Coacervates of Lactotransferrin and β- or κ-Casein: Structure Determined Using SAXS

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ABSTRACT: Lactotransferrin (LF) is a large globular protein in milk with immune-regulatory and bactericidal properties. At pH 6.5, LF ($M = 78$ kDa) carries a net (calculated) charge of +21. β-Casein (BCN) and κ-casein (KCN) are part of the casein micelle complex in milk. Both BCN and KCN are amphiphilic proteins with a molar mass of 24 and 19 kDa and carry net charges of −14 and −4, respectively. Both BCN and KCN form soap-like micelles, with 40 and 65 monomers, respectively. The net negative charges are located in the corona of the micelles. On mixing LF with the caseins, coacervates are formed. We analyzed the structure of these coacervates using SAXS. It was found that LF binds to the corona of the micellar structures, at the charge neutrality point. BCN/LF and KCN/LF ratios at the charge neutrality point were found to be $\sim 1.2$ and $\sim 5$, respectively. We think that the findings are relevant for the protection mechanism of globular proteins in bodily fluids where unstructured proteins are abundant (saliva). The complexes will prevent docking of enzymes on specific charged groups on the globular protein.

I. INTRODUCTION

The interaction and self-assembly of oppositely charged proteins is a topic of recent research. Historically, it starts with Tiebackx1 who first reported on the coacervation of gum Arabic (GA) and gelatin. However, it was Bungenberg de Jong and Kruyt2 who made the first systematic investigation into the phenomenon, using a positively charged protein (gelatin) and the negatively charged polysaccharide GA. The interaction of oppositely charged (bio)polymers has an important application in encapsulation. Again, Bungenberg de Jong and Kruyt2 were first to show encapsulation of carbon particles and dyes in the GA/gelatin system. The National Cash Register company developed carbonless copy paper in the early 1950’s based on this system. Nowadays, for encapsulation, the so-called layer by layer technology is often used.3 Recent reviews on the interaction of oppositely charged (bio)polymers are presented by Izmudov4 and in a special issue of Soft Matter entitled “Polyelectrolytes in biology and soft matter”.5

In Figure 1, we present a schematic illustration of the main types of coacervates. Systems containing a polyelectrolyte/polyasaccharide and an oppositely charged protein or colloid have been extensively researched and were reviewed by De Kruif et al.,6 Schmitt and Turgeon,7 and, more recently, Kayitzmayer.8 In general, complexes and coacervate phases are formed when the two colloids are on the opposite side of the isoelectric point (IEP) and at low(er) ionic strength. Proteins are patchy colloids and they may even form coacervates at the “wrong” side of the

Figure 1. Schematic illustration of the morphology of various coacervates. Red color: positively charged; blue color: negatively charged (or vice versa). A: chromatin, the DNA-histone complex (from Arcesi et al.15,17). B: gum Arabic + β-lactoglobulin (from Weinbreck et al.10). C: gum Arabic + gelatin (from Bungenberg de Jong and Kruyt2). D: BCN + lactotransferrin (from Pan et al.,14 Anema and De Kruif,12 and this paper). E: lysozyme in a polyelectrolyte brush (from Witteman and Ballauf18). F: complex coacervate core micelles (from Voets et al.19) or surfactant micelles + polyelectrolyte (from Chiappisi et al.20). G: ionic crystals made of PMMA spheres (from Leunissen et al.21). H: (fractal) aggregates of lysozyme and β-lactoglobulin (from Howell et al.22) or silica particles (from Kim and Berg,23 Rasa et al.24). Note: drawings are not to scale.
IEM. Small proteins (colloids) may decorate the polysaccharide as depicted by Weinbrenck et al.10

Croguennec and co-workers investigated the formation of hierarchical self-assembly of α-lactalbumin with lysozyme.11 Supramolecular structures are formed from tetrahedral protein building blocks containing two lysozyme and two α-lactalbumin molecules interacting through their oppositely charged patches. Interestingly any further supramolecular assembly seems to be driven by hydrophobic interactions.

