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Accumulation of small protein molecules in a macroscopic complex coacervate

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To obtain insight into the accumulation of proteins into macroscopic complex coacervate phases, the lysozyme concentration in complex coacervates containing the cationic polyelectrolyte poly-(*N*,*N* dimethylaminoethyl methacrylate) and the anionic polyelectrolyte polyacrylic acid was investigated as a function of the mixing ratio, protein concentration and ionic strength. Maximal protein enrichment of the complex coacervate phase was observed to require the presence of all three macromolecules. Under optimized conditions the protein concentrations in the complex coacervate were as high as 200 g L^{-1}. Such high concentrations are comparable to the protein concentration in the cytosol, suggesting that these interesting liquid phases may serve a suitable model system for the phase behavior of the cytosol and genesis and function of membrane-less organelles. The high stability of the complexes and the salt dependent uptake of protein suggest that complex coacervates may provide a way to store hydrated proteins at high concentrations and might therefore be of interest in the formulation of high protein foods.

Introduction

Oppositely charged macromolecules in an aqueous environment can form dense liquid-like phases, which are called complex coacervates.[1] Whether these oppositely charged macromolecules organize into complex coacervates (liquid-like) or polyelectrolyte complexes (glass-like) depends on the relaxation behaviour of the polyelectrolyte complexes. This relaxation behaviour strongly depends on the salt concentration, the molecular architecture and, when weakly charged polyelectrolytes are involved, on the pH of the system.[2,3]

For weakly charged polyelectrolytes the charge density of the polymer depends on the pH of the system. Optimal complexation between weak polyacids and polybases is found at their isoprotic point $\left(\mathrm{pI} = \frac{1}{2}(\mathrm{p}K_a + \mathrm{p}K_b)\right)$. For many weakly charged polyelectrolyte couples the pI $\approx$ 7 and often complex coacervation in these systems occur under physiological conditions (an ionic strength of $\approx$150 mM, pH $\approx$ 7). Proteins are a special class of weakly charged polyelectrolytes, having not only a net charge but also a charge sign which depends on the pH. Depending on the type of protein molecule and the pH of the system, protein molecules can form complex coacervates with both polyanions (at lower pH) and polycations (at higher pH). The complex coacervate formation of protein molecules and polyelectrolytes has been studied by many groups with fundamental interest in the interactions between polyelectrolytes and protein molecules and applications in e.g, food and pharma.[4–6]

Most complex coacervate systems studied so far are two-component systems containing oppositely charged proteins and polyelectrolytes or 2 oppositely charged polyelectrolytes. However, it has been shown that proteins can be incorporated into complex coacervates by mixing the aqueous solutions of polyanions, polycations and proteins, in three-component systems.[2,7–10] Mixing of aqueous solutions containing like charged homopolymer and protein molecules and solutions containing oppositely charged diblock copolymers (consisting of a neutral hydrophilic block and a charged block) results in polyelectrolyte complex micelles with protein molecules in the core.[2,7–9] It was further shown that lipase remained active during and after incorporation into these micelles.[11]

In the micelles the affinity of the proteins for the polyelectrolyte complex strongly depends on the ionic strength. The amount of protein molecules present in the polyelectrolyte complex micelles decreased as a function of the salt concentration,[8,9] at physiological ionic strength (0.15 M) no proteins were present within the micelles. This is a result of the difference in charge density between the protein molecules and the synthetic polyelectrolytes. The electrostatic attraction between the protein molecules and the oppositely charged polymer molecules is much weaker than the attraction between

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the oppositely charged polymers. The proteins are bulky and only bear a few charges in comparison to the polyelectrolytes. Above a certain ionic strength it is therefore entropically no longer favourable to incorporate proteins. To compensate for the loss of protein molecules the complex only needs to take-up a few charges (H^+, OH^-, cations and anions).

