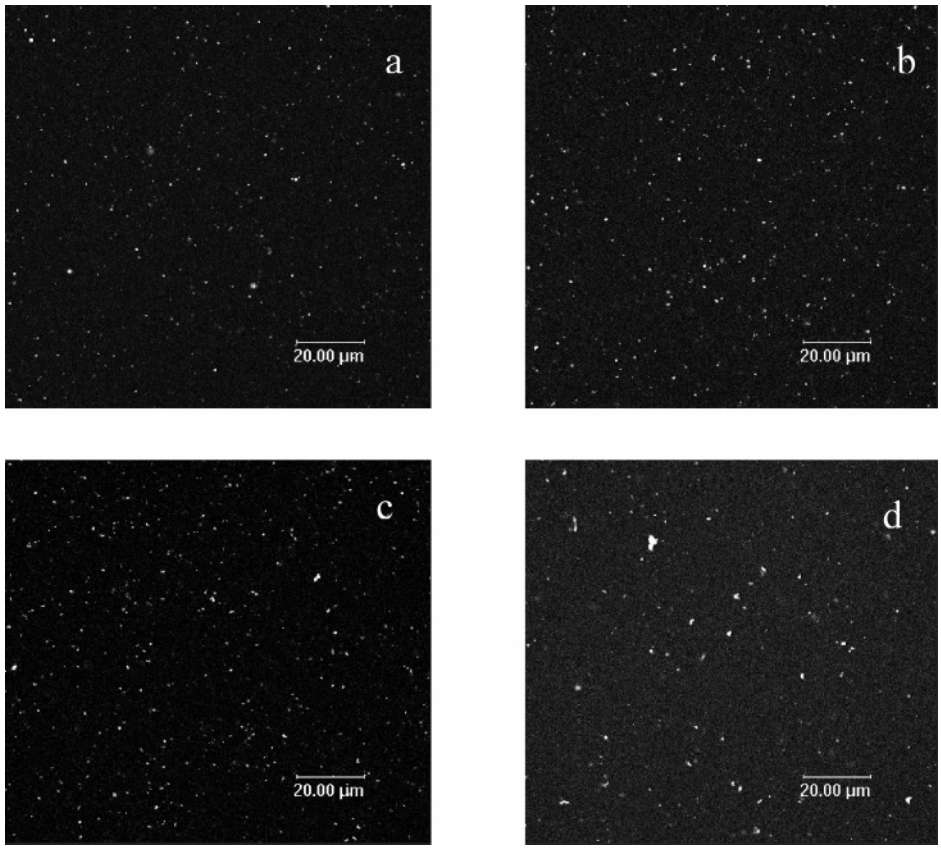

Figure 5. CSLM micrographs of mixtures containing 15 g/L κ-car and 0.2 g/L β-lg clusters with different sizes: $R_{gz} = 35$ nm (a), 100 nm (b), 270 nm (c), and 1 μm (d). The scale bar indicates 20 μm.

