Complex coacervation of lactotransferrin and β-lactoglobulin

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ABSTRACT

Hypothesis: Oppositely charged proteins should interact and form complex coacervates or precipitates at the correct mixing ratios and under defined pH conditions.

Experiments: The cationic protein lactotransferrin (LF) was mixed with the anionic protein β-lactoglobulin (B-Lg) at a range of pH and mixing ratios. Complexation was monitored through turbidity and zeta potential measurements.

Findings: Complexation between LF and B-Lg did occur and complex coacervates were formed. This behaviour for globular proteins is rare. The charge ratio’s of LF:B-Lg varies with pH due to changing (de) protonation of the proteins. Nevertheless we found that the complexes have a constant stoichiometry LF:B-Lg = 1:3 at all pH’s, due to charge regularization. At the turbidity maximum the zeta potential of complexes is close to zero, indicating charge neutrality; this is required when the complexes form a new concentrated liquid phase, as this must be electrically neutral. Complexes were formed in pH region 5–7.3. On addition of salt (NaCl) complexation is diminished and disappears at a salt concentration of about 100 mMol. The coacervate phase has a very viscous consistency. If we consider the proteins as colloidal particles then the formed complex coacervate phase may have a structure that resembles a molten salt comparable to, for example, AlCl₃.

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1. Introduction

Complex coacervation is the phase separation into a concentrated and (very) dilute phase of two oppositely charged macromolecules. The first observations of coacervates was made by Thiebax [1] and it was Bungenberg de Jong and Kruyt [2] who made a systematic study of the gelatin and gum Arabic system. Since these pioneering studies there is now a wealth of literature on the interaction and coacervation of proteins with (weak and strong) polyelectrolytes and among them the interactions with DNA. See reviews by Izumrudov [3], Rubinstein and Papoian [4], de Kruif et al. [5], Schmitt and Turgeon [6], and Kayitmazer et al. [7]. The interaction or complexation of globular proteins with other proteins is rather more scarcely studied. Recent publications are on the interaction of the globular proteins lactotransferrin (LF) [8] or lysozyme [9] with caseins, which can also be considered as a weak polyelectrolyte.

Reports on the complexation of globular proteins are even scarcer. One may distinguish four types of complexation. Firstly, there is the association of proteins such as insulin, [10] and β-lactoglobulin (B-Lg) into oligomeric assemblies [11,12]. Secondly, there is proteins self-assembly, such as the formation of protein virus coats and the formation of microtubules such as the cell cytoskeleton [13], as well as the formation of protein nano-tubes from partially hydrolysed α-lactalbumin [14]. Proteins can also self-assemble into fibril structures, for example heating B-Lg at low pH and high temperature leads to fibrils and even nanotubes [15]. Pure β-casein and κ-casein are known to form soap-like micellar structures with a hydrophobic core and a hydrophilic corona [16,17]. Many storage proteins also form larger complexes of (slightly) different proteins, e.g. soy proteins [18]. Thirdly, there is coacervation, i.e. the phase separation into a concentrated and dilute liquid may occur if there is a weak attractive interaction between the proteins (peptides). This occurs when the second virial coefficient is slightly negative. Recently it was shown that mussel foot protein (MFPp-35) undergoes a coacervation (i.e. liquid–liquid transition) in salt water [20]. In addition, the crystallization of proteins from solution is also an example of a new phase [19]. Fourthly, there is hetero-association and complex coacervation. For example, when apo-α-lactalbumin (negatively charged) is mixed with lysozyme (positively charged), large spherical aggregates can be formed under certain pH and concentration conditions [21–24]. Similar micron-sized

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microspheres could be formed when ovalbumin, avadin, or bovine serum albumin respectively were mixed with lysozyme [25]. These spherical particles were considered to form through a self-assembly process.

These examples of protein hetero-association do not show a phase separation. They lead to new assemblies but remain a one phase system. Here we aim to investigate the association of two proteins into a new liquid phase, similar to the coacervation of oppositely charged polyelectrolytes.