With these findings on protein-filled polyelectrolyte complex micelles in mind we now investigate the affinity of the protein molecules for macroscopic complex coacervate phases. The phase behaviour of complex coacervates is remarkably similar to the phase behaviour of liquid-like intracellular protein bodies.[12–15] These protein bodies are membrane-less organelles, which look like droplets, consisting of RNA and proteins, of which the function is unknown. We expect that the complex coacervates can serve as a model system for these cellular components. Further, the complex coacervates might be used to mimic molecular crowding and enforce close packing of protein molecules as observed in the crowded environment of the cytosol, the cellular fluid. Additionally, because it is easy to release protein molecules from the polyelectrolyte complex micelles, complex coacervates could be used to store hydrated protein molecules.

In this paper we study the macroscopic complex coacervation of poly(acrylic acid) (PAA_{140}), poly(*N*,*N* dimethylaminoethyl methacrylate) ($PDMAEMA_{108}$) and lysozyme, the polyelectrolytes in this system are both weak and their pI $\approx$ 6.7. Studying macroscopic complex coacervation with protein molecules instead of complex coacervate micelles[2,7,9] allows us to determine the amount of incorporated and non-incorporated protein molecules by UV-Vis. These experiments are very suitable for concentration determination, because the protein concentration of the non-incorporated and, after dissolving the complex by the addition of salt, incorporated protein molecules can easily be determined. Here we mix the different components, centrifuge the solution to speed-up the phase separation and determine the protein concentration in the supernatant.

Experimental

Materials

Lysozyme (L6876) was purchased from Sigma and used without further purification. The negatively charged polyelectrolytes poly(acrylic acid) P2006-AA (M_n = 10 000), having about 140 charges when fully charged and the positively charged polyelectrolytes poly(*N*,*N* dimethylaminoethyl methacrylate) P9740-DMAEMA (M_n = 17 000), having about 108 charges when fully charged, were purchased from Polymer Source, Inc., Canada. Sodium chloride was purchased from Sigma Aldrich.

Sample preparation

The polymer or protein molecules were dissolved in milliQ water and the pH was adjusted to 7 using either NaOH or HCl. These solutions were mixed to obtain samples of the desired protein concentration, ratios, salt concentration, *etc.*, all experiments were performed in duplicate. The total sample volume was 250 μL in all cases. The macromolecules were always mixed in the same order: first the like charged molecules (*i.e.*, lysozyme and PDMAEMA) were mixed and then the oppositely charged macro-ion (*i.e.*, PAA) was added. After mixing the samples were equilibrated for at least 8 hours. Samples were centrifuged for 20–30 minutes at 12 000–15 000 rpm and at 15 °C on a IEC Micromax RF centrifuge. After centrifugation the protein concentration in the supernatant was determined using the molar extinction coefficient of lysozyme at 281.5 nm: 2.635 L g^{-1} cm^{-1} [16] (the concentration of the lysozyme stock solution was also determined by UV). Spectra from 400–240 nm were recorded on a Shimadzu UV-2401PC spectrophotometer. When the total absorbence of the sample at around 400 nm was much higher than the intensity of the blanc, indicating the presence of complex coacervate droplets, the sample was centrifuged again a few days later and the UV-Vis experiment was repeated. This procedure was performed until there were no more complex coacervate droplets present in the supernatant.

To determine whether the protein molecules could freely diffuse out of the complex samples were prepared at 2 different salt concentrations (0.1 M and 0.15 M NaCl). After the complex coacervate phase had formed, these samples were centrifuged and the protein concentration in the supernatant was determined, to see whether the protein concentration in the supernatant had changed. The following procedure was used twice: the supernatant was removed and a 150 μL solvent with the same salt concentration was added and the sample was vortexed. After 2 days the sample was centrifuged and the protein concentration in the supernatant was determined.

The protein concentration in the complex coacervate phase was determined using the following procedure: the mass of eppendorf tubes was determined by weighing eppendorf tubes that were empty and when the complex coacervate phase was formed (after the removal of the supernatant). Typically the mass of the complex coacervate was 5–10 μg. The complex coacervate was dissolved at high ionic strength. The lysozyme concentration was determined using UV-Vis spectroscopy (see above).

