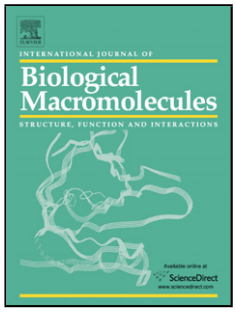

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Influence of β-lactoglobulin and calcium chloride on the molecular structure and interactions of casein micelles

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Highlights:

- Static and dynamic light scattering was applied to milk proteins
- Radii of hydration and gyration, molecular weights and second virial coefficients were estimated
- Casein micelles show deviation from a hard sphere under native conditions
- Hard spheres resulted at elevated calcium chloride concentrations or after removal of β-Lactoglobulin

Abstract

Targeted processing of casein micelles (CM) requires a basic understanding of their molecular structure as well as their interactions with each other and with other components. In this study, angle- and concentration-dependent static and dynamic light scattering is applied to investigate changes in the molecular weight, size, and intermolecular interactions of CM after the addition of β-lactoglobulin (ß-Lg) and calcium chloride. Addition of a surplus of ß-Lg impairs the colloidal stability of CM. In the presence of 0.5 wt% β-Lg and natural calcium chloride concentrations (10mM), the molecular weight of CM is reduced and the radius of gyration is increased. Both changes can be explained by the release of α_{S2}-casein and κ-casein, which were determined in higher concentration free in solution by High performance liquid chromatography. In contrast, the structure of casein micelles is not altered by the presence of β-Lg at elevated calcium chloride concentrations. The repulsive forces between the CM show no significant dependence on ß-Lg for all calcium chloride concentrations tested.

Keywords: β-lactoglobulin, casein micelle, calcium chloride, light scattering

1. Introduction

Caseins and β-lactoglobulin (β-Lg) are the most abundant proteins in cow milk and they have a range of beneficial functions. For example, they act as stabilizers of interfaces[1–3], carriers of bioactive substances[4–7], or raw materials for a number of gelled products[8–10]. Caseins, in particular, are natively unfolded proteins that form casein micelles (CM) in aqueous solution. Four types of casein (α_{S1}-, α_{S2}-, ß- and κ-casein) can be distinguished because of different primary structures and solubility in solutions containing calcium ions. The α_S- and ß-caseins are calcium insoluble and located inside the micellar structure while κ -casein is calcium soluble and mostly present on the micellar surface[11]. ß-casein mainly interacts via hydrophobic bonding and dissociates from the micelle at low temperature[12]. The highly phosphorylated α_S-caseins are cross-linked with calcium phosphate nanoclusters and show no temperature-dependent dissociation. Sedimentation experiments have characterized CM as spherical molecules with a molecular weight (M_W) of 2.8×10^8 g/mol and a mean hydrodynamic radius (R_H) of 77.8 nm[13]. Because CM have a broad range of sizes, size-fractionated samples containing CM are often used for studies aimed at revealing their structural details[14–16]. CM effectively behave as hard spheres at neutral pH (pH=6.7, 20°C) with repulsive (steric) interactions between them[17]. They are highly hydrated, with a moisture content of approx. 4g water per g protein[18–20]. The structure of CM is sensitive to the calcium ion concentration in the surrounding solution because calcium phosphate clusters hold the caseins together. Natural milk serum contains approx. 30mM calcium for which 10mM is diffusible and 2mM exists in ionized form[21,22]. The remaining calcium is incorporated in phosphate clusters within CM[23]. Removal of calcium leads to a release of soluble caseins and this results in CM instability[24]. In combination with drying forces, elevated calcium concentrations lead to aggregation of CM as surface-sensitive X-ray measurements in casein films showed[25]. Recent studies used high pressure to investigate structure and stability changes of CM at different calcium concentrations[26,27].

β-Lg is a well-folded globular protein, which takes up approx. 0.3 wt% in cow's milk[28,29]. It has the characteristic structural feature of a calyx made of two β-strands[30]. Depending on the pH, β-Lg undergoes a number of structural transitions[31] and adopts a range of oligomeric states[32]. β-Lg is also sensitive to serum Ca^{2+} concentration as denaturation and aggregation studies showed[33,34]. Heat-denatured β-Lg associates on the surface of CM or form complexes with κ-casein[35,36]. It was also shown by fluorescence spectroscopy that Ca^{2+} weakly but specifically binds also to native β-Lg[37]. In addition, ß-Lg adheres on the surface of CM during microfiltration and forms a deposit of interconnected CM with a porous mass-fractal structure on top of membranes[38]. Consequently, the mean specific fouling resistance of the casein layer is significantly reduced in the presence of β-Lg[39].

Static and dynamic light scattering are powerful tools to extract structural information, e.g. morphology, sizes and interactions, of milk proteins[40–43]. A recent study reported that multiangle laser light scattering and differential refractometer could be coupled with transmission electron microscopy and asymmetrical flow field-flow fractionation to characterize the size and morphology of heat induced casein micelle/whey protein aggregates[44]. However, little is known about the interaction between CM and β-Lg without thermo-treatment in solution.

