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Extraction of Lysozyme from Chicken Albumen Using Polyelectrolyte Complexes

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Cells use droplet-like membrane-less organelles (MLOs) to compartmentalize and selectively take-up molecules, such as proteins, from their internal environment. These membraneless organelles can be mimicked by polyelectrolyte complexes (PECs) consisting of oppositely charged polyelectrolytes. Previous research has demonstrated that protein uptake strongly depends on the PEC composition. This suggests that PECs can be used to selectively extract proteins from a multi-protein mixture. With this in mind, the partitioning of the protein lysozyme in four PEC systems consisting of different weak and strong polyelectrolyte combinations is investigated. All systems show similar trends in lysozyme partitioning as a function of the complex composition. The release of lysozyme from complexes at their optimal lysozyme uptake composition is investigated by increasing the salt concentration to 500 mM NaCl or lowering the pH from 7 to 4. Complexes of poly(allylamine hydrochloride) and poly(acrylic acid) have the best uptake and release properties. These are used for selective extraction of lysozyme from a hen-egg white protein matrix. The (back)-extracted lysozyme retains its enzymatic activity, showing the capability of PECs to function as extraction media for proteins.

1. Introduction

Cellular processes are extremely efficient and the most complex reactions occur in the blink of an eye. This efficiency requires extremely good spatio-temporal organization of molecules and compartmentalization of components is a way to achieve this. Traditionally compartmentalization has been assumed to occur in organelles surrounded by lipid membranes. However, recently membrane-less organelles (MLOs) have been suggested as spatio-temporal organizers. MLOs are dynamic droplet-like condensates formed by aqueous liquid–liquid phase separation. Several kinds of MLOs have been reported, such as stress granules, nucleoli, cajal bodies, paraspeckles, and more.[1–8] The exact role and mechanism of many MLOs is still unknown, but it is expected that the separate chemical environment provided by these condensates allows for specific processes and reactions to occur.[2,3,6,9]

Cellular MLOs typically consist of charged intrinsically disordered proteins (IDPs) and oppositely charged polynucleotides.[2,6,7] The careful interplay of electrostatic interactions and other interactions such as cation–pi interactions, hydrogen bond formation and hydrophobic interactions results in the formation of droplet-like condensates that are explicitly distinct from the surrounding fluid. A simple way to model these cellular condensates is by making use of oppositely charged polyelectrolytes. When aqueous solutions of oppositely charged polyelectrolytes are mixed, condensates will form that are distinctively different from the surrounding fluid. These polyelectrolyte condensates are called polyelectrolyte complexes (PECs). Factors that influence phase behavior of PECs include polycation to polyanion ratio, ionic strength, polyelectrolyte chemical structure, and pH. Unsurprisingly, the phase behavior of PECs and their response to changes in the local environment is similar to the reported phase behavior of MLOs.[10–14]

Once the PECs have formed, the partitioning of additional compounds, such as proteins, between the PEC and the dilute supernatant phase can be studied. The partitioning between PEC and supernatant is analogous to the partitioning between MLOs. For PECs it has been found that proteins can partition into the polyelectrolyte-rich phase.[15–21] The partitioning behavior of proteins also depends on the factors that influence the phase behavior of PECs.[17] This multi-parameter dependence on the partitioning of macromolecules makes understanding of the exact molecular details challenging.[17] However, if we understand and can control the partitioning of molecules with a similar selectivity and efficiency as MLOs, PECs can be used as aqueous extraction media.[17] These extraction media are expected to show a high uptake of a specific molecule from an aqueous solution and a controllable release suitable for the recovery of bio-active compounds.

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smll.202105147.

