Influence of the Ionic Strength on the Structure of Heat-Set Globular Protein Gels at pH 7. Ovalbumin

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ABSTRACT: The effect of the NaCl concentration (C_s) on the structure factor and the turbidity of heated ovalbumin solutions was investigated using cross-correlation dynamic light scattering. The heated systems are characterized by a correlation length, which depends on protein and salt concentration, beyond which they are homogeneous. At length scales below this correlation length, the systems have a self-similar, fractal, structure. For $C_s \geq 100$ mM, very turbid systems are formed with correlation lengths that show strong concentration dependence, and the correlation length rapidly rises above 1 μm at protein concentrations above 4 g/L. For $C_s \leq 30$ mM NaCl the systems remain transparent with relatively small correlation lengths (<20 nm). At 50 mM NaCl the correlation length first increases strongly with increasing protein concentration followed by a decrease above 8 g/L. At both 30 and 50 mM, an unexpected increase in the correlation length and turbidity is observed at high protein concentrations (above 40 and 30 g/L, respectively). From the present work we conclude that the structure of heated globular protein solutions and gels is generally determined by the interplay between the growth of the aggregates and the electrostatic interaction between the aggregates.

Introduction

Heat-induced denaturation of globular proteins in aqueous solution generally leads to aggregation of the proteins. Above a given critical protein concentration (C_g), which depends on pH, temperature, ionic strength, and type of protein, a gel is formed.[1,2] The structure of these gels depends on the strength of electrostatic interactions during heating. In the presence of strong interactions, i.e., far from the isoelectric point (pI) of the protein and at low ionic strength, the gels are transparent and consist of (thin) strands of aggregated proteins. When interactions are weak, i.e., near the pI of the protein and at high ionic strength, the gels become turbid and the aggregates are more branched and clustered. Under these conditions microphase separation may possibly occur, which renders the gels heterogeneous at large length scales.

In part I of this series[3] the effect of varying the NaCl concentration on the structure and the turbidity was reported for β-lactoglobulin solutions and gels after prolonged heating. It was shown that the length scale of the heterogeneity of the gels increases strongly from less than 20 nm below 50 mM NaCl to more than 1 μm above 200 mM. At intermediate salt concentrations (100 and 150 mM) the scattering wave vector (q)-dependent structure factor ($S(q)$) could be well described by the following simple equation:

$$S(q) = (1 + q^2 R_a^2/3)^{-1} \quad (1)$$

where R_a is an apparent radius of gyration in undiluted systems that characterizes the length scale beyond which the system is homogeneous. At intermediate salt concentrations, R_a increases with increasing protein concentration as the size of the aggregate increases but is smaller than the z-average radius of gyration of the aggregates, R_{gz}, because the aggregates interpenetrate. R_a reaches a maximum close to C_g, after which it decreases as the system becomes denser. The maximum value of R_a is about 300 nm at 100 mM and almost 2 μm at 150 mM.

In the present paper we study the influence of adding NaCl on the structure of heated ovalbumin solutions, which we will compare with the results obtained for β-lactoglobulin. Ovalbumin is the main protein component in egg white, with a molar mass of 45 kg/mol and a radius of about 3 nm.[4−6] At pH 7 ovalbumin associates in the form of linear chains with a degree of branching that depends on the ionic strength.[4,7,8] In the absence of added salt, gels are formed only at relatively high protein concentrations ($C_g \approx 60$ g/L). These gels are clear, and the structure factor measured by X-ray scattering ($0.1 < q < 4$ nm^{-1}) is characterized by a so-called interaction peak at small scattering wave vectors.[9] In the q range covered by light scattering ($0.002 < q < 0.025$ nm^{-1}), the scattered light intensity is independent of q, implying that the gels are homogeneous at length scales larger than about 15 nm. With increasing salt concentrations C_g decreases, and the gels become increasingly turbid for $C_s > 30$ mM.

Experimental Section

Materials. The ovalbumin used for this study was purified from egg white of freshly laid hens' eggs (less than 2 h old) based on the procedure of Vachier et al.[10] and contained a small fraction (6%) of heat-stable ovalbumin. The ovalbumin was dissolved in Millipore water with 3 mM NaN_3 added to prevent bacterial growth. The pH was set at 7 using a NaOH solution. NaCl solutions were used to set the ionic strength.