This study is intended to determine whether changes in the structure and interactions of CM already occur upon the addition of a surplus of β-Lg (in native state) in solution. Hence, we analyzed the stability of CM with and without presence of ß-Lg under various calcium concentration by changing the calcium chloride concentration. To achieve this, we estimated the molecular weights,

intermolecular interactions, and radii of gyration and hydration by performing angle- and concentration-dependent static and dynamic light scattering and measured the individual casein concentration by HPLC. This study expands the field of application of scattering techniques to more complex systems and could have implications for the processing of skim milk and milk protein concentrates in general.

2. Materials and Methods

2.1 Materials

WPI power (Type 895, Lot No. CT08), containing roughly equal fractions of variants A and B, was obtained from Fonterra (Auckland, New Zealand). Raw skim milk was purchased from Molkerei Weihenstephan GmbH & Co (Freising, Germany). Ultrapure water from a Millipore (Milli-Q Integral 3) water treatment plant (Damstadt ,Germany) was used for the preparation. All reagents with HPLC grade were received from Sigma Aldrich (Damstadt, Germany) unless described otherwise.

2.1 Sample preparation

Fig.1 indicates the workflow of sample preparation and analysis. Native β-Lg was obtained from WPI powder after an aggregation and membrane separation process of α-Lactalbumin following the procedure described elsewhere[45]. β-Lg concentration was adjusted to 0.5 wt% in all samples used for the later scattering experiments.

CM were extracted from raw skim milk by centrifugation for 65 min at 31,000 rpm with a fixed-angle laboratory ultracentrifuge (Beckman Coulter, Germany). Centrifugation and resuspension

steps were conducted at 20 °C. A Bis-Tris base buffer (50 mM Bis-Tris with 10 mM $CaCl_2$) was used to dissolve the pellets at pH 6.8.

After resuspension for 2 h, the dispersion was centrifuged at 12,000 rpm for 13 min and then the supernatant was obtained. A further centrifugation step at 17,000 rpm for 13 min resulted in a supernatant containing a second set of size-fractionated CM, which was stored to prepare casein/ß-Lg mixture.

CM and ß-Lg were mixed at 20 °C for 20 min. The concentrations of casein and ß-Lg in mixed solution were 4 and 5 mg/ml, respectively. Solutions containing casein only and casein/ß-Lg mixture were stored for light scattering experiments.

2.3 Light scattering experiments

SLS/DLS experiments were carried out with an ALV/CGS-3 Kompakt Goniometer System (ALV-Laser Vertriebsgesellschaft mbH, Germany) equipped with a 22 mW He-Ne laser source (λ = 632.8 nm) at T = 20 °C. Scattering experiments were carried out three times, at five scattering angles (θ = 20°, 30°, 40°, 50°, 60°) for 30 s each.

Zimm plot is used to analyze polymers or polymer complexes in a monodisperse nature determined by light scattering. A Zimm plot was constructed to derive the molecular parameters using eq. (1)[46]:

$$\frac{KC}{R_\theta(q,c)} = \frac{1}{M_W}\left(1 + \frac{q^2 R_g^2}{3}\right) + 2A_2C \qquad (1)$$

where c is the mass concentration of the protein, q is the scattering vector, R(θ) is the Rayleigh ratio, and A_2 is the second virial coefficient.

Measured points of Zimm plot were extrapolated to zero angle and zero concentration to obtain molecular parameters, i.e. radius of gyration (R_g), molecular weight (M_w) and second virial coefficient (A_2) in equation 1.

Arbitrary constant (K) includes all optical constants:

$$K = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 \quad (2)$$

where n_0 is the refractive index of the solvent, dn/dc is the refractive index increment of the solute in the solvent, λ is the wavelength of the laser beam in vacuum, and N_A is Avogadro's number.

The average decay rate ⟨Γ⟩ and the variance were determined by second-order cumulant fitting of the field correlation function. The hydrodynamic radius was calculated from the Stokes-Einstein relation:

$$R_H = \frac{kT}{6\pi\eta D} \quad (3)$$

The refractive index increment of $\frac{dn}{dc} = 0.185$ mL/g was taken from the literature[47].

We used a plot with a semi-logarithmic concentration axis as commonly used for dose-response curves to depict the effect of calcium.

2.4 HPLC determination of individual casein concentration

To analyze the stability of CM with and without mixing with ß-Lg, ultracentrifugation was used again to separate single caseins from CM in solutions with 10 mM $CaCl_2$. Due to the low stability

of CM under this condition, single caseins will be released from them into supernatant upon centrifugation. The concentration of single caseins could then be detected using HPLC.