DOI: 10.1002/smll.202105147

In this study we investigate the partitioning of the protein lysozyme in different model PEC systems. Lysozyme is a naturally occurring antibacterial enzyme of industrial importance and is used in the food and pharmaceutical industries as an additive to increase shelf life.[22–27] It is a relatively small protein with a MW of 14.3 kDa and an isoelectric point of 11.35, making it cationic (net charge +7) at neutral pH. Lysozyme is also part of the innate immune system and present in human tears, mother's milk, and saliva.[26,28,29] A potent source of lysozyme is chicken albumen (i.e., egg white). Approximately 10% of albumen is protein, and of these proteins ≈3–4% is lysozyme.[28,29]

Four model PEC systems were used in this study consisting of combinations of the polycations poly(allylamine hydrochloride) (PAH) and poly(diallyldimethylammonium chloride) (PDADMAC), and polyanions poly(acrylic acid) (PAA) and poly(4-styrene sulfonate) (PSS) as shown in **Figure 1**. PAH and PAA are weak polyelectrolytes with pH-dependent charge. PDADMAC and PSS are strong polyelectrolytes that bear their charge regardless of pH. All polyelectrolytes have a molecular weight (MW) approximately an order of magnitude larger than lysozyme to facilitate separation of the polyelectrolytes from lysozyme, when desired.

First, we show the formation of PECs by mixing two oppositely charged polyelectrolytes. The PECs form a macroscopic polyelectrolyte-rich solid-like phase distinct from the polyelectrolyte-poor aqueous supernatant phase. The partitioning of lysozyme between the PEC phase and the supernatant phase is then measured as a function of the PEC composition to determine the optimal condition for lysozyme take-up. After lysozyme partitioning at the optimal composition, lysozyme is back-extracted from the PECs by increasing the salt concentration or lowering the pH. The PEC system with the best back-extraction properties is then used to extract lysozyme from a chicken albumen solution. Finally, we show that the back-extracted lysozyme retains its enzymatic function. The results from this study show the potential of PECs as selective extraction media for proteins.

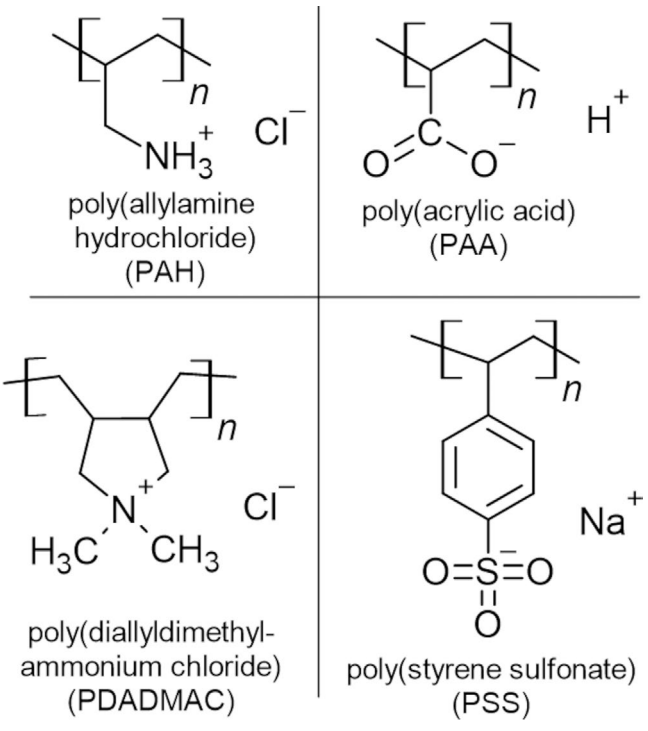

Figure 1. Structure of the polyelectrolytes with their respective counterion used in this study. Top: weak polyelectrolytes where the charge depends on pH. Bottom: strong polyelectrolytes with charge independent of pH. Left: polycations. Right: polyanions.

2. Results and Discussion

Previous studies have shown that lysozyme take-up by PECs can be very efficient and selective, that is, only lysozyme can be selectively extracted from an ideal mixture of two proteins by a PAH/PAA PEC system.[16] In this study we investigate the take-up and release of lysozyme by four different PEC systems enabling us to study the role of pH-dependent charge of the polyelectrolytes on the lysozyme partitioning. The goal is to identify the PEC system(s) that is/are most suitable for the extraction of lysozyme from a complex natural occurring protein mixture. Here we use a weak and a strong polycation, PAH and PDADMAC respectively, as well as a weak and a strong polyanion, PAA and PSS, respectively. These polyelectrolytes can be combined in four different ways allowing us to compare PECs consisting of weak/weak, strong/strong and two varieties of weak/strong polyelectrolytes. All polyelectrolytes have similar MWs (100–350 kDa) and their weights are an order of magnitude larger than the MW of lysozyme.