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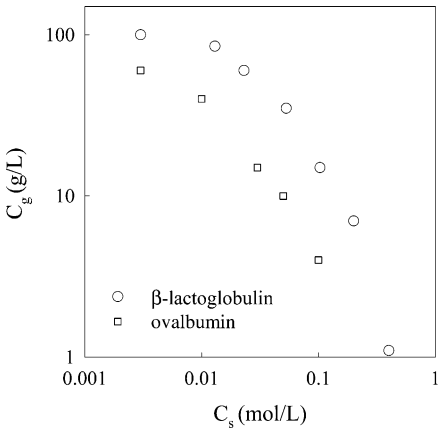

Figure 1. Critical gelation concentration (C_g) as a function of the NaCl concentration for ovalbumin and β-lactoglobulin at pH 7.

The solutions were filtered through 0.45 μm pore size filters (Millex-SV, Millipore Corp., Bedford, MA). Protein concentrations were measured using refractive index measurements and UV absorbance at 280 nm. Consistent results were obtained using refractive index increment $dn/dC = 0.181$ and absorption coefficient $\epsilon = 0.698$ L g^{-1} cm^{-1}.

Methods. The experimental methods are described in part I. Light scattering measurements were carried out at 20 °C using a commercial version of the 3D cross-correlation instrument. The small-angle X-ray scattering (SAXS) experiments were done on the DUBBLE beamline at the ESRF synchrotron facilities (Grenoble, France).

Confocal scanning laser microscopy (CSLM) images were recorded at NIZO food research (Ede, The Netherlands) on a LEICA TCS SP1 CSLM, equipped with an inverted microscope (model Leica DM IRBE), used in the single photon mode with an Ar/Kr laser. Ovalbumin was labeled with the fluorochrome rhodamine B for which the excitation wavelength and emission maximum are respectively 568 and 625 nm. Before heat treatment rhodamine B (20 μL, 0.1% w/w) was added to 1 mL of protein solution. Subsequently, the solutions were heated for 1 h at 78 °C in the CSLM sample cell and allowed to cool to room temperature before imaging.

Results

Ovalbumin solutions at pH 7 and different NaCl concentrations were heated at 78 °C for 24 h. After this prolonged heat treatment all proteins had aggregated except a small fraction (6%) of heat-stable ovalbumin. The critical protein concentration, C_g, beyond which the samples gelled or precipitated decreases with increasing salt concentration (see Figure 1). For comparison, we have included the results for β-lactoglobulin at pH 7 taken from ref 11. For 200 mM or higher NaCl concentrations precipitation was observed at low protein concentrations down to at least 1 g/L.

Diluted Ovalbumin Aggregates. For $C < C_g$, we determined the structure factor ($S(q)$) of the ovalbumin aggregates in dilute solution by measuring the q dependence of the relative excess scattering (I_r). From these data, the weight-average molar mass (M_w) and the z-average radius of gyration (R_{gz}) were determined as described in ref 4. The size of the aggregates increased with increasing protein concentration, and very close to C_g the aggregates became too large to measure. In ref 4 it was shown that the structure of ovalbumin aggregates is self-similar and is characterized by a fractal dimension (d_f), i.e., $S(q) \propto q^{-d_f}$ for $qR_{gz} \gg 1$.[12,13] We found that $d_f = 2.0$ at 100 mM and $d_f = 1.7$ without NaCl.

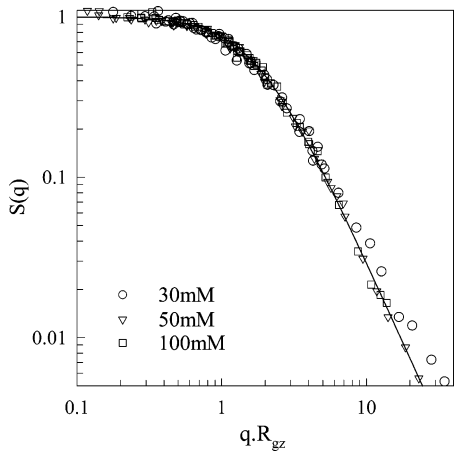

Figure 2. Structure factors of highly diluted ovalbumin aggregates formed after prolonged heating at different NaCl concentrations. The solid line represents $S(q) = (1 + q^2R_{gz}^2/3)^{-1}$.

