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PAPER

Co-acervates of lactoferrin and caseins

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On mixing positively charged lactoferrin (LF) with negatively charged caseins (*CN) it is observed that complexes are formed. The * stands for α, β, κ or Na. The size of the complex co-acervates appears to grow indefinitely and asymptotically near the point of charge equivalency. Away from the charge equivalent ratio it seems that build-up of (surface) charges limits complex size. We proposed a simple scaling law so as to predict the size of the complex. By assuming that surface charge density is constant or can reach only a maximum value, it follows that scattering intensity is proportional to $|(1 - x/x_{crit})|^{-3}$ where x is the mole (mass) fraction of the cationic protein and x_{crit} the value of the mole (mass) fraction at the charge equivalent ratio. Both scattering intensity and particle size obey this simple assumption. We investigated three different caseins, all of which formed co-acervate complexes with LF, but at different molar ratios. Critical composition varied inversely with pH, showing that charge neutrality is the determining factor. Sodium caseinate formed complexes as well but the growth was limited, presumably due to the intrinsic surfactant properties of whole casein. Adding NaCl diminishes the interaction and above 0.4 mol L^{-1} of NaCl no β-CN–LF complexes are formed. The charge neutral composition shifts to the LF side on adding NaCl, probably because the casein can wrap around the LF more effectively.

Introduction

Lactoferrin (LF) is an 80 kDa glycoprotein present in mammalian milk and is at very high levels in human milk, with more than 1 gram per litre. The concentration of LF in bovine milk is tenfold lower. LF is considered an important immunoregulatory protein and also has anti-microbial activity and, after proteolysis of LF in the stomach, lactoferricin has an even stronger anti-microbial activity. The microbiological activity of LF is attributed to its very strong iron binding capability and thus effectively sequestering its environment from Fe^{3+} ions.[1]

LF is a strongly cationic protein with a high pI (~8.9). It is known that LF can bind to anionic proteins such as β-lactoglobulin[2] but not to α-lactalbumin. It binds particularly strongly (at neutral pH) to osteopontin (OPN), which is a largely unstructured phosphorylated and sialated protein present in cheese whey.[1] OPN resembles the caseins present in casein micelles in mammalian milk that are responsible for calcium transport to the neonate. It was therefore conjectured that the LF formed co-acervate type complexes with the caseins in a similar way as osteopontin.[1] For recent reviews on (synthetic) polymeric co-acervates, see Voets *et al.*,[3] de Kruif *et al.*,[4] Weinbreck *et al.*[5] or Cooper *et al.*[6] In parallel with LF the enzyme lysozyme (LZ) is present in human milk at high levels as well. It is known that LZ binds to caseins.[7,8]

The isothermal titration calorimetry study of Yamniuk *et al.*[1] on the binding of LF to OPN is very interesting as it reveals in detail the thermodynamics of the binding. In addition they give an extensive overview of the biological activity of LF.

Recently we observed that adding LF to skim milk induced a disintegration of the casein micelles with the milk becoming more translucent with longer holding times.[9] However, on addition of LZ to milk, a completely different behaviour was observed as the milk flocculated.

Considering the contrasting behaviours of LF and LZ when added to milk, and also in the context of humanizing bovine milk, it was decided to investigate the binding of LF and LZ to the different caseins in some detail. We have reported on the interactions of the caseins with LZ in another paper.[10]

In this paper, we report the formation of co-acervates of LF with α-casein (ACN), β-casein (BCN), κ-casein (KCN), or sodium caseinate (NaCN). The similarity with polysaccharides is that the caseins are unstructured and could be viewed as a polymer or rather a poly-ampholytic, poly-electrolytic polymer. In addition, the caseins have a tendency to self-assemble into polymeric micelles. In that respect, the caseins resemble the di-block polymers that have been discussed by Voets *et al.*[3] The di-block polymers consist of a charged block and a hydrophilic but uncharged block. After co-acervation with another charged polymer the uncharged block stabilizes the complex by forming a steric coat.

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Materials and methods

Protein samples and solutions

The ACN and KCN were obtained from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The BCN was obtained from EURIA (Rennes, France). LF (>90% purity) and NaCN (NaCN) were obtained from the Fonterra Cooperative Group (Auckland, New Zealand). All proteins were used as supplied. Stock protein solutions of LF, ACN, BCN, KCN and NaCN of $\sim$10 mg mL^{-1} were prepared by mixing the proteins with water until dispersed. A small amount of sodium azide (0.02% w/v) was added as a preservative and then the protein solutions were filtered using syringe filters (0.4 μm) to remove dust and large aggregates. The protein concentration in each filtered protein solution was determined using UV absorption at 280 nm and the known extinction coefficients for each protein.[11,12] All experiments reported used the protein solutions on a w/w basis. Where required, the pH values of solutions were altered by the addition of 1 M HCl or 1 M NaOH while stirring.

Dynamic light scattering measurements

Dynamic light scattering (DLS) and conductivity experiments were performed using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, Worcestershire, U.K.). The details and methodology of this technique have been described previously.[13]

pH measurements

The pH of solutions were measured using a N61 Schott-Gerate combination pH electrode (Schott-Gerate, Hofheim, Germany) associated with a Radiometer PHM 92 Lab pH meter (Radiometer Analytical, Bronshoj, Denmark).

Results and discussion

After adding LF to skim milk it was observed that the milk samples became transparent with time.[9] We also observed that mixing pure caseins (*e.g.* KCN) with LF may lead to turbid suspensions or even a gelatinous sediment, depending on the mixing ratio. Thus it appeared that at neutral pH, the positively charged LF (pI $\approx$ 8.9) interacted with the negatively charged caseins (pI $\approx$ 4.6 to 4.8). From the experiments with the casein micelles in skim milk we found that the interaction was pH dependent.[9] It was therefore conjectured that the LF formed co-acervate type complexes with the caseins in a similar way as LF with osteopontin does.[1]

First we will present the data obtained by titrating LF into the different casein (ACN, BCN and KCN) and NaCN dispersions at approximately neutral pH, which is equivalent to the natural milk pH and about halfway between the pI of the LF and the casein proteins. During these additions we measure the size of the complexes and the scattering intensity at a backscattering angle (by DLS). These data show a clear complexation of the proteins and even an asymptotic growth of the complexes at the point of charge neutralization. It is observed that this critical composition, where both particle size and scattering intensity increase asymptotically, can be approached by titrating CN into LF or *vice versa*.