Anema and De Kruijff12,13 studied the interaction between folded proteins (lactotransferrin and lysozyme) with unstructured proteins (e.g., α-s-casein). These proteins form coacervates and lactotransferrin forms stable (overcharged) associates on either side of the charge neutrality point. α-s-Casein can be considered as poly(amide) electrolyte and then there is a great similarity with the coacervation of polysaccharides and oppositely charged colloids or proteins. Pan et al.14 interacted lysozyme with β-casein (BCN) and subsequently denatured the lysozyme. If the proteins (colloids) are large enough, the polyelectrolyte may wind around the protein. For instance, in the chromatin complex, DNA winds around the histones, therewith effectively folding the DNA into the cell. The folding comes with an energetic penalty, i.e., folding of the stiff polyelectrolyte chain. Park et al.,16 Arcesi et al.,17 and Anema and De Kruijff18 showed that one can add small amounts of lysozyme to BCN (at low salt content) without flocculation; however, adding BCN to lysozyme flocculated the system and it was suggested that this was due to the folding energy penalty of BCN around the very small lysozyme. If the polyelectrolyte forms a brush, e.g., by grafting to the surface of a colloid or because it has a hydrophobic tail, then the proteins are attached to the charged polymer corona, as shown by Witteman and Ballau,19 and in this work. Voets et al.19 reviewed the work on complex coacervate core micelles in which diblock polymers have a neutral tail, but an oppositely charged head. These systems form coacervates but cannot grow because of the neutral tail.

Biesheuvel et al.20 studied the interaction between two folded proteins, i.e., lysozyme and succinylated lysozyme which can be compared with the interaction of oppositely charged colloid particles, e.g., by Kim and Berg21 and Rasa et al.22 The work on oppositely charged colloids shows rich phase behavior, from fractal aggregates to ordered crystalline structures. Both Rasa et al.24 and Leunissen et al.21 used low dielectric solvents, in which the product of the Debye–Hückel screening parameter κ, and particle radius a, is large; i.e., κa ≫ 1. By manipulating the conditions, Leunissen et al.21 were able to mimic the crystalline structures of simple salts. In addition to the long-range attraction the PPMA particles have a short-range polymeric repulsion. In this paper we studied the adsorption of lactotransferrin to the polyelectrolyte brush of BCN micelles.

Lactotransferrin (LF) is a large (78 kDa) protein with pI ≈ 8.32 and has immune regulatory and bacteriostatic properties.6,27 BCN is an unstructured protein of 209 amino acids from milk. It is amphiphilic with a molecular mass of 24 kDa and a pI ≈ 5.6. The net number of charges on both proteins can be calculated using the primary sequence of the Swiss Protein Database.28

Early investigations on the temperature driven hydrophobic self-association of BCN were made by Payens29 during the 1960s using light scattering, analytical ultracentrifugation, viscosity measurements, and high pressure measurements. Kegeles30 proposed a so-called shell model for the self-association of BCN. Later, the work on BCN was extended often under varying and different conditions.31,32 Interestingly, Leclerc and Calmettes33 measured second virial coefficients of the micelles and found slightly negative values. This is consistent with the idea that there must be an overall attractive (cooperative) interaction in order to form micelles. Mikheeva et al.34 made an extensive investigation of the thermodynamics of the micellization of BCN confirming the shell model proposed by Kegeles.30,35 The micellization enthalpy is however endothermic, indicating that micellization is strongly entropy driven.36

Thus, BCN is monomeric at temperatures below 5 °C and forms polymeric micelles containing about 40 monomers at temperatures above 30 °C. Details will depend on the buffers used and the addition of “chaotropes”. For example, guanidine hydrochloride or urea will dissociate the BCN micelles. However, cross-linking the micelles using transglutaminase prevents the dissociation and leaves the micelle greatly intact.37,38 From small angle scattering experiments, the size of the micelles in a 25 mM phosphate buffer is about 15 nm (hydrodynamic radius and interaction radius) and the radius of gyration is 6 nm.39 Hence, it was concluded that the micelles had a relatively dense core of about 7 nm radius and a fluffy outside, being the hydrophilic N-terminus of BCN. In a recent paper, the association of BCN (and KCN) was studied using SANS and SLS.39 The (truncated) scattering spectra were fitted to the sum of an oblate ellipsoid and a Debye function, and are generally consistent with previous studies. Interestingly, Ossowski et al.39 studied the fibril formation on standing and applying shear.