Eight samples (each of approx. 1.5 ml) were added into 8 centrifugation tubes followed by an ultracentrifugation step (31,000 rpm) at T = 20 °C for 65 min. The supernatant for each sample was then collected and stored at T = 4 °C for the determination of casein concentration using HPLC.
A total of 0.4 ml of sample was mixed with 1.6 ml of guanidine buffer (6 M guanidine-HCL, 5.37 mM trisodium citrate, 19.5 mM DTT in 0.1 M Bis-Tris buffer) in an Eppendorf reaction tubes. After a reaction time of 30 min, samples were filtered through regenerated cellulose syringe filters with a pore size of 0.45 µm and injected into HPLC vials. The concentrations of the individual caseins within the CM were determined using an Agilent 1100 series RP-HPLC system (Agilent Technologies, Germany), as described elsewhere[48]. The chromatograms were evaluated using ChemStation (version B.04.03) for LC systems (Agilent Technologies, Germany).

2.5 Data analysis

The obtained HPLC results were analyzed with SigmaPlot (Version 12.3, Systat Inc., USA) using paired t-test for equity of variances. The significance level for all samples was set at a P-value of < 0.05.

3. Results and Discussion

3.1 Light scattering analysis on casein/ß-Lg mixture

We performed angle- and concentration-dependent scattering experiments on casein suspensions with and without added β-Lg. The filled rectangles in Fig.2a are the measured scattering data plotted in the Zimm plot. The data on the nearly horizontal line correspond to one sample measured

at different scattering angles, while the data along upwards directed lines represent casein suspensions of different concentrations measured at one and the same scattering angle. The lines are linear fits to the data. Filled circles correspond to data that were generated by extrapolation towards zero scattering angle (X-axis) and zero concentration (Y-axis). Values for M_W, the second virial coefficient A_2, and the radius of gyration R_g can be estimated from the fit and are summarized in Table 1.

The molecular weight of the CM is 2.89×10^8 g/mol, which is within the same range as values reported from an ultracentrifugation study[13]. The second virial coefficient is very small, at 2.65×10^{-10} mol·L^2/g^2, which indicates weak repulsive interactions between the micelles. The estimated molecular weight of β-Lg corresponds roughly to the dimeric state, with 36.7 kDa[49] expected for this pH range[31]. The repulsive interactions between the β-Lg molecules are stronger by two orders of magnitude than those between CM under the same conditions.

The scattering function of CM in Fig.2b was generated from the scattering data after extrapolation towards zero concentration. The clear angular dependence is an indication of a strong contribution of the micellar form factor to the scattering intensity. The solid lines in Fig.2b correspond to model fits with monodisperse hard spheres and random coils. The best fit describes the micelles as hard spheres in solution.

Furthermore, the effects of both 0.5 wt% β-Lg (with and without) and four different calcium concentrations on the structure and interactions of CM were tested. All corresponding scattering data are shown in a Debye plot in Fig.3, which plotted of Kc/R as a function of casein concentration based on results obtaining from Zimm plot. The ratio of Kc/R for CM without β-Lg (open symbols)

increased with an increase of casein concentration from 1 mg/mL upwards. This positive correlation indicates repulsive interactions between CM, which enlarge the distance between micelles and prevent CM from aggregation. This leads to a stable suspension. In contrast, the Kc/R ratio increase with a decrease in calcium concentration below a casein concentration of 1 mg/mL. Attractive interactions dominate between micelles at a low calcium concentration, which leads to disintegration of the internal structure of CM.

An arrow in the plot shown in Fig.3 indicates that the addition of calcium chloride stabilizes the CM in diluted suspensions. The critical concentration c* of $CaCl_2$, indicating the point of (de-) stabilization of CM, was shifted from c* = 1.3 to 0.5 mg/mL when the calcium chloride concentration was increased from 1 to 50 mM.

Fig.3 also shows how the addition of a surplus of ß-Lg (closed symbols) influenced the stabilities of casein suspensions. Disintegration occurs already at c* = 3 mg/mL, while pure CM exhibited instability only at c* = 0.2 mg/mL. A less pronounced but significant destabilizing effect of β-Lg could also be seen in the presence of 10 mM $CaCl_2$. In contrast, scattering data of CM with and without adding β-Lg did not demonstrate similar effects at 30 and 50 mM calcium chloride.

The data sets obtained at a calcium chloride concentration of 1 mM are excluded from further consideration. It is because the instability/disintegration of the CM occurs throughout nearly the entire casein concentration range in this study.

We estimated characteristic sizes for CM for the various experimental conditions, namely, using eq. (1) for determining the radius of gyration (Rg), and eq. (3) for calculating the radius of

hydration(R_H). Without β-Lg, the radii of gyration for CM showed no dependence on calcium concentration and were distributed around Rg = 100 nm (Fig.4a). A difference in CM with added β-Lg only occurred when calcium chloride concentration was at 10 mM. The increased radius of gyration for CM under these conditions can be explained by the dissociation of material originating from the core of the CM, by the mere transfer of mass to the outside, or by an expansion due to the association of material. The latter can be ruled out since the corresponding radius of hydration with 10 mM calcium chloride + β-Lg did not significantly increase (compared with Fig.4b). In general, the hydrodynamic radii for CM are distributed around 120 nm and show no dependence on either β-Lg or calcium chloride. To explain the increase in the radius of gyration, it is thus necessary to consider the molecular weights.