Recently we briefly reviewed the different forms of hetero-complexes which can be assembled in a water environment including non-protein aggregates [26]. We further showed that LF and β-casein or κ-casein form complexes. The structure of the complexes was determined using SAXS and it was shown that LF binds to the corona of the micellar structures formed by both β-casein and κ-casein, indicating that these are hierarchical structures [26].

Rasa et al. [27] studied the complexation of negatively charged silica spheres with positively charged alumina coated silica spheres in low salt ethanol solutions, which allows a long range interaction. At some mixing ratios stable dispersions were formed due to overcharging of the complexes, whereas sediments were formed at mixing ratios where the complexes were charge-neutral. Hetero-aggregation of oppositely charged colloids is reviewed by Islam et al. [28] and more recently by Lopez-Lopez et al. [29]. These reviews focus on the kinetics and structures of the formed aggregates.

Here we will study the interaction of two globular proteins, i.e. B-Lg and LF, which is called hetero protein association. We find that the complexes show all the hall marks of a coacervate rather than associate, aggregate or precipitate. While working on our paper we became aware of a recently published study by Yan et al. [30]. They studied a system that is nominally the same as ours; however, they stated that interaction only occurred over a narrow pH range and they presented a complex interpretation of the results based on both kinetic and equilibrium arguments. Our results, obtained independently, show complexation over a wide pH range, and the results allow for a simple and straightforward interpretation and quantification.

LF is a large globular protein in milk with immune-regulatory and bactericidal properties. LF (M = 78 kDa) has a pl of 8.3 and at lower pH it can have several 10s of positive charges. B-Lg is a globular protein in milk and is the most abundant protein in cheese whey. B-Lg has a pl of 4.8 and a molar mass of 18 kDa. It easily forms dimers and some larger oligomers depending on pH, temperature and concentration [11]. On mixing LF with B-Lg complexes or coacervates are formed. Here we aim to present our new results and outline the origin of the differences between our results and those of Yan et al. [30].

Stock samples of LF and B-Lg were prepared at a concentration of ~20 mg/mL. The concentration was checked by UV spectroscopy at 280 nm using the known extinction coefficients for LF and B-Lg [32]. Working solutions were prepared by diluting the stock solutions by a factor of 4 to give concentrations of ~5 mg/mL. The pH of the working solutions was adjusted to the desired values using 1 M HCl or 1 M NaOH. The conductivities of the working solutions were 0.3 and 0.4 mS/cm for the B-Lg and LF respectively. Based on a standard curve of conductivity versus [NaCl], this corresponded to an ionic strength of approximately 0.002 and 0.003 M for the B-Lg and LF, respectively. This is about 10 and 50-fold more than the molar concentration of the proteins but very low on an absolute scale and the Debye–Hückel length would be about 5 nm or the size of LF.

2.2. pH measurements

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

2.3. Turbidity/Transmission measurements

Transmission or absorbances of samples were measured at 900 nm in 1 cm pathlength plastic cuvettes using a Jasco V580 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan). All pure B-Lg and LF solutions had transmissions close to 100% in both water and the salt solutions used. In a few experiments, the scattering intensities were monitored with temperature using dynamic light scattering (DLS) in a Malvern Zetasizer nano (Malvern Instruments, Malvern, Worcestershire, UK), as has been described previously [8,9].

2.4. Zeta potential measurements

The zeta potential of the particles in solution was measured using a Malvern Zetasizer nano and the associated disposable folded capillary cells (Malvern Instruments, Malvern, Worcestershire, UK). Only samples in the turbid region were monitored and no further dilution or sample preparation was performed.