We first investigated the partitioning of lysozyme as a function of the PEC composition quantified as the charge fraction (F^-). F^- is defined as the anionic fraction of polyelectrolyte charges (Equation 1) and it follows that at $F^- = 0$ only polycations are present, at $F^- = 1$ only polyanions and $F^- = 0.5$ indicates charge stoichiometry. The PEC forms a distinct macroscopic solid-like phase in contrast to the aqueous supernatant phase (Figure S1, Supporting Information). The lysozyme concentration in the supernatant phase was measured using UV–vis spectrophotometry. The composition at which optimal partitioning occurs is the composition where the lysozyme concentration in the supernatant phase is the lowest.

The partitioning of lysozyme as function of the composition for the four different systems can be found in **Figure 2**. For all PECs the general profile follows a similar shape. Starting at the cationic side ($F^- < F^-_{optimal}$) with a plateau region, at this composition range lysozyme remains in the supernatant. At a certain composition a steep decrease is observed where a minimum amount of lysozyme is found in the supernatant. At this composition optimal lysozyme take-up occurs and we will refer to this composition at $F^-_{optimal}$. Finally, a more gradual increase in lysozyme concentration is observed at the anionic side ($F^- > F^-_{optimal}$) until no lysozyme take-up occurs. In all combinations there was an optimal PEC composition where nearly all lysozyme was partitioned in the PEC.

$F^-_{optimal}$ varied per system, from approximately charge stoichiometry for PAH/PAA ($F^- = 0.53$) and PAH/PSS ($F^- = 0.50$) to systems containing noticeably more negative charge for PDADMAC/PAA ($F^- = 0.60$) and PDADMAC/PSS ($F^- = 0.61$). Similar profiles have been reported before.[15,16] For all the evaluated PEC systems in Figure 2, the presence of both polycations and polyanions is necessary for partitioning of the lysozyme. At both $F^- = 0$ or 1, where the protein is only present with one