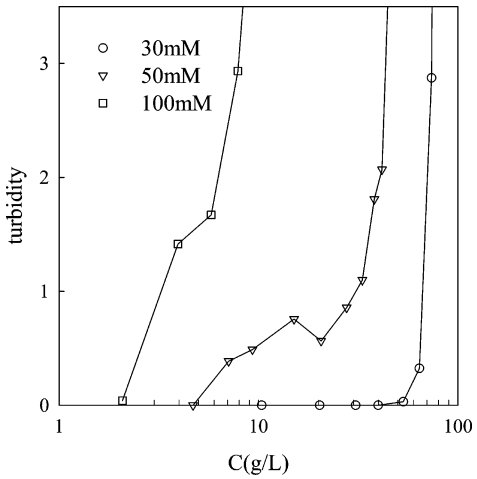

Figure 3. Concentration dependence of the turbidity of heated ovalbumin solutions at different NaCl concentrations.

Figure 2 compares the structure factors at 30, 50, and 100 mM NaCl. At 50 and 100 mM the results can be well described by $S(q) = (1 + q^2R_{gz}^2/3)^{-1}$ over the whole q range, but at 30 mM the data deviate upward from this equation at large q values, an effect similar to that reported in ref 4 for systems without NaCl. Cryo-TEM images of the aggregates show that the aggregates consist of linear strands that are increasingly branched with increasing ionic strength.[8] SAXS measurements show that the thickness of the strands is independent of the ionic strength but that the persistence length varies.[8]

Undiluted Ovalbumin Systems. In the absence of NaCl, heated ovalbumin solutions at pH 7 were clear over the whole concentration range up to at least 100 g/L. Figure 3 shows the concentration dependence of the turbidity of the heated protein systems at 30, 50, and 100 mM NaCl. At 30 mM, the gels were transparent except for $C > 80$ g/L where we observed, unexpectedly, a dramatic increase of the turbidity. At 50 mM, the turbidity increases to a maximum at $C \approx 20$ g/L, after which it decreases a little, but again there was a strong increase in turbidity at high protein concentrations. At 100 mM, the turbidity increased strongly already at low protein concentrations. We did not anticipate the increase at high protein concentrations at 30 and 50 mM NaCl since it was not observed for heated β-lactoglobulin

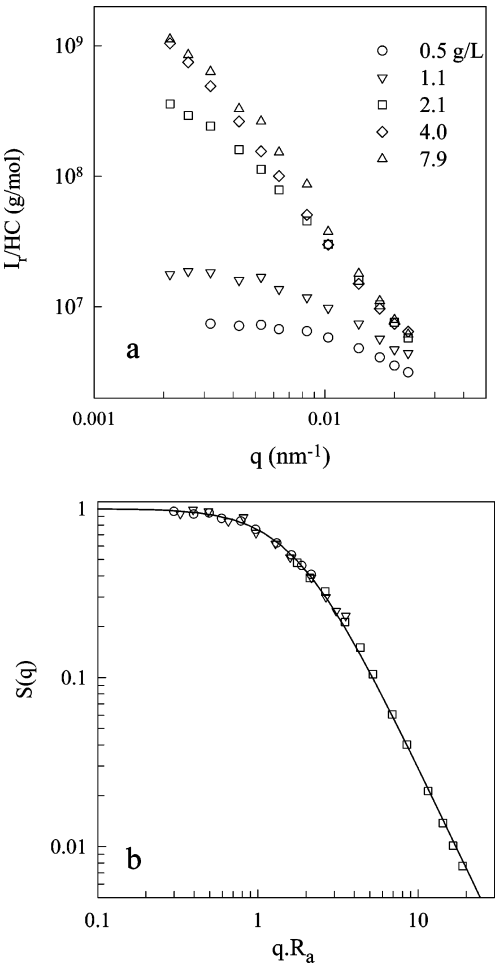

Figure 4. (a) q dependence of I_r/HC of ovalbumin solutions in 100 mM NaCl at different protein concentration after prolonged heating. (b) Structure factor obtained by normalization of the data shown in (a). The solid line represents eq 1.