3. Results and discussion

3.1. pH titration

We titrated a ~5 mg/mL LF into a ~5 mg/mL B-Lg and measured the pH. Since the protein concentrations in both stock solutions were nearly equal, the total protein concentration is constant. Also the initial pH of both solutions was the same (~pH 6.7). Nevertheless the titration curve, i.e. pH as a function of the [LF]/(B-Lg + LF)] ratio in Fig. 1, shows a very typical behavior. Initially pH goes up, then down and then returns to the original level. The reason for this behavior is charge regularization [33]. LF binds an excess of B-Lg (compared to the strict stoichiometric ratio of the charges). The excess B-Lg that binds to LF can set free some protons and as a result pH drops somewhat. Note that when there is an excess of B-Lg some extra protons are bound. The precise shape of the curves depends on where the pH is between the two pl's. If halfway a more symmetrical curve is obtained with a maximum and a minimum [8]. It is noted that the strongest interaction occurs at a LF mole fraction of 0.25 indicating that 1 LF binds three B-Lg. This pH titration curve clearly shows that there is an interaction between the two proteins.
3.2. Turbidimetric titration

In the first turbidity experiments, a series of solutions with different ratios of B-Lg and LF were prepared in separate cuvettes and the turbidity at 900 nm of the solutions were determined after mixing for 30 min. In Fig. 2 we show examples of the samples in cuvettes with increasing \([LF]/([LF] + [B-Lg])\) mole fractions. In all cases the solutions were completely transparent at high and low \([LF]/([LF] + [B-Lg])\), but displayed a region of turbidity at intermediate levels. The level of LF required to produce a turbid solution when mixed with B-Lg increased as the pH of the solution increased.

In Fig. 3 we show the change in turbidity of the mixture as a function of the mole fraction \(x = [LF]/([LF] + [B-Lg])\) where the concentrations are expressed as molar concentrations. Turbidity of a sample is defined as \(s = -\ln(I/I_0)\) where \(I\) is the transmitted intensity and \(I_0\) the incident intensity. The turbidity of a dilute dispersion can be written as: \(s = \text{constant} \cdot c \cdot M\), where the constant depends on the optical properties of the system, \(c\) is the mass concentration and \(M\) the molar mass. Assuming that at maximum turbidity the protein binding is optimal, it follows that the turbidity at the maximum is a good measure for the molar mass of the complex, since protein concentration is a constant.

We have chosen to present the data as a function of mole fraction because that is more directly related to molecular interaction.

![Fig. 1. pH as a function of \([LF]/([LF] + [B-Lg])\) on titrating LF into B-Lg. Initial pH of both solutions was ~6.7 [34].](image1)

![Fig. 2. Visual turbidity of samples of LF mixed with B-Lg at pH 6.3 and pH 6.5. The number above each cuvette is the mole fraction of LF (see text for explanation).](image2)

![Fig. 3. Turbidity as a function of \([LF]/([LF] + [B-Lg])\) on titrating LF into B-Lg. Initial pH of both solutions ranged from 5.3 to 7.3.](image3)

![Fig. 4. Turbidity as a function of \([LF]/([LF] + [B-Lg])\) on mixing LF with B-Lg by three different methods. The initial pH of both solutions was pH 6.5.](image4)
the correct mixing ratio, but once the complex has formed, it dissociates more slowly when the mixing ratio is exceeded from either side.

3.3. Coacervates of B-Lg–LF

The first question may be: what is the nature of the complexation? We observe the following. Complexation seems to be an equilibrium system. It is reproducible and does not depend from which side the titration is made (Fig. 4). In addition centrifugation of the turbid dispersion gave a clear and very viscous liquid (see photographs in Fig. 5). Therefore we think that complex coacervates are formed. The definition of complex coacervation is coacervation caused by the interaction of two oppositely charged colloids [36]. Coacervation is the separation into two liquid phases in colloidal systems [36]. The phase more concentrated in the colloid component is the coacervate, and the other phase is the equilibrium solution.