Fig.5 shows the variation of the molecular weight of CM with calcium chloride concentration. Addition of β-Lg reduces the molecular weight of CM at 10 mM calcium chloride to two-thirds of its original value. This suggests that these conditions cause weakly bound caseins to dissociate from the micellar interior and disperse into the solution. A possible reason for this is the calcium sensitivity of β-Lg[37], which competes with the caseins for the micellar calcium. The withdrawal of calcium leads to dissociation of caseins from the micellar interior and changes the mass distribution of CM. The resulting larger mass density near the surface results in an increase in the radius of gyration. These structural changes lead to deviations from the hard sphere model. The ratio Rg/R_H (Fig.4c) does not correspond exactly to the theoretical value of a hard sphere in the presence of β-Lg and 10 mM calcium chloride concentrations in our samples and indicates a more open structure instead. Without β-Lg, an increase in salt concentration causes a slight reduction in the molecular weight. This takes place without a noticeable change in the overall size and density

distribution because the radii of CM in the absence of β-Lg do not change at different calcium chloride concentration (see Fig.4). High pressure experiments provide an explanation for this observation based on changes in intermolecular interactions. High pressure dissociation experiments of CM revealed a calcium-dependent transition of CM between 10 and 50 mM, which was accompanied by the formation of new intermolecular interactions, such as calcium phosphate bonds and hydrophobic contacts[26]. Additionally, the more compact structure of pressure-treated CM was explained by a pressure-induced increase in the calcium concentration and a subsequent enhancement of the hydrophobic interactions[50]. As a result of elevated calcium concentrations, the micelles would release water that was previously bound at the protein interface or trapped in voids. Based on these considerations, $2.8 \cdot 10^6$ water molecules would separate from a single CM after the addition of 30 mM calcium chloride. These would account for approx. 40% of the bound water molecules.

Fig.6 shows the calcium chloride dependence of the second virial coefficient. It describes the non-ideal solution behavior and is often used to quantify the solute-solute interactions. The weak steric repulsion forces between CM show no significant dependence on β-Lg, but increase by a factor of two at elevated salt concentrations (30 and 50 mM). The latter does probably not reflect changes of the micellar interaction but is rather a consequence of contributions of protein-cosolute interactions[51,52].

3.2 Concentration of individual caseins in casein and casein/ß-Lg mixture at natural calcium chloride concentrations

This set of experiments aims to reveal the impact of adding ß-Lg to the stability of CM at a $CaCl_2$ concentration of 10 mM as mimics of natural milk serum. Specifically, ultracentrifugation is used

to induce the sedimentation of micellar caseins into a pellet and we can then determine the concentration of free residual caseins remaining in the supernatant using HPLC. Fig.7a shows the concentration of individual caseins with and without the addition of ß-Lg after centrifugation in supernatant with 10 mM $CaCl_2$.

The concentrations of free κ- and α_{S2}-casein in the solution containing also β-Lg were significantly higher than the one without the addition of ß-Lg (Fig.7b). Interestingly, the concentrations of two other caseins (α_{S1}- and ß-casein) remained unchanged.

We explain this observation by the reduced stability of CM due to the competition of calcium in the presence of surplus ß-Lg. It destabilized the calcium phosphate center and CM tend to lose their integrity, which results in an open structure via the release of α_{S2}-casein and κ-casein[53]. This is consistent with the lower molecular weight of CM in the presence of ß-Lg at 10 mM $CaCl_2$, as shown in Fig.5 and the higher R_g/R_h ratio as shown in Fig.4c. The release of both proteins in the presence of β-Lg creates an inhomogeneous inner structure (as shown in Fig.7c) compared with the CM structure under native conditions and leads to a higher R_g value upon the measurement of light scattering (see Fig.4a).

4. Conclusion

The structure of CM shows deviations from a hard sphere model when the amount of β-Lg is increased compared to the native concentration ratios found in milk. In contrast, CM behave as hard spheres either in the absence of β-Lg at calcium concentration of 10mM or at an elevated calcium concentration, irrespective of whether β-Lg is present. The structural transformation involves changes in the radius of gyration and the molecular weight, suggesting that CM contain a

higher proportion and a different composition of caseins under hard sphere conditions. These changes may have consequences for food processing, in which the concentrations of whey protein and calcium chloride change. For instance, these findings may be relevant to the process of producing milk concentrates using nano-filtration, where part of the ionic components present in the milk serum get lost into the permeate. Specifically, CM as deposits on a membrane are in contact with whey protein when the salt concentration is high during the filtration process. Two different mechanisms can be identified to explain the structural changes induced by β-Lg and calcium chloride. First, β-Lg competes with caseins for micellar calcium in solution. A surplus of ß-Lg weakens contacts between caseins in the micellar interior and induces the dissociation of a proportion of caseins which results in a reduction of the molecular weight of CM. Second, elevated calcium concentration induces the formation of new intermolecular interactions between caseins and the release of water from casein interfaces. The finding that the strength of the repulsive forces between CM does not change in the presence of β-Lg underlines the fact that the structural changes mainly involve the core of the micelle. Despite the release of κ-casein, the intermolecular interactions remain largely unaffected. For future studies, it would be interesting to investigate the consequences of the ß-Lg effect on the colloidal stability of CM in real milk samples.