We made a few titrations at different pH *e.g.* pH = 6.0 (data not shown) and as expected the critical composition shifted towards the casein side because at lower pH the total charge of the casein will diminish. All these data show that the interaction and complexation of LF with the caseins is of an electrostatic character and relates to the charges on the proteins at the respective pHs. On adding NaCl the interaction of LF with the caseins weakens considerably, again indicating that complexation is mainly entropy driven through the release of the counter ions. In the final experiments we will present some additional data on the effects of temperature variations and dilutions on the interactions between the LF and the casein proteins.

Titration of KCN and LF

We titrated KCN (8.0 mg mL^{-1}) with LF (10.8 mg mL^{-1}) and *vice versa*, with the initial pH of the proteins adjusted to pH = 6.55. We measured the apparent particle size and the scattering intensity changes (Fig. 1A) as well as the pH changes (Fig. 1B) of the dispersion during the titration. The drawn line is according to eqn (3) and will be discussed below. It is clear that during titration, the pH is changing due to the release of extra protons into the solution. The protons must come from the carboxylic groups of the KCN molecules. We suppose that hydrogen bridges are formed between the amino and carboxylic groups. If the pH is chosen midway between the pK_as of the carboxylic groups (about

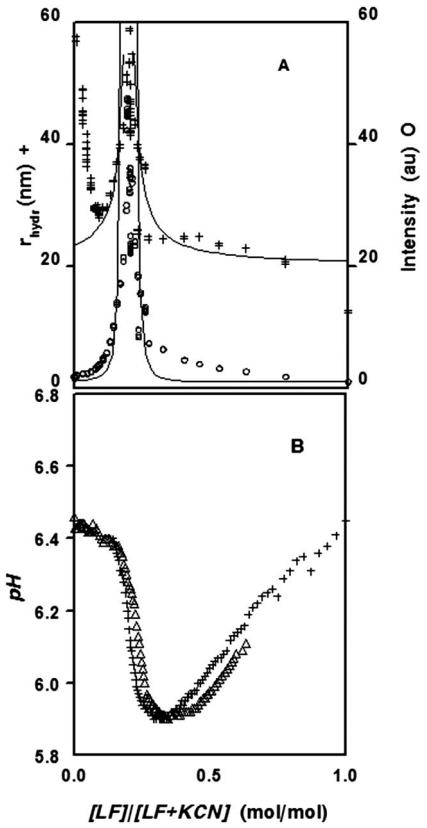

Fig. 1 (A) Measured scattering intensity (○) and apparent r_{hydr} (+) on titration of KCN into LF (and *vice versa*). Intensities were scaled to the LF scattering intensity. (B) Change in pH on titration of KCN into LF (+) and LF into KCN (△). Both solutions had an initial pH of 6.55.

pH 4) and the amine groups (about pH 10), then the curve would be more symmetrical and would show an increased pH at the casein side due to the binding of protons by the poly-cation.[3]

The abscissa is the mole fraction of LF in solution with respect to the total protein present. It is observed that at $x = 0.2$ mol mol^{-1} or 0.6 w/w (where x = LF/(LF + KCN)) the particle size and scattering intensity go through a maximum (Fig. 1A). This is the point where the number of positive and negative charges is equal and this corresponds to the inflection point in the pH curve (Fig. 1B). Since the molar mass of LF is about 6 times greater than that of KCN, the inflexion is at a molar ratio of LF/KCN of ~1/4 and thus at $x = 0.2$, suggesting that 4 KCN bind to one LF.

However, the size increases suggest that much larger particles are formed. In fact, near the equivalent point the system becomes almost milk white and clearly shows signs of flocculation. In the case of KCN, the flocculated particles do not completely re-disperse on continued titration. Nevertheless the pH curves from either side (*i.e.* KCN into LF or LF into KCN) overlap and the exchange of protons continues. The system does not re-disperse away from the equivalent point probably due to hydrophobic bonding of the KCN as the hydrophilic and sialated glyco-macro-peptide part of KCN will bind to the LF. Furthermore from the size measurements (see Fig. 1A) and electrophoretic analyses, using the zeta sizer, it is apparent that the KCN is polymerized through disulfide bonds and hydrophobic interactions; the latter may be partly disrupted on the addition of LF.

Titration of ACN and LF

A dispersion of ACN (9.8 mg mL^{-1}) was titrated into LF (10.8 mg mL^{-1}), and *vice versa*, and the particle size and scattering intensity (Fig. 2A) and also pH changes (Fig. 2B) were monitored. As with the KCN/LF experiments, the particle size, scattering intensity and pH changed during the titration. For the ACN/LF system, the equivalent point is at about 0.6 mol mol^{-1} or about 0.85 w/w. Since the molar mass of LF is about 3 times higher than that of ACN, the molar ratio of LF/ACN ~2 at the equivalent point. It is probably coincidence that this ratio appears to be a whole number as the complexes consist of many (tens of) molecules and the ratio is determined by the charge equivalency of the proteins.