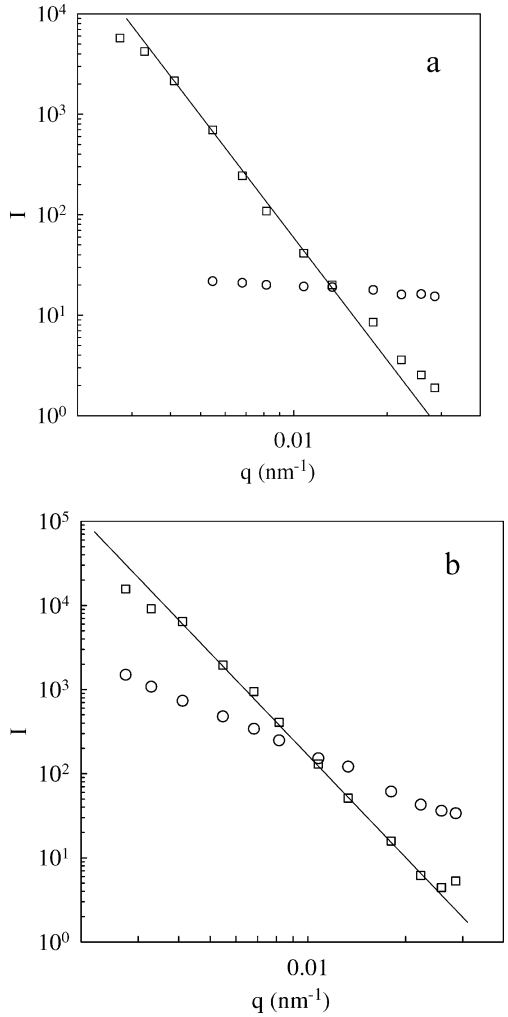

Figure 6. Dependence of the scattered light intensity on the scattering wave vector for 0.2 g/L β-lg clusters (circles) in the absence of κ-car and microdomains (squares) in mixtures. In Figure 6a the results are shown for clusters with $R_{gz} = 35$ nm and 7 g/L κ-car and Figure 6b those for clusters with $R_{gz} = 1$ μm and 4 g/L κ-car. The solid lines have slope -4.

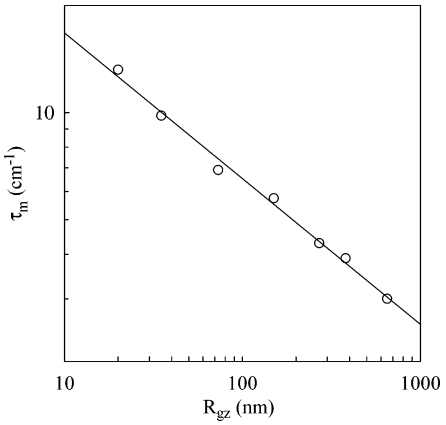

Figure 7. Dependence of the maximum turbidity on the β-lg cluster size in mixtures with 2 g/L β-lg.

repulsion. Moreover, the clusters are polydisperse in size and shape; they are flexible and they have a fractal structure.

For all cluster sizes, phase separation occurs above the overlap concentration of the polysaccharide where the correlation length (ξ) decreases with increasing κ-car concentration following a power law.[35] Comparison of ξ of the semidilute κ-car solutions at C_m with the cluster size shows that in each case R_{gz} is at least 10 time larger than ξ and that there is no simple relationship between the two parameters.

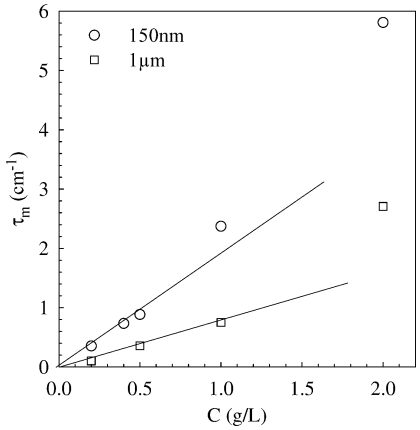

Figure 8. Dependence of the maximum turbidity on the β-lg concentration mixtures for two β-lg clusters sizes.

The microdomains sediment after several days and the supernatant has been analyzed by size exclusion chromatography as explained in ref 7. The fraction of proteins that phase separates (F) increased with increasing κ-car concentration starting at C_m and approaches unity at high κ-car concentrations. However, the turbidity rises more steeply to its maximum value than F. This is shown in Figure 3 where we compare F with τ/τ_m. The implication is that the size or the structure of the microdomains varies with increasing κ-car concentration. Indeed, it was shown in ref 7 that the microdomains are larger if the κ-car concentration is close to C_m. In the present investigation, we confirmed this observation using CSLM. In addition, any effect of interactions between the microdomains increases with increasing F and may influence the results at larger F. The effect of the protein concentration on the turbidity will be discussed below.