On increasing the concentration, the BCN dispersions become very viscous, but viscosity decreases exponentially with temperature and increases with ionic strength.40

BCN micelles have been claimed to have chaperone-like properties.41,42 It remains to be seen whether this is a real chaperone property, i.e., preventing proteins from aggregation during unfolding/folding, or just the generic surfactant property of BCN in stabilizing hydrophobic particles like emulsion droplets. Actually, experiments on heating lysozyme in the presence of BCN showed that refolding was inhibited in the presence of BCN.43 We have used caseins to stabilize gold sols and whey protein particles (unpublished results), and BCN is known to absorb small molecules such as vitamins and drugs.44 κ-Casein (KCN) is famous for its stabilization of the casein micelle. Often it is claimed to be of electrostatic origin but that is improbable, because KCN carries only few net charges and, in addition, the Debye–Huckel screening parameter in milk is only 1 nm. A quantitative calculation of the steric, electrostatic, and van der Waals potential was given by Tuner and De Kruijff45 showing that electrostatic contributions are small. Numerous publications discuss the interaction of KCN with the (heated) whey proteins or with added polysaccharides. There are few papers on the micellization of KCN. KCN forms micellar structures, and the size and number of monomers (∼30) is largely independent of temperature and ionic strength, which contrasts with BCN. Much of the early work on KCN was done by Vreeman and co-workers.46–49 Thurn et al.31 measured SANS spectra and concluded that KCN formed micellar structures just like BCN, but these probably associated through (disulfide) bonding at the hydrophilic N-terminus. De Kruijff and May40 measured SANS spectra of KCN micelles in which the disulfide bonds were reduced with dithiothreitol. They found structures with a dense core and a fluffy corona with a radius of 12–15 nm depending on the measurement method. Later, De Kruijff et al.47 did further measurements using SANS confirming the earlier data and showed, using NMR, that BCN micelles are less dense and more dynamic than KCN micelles. Ossowski et al. report a
temperatures, independent association number of 30–35.39 Hence, KCN appears to be a micelle with a dense core and a (very) fluffy corona. From a structural point of view, both micelles appear to be very similar.

Often, micelles, such as those from BCN, are used to adsorb materials in the hydrophobic core. However, for positively charged solutes adsorption in the corona of the micelles is present as well. Here, we investigate the adsorption of lactotransferrin, a basic protein, into the corona of the micelles but also at temperatures where the BCN micelles are dissociated. We will use SAXS measurements to unravel the structure of the coacervates of lactotransferrin with BCN or KCN. We will analyze the SAXS spectra using an appropriate model for the system at hand and measure the scattering of both lactotransferrin and BCN (the last one also as a function of temperature).

II. EXPERIMENTAL METHODS

Experimental Solutions. Lactotransferrin (97% purity) was obtained from DMV International (Veghel, The Netherlands). KCN (>95% purity) was prepared according to the method of Leaver and Law.51 BCN (>95% purity) was prepared according to the method of Payens and Heremans.29 Proteins were dissolved in Milli-Q water NanoSTAR (Bruker AXS, Karlruhe, Germany) with a pinhole 0.95 cm^2.mg^{-1}. The low-Q values vary only slightly with temperature, whereas the value of S(Q = Q_{max}) increases with temperature. The high concentration data show a clear sigmoidal increase attributed to an increasing micellization, which is then fully consistent with SANS data of Leclerc and Calmettes,50 calorimetry data of Mikheeva and Grinberg,34 and dynamic light scattering results of O’Connell, Grinberg, and De Kruif.38 O’Connell and De Kruif,53 and De Kruif and Grinberg.53 The value of Q_{max} is 310 Å^{-1} and is independent of temperature and the same for the two concentrations. It must be remarked that there are some quantitative differences with the references mentioned. We attribute this to the fact that we did not use a 25 mM sodium phosphate buffer in this investigation. Adding salt further promotes micellization (data not shown), but we intended to investigate the binding of lactotransferrin and therefore did not add salt as this would reduce the interactions.