Results and discussion

Turbidity and light scattering are common methods to study polyelectrolyte complex formation.[17–20] In these techniques the turbidity or light scattering intensity is measured as a function of a variable, *e.g.*, the ratio between the macroions, concentration, pH or ionic strength. When light scattering is used one can further obtain information about the hydrodynamic radius of the objects that are formed, which is very useful when one is interested in the formation of *e.g.*, polyelectrolyte complex micelles.[21] In this study we are interested in the incorporation of protein molecules into the complex coacervate phase. UV-Vis experiments are in this case very suitable, because the protein concentration of the non-incorporated protein molecules can easily be determined. Here we mix the different components, centrifuge the solution to speed up the phase separation and then determine the protein concentration in the supernatant.

The lowest protein concentration in the supernatant corresponds to optimal complex formation. This is opposite to the trend observed in light scattering or turbidity experiments where a highest intensity corresponds to the optimal complex composition. Spectra recorded from 400–240 nm additionally give information about the presence of aggregates in the supernatant. Large complexes will scatter light resulting in a higher absorption than the solvent baseline. So additionally these UV experiments give information about the relaxation behaviour and relaxation times of the complex coacervate formation.[2,3]

The ratio between the oppositely charged macroions is an important variable in determining the amount of proteins that can be incorporated into the complex coacervate. If one starts with a solution containing only one type of the charged species, let us say only negatively charged species and starts adding positively charged species the system goes through different regimes. First soluble complexes, with a net negative charge are formed. These soluble complexes repel each other and remain therefore in solution. Near the equal charge ratio, a macroscopic phase is formed. At a low ionic strength this macroscopic phase is a precipitate and at a higher ionic strength a complex coacervate (at a very high salt concentration no complexation occurs because the charges on the macromolecules are screened). If one continues to add positively charged species to the system soluble complexes will be found with a net positive charge.[3]

We define this complex composition $F^- = \frac{[n_-]}{[n_-] + [n_+]}$, where n_+ and n_- are the positive and negative charge concentration. At F^- = 0 the system only contains the positively charged species, at F^- = 1 only the negatively charged species. Electroneutralitly is found at F^- = 0.5. Deviation from this value in experiments is found because the actual number of charges on weakly charged polyelectrolytes is unknown. The charge of these polymers is a function of the pH and they can influence each other's dissociation behaviour.[22,23] To calculate F^-, the number of charges on the fully charged polyelectrolytes, 140 for PAA and 108 for PDMAEMA, and the actual charge of lysozyme determined from a pH titration ($\approx$+7 charges at pH = 7)[24] were used.

In Fig. 1 the concentration of lysozyme in the dilute phase (after centrifugation) for 2 different starting concentrations of lysozyme is presented as a function of the complex composition. Intuitively, one would expect that the maximal number of incorporated (positively charged) lysozyme molecules would be found when only the negatively charged polyacrylic acid molecules are present ($F^- \approx 1$). However, a pronounced minimum for both protein concentrations is found at around $F^- \approx 0.63$, meaning that maximal incorporation of the protein molecules occurs when positively charged polycations are present. Two-component systems of PAA and lysozyme form a precipitate instead of a complex coacervate (very close to F^- = 1). Complex coacervates are formed in all cases when PDMAEMA is present in the system, so in the presence of three components (PAA, lysozyme and PDMEAMA). It seems to be more favourable for lysozyme to be part of a complex coacervate with a net negative charge ($F^- > 0.5$). Above a certain PDMEAMA concentration $F^- \approx 0.5$ no more lysozyme molecules are incorporated, but the

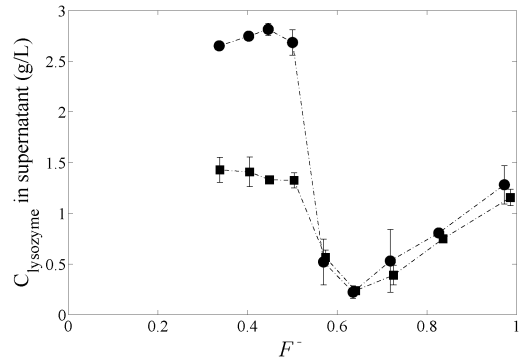

Fig. 1 Lysozyme concentration in the supernatant as a function of the composition (F^-). ● = 2.8 g L^{-1} lysozyme and ■ = 1.4 g L^{-1} lysozyme (protein concentrations at the start of the experiment), pH = 7, C_{NaCl} = 0.1 M, the concentration of PAA (3.6 g L^{-1}) and lysozyme were fixed and the concentration of PDMAEMA was varied. The error bars show the standard deviation over two independent experiments.