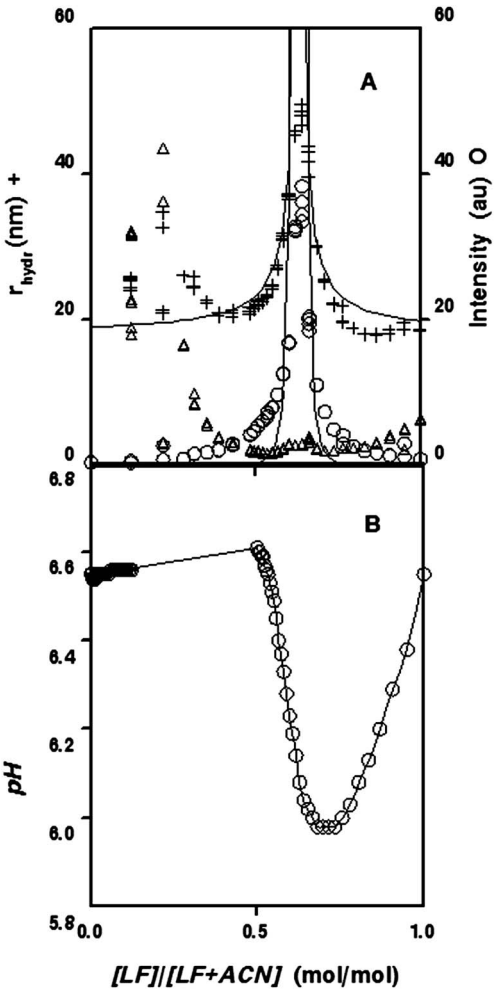

Fig. 2 (A) Measured scattering intensity (○) and apparent r_{hydr} (+) on titration of ACN into LF (and *vice versa*). Intensities were scaled to the LF scattering intensity. Triangles are: r_{hydr}^3/I_{scat} which is predicted to be approximately constant in the critical region (see text). (B) Change in pH on titration of ACN into LF (○) and LF into ACN (+). Both solutions had an initial pH of 6.55.

Titration of BCN and LF

The particle size and scattering intensity changes (Fig. 3A) and also pH changes (Fig. 3B) were followed during the titration of BCN (14.9 mg mL^{-1}) and LF (10.8 mg mL^{-1}). The pH-titration curve shows that the pH initially increased at low BCN/LF ratios and then passed through a minimum (Fig. 2B). The inflection point in pH corresponds closely to the asymptotic increase in scattering intensity, which was at $x = 0.5$ (mol mol^{-1}).

The ratio of r_{hydr}^3/I_{scatt} is constant especially in the region where the peak occurs. Thus the scattering intensity scales with particle size at a constant overall concentration and particles grow at the expense of the number of particles. Swelling of the particles would lead to a larger size but to a decrease in scattering intensity as the measurements are performed in a backscattering geometry. The form factor of larger particles already drops off at lower wave vectors. The Guinier slope increases while the ordinate remains constant for a swelling particle.

Titration of NaCN and LF

The titration experiments between NaCN and LF, as measured by DLS, are shown in Fig. 4A. The data are presented as a function of the mixing fraction on a w/w basis because the mole fraction is not defined. The apparent particle diameter of the pure NaCN solution was ~250 nm. This is not a realistic value as a solution with particles of this size should have been very turbid; however, the NaCN solutions were only very slightly opaque. In the DLS correlation function slow relaxation (diffusion) of caseins seems to dominate. After the first addition of LF (see second cuvette in Fig. 4B) the apparent size has dropped to ~45 nm.

The NaCN/LF system only showed a very weak complexation/aggregation between the proteins. Even at the critical mixing ratio of $x = 0.77$ w/w only a small increase in turbidity was observed (Fig. 4A). This is more clearly evident when comparing

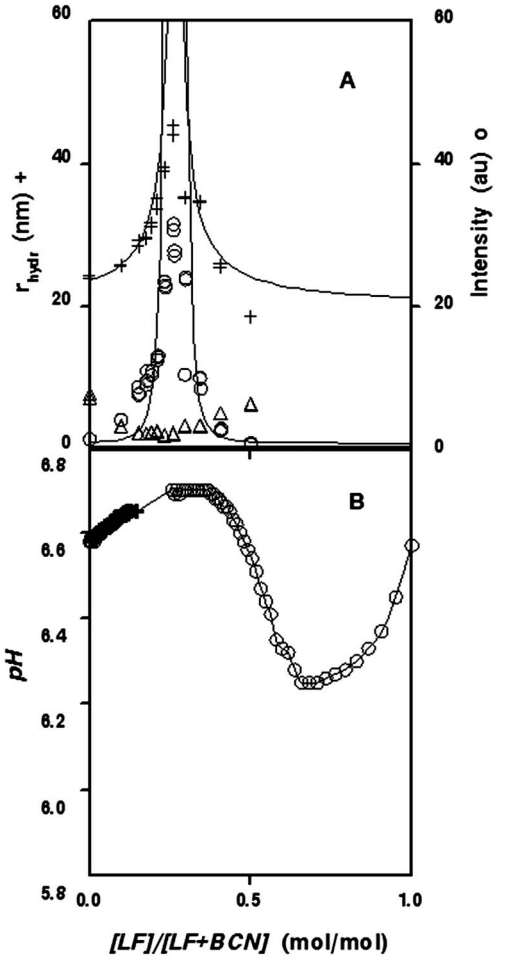

Fig. 3 (A) Measured scattering intensity (○) and apparent r_{hydr} (+) on titration of BCN into LF (and *vice versa*). Intensities were scaled to the LF scattering intensity. Triangles are: r_{hydr}^3/I_{scat} which is predicted to be approximately constant in the critical region (see text). (B) Change in pH on titration of BCN into LF (+) and LF into BCN (○). Both solutions had an initial pH of 6.55.

the turbidity of the NaCN/LF system (Fig. 4A) with those of the pure caseins (Fig. 1A, 2A and 3A), as the latter turned almost milk white at the critical mixing ratio. The NaCN/LF system showed some changes in turbidity but only slightly as illustrated in Fig. 4B. We suspect that the weak complexation of NaCN with LF was due to the higher ionic strength of NaCN solutions as the sample had a conductivity of 0.54 mS cm^{-1}, which is equivalent to a salt solution of 50 mmol L^{-1}.