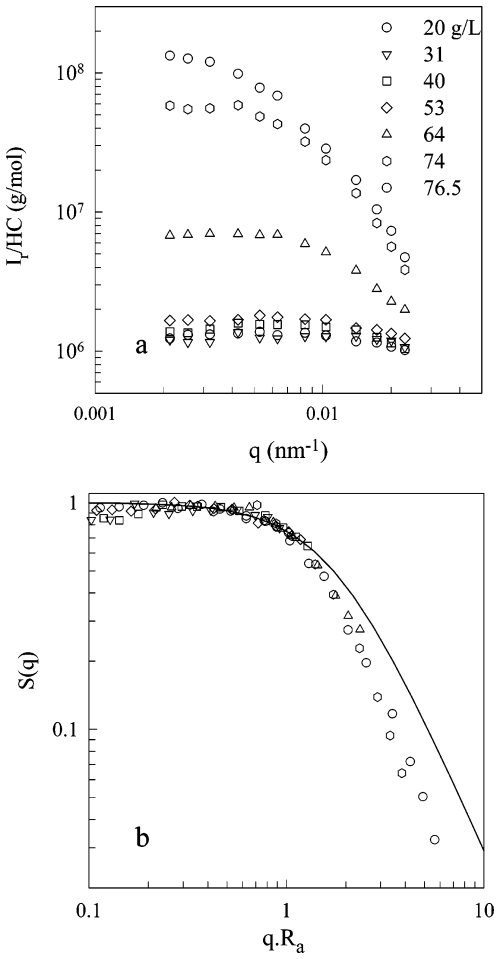

Figure 5. (a) q dependence of I_r/HC of ovalbumin solutions in 30 mM NaCl at different protein concentration after prolonged heating. (b) Structure factor obtained by normalization of the data shown in (a). The solid line represents eq 1.

systems (see part I). This phenomenon will be discussed in the Discussion section.

The q dependence of I_r/HC was determined for a range of protein concentrations at different NaCl concentrations, where H is an optical constant (or contrast factor).[4] Extrapolation of I_r/HC to $q = 0$ yields an apparent molar mass, M_a, which is inversely proportional to the osmotic compressibility. The structure factor is determined by plotting $S(q) = I_r/(HCM_a)$ as a function of qR_a. R_a was obtained by fitting the initial q dependence to eq 1. The results obtained at 100, 50, and 30 mM NaCl are shown in Figures 4, 6, and 5, respectively. In the absence of NaCl, I_r is independent of q over the whole concentration range investigated.

At 100 mM NaCl, the q dependence of I_r/HC becomes more important with increasing concentration, and for $C = 4$ and 7.9 g/L we observe within the experimental error the same power law behavior over the whole accessible q range (see Figure 4a). This implies that the correlation length of the gels is larger than 1 μm for $C \geq 4$ g/L, which explains the high turbidity of these systems. The structure factor can only be determined over a limited concentration range because the turbidity becomes too high for $C > 8$ g/L. Figure 4b shows that within the experimental error the structure factor is independent of the concentration and can be described by the same simple equation (eq 1) used in part I for β-lactoglobulin.

At 30 mM NaCl, and protein concentrations up to 50 g/L, I_r/HC showed no q dependence in the range covered by light scattering and is only weakly dependent on the protein concentration (see Figure 5a). At higher concentrations ($C > 50$ g/L), we observe a significant increase of I_r/HC, which also becomes q-dependent (see Figure 5a). Figure 5b shows that the structure factors at these higher protein concentrations are not independent of the concentration and cannot be well described by eq 1. The q dependence of $S(q)$ is stronger than predicted by eq 1 and appears to increase with increasing protein concentration, indicating a denser structure.