The (excluded volume) correlation peak at Q = 310 Å^{-1} corresponds to a half-distance (radius) of 20 nm. This amplitude increase is to be expected when volume fraction, \( \phi \), of the micelles increases at the expense of monomers. For noninteracting particles, the ordinate would be proportional to \( \phi \). For interacting particles, the ordinate would be proportional to \( \phi \times S(Q = 0, \phi) \), where \( S(Q, \phi) \) is the structure factor of the interacting particles, assuming that the form factor \( P(Q) \) does not change.

For a hard sphere or repulsive particle system, \( S(Q = 0, \phi) \) decreases exponentially with increasing \( \phi \). For an attractive interaction, \( S_{abs}(Q = 0, \phi) \) increases with increasing attraction and eventually diverges at phase separation. We therefore used an adhesive hard sphere (Ahs) structure factor with the condition that \( \phi \times S_{abs}(Q = 0.01 Å^{-1}, \phi) \) is constant. This is realized for a second...
Figure 3. Scattering spectra of BCN at temperatures between 2 and 30 °C. Correlation peak at intermediate wave vectors increases with temperature. (A) BCN 4 g/L. (B) BCN 16.1 g/L. The fitted spectra (drawn lines, see text) were offset by a factor of 4, for clarity.

Figure 4. Relative amplitudes of the scattering spectra at low Q-values and at the maximum of the correlation peak (S(Q = Q_{max})) as a function of temperature. ○, S(Q = Q_{max}) 16.1 g/L; □, S(Q = Q_{max}) 4 g/L; closed symbols experimental S(Q)-values at the three lowest Q-values.

virial coefficient $B_2/V_{hs} = -2.36$, where $V_{hs}$ is the hard sphere volume $4/3 \pi (20 \text{ nm})^3$. After some preliminary data fitting, it appeared that $B_2/V_{hs} = 0$ gave an excellent fit of the data. It is interesting to note that the effective second virial coefficient is zero, which is to be expected for particles in equilibrium with a monomeric system. The osmotic compressibility ($S(Q = 0, \varphi)$) of the particles must be very small. Adding particles will tend to dissociate them into monomers or the increased number density will be compensated by attractive interactions and as a result the osmotic pressure does not increase. Leclerc and Calmettes measured second virial coefficients as a function of temperature and found slightly negative values.

We fitted the data to the following expression:

$$FF(Q, a) = \ln[(a_0 \times \text{IAHS}(Q, a_1, a_2) + a_3 \times \text{PDebye}(Q, a_4))]$$

(1)

$$\text{IAHS}(Q, r_{0}, a_2) = \text{Ihspl}(Q, r_{0}, a_2) \times S(Q)$$

(2)

$$\text{Ihspl}(Q, R_g, a_1) = a_1 \times \exp \left[ -\frac{Q^2 \cdot (R_g)^2}{3} \right]$$

$$+ a_2 \left( \frac{\text{erf}(Q \cdot \frac{R_g}{\sqrt{6}})}{Q} \right)^{P1}$$

(3)

Equation 3 is the Beaucage result. Thus the fitting function $FF(Q, a)$ is the sum of an adsorbable hard sphere contribution $\text{IAHS}(Q, r_{0}, a_2)$ including a structure factor $S(Q)$. The symbols represent the following: $a$ is a matrix containing elements $a_0$ to $a_4$ where $a_0$, $a_1$, and $a_2$ are amplitudes; $a_3$ is the radius of gyration of the particles, $R_g$ and $a_4$ is the polymer radius of gyration, $R_g$.