The titration between NaCN and LF (Fig. 4A) can be directly compared to that of BCN with LF on a w/w basis (Fig. 5A). It is interesting that the critical mixing ratio for the NaCN/LF system is the same as that for the BCN/LF system (*i.e.* $x = 0.77$ w/w).

Considering all the experiments on the titration of the caseins (ACN, BCN, KCN, and NaCN) with LF, it seems that LF binds to the caseins but that the system is "self-stabilising" in that there is no unlimited growth at the critical mixing fraction. This resembles the behavior of complex core co-acervate micelles of polymers with a neutral but hydrophilic tail.[3,14] In these systems the hydrophilic but uncharged tail prevents unlimited growth and provides steric stabilization.

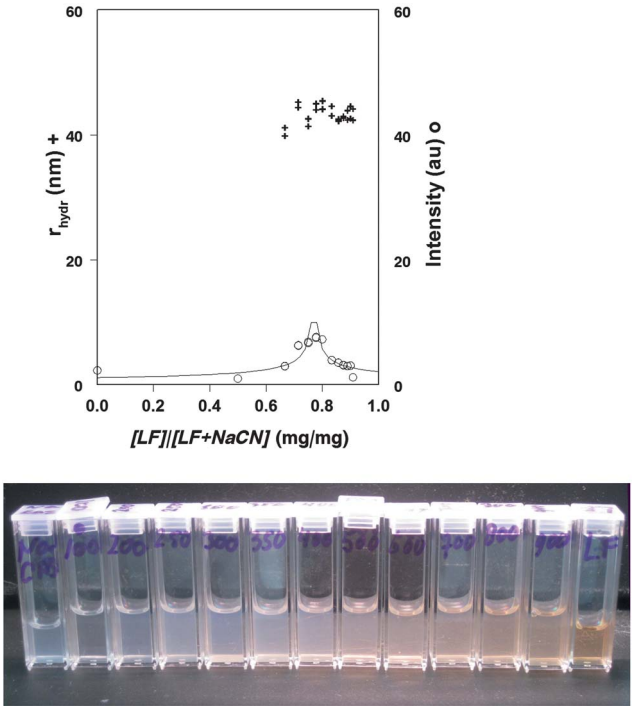

Fig. 4 (A) Measured scattering intensity (○) and apparent r_{hydr} (+) of a mixture of 5 mg mL^{-1} NaCN and LF in a total volume of 1.5 mL. (B) Photograph of cuvettes containing from left to right increasing amounts of LF. Outmost cuvettes contain pure NaCN and LF respectively. Note the decreased turbidity of the second cuvette.

Effect of NaCl on the interaction between BCN and LF

It was expected that the complexation would depend on salt concentration, and the level of salt in the NaCN/LF system was suspected to account for the low complexation between these proteins. The salt levels of the unmixed ACN, BCN and KCN

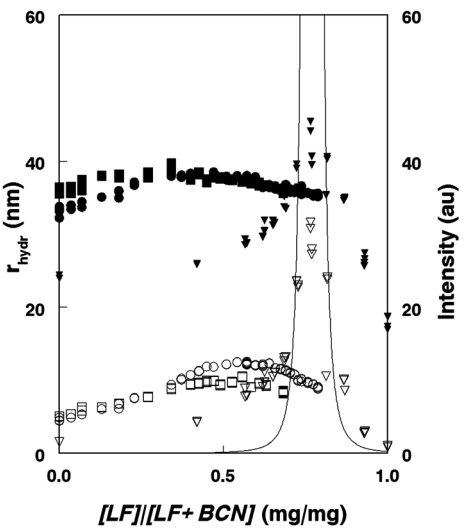

Fig. 5 Effect of NaCl concentration on the scattering intensity and apparent r_{hydr} when LF is titrated into BCN. The [NaCl] were: (□, ■) 0.14; (○, ●) 0.073; and (▽, ▼) 0.01 mol L^{-1}. Open symbols denote scattering intensity and filled symbols, apparent hydrodynamic radius.

solutions were estimated by measuring conductivity. Normalized on 10 mg mL^{-1} we found that the conductivity of these solutions was equal to or lower than a 10 mmol L^{-1} NaCl solution. This can be considered as the salt content of the serum as the protein would contribute only slightly to the conductivity. The Debye–Huckel length, $1/\kappa$, is 3.3 nm and is therefore about the same as the protein dimension. As a result, the protein charges will strongly interact.

The effect of NaCl on the size and scattering intensity when LF and BCN were mixed is shown in Fig. 5. Clearly the addition of NaCl has a dramatic effect on the complexation. Firstly, the size of the complexes remains small but larger than without NaCl. Secondly, the divergence at x_{crit} is absent when NaCl is present. It seems that low levels of NaCl break up the complexation. At salt levels of 0.073 M and 0.14 M, the Debye–Huckel lengths are reduced to 1.1 nm and 0.8 nm respectively.

Interestingly, in the samples with added NaCl, the maximum in size occurs at a smaller x than the maximum in scattering intensity, respectively at $x \approx 0.35$ and $x \approx 0.58$. This could be explained as follows. We are measuring at a backscattering angle of 177°. DLS measurements indicate an increasing particle size, which may be due to a growth in mass of the complex. If, however, this is due to swelling only, then this will lead to a lower scattering intensity because the form factor of the particle (in the case of swelling) drops off at a lower scattering angle. So it appeared that maximum scattering intensity was reached at $x =$ 0.42, 0.58 and 0.77 w/w with NaCl levels of 0.14 M, 0.073 M and 0.01 M, respectively.

After titration of BCN into LF (or *vice versa*) we took the final turbid sample and then titrated NaCl in to see how stable the formed complex was. Several samples with different compositions and initial turbidities were tested (Fig. 6A). On adding NaCl to these turbid BCN–LF samples, the intensity goes down and then levels off when the NaCl concentration exceeds about 0.4 mol L^{-1}. This corresponds to $1/\kappa = 0.5$ nm, which is about the distance between charged groups in the protein. The particle size (not shown) also decreased, often below that of the BCN suspension indicating that initial BCN clusters were dispersed during complexation.