At 50 mM NaCl the concentration dependence of I_r/HC is more complex. For clarity, we show the low and high concentration data in separate figures (Figure 6a,b). Figure 6a shows that when increasing the protein concentration up to about $C_{max} \approx 7$ g/L, the q dependence of I_r/HC increases similarly to that at 100 mM NaCl as shown in Figure 4a. However, between 7 and 28 g/L, I_r/HC decreases with increasing protein concentration, and its q dependence becomes weaker (Figure 6b). So far, this behavior is similar to that observed for heated β-lactoglobulin solutions at 100 mM NaCl. However, when we increase the protein concentration even further, I_r/HC increases again and its q dependence becomes stronger. The increase of the correlation length takes place simultaneously with the increase in turbidity for $C > 20$ g/L. Below 7 g/L the structure factor can

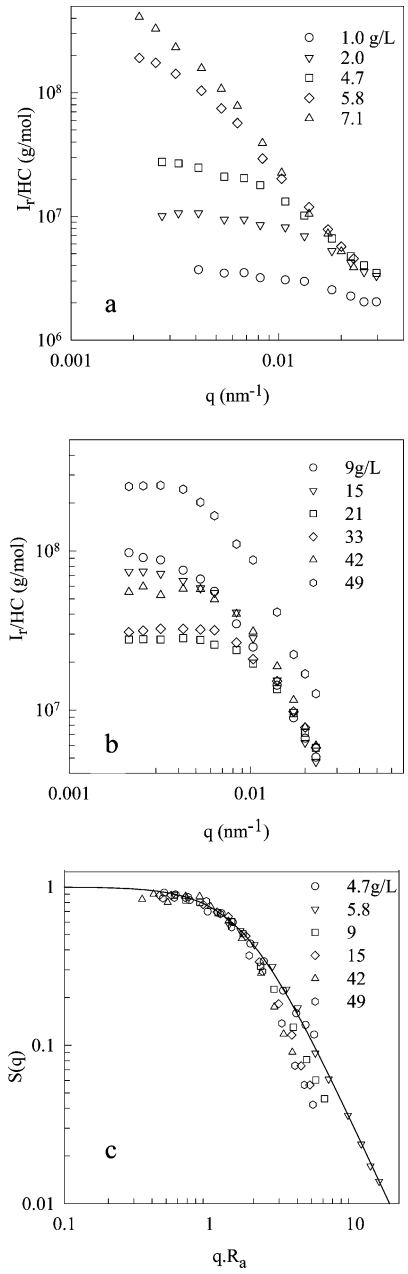

Figure 6. (a, b) q dependence of I_r/HC of ovalbumin solutions in 50 mM NaCl at different protein concentration after prolonged heating. (c) Structure factor obtained by normalization of the data shown in Figure 5a,b. The solid line represents eq 1.

be described by eq 1, but at higher concentrations we observe a clear deviation (see Figure 6c). The q dependence becomes stronger with increasing protein concentration, again indicating a denser structure. In fact, the results for $C > 30$ g/L (50 mM NaCl) resemble those obtained at 30 mM NaCl for $C > 50$ g/L.

From the initial q dependence of I_r/HC shown in Figures 4−6, we can still determine M_a and R_a using eq 1 even if the full q dependence is not described by this equation. The concentration dependence of R_a at different NaCl concentrations is plotted in Figure 7 and is compared with that of R_{gz} of the diluted aggregates. The latter diverges at C_g, where a gel is formed. At very low concentrations, interaction between the aggregates is small and $R_a \approx R_{gz}$. With increasing concentration interaction increases, and the aggregates start to interpenetrate. As a consequence, R_a becomes smaller than R_{gz}.