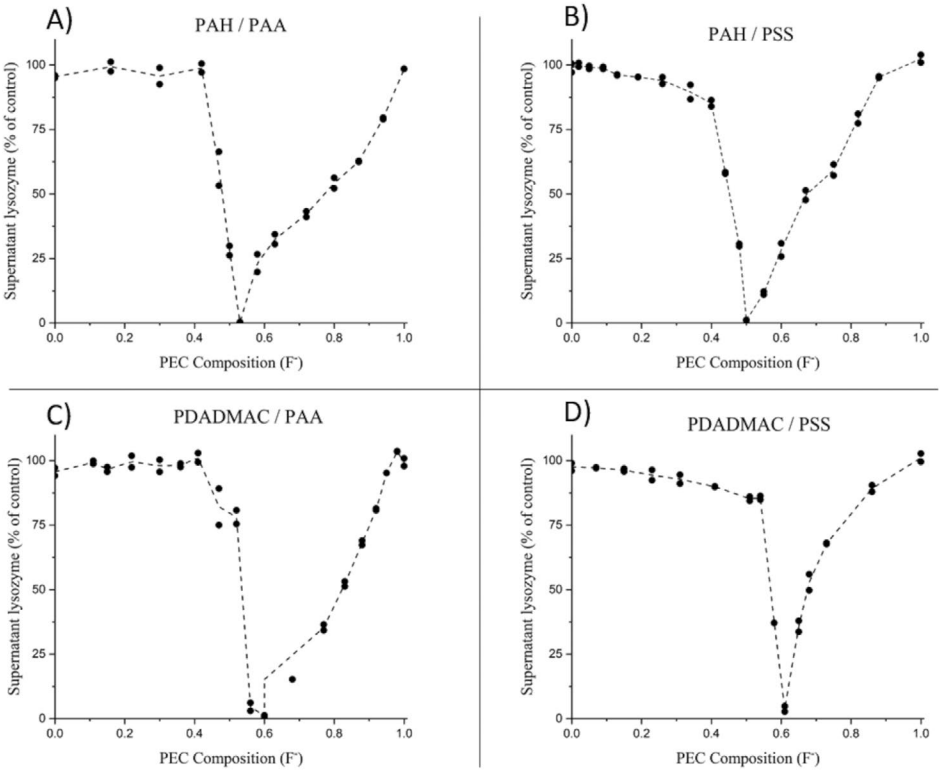

Figure 2. The partitioning profiles of lysozyme in various PECs at pH 7; A) weak/weak PAH/PAA, B) weak/strong PAH/PSS, C) strong/weak PDADMAC/PAA, D) strong/strong PDADMAC/PSS, at different PEC compositions as expressed in F^-. A low supernatant lysozyme content corresponds to a high PEC lysozyme content and vice versa. Values represent individual measurements; lines connect averages of duplicates.

polyelectrolyte, the solutions are optically clear indicating there are no soluble PECs present.

PEC systems containing PDADMAC consistently showed $F^-_{optimal}$ at slightly anionic conditions (F^- = 0.60–0.61) compared to PAH systems at approximate charge stoichiometry. This suggests that the partitioning is not solely a charge-driven process, in which case the expected $F^-_{optimal}$ would be found at charge stoichiometry for all PEC systems.[16] Including the charges of lysozyme does not significantly change the F^-.[16] The fact that no partitioning occurs at either F^- = 0 or 1 but instead is optimal at a very specific F^- suggests that specific interactions between the polyelectrolytes are an important step for the partitioning of lysozyme. The preferential interaction of proteins with polyelectrolytes at a more anionic charge ratio has previously been observed for some polyelectrolytes, although the exact reason remains unknown.[30,31] For the determination of F^- (using Equation 1), the assumption has been that all polyelectrolyte monomers were charged. Recently, a study has demonstrated a method to measure the charge fraction of polyelectrolytes in PECs.[32] This technique may be applied in the future to further elucidate the exact role of charges on partitioning of proteins in PECs.

When the starting concentration of lysozyme is increased, the PEC becomes saturated and at high enough concentrations a fraction of the lysozyme will remain in the supernatant, even at the $F^-_{optimal}$.[16] This could be compensated for by increasing the total amount of PEC.[16] A previous study has suggested that within PECs the lysozyme concentration can be enriched up to 200 g L^{-1}.[15] This behavior suggests a model of PECs as a phase that can be "filled" with lysozyme and upon saturation the remaining lysozyme remains in the supernatant phase. In all the PEC systems of Figure 2, we see a clear F^- value where the supernatant lysozyme approaches zero. This suggests that for none of our systems we have reached the PEC saturation point.

All four evaluated PECs show effective lysozyme take-up at $F^-_{optimal}$. For extraction processes, recovery of lysozyme from the PEC is equally important. We therefore study the release of lysozyme from the PECs by addition of salt or lowering of pH. First, we prepare PECs with partitioned lysozyme at their $F^-_{optimal}$ found in Figure 1. Then, we replace the supernatant with either 500 mM NaCl or water with a pH of 4. We evaluated the concentration of lysozyme in the refreshed supernatant and express this value in the percentage of lysozyme released from the PEC. The process of releasing lysozyme in new supernatant is referred to as back-extraction. Salt ions can screen charges on the polyelectrolytes and proteins. By increasing the salt concentration the interactions between the charged macromolecules can weaken and can result in the release of proteins.[33] Altering the pH will affect the degree of ionization of weakly charged groups on the polyelectrolytes and proteins. If the interactions between the proteins and polyelectrolytes weaken as a result of a change in pH, proteins can be released from the PEC into the supernatant phase.[16,20] Previously, we have reported that a decrease in pH from 7 to 4 gave the best result.[16]