We normalized the data by a factor $x + 4*(1 - x)$ because the scattering intensity of pure BCN was 4 times stronger than that of pure LF. As a result of this normalization, the data collapsed onto a single curve showing that differences in intensity levels were due to differences in composition and scattering power rather than a different behaviour on adding NaCl (Fig. 6B). The samples in Fig. 6 represented by the (inverted) triangle symbols are both samples (with $x = 0.42$) but repetitions of the same experiment on different days. However in the experiment with the inverted triangles we started the DLS measurement immediately after mixing and collected time averaged data for 120 s. After that a second measurement of 120 s was started. The apparent particle size in the first run was slightly smaller than that in the second run and constant after that and equal to the experiment conducted a few days previously. We therefore suggest that the particle size was relaxed to an equilibrium value after the second run. From this we conclude that the relaxation time of the system is a few minutes on adding NaCl.

It was remarkable to see that in some of the samples, the intensity initially increased on adding NaCl. This can be

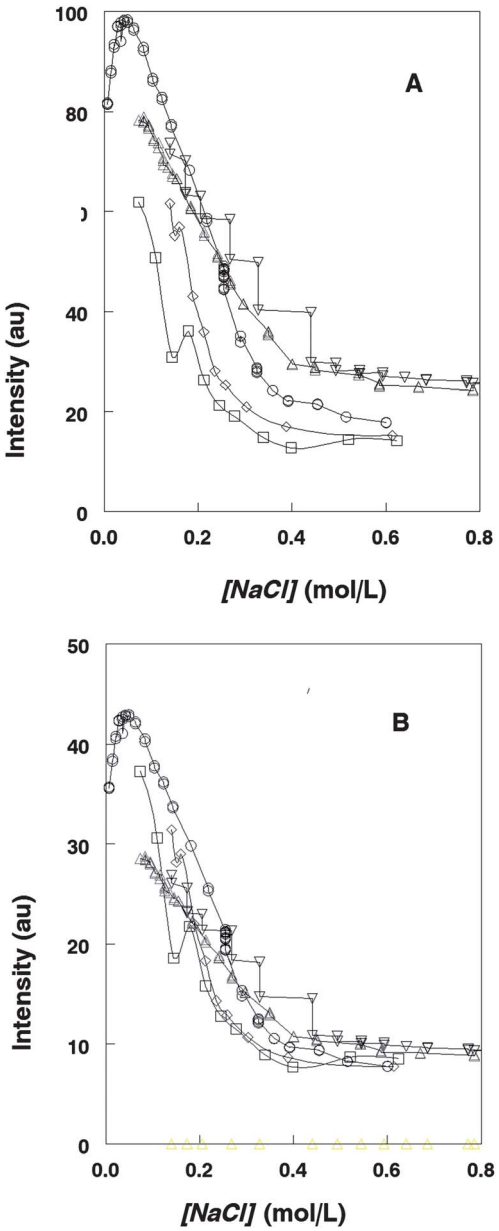

Fig. 6 (A) Measured intensity decrease as a function of [NaCl] (mol L^{-1}) for BCN and LF mixtures at various complex compositions. (B) Intensity scaled on scattering of the pure components. Mole fractions of the proteins were: △, ▽, $x = 0.42$; □, $x = 0.43$; ○, $x = 0.57$; ◇, $x = 0.68$; and □, $x = 0.78$. The first point corresponds to the initial salt concentration.

understood as follows. For a sample with low NaCl and at an x-value smaller than x_{crit} the scattering intensity is low. On adding salt, the x_{crit} moves to lower x-values so the samples move towards the actual x-value and as a result intensity increases. On adding more NaCl, the x_{crit} is reduced further and so the intensity will drop again when the x_{crit} passes the actual x-value.

From the data collected, we assembled a 3D picture so as to illustrate the state diagram of the BCN–LF–NaCl system (Fig. 7). This 3D picture illustrates the scattering behaviour as a function of composition, but is not a 3D fit to the data but rather a mesh placed over the available data. Along an iso-ionic

strength (NaCl) line the scattering intensity goes through a maximum, and the position of this maximum moves toward a higher x, *i.e.* toward the LF side, as the NaCl levels increase. Going along isopleths near $x = 0.55$ (*i.e.* a line of constant composition) the scattering intensity may increase and then subsequently decrease as well.

Stability of the BCN–LF complexes

The stability of the complexes decreases with increasing NaCl concentrations. We therefore assumed that the complexes obey a mass action law which may be written as:

$$p[\mathrm{BCN}] + q[\mathrm{LF}] \leftrightarrow [\mathrm{BCN_pLF_q}]$$

and an associated equilibrium constant (K_{coac}) that will depend on the NaCl concentration in solution.

We therefore mixed BCN and LF at pH 6.56 and at $x_{crit} = 0.77$. We then diluted this dispersion with 10 mM of NaCl, which is the estimated ionic strength of the protein mixture as determined from conductivity measurements. In Fig. 8 we present the measured intensity and size data. We corrected the data by subtracting the scattering intensity of a 1000-fold diluted sample and by dividing the scattering intensity by the concentration factor, so as to correct for the fact that there are fewer particles in the scattering volume.

The scattering intensity decreases by a factor of 2 on diluting 100 times with 10 mM of NaCl. Although it may seem that the complexes dissociate, if it was a mass action law equilibrium the slope should have been much steeper. We therefore repeated the experiment using de-ionized water as the dilutant. In this case the scattering intensity and size increased. We therefore think that using the 10 mM NaCl may have effectively increased the ionic strength slightly, which caused dissociation of the complexes, whereas adding water decreased the ionic strength and the co-acervation is promoted, notwithstanding the dilution.

The scattering intensity also increased on adding a mixed cation–anion exchange resin, which will lower ionic strength in the solution and therefore promote complexation. Thus in order to determine the mass law equilibrium it will be necessary to equilibrate extensively against a bath of constant ionic strength or use the filtrate of the protein solution for dilution.