polymers do form a complex coacervate phase. The driving force for the complex formation between lysozyme and the polymers is likely to be electrostatic in nature. If hydrophobic interactions or hydrogen bonding were the dominating interactions the addition of positively charged polymers would not lead to the release of all the protein molecules.

Salt is a very important parameter to regulate the interactions within a polylectrolyte complex. We have therefore detemined how the salt concentration affects the amount of incorporated proteins at the optimal composition for the polymer molecules ($F_{optimal}{}^-$). In Fig. 2 the lysozyme concentration in the supernatant is shown as a function of the salt concentration for samples with $F_{optimal}{}^-$. We start at a salt concentration of 0.1 M NaCl, since below this concentration a glass-like polyelectrolyte complex phase is formed and not a liquid complex coacervate. The complex coacervate phase dissolves above a salt concentration of 2 M NaCl. There is a sharp increase in the protein concentration in the dilute phase between 0.1 M and 0.2 M NaCl, at 0.1 M the majority of the protein molecules are incorporated and above 0.2 M NaCl all protein molecules are free in solution.

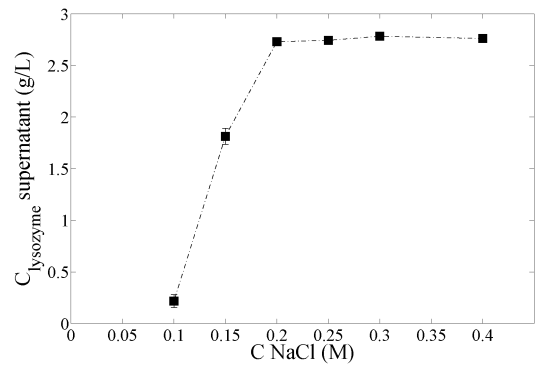

Fig. 2 Lysozyme concentration in the supernatant as a function of the NaCl concentration. pH = 7, $C_{PDMAEMA}$ = 4.5 g L^{-1}, C_{PAA} = 3.6 g L^{-1}, $C_{lysozyme}$ = 2.8 g L^{-1}. The error bars show the standard deviation over two independent experiments.

When polyelectrolyte complexes form the gain in entropy due to the release of counterions is smaller for shorter polyelectrolytes and macro-ions with a low charge density (like proteins) than for long polyelectrolytes. Addition of salt to polyelectrolyte complexes or complex coacervates is known to shift the equilibrium from the dense complex phase towards small soluble complexes or single polyelectrolytes in solution.[25] For shorter polyelectrolytes and macro-ions with a low charge density the ionic strength at which this happens is expected to be lower, because less salt ions are needed to screen the charges on these molecules. For polyelectrolyte complex micelles with lipase incorporated into the core it was found that first the lipase molecules were released at a low salt concentration (0.12 M NaCl), but the micelles remained intact and only completely disintegrated above 0.5 M NaCl.[8] Here we are dealing with a macroscopic complex coacervate phase and that is most likely the reason why protein molecules are still present inside around 0.15 M NaCl.

By changing the ionic strength we can reversibly store protein molecules in the complex coacervates. In the cytosol and the protein bodies, protein molecules are mobile. A question that arises is whether the protein molecules can diffuse out of the complex coacervates? A way to study this is to remove the supernatant and add new buffer. For this experiment two samples were made (in duplicate): $C_{PDMAEMA}$ = 9 g L^{-1}, C_{PAA} = 7.2 g L^{-1}, $C_{lysozyme}$ = 4.8 g L^{-1} at 0.1 M and 0.15 M NaCl. The protein concentration in the supernatant was determined at the start (start) and then replaced by MilliQ water with the same pH and salt concentration. After an equilibration time of 2 days the protein concentration was determined (1× removal). This procedure was then repeated (2× removal). The results for these three steps can be found in Fig. 3.