In **Figure 3**A,B lysozyme back-extraction via the addition of salt or decrease in pH are shown, respectively. For both methods the supernatant phase was removed and replaced

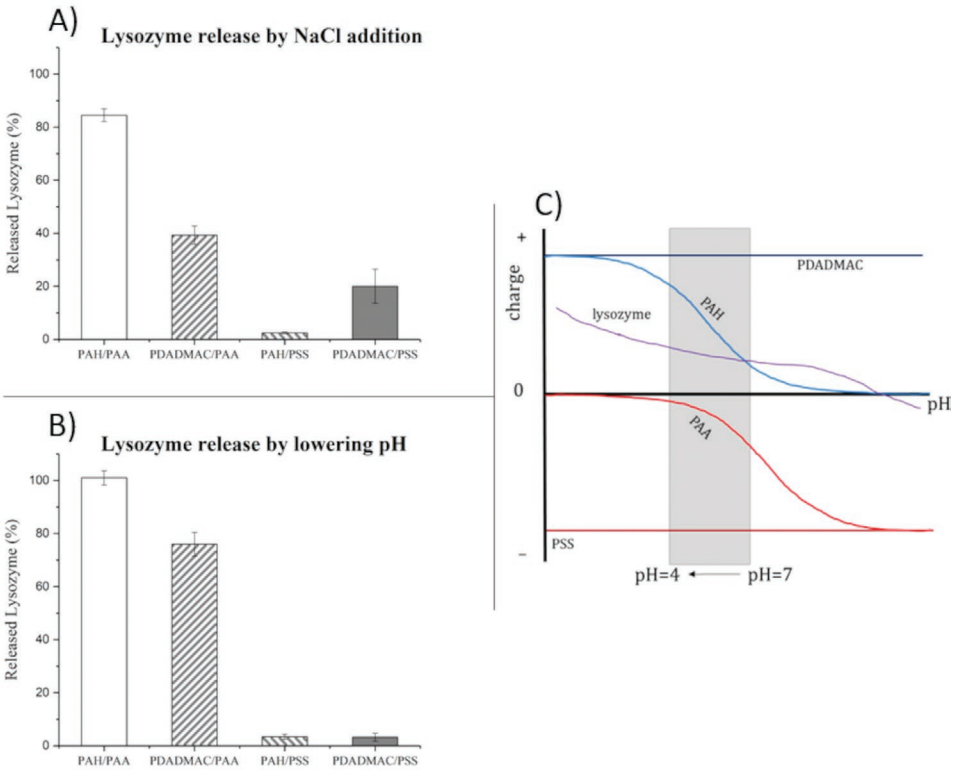

Figure 3. The back-extraction of lysozyme from the different PEC systems using A) 500 mM NaCl, B) 4 mM HCl (pH decrease of 7 to 4), C) schematic sketch that illustrates how the charge of the polyelectrolytes and lysozyme changes as a function of pH.[34] Adapted with permission.[35] Copyright 2019, The Authors. Published by Elsevier B.V. Columns represent the average of $n = 4$ with error bars indicating standard deviation.

by new aqueous supernatant with either a 500 mM NaCl concentration, or a lower pH (≈4). The different PEC systems show widely different releases of lysozyme (Figure 3A) at 500 mM NaCl. The lowest release is found for PAH/PSS with 2.5 ± 0.3% of lysozyme released. Second lowest is PDADMAC/PSS with 20.0 ± 6.4%. Second highest is PDADMAC/PAA with 39.3 ± 3.5% released and the greatest back-extraction efficiency was found for PAH/PAA with 84.5 ± 2.4% of lysozyme released.