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Statement of conflict of interest

The authors declare no competing financial interest.

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FIGURE CAPTIONS

Figure 1. Schematic representation of the experimental design.

Figure 2. Analysis of the static light scattering of CM. (a) Zimm plot representation of the data with lines used to extrapolate to zero angle (X-Axis) and zero concentration (Y-Axis). (b) Extrapolated scattered intensity to zero concentration as a function of the scattering vector q. The solid lines are predictions of the monodisperse hard sphere and coil models.

Figure 3. Concentration dependence of K·c/R of CM only (open symbols) and in the presence of 0.5 wt% β-Lg (closed symbols) at four selected calcium chloride concentrations.

Figure 4. Radii of gyration (a), radii of hydration (b), and radii ratio R_g/R_H (c) for CM (open symbols) and CM+β-Lg (closed symbols) as a function of calcium chloride concentration. Dashed lines in c) indicate the symbols as theoretical values for hard spheres (bottom) and random coil (top).

Figure 5. Molecular weights for CM (open symbols) and CM+β-Lg (closed symbols) at different calcium chloride concentrations.

Figure 6. Second virial coefficients for CM (open symbols) and CM+β-Lg (closed symbols) at different calcium chloride concentrations.

Figure 7 a) Effects of ß-Lg on concentrations of non-micellar caseins at 10 mM $CaCl_2$. Changes of k-casein and α_{s2}-casein concentrations are shown in an enlarge view in b). Structural changes of CM are shown in the schematic representation under c).