In view of the presence of BCN polymers, we added a Debye polymer scattering term $P\text{Debye}(Q, a_0)$ under the assumption that the two contributions are not correlated. For the calculation of the structure factor of adhesive hard spheres. Volume fraction $\varphi$ increased with temperature (2 to 30 °C) from 0.12 to 0.2 for the 16.1 g/L data, which corresponds to a voluminosity of 12 mL/g, a reasonable value in view of the low salt concentration. We found a value of 3.5 mL/g in a phosphate buffer. Panouille et al. observed a divergence of the viscosity at 90 g/L (in salt buffer), which would correspond to a voluminosity of 0.63/0.09 = 7 mL/g. Sood et al. found from sedimentation experiments a value of 3 mL/g for the monomer. These voluminosity results indicate that the BCN micelles are quite fluffy objects. Here we used a voluminosity of 12 mL/g for all the experiments. For the 4 g/L data, we divided volume fractions of the 16.1 g/L data by 4. With these values, we fitted the experimental data to eq. 1.

Equation 3 contains 5 fit parameters: $a_0$, the amplitude of the Guinier function; $a_1$, the radius of gyration; $a_2$ the amplitude of the power law function, which for a given structure should be approximately constant. In fitting the data, we found that the contribution of the power law function was very small. Actually, small negative values were also found, which is not physically realistic; therefore, we set $a_2 = 0$. The amplitude of the Debye function is $a_4$, and $a_4$ is the radius of gyration of a polymer chain. $P1$ is the power law exponent and should be 4 in the Porod limit. We used $P1 = 4$, but results are rather insensitive to the precise value of $P1$. It appeared that a value of $\tau$, the “adhesiveness” parameter, in the expressions of $S(Q, \varphi)$ of 0.25 gave a very good fit of the data, especially the low concentration data. Setting the potential well width to 0.1 then gives a value for $B_2/V_{hs} = 0$. This value was used for all calculations and the volume fraction was determined by varying the temperature. The correlation length was set to 20 nm because of the position of the correlation peak in Figure 3. Therefore, the fit parameters for the data in Figure 3 were the size ($R_g$) of the micelles, the radius of gyration of the
polymer, and the scattering amplitudes. Equation 1 gave a very good fit to the data.

We evaluated the fit parameters of both samples by plotting them as a function of temperature (Figure 5). The amplitude $a_0$ increases with temperature indicating the increased number of micelles, but the amplitude will also depend on the precise nature of the interparticle (20 nm radius) interaction. We observed that the $R_g$ of the particle is very small (about 4 and 2 nm), but it is questionable whether this has any physical significance, as the low $Q$-data are multiplied by the $S(Q)$ values. It is noted that the calculated spectra show a much more detailed variation due to the use of a monodisperse structure factor $S(Q, \phi)$. Polydispersity in size and the distribution in wavelengths of the SAXS will effectively smooth the data. From SANS measurements (in 25 mM sodium phosphate buffer) we found a $R_g$ of 8 nm and an interparticle radius of 15 nm. Fitting the data with the Beaucage function only gives an $R_g$ of 15 nm. The volume fraction (number) of the micelles increases up to 30 °C. The micelles have a small radius of gyration and a large specific volume with a compact core and a fluffy “hairy” outside. In order to resolve the precise mass distribution in the BCN micelles, it would be required to do contrast variation using a technique such as SANS.

**BCN into Lactotransferrin.** We titrated BCN into lactotransferrin and measured the scattering spectra at each composition. The results are presented in Figure 6 and the drawn lines are fits to the data using the Beaucage function with two “levels”. For comparison we have plotted the spectra of BCN and lactotransferrin at 25 °C as well.

In Figure 7 we plot the coefficients of the fit. The data were fit to a Beaucage function with two Guinier and two power law regimes. The fitting function $FF(Q, a)$ is given by eq 4, following Pignon et al. 59

\[
FF(Q, a) = \ln \left[ a_0 \times \exp \left( -Q^2 \times \frac{(a_1)^2}{3} \right) + a_1 \left( \frac{\exp \left( \frac{Q \times a_1^2}{3} \right)}{Q} \right)^{\frac{1}{\gamma_1}} \right] + \ln \left[ a_2 \times \exp \left( -Q^2 \times \frac{(a_2)^2}{3} \right) + a_2 \left( \frac{\exp \left( \frac{Q \times a_2^2}{3} \right)}{Q} \right)^{\frac{1}{\gamma_2}} \right]
\]

(4)

In total there are 6 fit parameters. $a_1$ and $a_2$ are the radii of gyration. The other parameters are amplitudes. We plot the fit parameters in Figure 7 as function of the mixing mole fraction.