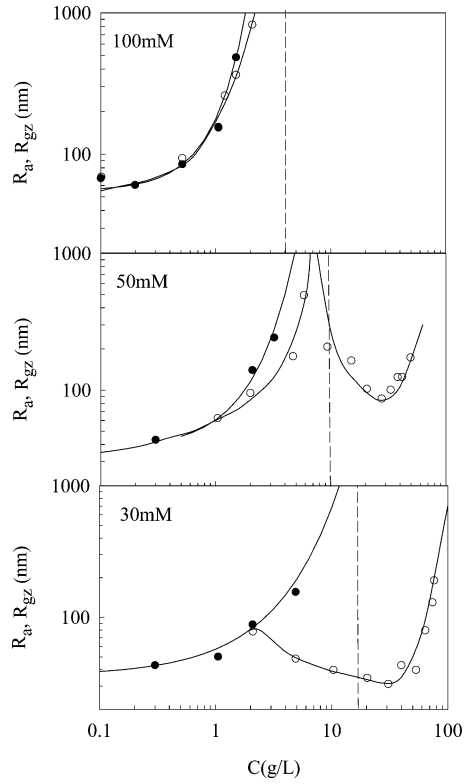

Figure 7. Concentration dependence of R_{gz} for diluted aggregates (filled symbols) and of R_a for undiluted heated ovalbumin systems (open symbols), at different NaCl concentrations. Dashed vertical lines indicate C_g, and the solid lines are guides to the eye.

At 100 mM NaCl, R_a increases strongly with increasing protein concentration and becomes larger than 1 μm for $C > 2$ g/L. The gels are heterogeneous on the micron length scale at least up to a protein concentration of 8 g/L, beyond which the gels are too turbid to allow accurate measurements. At 50 mM NaCl, R_a also increases strongly with increasing protein concentration and becomes larger than 1 μm at $C_{max} \approx 7$ g/L, but then R_a decreases with increasing concentration to $C \approx 30$ g/L, where R_a is reduced to about 80 nm. At higher concentrations R_a increases again. At 30 NaCl, a maximum is observed at $C_{max} \approx 2$ g/L, where $R_a \approx 80$ nm. From 2 g/L to about 30 g/L R_a decreases to about 35 nm, and above that concentration it increases again. We note that the maximum occurs close to the gel point at 50 mM NaCl and well below the gel point at 30 mM.

In Figure 8 we have plotted M_w as a function of R_{gz} for the diluted aggregates and M_a as a function of R_a for the undiluted systems. For self-similar aggregates one expects to find $M_w \propto R_{gz}^{d_f}$, where the prefactor depends on the elementary unit of the fractal structure. Within the experimental error the results at different salt concentrations have the same power law dependence with exponent 2, but the results at 30 mM are not incompatible with an exponent 1.7. The dependence of M_a on R_a as presented in Figure 8 shows two different behaviors. The data obtained at lower protein concentrations fall on top of those of the diluted aggregates (R_a, $R_g \leq 100$ nm). For these systems the structure factor is the same as that of the diluted aggregates. At higher protein concentrations M_a is systematically

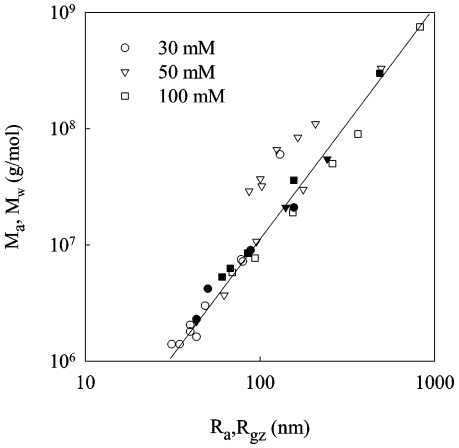

Figure 8. Dependence of R_{gz} of diluted aggregates (filled symbols) and of R_a of undiluted heated ovalbumin solutions (open symbols) on M_w and M_a, respectively, at different NaCl concentrations. The line has slope 2.

larger for a given value of R_a ($R_a > 100$ nm), implying that these systems are denser, which is consistent with the structure factors of these systems (see Figures 4−6). Nevertheless, M_a apparently has the same scaling dependence on R_a over the limited range of experimental data.

Discussion

In this study, the effect of salt concentration on the structure and turbidity of heated ovalbumin solutions was investigated and compared with that of β-lactoglobulin. Apart from the strong upturn of the turbidity and R_a at high protein concentrations, results obtained for ovalbumin were similar to those obtained for β-lactoglobulin as presented in part I.