An increase of ionic strength as a result of added salt ions is known to induce screening effects between oppositely charged polyelectrolytes, preventing the polyelectrolyte from interacting (as strongly) with each other. Different PEC systems containing proteins respond to an increase in ionic strength differently. PEC micelle systems can either release the protein while remaining intact, or disintegrate completely and release the protein this way based on the weak/strong nature of the polyelectrolytes[33,36] There are several examples in literature where PECs consisting of weak polyelectrolytes versus PECs consisting of strong polyelectrolytes show a different resistance toward an increase in ionic strength.[37–40] In general, complexes consisting of only weak polyelectrolytes disintegrate at a lower ionic strength than complexes consisting of strong polyelectrolytes.[37] When weak and strong polyelectrolytes are combined the degree of ionization and thus the pH of the system becomes important. In our case the lowest release was found with PAH/PSS. This is consistent with earlier reports that show that PAH/PSS PECs may even gain stability in the presence of up to 3 or 4 M NaCl.[41,42] PDADMAC/PSS PECs or multilayers have demonstrated the ability to form stable PECs in the presence of up to 2 M NaCl.[38,39] Lysozyme release from PDADMAC/PSS PECs is then more likely a result of reduced attraction between the PEC and lysozyme, which has a much lower charge density than the polyelectrolytes. PDADMAC/PAA PECs consisting of larger polyelectrolytes have been shown to be stable up to 1 M NaCl.[43] Interestingly, an increase in NaCl concentration does not result in a linear lysozyme release profile for PAH/PAA for the range of 0 to 1 M NaCl (Figure S2, Supporting Information).

A change in PEC size as a result of added salt or lowering pH was observed (Figure S3, Supporting Information) for both salt and lower pH in most PEC systems. The PECs either swelled or decreased in size. However, there was no clear correlation between change in PEC size and released lysozyme (Table S1, Supporting Information). For example, in the case of PAH/PAA PECs where all lysozyme was released by lowering the pH, the PEC size increased by ≈10%. Earlier reports on water content in PECs suggests that minor changes in PEC composition can affect their water content. This could explain the observed variations in PEC size as a result of pH change.[17] Size changes at increased salt concentration could be explained by the PEC network loosening due to screened polyelectrolyte charges and as a result more water is taken up by the PEC. An interesting observation was found for PDADMAC/PAA PECs, where lowering the pH to 4 resulted in a nearly sixfold increase in PEC size. Visually the PDADMAC/PAA formed a hydrogel structure specifically at lower pH values, possibly due to hydrogen bonding.[44] For soluble PDADMAC/PSS PECs

at stoichiometric compositions, an NaCl concentration of 0.5 M has been found to sharply increase the PEC hydrodynamic diameter.[45]

A decrease in the partitioning of proteins at higher NaCl concentrations has been reported earlier and it was hypothesized that the salt ions weaken the electrostatic and entropic interactions between the proteins and the polyelectrolytes.[18] That the presence of salt ions influences the interaction between proteins and singular polyelectrolytes has been widely established and it stands to reason that salt ions can similarly influence the interactions between proteins and PECs.[46] It has been shown that protein charge is important in protein partitioning in PECs, and salt ion screening can influence the degree to which protein and PEC charges are able to see and interact with each other.[16,18]

Figure 3B presents the back-extraction of lysozyme when the pH of the supernatant is decreased from pH 7 to pH 4. This figure shows that a decrease in pH has a different effect on the four investigated systems. Both PAH/PSS and PDADMAC/PSS show the least amount of released lysozyme with 3.4 ± 1.0% and 3.2 ± 1.5%, respectively. PDADMAC/PAA released 76.0 ± 4.5% and PAH/PAA released 101.0 ± 2.7% of lysozyme.

For the strongly charged PDADMAC/PSS PECs it is expected that a change in pH will have little effect on the protein release as both polyelectrolytes bear a charge independent of pH. By changing the pH the charge of protein will become more positive, the net charge of protein shifts from +7 to + 12. In Figure 3B it can be seen that the amount of lysozyme in the supernatant for this system at pH 4 is very low. The lysozyme that is released is likely the result of the change in lysozyme charge due to the decrease in pH.[34]