Only the first amplitude changes with the molar fraction of BCN, which means that the number of particles increases up to a maximum. That maximum is at a mole fraction of ~0.55. In addition, the scattering intensity at the maximum is 46 times higher than that of lactotransferrin. Since the weight concentration is almost constant during titration (BCN is 16.1 g/L and lactotransferrin is 14.3 g/L) it means that the molar mass of the aggregate is approximately 46 × 78 kDa = 3588 kDa. If the aggregate contains $n$ molecules, than (0.45 × $n$ × 78 kDa) + (0.55 × $n$ × 24 kDa) = 3588 Da and it follows that $n$ is about 74. Hence, each complex contains 33 lactotransferrin and 41 BCN molecules. This is an interesting result, as the number of BCN monomers in a pure BCN micelle is on the order of 30 to 40.62,54,55 This result seems to suggest that lactotransferrin binds into “the hairs” of the BCN micelle (Figure 8 gives an artist impression).

Figure 5. Plot of the fit parameters (of Figure 3, BCN) as a function of temperature. For the 4 g/L (left pane) and 16.1 g/L (right pane): $0$, amplitude of the Guinier function; $\Delta$, $R_g$ of sphere; $\bullet$, amplitude Debye function; $+$, $R_g$ Debye. The amplitude of the power law function was set to zero.

Figure 6. Plot of the scattering spectra on titrating BCN into lactotransferrin at 25 C. For comparison we plotted the scattering spectra of BCN and lactotransferrin as well. Lines are fits through the data. Low $Q$ scattering is maximal at equimolar composition. Fit parameters are presented in Figure 7.

Figure 7. Plot of the fit parameters of eq 4 (spectra of Figure 6) as a function of the mole fraction BCN. $Q$, amplitude Guinier; $\Delta$, $R_g$ (nm); $\square$, amplitude erf$1$; $+$ amplitude second Guinier level; $\triangle$, $R_g$ (nm); $\bullet$, amplitude erf$2$.
This is similar to the interaction depicted by Jackler et al.\textsuperscript{60} for bovine serum albumin when bound to spherical polyelectrolyte brushes composed of a polystyrene core and a poly(acrylic acid) shell.

We measured the changes in scattering intensity at mole fraction $x \approx 0.5$, so close to the electroneutrality point, as a function of temperature (Figure 9). The data fitting was good with the exception at the lowest $Q$-values. Clearly the Beaucage function cannot “depress” scattering intensity at low $Q$ due to the particle interactions which are incorporated in the structure factor. Nevertheless, it is of interest to present the fit data in Figure 9 together with the experimental low $Q$ scattering (we averaged the 4 lowest $Q$-values).

The averaged experimental low $Q$ values show a clear dissociation of the micelles when going to lower temperature (Figure 10). Curve fit is a sigmoidal function. Of the 6 fit parameters of the two-level Beaucage function, we notice that the first amplitude follows the experimental data, but due to the poor fit, at low $Q$ it is above the experimental amplitude. The $R_g$ is 12 nm and larger than the value from the combined Beaucage-Debye fit. It illustrates that parameters are correlated and depend on the chosen fit function.

Obviously the complexes dissociate and probably into monomeric complexes, i.e., one BCN plus one lactotransferrin at lower temperatures. The micellization of BCN is promoted by the binding of the lactotransferrin as the dissociation temperature is shifted to lower temperatures after binding. For pure BCN (Figure 4), the inflection point is at 14 °C, but in the presence of lactotransferrin, the inflection point is at 9 °C (Figure 10).