The measurements were repeated at a few other protein concentrations. At the lowest protein concentration investigated (0.2 g/L), the mixtures remained transparent even when phase separation occurred, and C_m was determined by measuring the variation of scattered light intensity. The effect of varying the protein concentration was found to be relatively weak, and the dependence of C_m on the cluster size was the same within the experimental error. To emphasize the latter, we have plotted in Figure 4 C_m normalized by the value at $R_{gz} = 100$ nm (C_m-(100)) for different protein concentrations $C_m(100) = 3.5$, 3.0, and 2.0 g/L for 0.2, 2, and 20 g/L protein clusters. The values of $C_m(100)$ vary by less than a factor of 2, whereas the protein concentration varies over 2 orders of magnitude between 0.2 and 20 g/L. To explore the full phase diagram, even lower protein concentrations need to be studied. However, the relative scattering intensity of the microdomains becomes very weak at lower concentrations and is difficult to distinguish from that of the polysaccharides.

Structure of the Microdomains. CSLM micrographs (Figure 5) show at low protein concentrations individual microdomains with radii close to the limit of the resolution, i.e., approximately one micron, if the κ-car concentration is much larger than C_m. If the κ-car concentration is close to C_m, we observed larger domains.[7] The domain size does not seem to be significantly influenced by the size of the protein aggregates even if the latter is itself about a micron. Using dynamic light scattering, hydrodynamic radii are found in accordance with microscopy. In ref 7, it was shown that the structure factor of the microdomains determined by static light scattering decreases

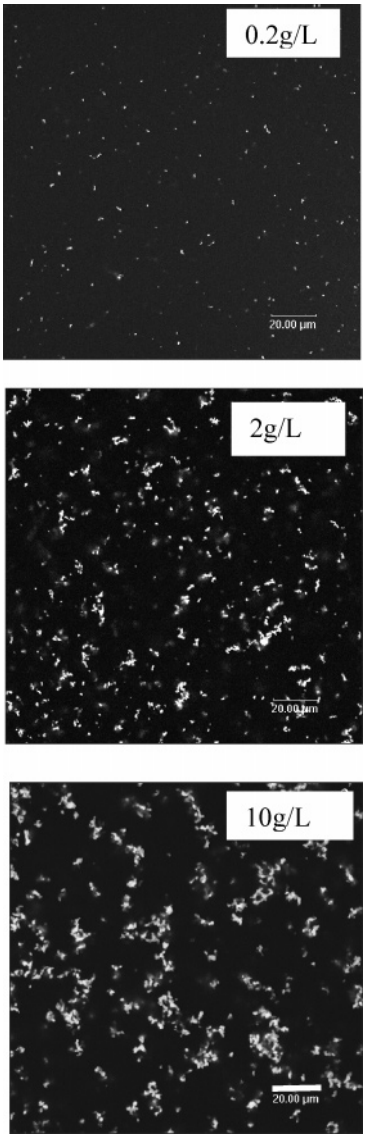

Figure 9. CSLM micrographs of mixtures containing 10 g/L κ-car and β-lg clusters with R_{gz} = 100 nm at three concentrations. The white scale bar indicates 20 μm.

as power law with the scattering wave vector: $S(q) \propto q^{-4}$, indicating homogeneous domains. In the present study, we confirmed this observation for other protein cluster sizes, see Figure 6. Deviations occurred at large q values indicating a lower limit of the homogeneous structure at a few tens of nanometers.

Figure 1 shows that the maximum turbidity in the presence of excess κ-car decreases systematically with increasing aggregate size. The dependence of τ_m on R_{gz} is plotted in Figure 7 and is compatible with a power law: $\tau_m \propto R_{gz}{}^{-0.4}$. In general, the turbidity is a function of the concentration, the weight average molar mass and the q-dependent structure factor ($S(q)$) of the clusters

$$\tau = HCM_w \int_0^{2\pi} \int_0^{\pi} S(q)(1 + \cos^2\theta)\sin\theta \, d\theta \, d\varphi \qquad (1)$$

where H is a constant for a given system.[38,39] If interactions between the microdomains can be ignored, then we need to consider only the structure factor of the microdomains. In fact, we will show below that at 2 g/L the effect of interactions is not negligible, but it is approximately the same for different cluster sizes. The amount of phase separated protein is close to the total protein concentration and is the same for each cluster