The coacervate phase could be rapidly deposited by mild centrifugation (500g, 10 min); the requirement to apply mild centrifugal forces to obtain a homogenous phase is not uncommon. This has further implications because if the complexes form a new phase then this is a fine dispersion of the concentrated protein phase in a dilute phase similar to phenomena in so-called spinodal decomposition. The coalescence of the liquid micro-droplets is a very slow process but can be accelerated by centrifugation. The new complex coacervate phase must be electrically neutral and as a consequence the micro droplets and the complexes therein must be electrically neutral as well. At maximum coacervation, the coacervates are charge neutral, i.e. the zeta potential should be close to zero. Coacervation is largest at pH = 6.3 which is slightly closer to the pI of B-lg, as halfway between the respective pI's would be pH 6.55 (= (4.8 + 8.3)/2). The complexation becomes very weak at pH = 7.3 which is still 1 pH unit away from the pl of LF.

3.4. Charges of B-Lg and LF

We calculated the number of net charges on the proteins as a function of pH based on sequences downloaded from the Swiss Protein Data Bank [37], and used the Scripps protein calculator to calculate net charges (Fig. 6).

In the pH range 5–8, the two proteins are oppositely charged. Note that this is a calculated charge, which may not fully apply for a folded protein. For LF we count 102 basic amino acids and 76 acidic amino acids, whereas for B-Lg there are 21 and 28 respectively. These amino acids are dissociated, and the degree of dissociation is (partially) a function of pH. The net charge is the difference between these large numbers.

3.5. Zeta potential

We measured the zeta potential as a function the mixing mole fraction, [LF]/([LF] + [B-Lg]) on titration of LF into B-Lg (Fig. 7). Note these were the mixing ratio’s and not (necessarily) equal to the composition of the complexes. The measurement of a zeta potential on these complexes may be questioned and we show the data to illustrate the behaviour. The zero potential (charge neutrality) compositions were obtained by interpolation, using a cubic spline fit, of the data and these are plotted against pH in Fig. 8.

3.6. Overall composition and charge neutrality of the coacervates

We used the turbidity data in Fig. 3 to estimate the initial mixing mole fraction or the ratio B-Lg/LF (mol/mol) at maximum turbidity for each pH. The composition of the complexes may be different from the mixing mole fraction due to charge regularisation at each pH.

Fig. 8 shows that the overall system is charge neutral at maximum complexation even in the absence of counter ions. Or stated
differently, the number of positive and negative counter ions of both proteins are about equal. Complexes must be charge neutral as well because if the coacervates are NOT neutral the build-up of charge would prevent growth of coacervates into a single liquid phase. In practice the initially formed coacervate droplets seem to coalesce (very) slowly. However, just as with the coacervates of B-Lg and gum Arabic [38], they will form on standing or on light centrifugation, which is a requirement for a real complex coacervate system according to the definition.

We measured the composition of the coacervate after mild centrifugation (using the samples represented in Fig. 5). Fig. 9 shows very surprisingly that the composition of the coacervate is constant and independent of pH at x = 0.25 or LF:B-Lg = 1:3, the same value as follows from Fig. 1. This was a surprising finding because the coacervates must be charge neutral at all pH. It therefore seems that charge regularisation makes the complexes neutral. What this means is that the in the complexes at e.g. pH = 6.7 some protons are dissociated from B-Lg to compensate the charge of LF. This causes the sigmoidal shape of the titration curve in Fig. 1 [8,31]. The results shown in Fig. 9 suggests that optimal complex coacervation would be at mixing ratio LF:B-Lg = 1:3, which then equals the complex composition. This indeed occurs near pH = 6.3 where turbidity is maximum.

A B-Lg molecule is smaller than LF, which is relatively highly charged and so we may speculate that B-Lg decorates the outside of a LF protein. However a single B-Lg molecule can only touch or kiss on one side of the molecule and therefore part of its charges will be uncompensated. It can be estimated [39] that LF can be coordinated by about 6 spheres (about 1.7 times smaller than a LF sphere). In that situation LF must share each B-Lg molecule with another LF molecule to attain overall charge neutrality. It was stipulated by Desfougeres et al. [25] that both charge difference and size difference are essential for the formation of microspheres from oppositely charged proteins. In contrast to the results of some previous studies where microspheres are formed [11,21–24] we find a continuous and separate phase of coacervates. It appears indeed that the two proteins form a liquid phase in which the proteins are distributed at random. The mole fraction is independent of pH.