The effect of the protein concentration on the turbidity and R_a, shown in Figures 3 and 7, can be explained as follows. When the proteins are heated they denature, which leads to aggregation. The aggregates grow in number and size until all native proteins are consumed. The heat treatment of the samples studied here (and also in part I) was such that almost all native proteins were aggregated. As shown in Figure 7, the number and the size of the aggregates after heat treatment increase with increasing protein concentration, which causes R_a to increase. At low concentrations interaction between the relatively small aggregates is negligible, and R_a is approximately equal to the z-average radius of gyration of the aggregates. However, with increasing concentration repulsive interaction becomes more important, and the aggregates also interpenetrate, so that $R_a < R_{gz}$. Beyond a certain protein concentration the effect of increasing interaction starts to dominate over the effect of increasing size and number of the aggregates. As a consequence, R_a passes through a maximum and decreases with increasing protein concentration at higher concentrations. It is obvious that the value and position of the maximum, R_{max} and C_{max}, respectively, depend on the strength of the interaction and thus on salt concentration, type of protein, and pH.

Qualitatively, the effect of varying the salt concentration may be understood in terms of varying electrostatic interaction. Using cross-correlation dynamic light scattering in undiluted protein systems, one measures the combined effects of growth of the aggregates and increasing repulsive interaction between the aggregates. On one hand, stronger electrostatic repulsion, i.e., lower salt concentrations, causes the protein to aggregate less easily, so that the aggregate size increases more weakly with increasing protein concentration. This is why the critical gelation concentration (C_g) increases with decreasing ionic strength. On the other hand, interaction between the aggregates increases with decreasing ionic strength. As a consequence, the decrease of the scattering intensity caused by repulsive interaction dominates over the increase caused by aggregate growth at lower protein concentrations if the salt concentration is lower. Therefore, one expects that the value of R_{max} will decrease with decreasing ionic strength (R_{max} at 50 mM $\approx$ 1 μm; R_{max} at 30 mM $\approx$ 80 nm). A shift of C_{max} (C_{max} at 50 mM $\approx$ 7 g/L; C_{max} at 30 mM $\approx$ 2 g/L) was observed. The value of C_{max} is determined by the interplay between the concentration dependence of the aggregate size, the strength of the interaction, and the structure of the aggregates. There is no direct correlation between C_{max} and C_g; at 30 mM $C_{max} < C_g$ and at 50 mM NaCl $C_{max} \approx C_g$. The stronger the interaction is, the smaller is C_{max} compared to C_g.

Comparing ovalbumin and β-lactoglobulin, we find that the critical concentration for gelation is smaller for ovalbumin over whole range of NaCl concentrations investigated (see Figure 1). Furthermore, the transition from transparent (homogeneous) to turbid (heterogeneous) gels occurs at lower NaCl concentrations for ovalbumin (between 30 and 100 mM NaCl) than for β-lactoglobulin (between 50 and 200 mM NaCl). It appears that the effect of NaCl on the general features of heated ovalbumin solutions is the same as for β-lactoglobulin but shifted to lower NaCl concentrations; the protein concentration dependence of R_a for ovalbumin at 50 mM is remarkably similar to that for β-lactoglobulin at 150 mM. The origin of the different sensitivity to added salt may be due to different aggregation mechanisms of the two proteins. As mentioned in part I, the aggregation of β-lactoglobulin at pH 7 occurs in two steps; in the first step well-defined primary aggregates containing about 100 monomers are formed, which associate in a second step to form large fractal aggregates or a gel. Ovalbumin forms a linear string of monomers or dimers with a degree of branching that increases with increasing salt concentration.

Apart form the higher sensitivity of ovalbumin to NaCl, the main difference between β-lactoglobulin and ovalbumin is the unexpected upturn of the turbidity and R_a at high protein concentrations for the latter. The upturn starts at higher protein concentrations if we decrease the NaCl concentration (30 and 40 g/L at 50 and 30 mM NaCl, respectively). We have observed an increase of M_a even in salt-free water for $C > 80$ g/L although the solution remains clear up to at least 110 g/L. The light scattering results inform us that the gels formed at high concentrations are increasingly heterogeneous with increasing protein concentration.