Figure 1

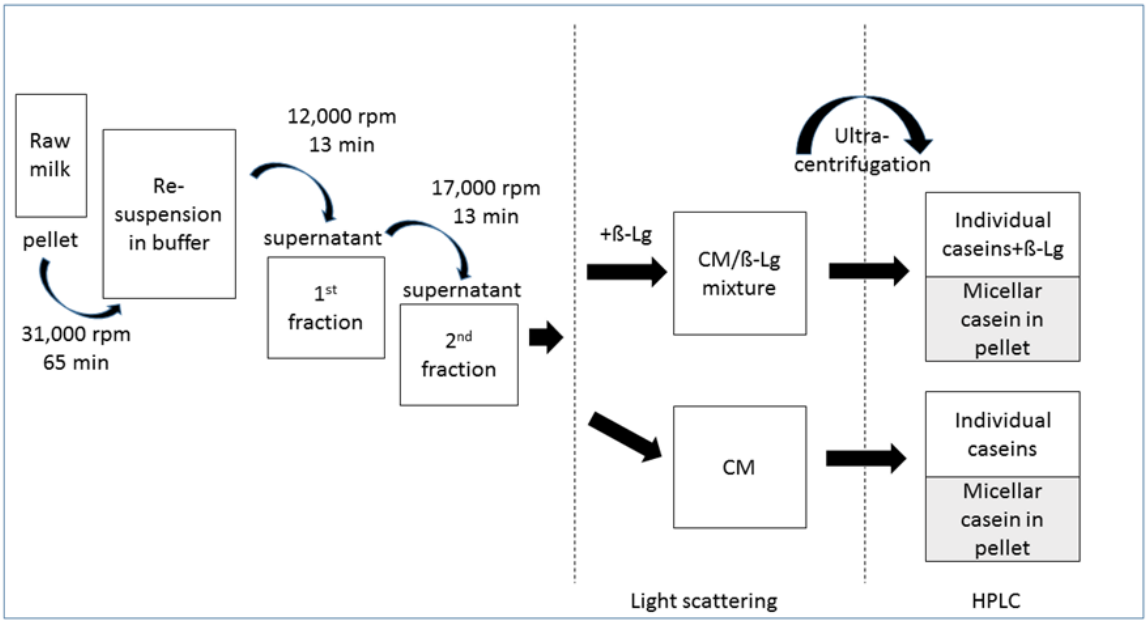


Figure 2

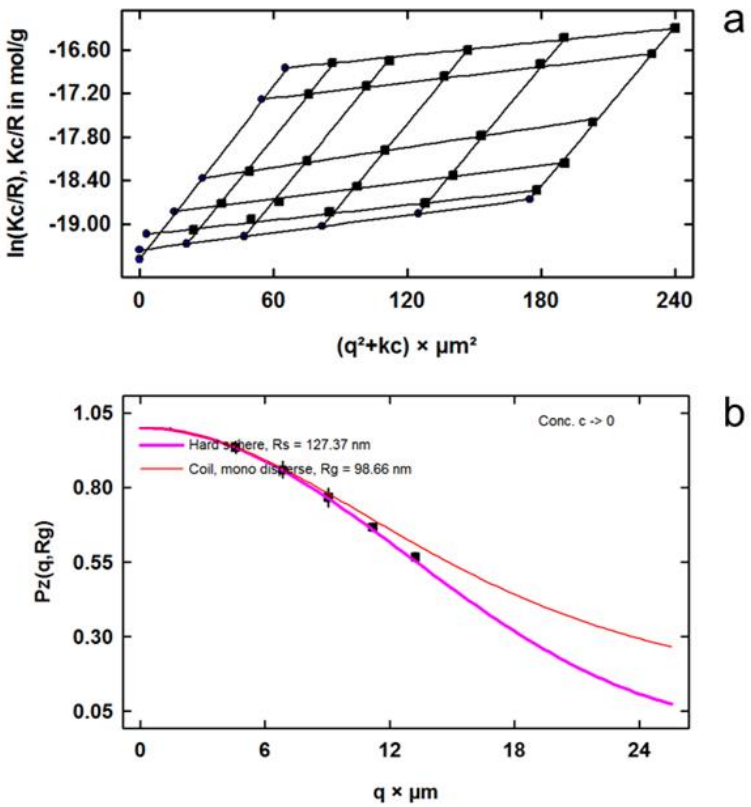


Figure 3

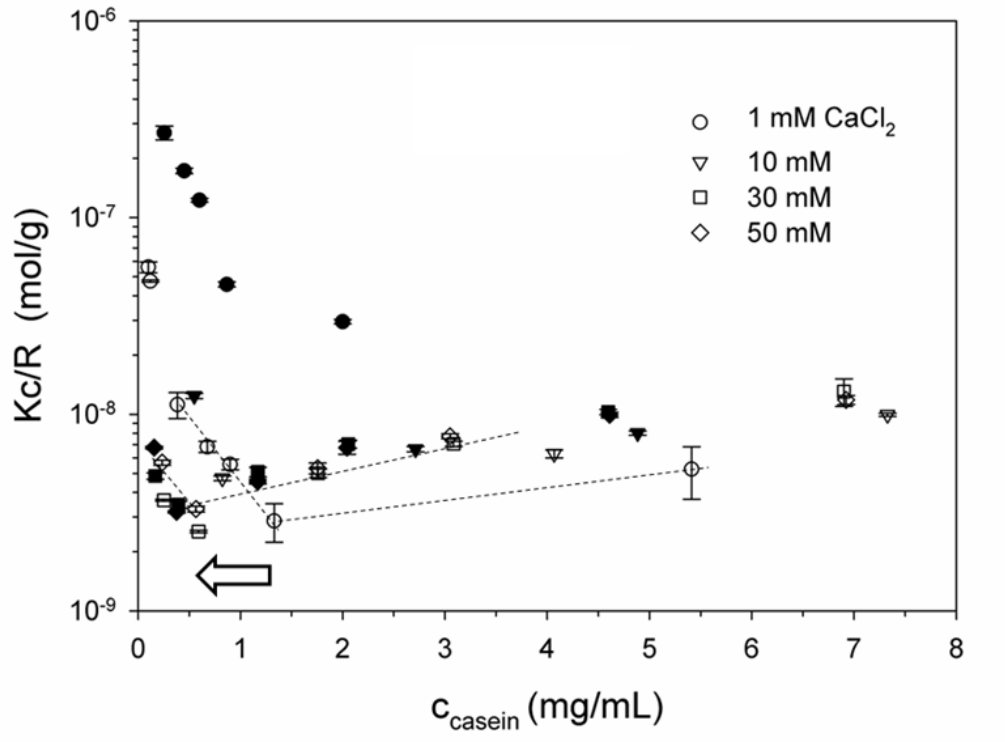


Figure 4

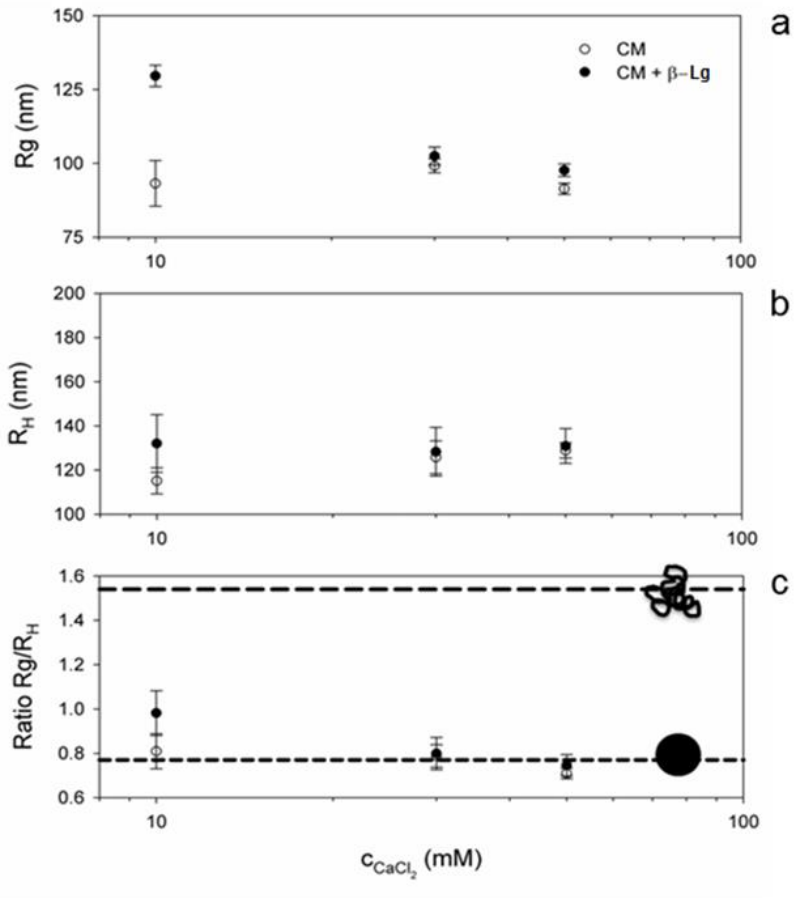