We made model calculations of the association constant defined by:

\[ 3\text{B-Lg} + \text{LF} \leftrightharpoons \text{LF-B-Lg}_3. \]

At a given pH and at the maximum of the complexation we mix X Mol of B-Lg and Y Mol of LF that then form Z Mol of LF–B-Lg3. Therefore the equilibrium constant is given by:

\[ [X - 3Z^3] \cdot \frac{[Y - Z]}{[Z]} = K_{ass}. \]

The value of \([Z]\) can be estimated from the turbidity by assuming that \([Z] = a_1\tau\), where \(a_1\) is a proportionality constant and \(\tau\) the turbidity. Since \(X\) and \(Y\) are known we could fit the turbidity data as a function of \(X, Y\) at each pH with two constants, i.e. \(a_1\) and \(K\) which is a function of pH. The value of \(a_1\) was somewhat variable, but had no great influence on \(K_{ass}\). In Fig. 10 we plot \(K_{ass}\) as a function of pH.

Thus with increasing pH the complexion strength increases and seems to go through a maximum. It is not clear what the origin of this behaviour is, but we surmise that it has to do with the increase of entropy on “freeing” of counter ions just as in complex coacervation of polyelectrolytes and proteins. Anyway the change of \(K_{ass}\) with pH accounts for the fact that the complexes have constant stoichiometry of LF:B-Lg = 1:3. If we consider the complex in a similar way as the structure of a halide of a trivalent cation (e.g. AlCl3) we would have a 6-fold coordination of LF by B-Lg where each B-Lg is shared with another LF. The structure of the coacervate phase could then be considered as a molten salt structure.

The estimated overall protein concentration is over 50% w/w, which is much higher than observed for coacervates from proteins and polysaccharides [38], and is probably related to the more efficient packing of the globular proteins and possibly the absence of counter ions. It would be of interest to study the internal structure of the coacervate using small angle scattering, by measuring partial

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**Fig. 8.** Mixing ratio’s B-Lg/LF (mol/mol) of the coacervates at the turbidity maximum (from Fig. 3) or from the zero zeta potential (from Fig. 7). The drawn line is the calculated charge neutrality based on the charge data in Fig. 6.

**Fig. 9.** Composition of coacervates. (●): Calibration curve from electrophoresis; (○): measured composition against nominal composition.

**Fig. 10.** Complex association constant of complexes at turbidity maximum as a function of pH.
structure factors. This would allow us to see the correlation/coordination of the proteins with themselves and with each other.

3.7. Salt dependence

Initially, we took the samples at pH 6.7 and at a range of mole fractions and titrated in 1 M NaCl and measured the turbidity. The turbidity decreased as the salt concentration increased, but the position of the maximum did not change. Next, we took the samples at maximum turbidity (maximum complexation) and titrated in 1 M NaCl. With increasing ionic strength the turbidity decreases. We plotted log(turbidity), proportional to molar mass of the complex, against \( I^{1/2} \) based on the assumption that molar mass would be a function of potential (electrostatic) interactions (Fig. 11). We assume a screened Coulombic interaction, which would be proportional to:

\[
\exp(-\kappa \cdot R),
\]

where \( \kappa \) is the Debye-Huckel screening length, which for a monovalent salt is:

\[
\kappa^{-1} \text{(nm)} = 0.3 / I^{1/2},
\]

where \( I \) is the ionic strength in molar concentration. The distance between charges is \( R \).