As expected the lysozyme concentration in the supernatant was highest at 0.15 M NaCl at the start of the experiment. After the first and second replacement the lysozyme concentration in the supernatant at 0.1 M NaCl is very low, indicating that it is more favourable for the protein molecules to remain in the complex coacervate at this ionic strength. At 0.15 M NaCl, the protein concentration in the supernatant is much lower than after the first removal step. For both salt concentrations it seems to be more favourable for the protein molecules to remain in the dense phase. The interactions between the protein molecules and the polyelectrolytes probably make outwards diffusion very slow.

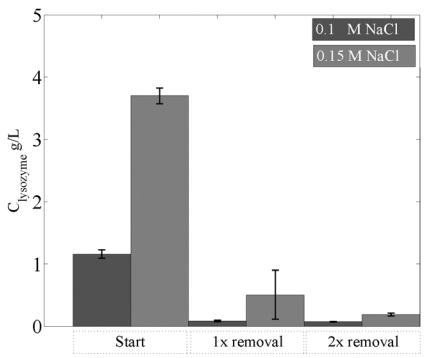

Fig. 3 Protein concentration in the supernatant in a supernatant removal experiment. The error bars indicate the standard deviation over two independent experiments.

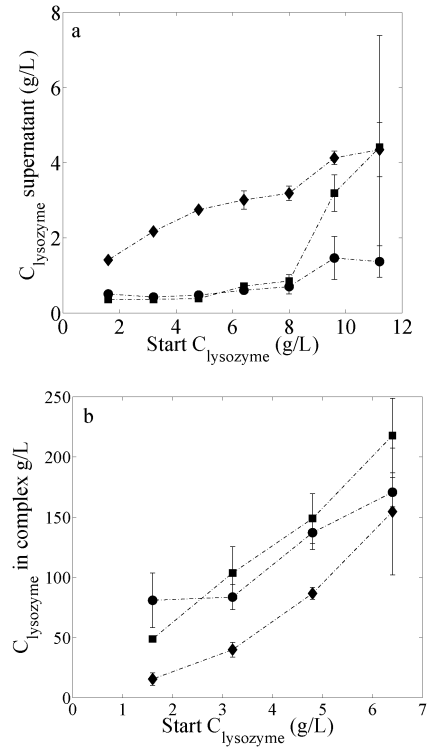

Fig. 4 (a) Lysozyme concentration in the supernatant as a function of the lysozyme start concentration. (b) Lysozyme concentration in the complex coacervate (determined after dissolving the complex coacervate). ● = $C_{PDMAEMA}$ = 4.5 g L^{-1}, C_{PAA} = 3.6 g L^{-1} and C_{NaCl} = 0.1 M, ■ = $C_{PDMAEMA}$ = 3.0 g L^{-1}, C_{PAA} = 2.4 g L^{-1} and C_{NaCl} = 0.1 M and ◆ = $C_{PDMAEMA}$ = 4.5 g L^{-1}, C_{PAA} = 3.6 g L^{-1} and C_{NaCl} = 0.15 M. The error bars show the standard deviation over two independent experiments.

The cytosol is a very crowded environment, with a protein concentration up to 300 g L^{-1}. Can the protein concentration in the complex coacervate become as high as in the cytosol? To estimate the maximal number of lysozyme molecules that can be incorporated into the complex coacervate phase, the lysozyme concentration in the supernatant and in the complex coacervate was studied as a function of the protein concentration. In Fig. 4a the amount of protein molecules in the supernatant as a function of the protein concentration at the start is given for 3 different situations: $F_{optimal}^{-}$ at 0.1 M NaCl, $F_{optimal}^{-}$ at 0.15 M NaCl and $F_{optimal}^{-}$ at 2/3 of the complex concentration (*i.e.*, with a lower polycation and polyanion concentration).