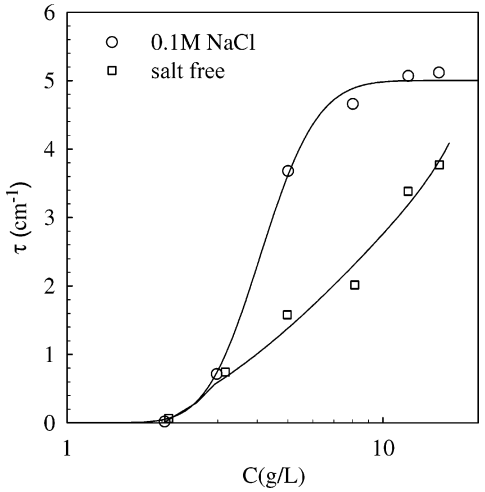

Figure 10. Comparison of the dependence of the turbidity on the κ-car concentration for mixtures containing 2 g/L β-lg clusters with R_{gz} = 200 nm with and without added salt. The solid lines are guides to the eye.

size. In addition, it appears from CSLM that the size of the microdomains is not significantly different. If we assume that the size and the shape of the domains are independent of the cluster size, then τ_m is proportional to the density (ρ) of the domains. Of course, the small scale structure is not the same for microdomains formed with different protein cluster sizes. However, the contribution of the small values of $S(q)$ at large q to the integral in eq 1 is negligible.

If the microdomains are simply close packed assemblies of monodisperse clusters, then their density is equal to the density of the clusters. For self-similar clusters $M \propto R_g^{d_f}$ so that their density is given by

$$\rho \propto M/R_g{}^3 \propto R_g^{d_f-3} \qquad (2)$$

Since $d_f = 2$, it would follow that $\tau_m \propto R_g{}^{-1}$. Experimentally, a weaker dependence of τ_m on R_{gz} is observed. One possible origin for the weaker dependence is the polydispersity of the clusters that increases with increasing R_{gz}. Small clusters can penetrate the larger clusters and thereby increase the density compared to a monodisperse system of the larger clusters. This also explains why the clusters are homogeneous on length scales smaller than R_{gz}.

Figure 8 shows τ_m as a function of the protein concentration for two different aggregate sizes. Initially, τ_m is proportional to the protein concentration, which confirms that the phase separation is not very sensitive to the protein concentration. However, τ_m clearly deviates upward above 1 g/L. The relative increase is probably caused by clustering of the microdomains. The increased clustering with increasing protein concentration is illustrated in Figure 9 where we compare CSLM micrographs of micro-phase separated mixtures at different concentrations of protein clusters with R_{gz} = 100 nm. The images at higher protein concentrations are similar to those of heated mixtures of globular proteins and polysaccharides indicating that similar micro phase separation occurs in-situ when the aggregates have grown sufficiently large.[12−15]

The influence of added salt is illustrated in Figure 10, where we compare the variation of the turbidity in the presence or absence of 0.1 M NaCl. In the absence of salt, the increase is weaker, but it starts at the same κ-car concentration. We verified that C_m was the same with and without added salt for different cluster sizes, see Figure 3. A more detailed study of the structure

of the microdomains at different ionic strengths is needed in order to explain the weaker increase of the turbidity with the κ-car concentration in the absence of added salt.

Conclusion

β-lg clusters phase separate into microdomains when mixed with κ-car at pH 7. The minimum κ-car concentration that is needed to induce phase separation decreases with increasing cluster size, but the effect of the cluster size becomes less for larger clusters. The effect of the protein concentration is small in the range between 0.2 and 20 g/L, where C_m varies by less than a factor of 2. The fraction of protein clusters that phase separates increases with increasing κ-car concentration and is almost unity for $C > 15$ g/L. The average radius of the microdomains is about 1 μm at high κ-car concentrations independent of the cluster size. However, the density of the microdomains decreases with increasing cluster size.

Acknowledgment. Authors are grateful to Réseau National Matériaux et Procédés (France) for the financial support of this work.

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BM050662M