Whether the representation of log(Turbidity \times M) against \( I^{1/2} \) is the correct scaling is uncertain. Nevertheless, the data tend to become superimposed. The data clearly show that for \( I^{1/2} > 0.3M^{1/2} \) turbidity vanishes and the coacervates are not formed, thus at 100 mMol NaCl coacervation is suppressed.

3.8. Temperature dependence

Theoretical models to describe complex coacervation of oppositely charged macromolecules usually contain the electrostatic Coulombic interaction and entropy term which accounts for the fact that counter ions are ‘freed’ and therefore acquire a contribution in their translational entropy. Adding an excess of salt suppresses not only the Coulombic interaction but also the entropic contribution of lowering the Gibbs free energy. We made a simple experiment, using a DLS instrument, in which we measured the scattering intensity at the turbidity maximum for samples at pH = 6.3 and varied the temperature to see whether temperature had a strong influence. Scattering did not systematically vary with temperature and we therefore concluded that the interaction is electrostatic (which has low temperature dependence) in nature in view of the charge distribution, but that in view of the salt dependence there will be an entropial contribution as well.

The complexes formed from B-Lg and LF mixtures show all the hallmarks of a macromolecular complex coacervate, i.e. the formation of a liquid phase concentrated in both proteins in equilibrium with a dilute phase. The formation of the concentrated liquid phase is very slow but can be aided by light centrifugation. We hypothesize that during titration the initial formation of complexes is limited by charge build up (under- or over-charging) on the complexes. This may be called the soluble complexes, but are actually small charged complexes [9]. On further titration these complexes serve as a nucleus for further growth until they reach charge neutrality upon which a new electro-neutral phase can be formed. So we think there is an essential difference between soluble complexes and coacervates.

At each pH both proteins carry a different net charge. Nevertheless, the complexes/coacervates composition was constant at a ratio LF:B-Lg = 1:3. This constant ratio is maintained probably because the 3:1 coordination of LF by B-Lg is highly favorable leading to a hypothetical molten salt structure. The coacervates shown in Fig. 5 are very viscous and are very similar to those described by Weinbreck et al. [38]. It would be of interest to study the viscosity and the (partial) structure factors of the coacervate by small angle scattering and the distribution of ions using Donnan potentials. We speculate that the structure of the coacervates resembles a molten salt structure and is probably devoid of counter ions, which on “liberation” increase the entropy of the system.

In contrast to our results, Yan et al. [30] find a very complex behavior of a system that is nominally and seemingly the same. Complexation is limited, as these authors remark, to extremely low salt <20 mMol and pH ranges 5.7–6.2. (here pH = 5.7–7.3 and salt <100 mMol). The difference may be the source of B-Lg. Yan et al. [30] remark that their B-Lg source contains partially denatured protein and this may affect considerably the formation of complexes and aggregates, which some of the authors had observed earlier in the formation of complexes of B-Lg and gum Arabic. Indeed Weinbreck et al. [40] showed that there is a huge difference in behavior between a purified B-Lg (used herewith) and when a non-purified commercial B-Lg source is used (as used by Yan et al. [30]). Actually, these are essentially two different systems, as was already concluded by Schmitt et al. [41].

4. Conclusions

When LF and B-Lg were mixed complex coacervates were formed at defined mixing ratios dependent on the pH’s of the initial solutions. Complexes were formed in pH region 5–7.3, and at the turbidity maximum, the complexes were charge neutral. Although the charge ratios of LF:B-Lg varies with pH, we found that the complexes have a constant stoichiometry of LF:B-Lg = 1:3 at all pH’s; this is due to charge regularization. On addition of salt (NaCl) complexation is diminished and disappears at a salt concentration of about 100 mMol. If we consider the proteins as colloidal particles then the formed complex coacervate phase may have a structure that resembles a molten salt comparable to, for example, AlCl₃. The results obtained herewith on purified B-Lg and LF can be understood by simply assuming charge neutralization of the complexes as would be required if the complexes form a new, and necessarily electro-neutral, phase.

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