Figure 5

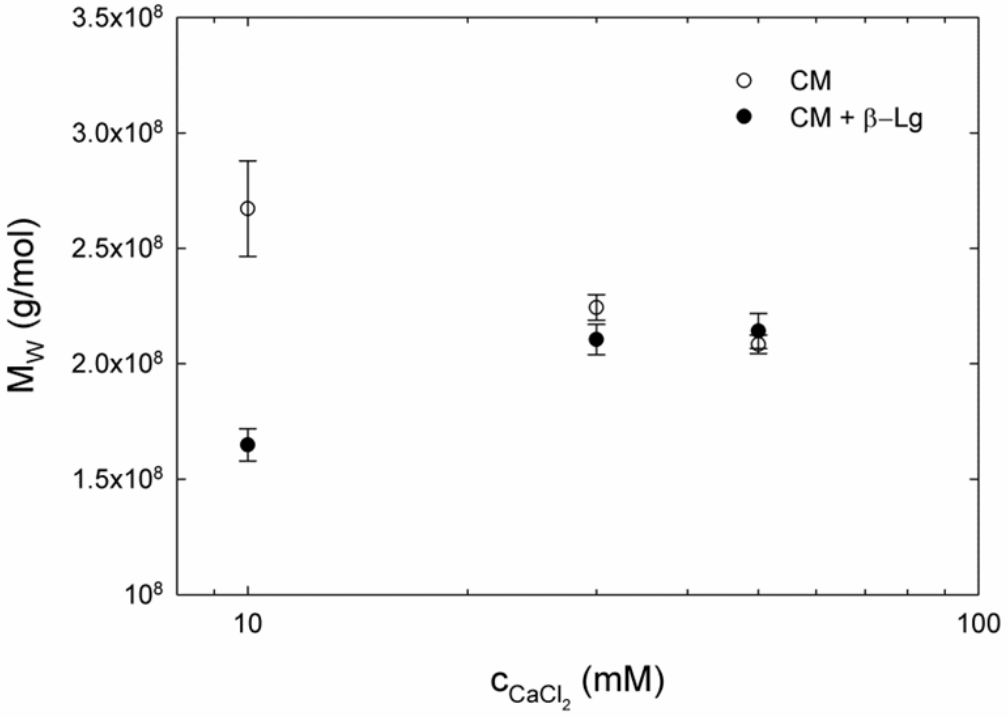


Figure 6

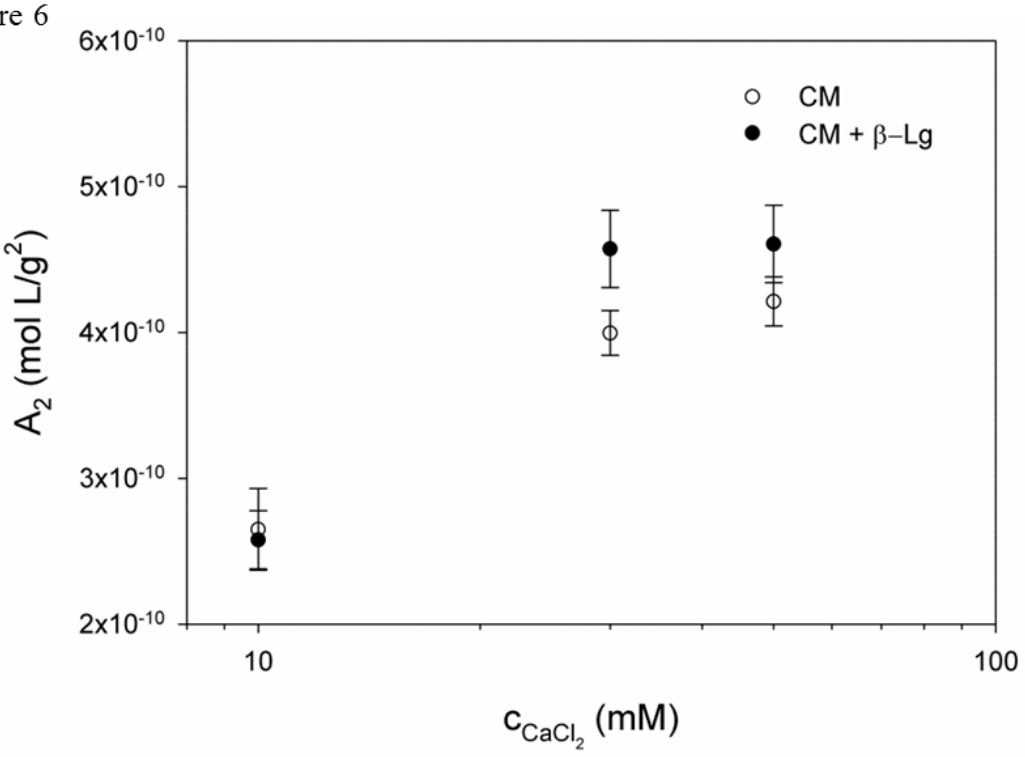
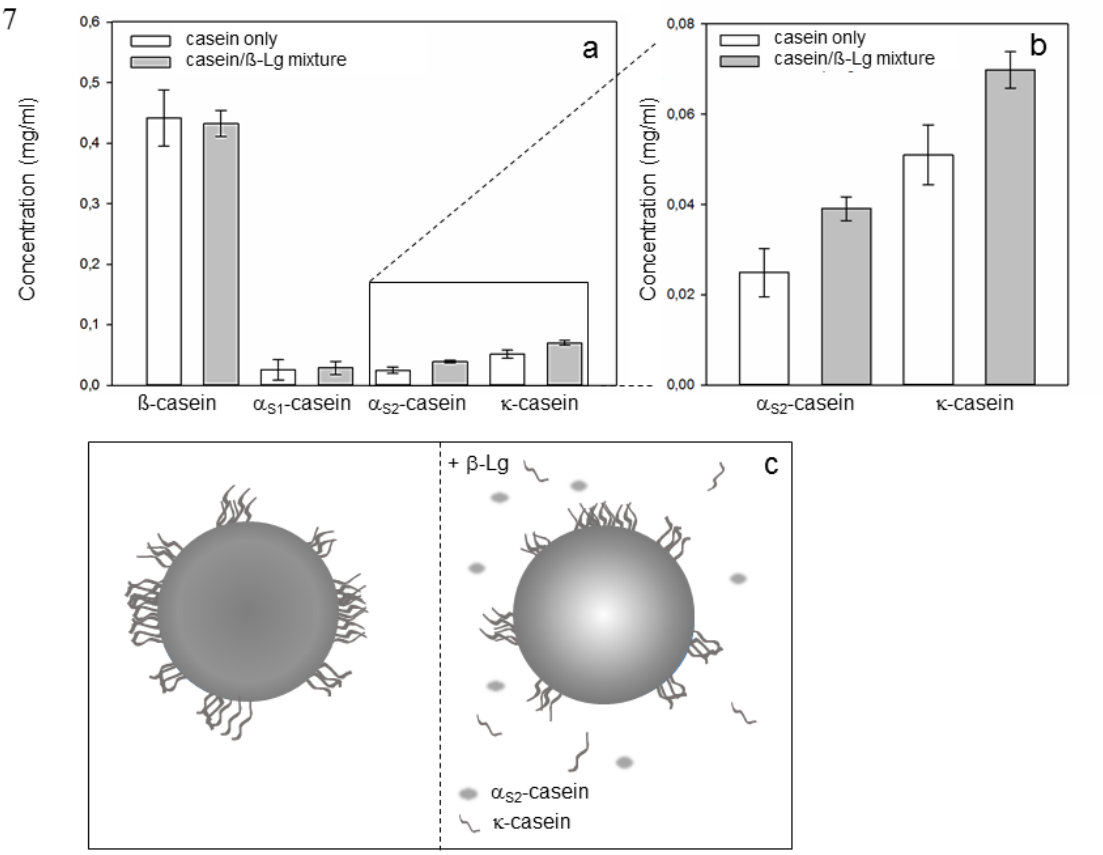


Figure 7



TABLES

Table 1. Values for the molecular weight M_W and second virial coefficient A2 extracted from static light scattering on suspensions of CM and β-Lg at pH 7.2.

parameters	CM	β-Lg
M_W	2.89×10^{8} g/mol	4.57×10^{4} g/mol
A_2	2.65×10^{-10} mol·L^2/g^2	7.27×10^{-8} mol·L^2/g^2