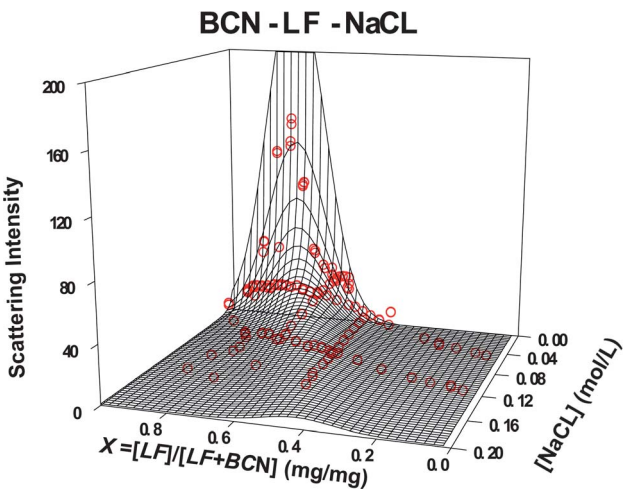

Fig. 7 State diagram of scattering intensity as a function of protein composition x and salt concentration. Note that the mesh is not a fit to the data but an overlay.

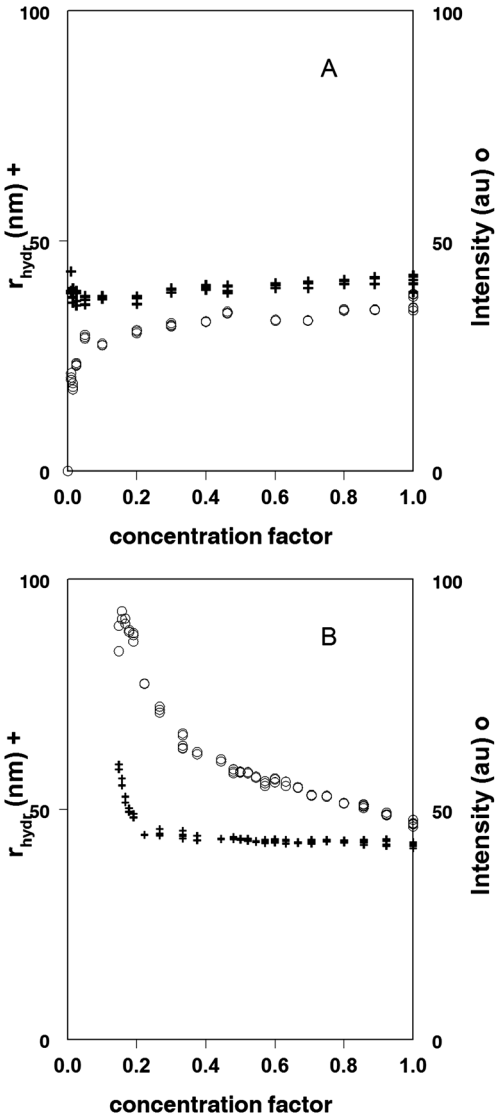

Fig. 8 Measured particle size (+) and scattering intensity (○, divided by the volumetric concentration factor) of coacervates of LF and BCN. (A) On dilution with 0.010 mol L^{-1} NaCl and (B) on dilution with de-ionized water.

Discussion and modelling of the experimental results

The mixing (mole) fraction:

$$x = [\mathrm{LF}]/([\mathrm{LF}] + [{*}\mathrm{CN}]) \quad (1)$$

is the relevant quantity as it determines the number of (positively) charged proteins over the total number of protein particles present. The * stands for A, B, K or Na, and therefore represents the different casein samples. Voets *et al.*[3] define a charge fraction f^+ (and $f^- = 1 - f^+$), which is the number of chargeable groups in the polymers with respect to the total, independent of the actual

dissociation state. Here we have proteins with both positive and negative chargeable groups and with varying pK. We therefore use the unambiguously defined mass fraction (or mole fraction) as shown in eqn (1), where [LF] and [*CN] may be either in mol L^{-1} or in g L^{-1} (or mg mL^{-1} or w/w) as indicated.

At a critical fraction, x_{crit}, the number of positive and negative charges is just equal. This critical value depends on the degree of dissociation for a weak polyelectrolyte and thus on how far the pH is from the pK of the dissociating group. For the moment we assume that we are about halfway between the pK_a and pK_b. In the case of caseins plus LF we chose a pH near 6.5–6.6 as this is about halfway between the respective isoelectric points of the caseins and LF and also because it is close to the pH in a natural milk system.

If the ratio, x, is away from x_{crit} then the complexes formed will be charged as there is a surplus of either polyelectrolyte (PE). It is an observable fact that the complexes formed are stable and remain constant in size with time and on changing the temperature. We did several experiments (data not shown) where we varied the temperature from 5 to 45 °C. There was, however, hardly any change in particle size and or scattering intensity. Therefore the complexes cannot be neutral. However, the charging of the complexes is limited because of the internal repulsion. If a *CN and a LF molecule carry p and q elementary charges respectively then the charge of a complex with composition x will be given by $p + x(q - p)$. At x_{crit} the net charge is zero so $(q - p) = -p/x_{crit}$. At a composition x, the number of excess charges is proportional to $p(1 - x/x_{crit})$. Now we assume that the growth of the complexes to a volume $4/3\pi r^3$ is limited by the accumulation of surface charge. Thus for a complex of size r, the surface charge is: $|(1 - x/x_{crit})|r^3/r^2 =$ constant, or

$$r^3 \approx 1/|(1 - x/x_{crit})|^3 \quad (2)$$

We use this reasoning intuitively, but it was shown by Park *et al.*[15] that oppositely charged macro-ion complexes overcharge spontaneously. It is therefore of interest to summarize the results of their calculations. Park *et al.*[15] considered the charge state of polymers interacting with oppositely charged (cylinders and) spheres. In the low salt limit, they apply charge renormalization, which means that the macro-ions are not fully charged because a number of the counter ions are "bound/condensed" to the macro-ion. This is the so-called Manning condensation.