The appearance of large-scale heterogeneity at high protein concentrations can be observed with CSLM, as is shown in Figure 10 at 30 and 50 mM NaCl. At low concentrations the gels that are formed after heat treatment are fully transparent and homogeneous, but at high concentrations the gels are heterogeneous even on the micron scale. The heterogeneity appears at lower concentrations at 50 mM than at 30 mM NaCl. At salt concentrations of 100 mM NaCl or higher, CSLM shows that the gels are heterogeneous on the micron scale even at low protein concentrations.

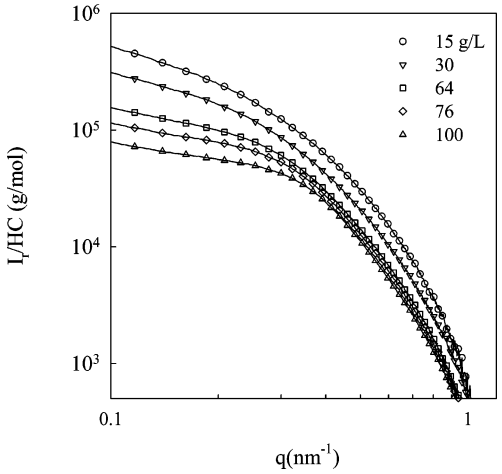

Figure 9. Wave vector dependence of I_r/HC of heated ovalbumin solutions in 30 mM NaCl at different protein concentrations obtained from SAXS measurements.

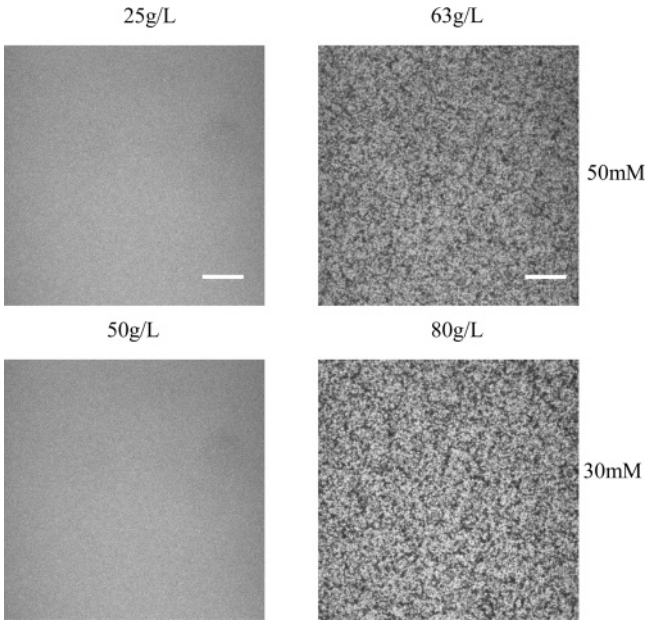

Figure 10. CSLM photos of ovalbumin gels formed by heating at 78 °C for 1 h at the different protein concentrations and NaCl concentrations indicated in the figure. The bar represents 25 μm.

To establish whether there is a local rearrangement of the aggregates at high protein concentrations, we used SAXS to determine the structure of the heated systems at 30 mM NaCl at small length scales. The q dependence of I_r/HC is shown in Figure 9 for different protein concentrations. At low protein concentrations the structure factor is characteristic for that of dilute semiflexible chains.[8] With increasing concentration the scattering intensity at low q values decreases due to increasing repulsive interactions, but we do not observe the dramatic increase of the scattering intensity for $C > 50$ g/L in the q range covered by SAXS as was observed with light scattering. This implies that the local structure is still controlled by the strong electrostatic repulsion between the aggregates even if at large length scales the systems become heterogeneous. Clearly, more research is needed to understand the origin of the large-scale heterogeneity that appears at high concentrations of ovalbumin, but that is not observed for β-lactoglobulin.

The main conclusion from the present work and that reported in part I is that the structure of heated globular protein solutions and gels is generally determined by the interplay between the growth of the aggregates and the electrostatic interaction between the aggregates. This interplay leads to a maximum in the turbidity at a certain protein concentration where the heterogeneity is maximum. The degree of heterogeneity, and thus the value of the turbidity at the maximum, increases with decreasing electrostatic interaction. The unexpected upturn of the turbidity at high protein concentrations has not been observed for β-lactoglobulin systems and is not a universal feature for globular protein gels.

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