**Lactotransferrin into KCN.** We titrated lactotransferrin into KCN and the results were very similar to those for lactotransferrin into BCN. Therefore, we will not repeat the discussion, but present the results only. The scattering spectra are given in Figure 11, and in Figure 12 we present the fit coefficients as a function of mole fraction.

The scattering intensity at the max ($x \approx 0.83$) is about 80/2.783 = 28.7 times higher than for lactotransferrin. So, the molar mass of the complex is $78 \times 28.7 = 2242$ kDa. The molar fraction of KCN in the complexes is 0.83, so there are about 5 KCN molecules per lactotransferrin molecule. Thus, we have 65 KCN molecules binding with 13 lactotransferrin proteins. The molar mass of a KCN micelle would then be 1235 kDa, compared to 840 kDa for BCN. The $R_g$ of KCN and BCN is virtually the same at 14 nm. This implies that KCN micelles are more compact than BCN micelles. The micellization of KCN is not temperature dependent while that of BCN is, and so one would expect a stronger interaction and more compact structure within the KCN micelle.

**Charge Neutrality of the Complexes.** We downloaded the amino acid composition of BCN and KCN from the Swiss Protein Data Bank.\textsuperscript{28} We modified the sequence in that we added

![Figure 8](image1.png)  
**Figure 8.** Cartoon of the binding of lactotransferrin to BCN micelles. Lactotransferrin is depicted as a dumbbell according to protein structure in the Swiss Protein Data Bank.\textsuperscript{28}

![Figure 9](image2.png)  
**Figure 9.** Scattering spectra of lactotransferrin-BCN complexes as a function of wave vector at temperatures between 5 and 30 °C. The sample was equimolar ($x \approx 0.5$). A few fit curves are included.

![Figure 10](image3.png)  
**Figure 10.** Scattering intensities at the 4 lowest $Q$-values as a function of temperature, and the values of the fit parameters from eq 4. The sample was equimolar ($x \approx 0.5$) lactotransferrin and BCN. Panel A, all data; panel, B detail of panel A. ○, experimental scattering intensities at lowest $Q$-values; □, amplitude of Guinier fit; ◊, $R_g$; □, $a_2$, amplitude power law; ▼, $a_3$, amplitude of Guinier 2 fit; ●, $a_4$, $R_g$; ■, $a_5$, amplitude power law 2.

![Figure 11](image4.png)  
**Figure 11.** Scattering spectra of LF-KCN complexes and fits of eq 4. For comparison also the scattering functions of KCN and LF are presented (the lower scattering spectra).
From SAXS measurements we concluded that 1.2 BCN bind 1 lactotransferrin when charge neutrality is reached. The micellization of BCN is promoted by the binding of the lactotransferrin as the dissociation temperature is shifted to lower temperatures. KCN micelles bind lactotransferrin as well and in way similar to BCN. However, KCN carries only about 4 net negative charges and therefore 5 KCN are needed to neutralize the 21 positive charges of lactotransferrin. It is not easy to imagine how the 5 KCN molecules are arranged on a relatively small protein measuring about 5 nm, even more so because KCN is arranged in micellar structures. In a study of the binding of LF to native casein micelles we found (JAFCS 2013) that by increasing LF concentration from 1% to 2% w/w the ratio increased from 1 to 2. Adding higher levels may have increased the ratio further.

Now, one may ask (as one of the reviewers rightfully did) what’s the use of all this? We think it is extremely relevant to see that a pair of structured and unstructured proteins behaves quite similarly to a structured protein and a (large) polyelectrolyte molecule, e.g., gum Arabic, carrageenan, and DNA. Since there are many unstructured proteins in body fluids (e.g., saliva) there is a good reason to assume that these proteins bind to globular proteins as well and therewith serve protective functions, e.g., against enzymatic degradation which is often based on docking of charged groups on the protein. We are hesitant to call this a chaperone activity as no folding is involved. More generally, the complexes could be used to protect proteins during processing and drug delivery, even more so because the unstructured proteins can be cross-linked easily.

**REFERENCES**