A PE polymer will then bind to the spherical macro-ion because, on binding, the counter ions and the condensed ions are released.[15,16] So even if there is no matching in charges, the PE will bind and the complex will have the charge of the polymer. By taking into account the bending energy of the polymer that wraps around the sphere (cylinder), the complex will be undercharged for a high bending energy. Thus the complex may be overcharged, neutral (by coincidence) or undercharged according to Park *et al.*[15] Experiments on DNA–protein complexes and computer simulations confirm this (counter-intuitive) picture.[15] Although DNA and proteins may have specific interactions, it still shows that overcharging may occur. Also it gives a ground for our intuitive notion that the charge state of the complexes will be somehow limited.

Since we titrate one PE into the other, the total mass concentration (*i.e.* mg mL^{-1}) is about constant as the two PEs usually have similar concentrations. For scattering particles that change their association behaviour at constant total concentrations, the scattering intensity is proportional to $\varphi^* r^3$, where φ is the volume fraction and about constant here. Complex co-acervates have a density equal or even higher than the primary particles.[4] Thus scattering would be described by:

$$I_{scat} \approx |(1 - x/x_{crit})|^{-3} + \text{constant} \quad (3)$$

It is clear that salt concentration has an influence as well, because on adding NaCl, the complexation diminishes and the release of counter ions contributes less to the free energy gain. At a critical NaCl concentration the complexes fully disintegrate and the dispersion becomes clear again. So it seems that the complexation is entropy driven. We expect that the power −3 applies for a given (low) salt condition and probably in the regime where counter ion condensation occurs because that limits the charge of the complexes. Eqn (3) cannot be correct at $x = 1$ or at $x = 0$ as it would predict a scattering level proportional to x_{crit}^{-3}. At $x = 0$ and $x = 1$, the scattering level is determined by the pure protein (including some aggregates); however, on approach of x_{crit}, the scattering goes up by an order 10 at least. We therefore add a constant level to eqn (3) so as to fit the whole scattering intensity curve. This constant prevents an easy determination of the exponent in a log–log plot but we found that the exponent is −3 or even higher.

In DLS a weighted average particle size is measured by $r_{hydr} = \langle r^6\rangle/\langle r^5\rangle$, hence larger particles, if present, are heavily weighted. Also the presence of initial clusters, as in the case of KCN will cause irregularities. The particle size would, in the present situation, thus scale as the cube root of the intensity, or $r_{hydr}^3/I_{scat} =$ constant. Indeed this ratio appears to be constant and particularly in the critical complex region (see the triangles in Fig. 2A and 3A). Deviations occur only at x-values close to the pure components as a result of clusters in the stock samples. Thus even though the model is simple, it is self-consistent.

After a titration experiment we used the resulting samples to test the temperature dependence of the particle size and scattering intensity. For instance, for the KCN/LF system, $x = 0.8$ w/w we did not see a notable change on varying the temperature from 40 °C to 5 °C. Similarly for the ACN/LF system there was virtually no temperature dependence of the scattering intensity. However, for the BCN/LF system we found that the scattering intensity decreased by a factor of two in the temperature range of 5 to 35 °C, for $x_{crit} = 0.77$, so just at the critical composition of the complex. This suggests that the hydrophobic tails of BCN contribute to the complexation as BCN is monomeric at 5 °C and forms micelles at 35 °C. On the LF side, at $x = 0.81$ the variation was smaller than a factor of two indicating that the temperature dependence decreases away from x_{crit} where charge dominates as proposed.

Van der Burgh *et al.*[17] studied complex core micelles of oppositely charged polymers. The difference with the present study is that one of their PEs had a neutral but hydrophilic segment. This neutral segment limited aggregation by providing the co-acervate core with a hydrophilic corona. They proposed a state diagram of the composition of the complexes. They defined a preferred micellar composition (PMC) which is equivalent to charge neutrality of the complexes. Away from the

PMC (with higher concentration of the negative di-block) micelles are formed with an excess negative charge. On further lowering the mole fraction the micelles disintegrate until they form soluble complex particles. This occurs at a critical excess anionic charge at which free di-block polymers become present.

Although our system is different in that there is no neutral block present, we propose that aggregation is limited by the build-up of surface charge and not by the presence of a neutral block. In addition, if the (more or less) neutral block would hinder further association (as proposed by Van der Burgh *et al.*[17]), it is noted that the blocks in our casein systems are hydrophobic and not hydrophilic. Thus it is difficult to imagine that association will be limited by the neutral blocks. Our light scattering results, particle size measurements and titration data show great similarity with the results of van der Burgh *et al.*[17] but the details are different. Here we account for the results assuming this straightforward and simple model of limited charge accumulation.

In their extensive review paper Voets *et al.*[3] discuss in great detail the formation of complex co-acervate core micelles in which one of the PEs has a neutral hydrophilic block. Our system has some similarity with that, if we consider LF as the cationic PE and the caseins as the di-block polymers. Pure caseins can form micelles. For example BCN forms micelles of a few dozen monomers,[18–20] whereas KCN has a large micellisation constant.[20,21] However, for the caseins, their slightly charged blocks are hydrophobic, especially in the case of KCN. Thus on complexation of the charged groups, a hydrophobic tail sticks out. As expected this leads to extensive aggregation in the order KCN > BCN > ACN as is observed. The hydrophobic aggregation is then limited by surface charge build-up. BCN is micellar at 30 °C, but these micelles dissociate at low temperatures so that BCN is monomeric at a few °C. This is the reason we see an effect of temperature on the LF–BCN complexes that were close to x_{crit}.

Lindhoud *et al.*[14] studied the influence of salt on the formation of complex core co-acervate micelles using SANS and light scattering and found that the complexes disintegrate at relatively high salt concentrations. They concluded, in view of the high salt concentration, that the driving force of complexation is not driven by electrostatics. It is interesting to note that Lindhoud *et al.*[14] observed a plateau value in their scattering intensity, accompanied by an increase of particle size on adding salt. This is fully consistent with a swelling particle due to diminished (internal) interactions.