At 0.1 M NaCl the supernatant of both complex compositions contains a constant protein concentration up to 8 g L^{-1} (lysozyme concentration before complexation). Above this total protein concentration, the lysozyme concentration in the supernatant for the complex coacervate prepared with 2/3 of the amount of complex increases significantly. This indicates that the complex coacervate cannot accommodate any more protein molecules.

Since there is a more complex coacervate phase present in the other system, and hence more available space, the complex coacervate is not filled-up with protein molecules and more protein can still be incorporated. As expected the protein concentration in the supernatant is higher at 0.15 M NaCl.

To determine the protein concentration in the complex coacervate phase, the supernatant was removed and the amount of complex coacervate was determined by taking the weight difference between empty tubes and tubes with complex coacervates. We further assume that the complex coacervate phase has the same density as water. As a result of these assumptions we estimate that the error is 10–20%.

Then the complex coacervate phase was dissolved in 2 M NaCl and the lysozyme concentration of the dissolved complex coacervate was determined with UV (281.5 nm, $\varepsilon = 2.635$ L g^{-1} cm^{-1}).[16] This procedure worked for the samples with a starting protein concentration below 8 g L^{-1} and for these samples we did not observe any absorbance at 400 nm in the UV-spectrum that would be caused by the presence of protein aggregates or complexes. We therefore conclude that the complex coacervates are completely dissolved and no protein aggregates are present in these samples.

Above this concentration, the complex coacervate phase became cloudy, where at lower protein concentrations it is optically clear. After the addition of 2 M NaCl to the cloudy complex coacervates a small precipitate was found at the bottom of the tube. Apparently the protein molecules at the higher protein concentration aggregated. The estimated amount of soluble lysozyme in the complex coacervate can be found in Fig. 4b.

The highest protein concentration in the complex coacervate of 150 to 200 g L^{-1} is found at 0.1 M NaCl and a lysozyme starting concentration of 6.5 g L^{-1}. This range of protein concentrations is similar to the protein concentration in the cell's cytosol.[26] The solubility of lysozyme in buffer is $\approx$210 g L^{-1}.[27] It appears to be possible to get more than 200 g L^{-1} lysozyme in the complex coacervate, but at concentrations higher than 200 g L^{-1} the interactions of the proteins with the complex do not prevent protein aggregation and cloudy complex coacervates are obtained.

Knowing the protein concentration in the complex coacervate ($\approx$150 g L^{-1}), we can make a rough estimate for the distance between the protein molecules. The molar mass of lysozyme is 14 300 kDa and the radius of the molecule is $\approx$2 nm.[28] This means that the distance between the lysozyme molecules is $\approx$3.4 nm, which is equal to about 1.5 lysozyme molecules. If we now include the volume occupied by the polymer molecules the intermolecular distance in the complex coacervate will be less than one molecule, which is smaller than the intermolecular distance in the cytosol.[29]

Concluding remarks

To conclude, we have shown that lysozyme can easily be incorporated into complex coacervates of PAA and PDMAEMA. The amount of incorporated lysozyme can be tuned by changing the complex composition, NaCl concentration and the starting concentration of the protein molecules. These protein molecules can be reversibly stored inside the complex coacervates below a total protein concentration of 8 g L^{-1} and their incorporation can be tuned by changing the ionic strength, thereby showing a phase behaviour similar to liquid-like intracellular protein bodies.[12] The salt dependence of the protein incorporation into the the complex coacervates indicates that they can be used as a storage facility for protein molecules and could be applied as a new method to formulate high protein foods for the ageing population. The protein molecules remain in the complex coacervate, outwards diffusion is very slow and probably very difficult because of the high concentration of macromolecules in the complex coacervate and the interactions between them. Protein concentration and estimated intermolecular distance in the complex coacervate are similar to the cytosol, indicating that these dense liquids could serve as a molecular crowding agent. Future tests on the biological activity of the protein molecules and their diffusion inside the complex coacervate phase will shed more light on the resemblance of complex coacervates to liquid-like intracellular components.

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