Weinbreck *et al.*[5] did similar experiments on the β-lactoglobulin/gum Arabic system, which formed co-acervate complexes near pH 4.0. The state diagram of complex formation shows a clear salt dependence. Weinbreck *et al.*[5] also performed SAXS experiments on the concentrated co-acervate phase. The scattering intensity decreased with increasing salt concentrations. The interpretation given was that the (electrostatic) repulsion between complexes decreased. As a result, osmotic compressibility of the (concentrated) system decreased, which was found to be linear with salt concentration. In other words, (electrostatic) repulsion keeps the complexes apart.

This observation was confirmed by rheological measurements on the co-acervate phase.[5] In addition, it was shown that the protein and the polysaccharide in the complex diffused slowly and independently, allowing the system to change slowly with time towards an equilibrium situation. De Vries *et al.*[22] developed a theoretical model in which they showed that complexes can be formed through the patch-wise distribution of charges, even at the 'wrong' side of the pI.

Conclusions

In summary, it seems that complexation between oppositely charged proteins as studied herewith is mainly entropy driven as was originally proposed by Voorn[23] and Overbeek and Voorn,[24] based on the experimental work of Bungenberg de Jong and Kruyt.[25–27] Here we propose that the build-up of surface charges limits growth of the protein complexes until complete charge neutralization is achieved at a critical value, x_{crit}. If the x_{crit} value changes with pH, then the stoichiometry of the complexes derives from the charge neutrality and is not linked to a molar stoichiometry, unless the excluded volume (steric) restricts the charge stoichiometry.

Notes and references

1 A. P. Yamniuk, H. Burling and H. J. Vogel, *Mol. Immunol.*, 2009, **46**, 2395–2402.
2 F. Lampreave, A. Piñeiro, J. H. Brock, H. Castillo, L. Sánchez and M. Calvo, *Int. J. Biol. Macromol.*, 1990, **12**, 2–5.
3 I. K. Voets, A. de Keizer and M. A. Cohen Stuart, *Adv. Colloid Interface Sci.*, 2009, **147–148**, 300–318.
4 C. G. de Kruif, F. Weinbreck and R. de Vries, *Curr. Opin. Colloid Interface Sci.*, 2004, **9**, 340–349.
5 F. Weinbreck, R. H. Tromp and C. G. de Kruif, *Biomacromolecules*, 2004, **5**, 1437–1445.
6 C. L. Cooper, P. L. Dubin, A. B. Kayitmazer and S. Turksen, *Curr. Opin. Colloid Interface Sci.*, 2005, **10**, 52–78.
7 A. L. de Roos, P. Walstra and T. J. Geurts, *Int. Dairy J.*, 1998, **8**, 319–324.
8 J. L. Thapon and G. Brule, *Lait*, 1986, **66**, 19–30.
9 S. G. Anema and C. G. de Kruif, *Biomacromolecules*, 2011, **12**, 3970–3976.
10 S. G. Anemaand C. G. de Kruif, submitted.
11 H. E. Swaisgood, in *Developments in Dairy Chemistry*, ed. P. F. Fox, Elsevier Applied Science, London, 1982, vol. 1: Proteins, pp. 1–59.
12 H. E. Swaisgood, in *Advanced Dairy Chemistry*, ed. P. F. Fox, Elsevier Applied Science, London, UK, 3rd edn, 1992, vol. 1: Proteins, pp. 63–110.
13 S. G. Anema and Y. Li, *J. Dairy Res.*, 2003, **70**, 73–83.
14 S. Lindhoud, L. Voorhaar, R. de Vries, R. Schweins, M. A. Cohen Stuart and W. Norde, *Langmuir*, 2009, **25**, 11425–11430.
15 S. Y. Park, R. F. Bruinsma and W. M. Gelbart, *Europhys. Lett.*, 1999, **46**, 454–460.
16 A. Wittemann and M. Ballauff, *Phys. Chem. Chem. Phys.*, 2006, **8**, 5269–5275.
17 S. van der Burgh, A. de Keizer and M. A. Cohen Stuart, *Langmuir*, 2004, **20**, 1073–1084.
18 L. M. Mikheeva, N. V. Grinberg, V. Y. Grinberg, A. R. Khokhlov and C. G. de Kruif, *Langmuir*, 2003, **19**, 2913–2921.
19 J. E. O'Connell, V. Y. Grinberg and C. G. de Kruif, *J. Colloid Interface Sci.*, 2003, **258**, 33–39.
20 C. G. de Kruif, R. Tuinier, C. Holt, P. A. Timmins and H. S. Rollema, *Langmuir*, 2002, **18**, 4885–4891.
21 C. G. de Kruif and R. P. May, *Eur. J. Biochem.*, 1991, **200**, 431–436.
22 R. de Vries, F. Weinbreck and C. G. de Kruif, *J. Chem. Phys.*, 2003, **118**, 4649–4659.
23 M. J. Voorn, *Recl. Trav. Chim. Pays-Bas*, 1956, **75**, 317–330.
24 J. T. G. Overbeek and M. J. Voorn, *J. Cell. Comp. Physiol.*, 1957, **49**, 7–26.
25 H. G. Bungenberg de Jong, in *Colloid Science*, ed. H. R. Kruyt, Elsevier, Amsterdam, 1949, vol. II, ch. VIII, pp. 232–258.
26 H. G. Bungenberg de Jong, in *Colloid Science*, ed. H. R. Kruyt, Elsevier, Amsterdam, 1949, vol. II, ch. VIII, pp. 335–432.
27 H. G. Bungenberg de Jong and H. R. Kruyt, *Proceedings of the Koninklijke Akademie Van Wetenschappen Te Amsterdam*, 1929, vol. 32, pp. 849–856.