For the three other systems, the charge of one or two of the polyelectrolytes present will depend on the pH of the system. To illustrate, Figure 3C shows a sketch of the charge of the polyelectrolytes and lysozyme as a function of the pH.[34] Weakly charged polycations are fully charged at low pH and uncharged at high pH while the opposite will be found for weak polyanions. If we first consider the system consisting of two weakly charged polyelectrolytes (PAA/PAH), a pH decrease from 7 to 4 will result in a decrease of negative charge on the anionic PAA and an increase in positive charge on cationic PAH. For this system we find almost complete lysozyme release, as has been reported earlier for PECs consisting of shorter PAA and PAH.[16] The protein release can be explained by a change in complex composition that occurs when the net charge on the polyelectrolytes changes when the pH is decreased. Effectively the system obtains a different composition than F^{-}_{optimal} and less protein can be taken up by the complex (Figure 2).[16] In principle an increase in pH will have the same, but opposite effect on the charge of the polyelectrolytes, now the polycation becomes less charged and the polyanion more. For lysozyme, with an isoelectric point of 11.65 a pH increase makes the protein less charged other interactions between the protein and the polyelectrolytes become stronger and make back-extraction at high pH difficult.[16]

The PAH/PSS and PDADMAC/PAA systems both consist of a weak and a strongly charged polyelectrolyte, but a clear difference in protein release is observed. The relatively large amount of lysozyme released by PDADMAC/PAA PECs and relatively small amount of lysozyme released by PAH/PSS can be explained when considering the sketch presented in Figure 3C. At pH 4 PAA will become less charged and the interaction between the PEC and the protein become weaker and more lysozyme is released. For the PAH/PSS almost no lysozyme is released by a decrease in pH. In this system a decrease in pH will result in a stronger interaction between PAH and PSS, as PAH becomes more charged.

Protein release from PECs might be dependent on system parameters apart from an increase in ionic strength or decrease in pH. For the lysozyme release as investigated in Figure 3, there was an abundance of supernatant phase compared to PEC phase of a factor of 30–60. In addition, lysozyme has high solubility in water. Together, this ensured that full back-extraction of lysozyme could be observed. However, care must be taken to design the back-extraction process for proteins with lower water solubility, or when working with different polyelectrolyte and protein concentrations.

The four PECs presented in this study display similar maximum lysozyme take-up (Figure 2). Contrary, the salt and pH-dependent release properties of these complexes are very different leading to strongly differing back-extraction efficiencies (Figure 3). Of the four polyelectrolyte combinations, PAH/PAA PECs demonstrated the greatest potential for lysozyme back-extraction by releasing all of its lysozyme with a decrease in pH. For this reason we continued with PAH/PAA PECs to evaluate whether they can be used to selectively extract lysozyme from a complex protein mixture. The protein mixture in this study is a lyophilized albumen powder, which is a commercially available protein mixture obtained by freeze-drying hen-egg white. It is commonly used for baking and cooking.

To extract lysozyme from albumen, PAH and PAA were added to a solution of albumen powder at the optimal lysozyme partitioning composition of $F^{-} = 0.53$ (Figure 2A). The supernatant phase was then refreshed and the protein(s) taken up by the PEC were released by lowering the pH from 7 to 4, similar to Figure 2. The protein composition is analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins based on mass (**Figure 4**). Columns on the gel represent different samples and bands within the columns represent proteins of similar mass.

In Figure 4, the protein composition of various steps in the lysozyme extraction process are shown. The three left columns contain reference samples of known protein sizes (R), pure lysozyme (A), and the albumen solution (B). Column (F) contains only PAH/PAA PECs. Columns (C–E) are triplicates of the supernatant phases after the addition of PAH and PAA at $F^{-} = 0.53$. Columns (G–I) are triplicates of the back-extraction.

In the albumen solution (B), a band indicating the presence of lysozyme, with a known MW of 14.3 kDa, is visible between 10 and 15 kDa. Lysozyme is the only protein present in albumen with this molecular mass.[28] Other common albumen proteins are indicated in Figure S4, Supporting Information. When PAH and PAA are added (C–E) the protein band representing lysozyme becomes less intense, indicating a decrease in lysozyme. After the back-extraction (G–I), we find only lysozyme in the supernatant. This suggests that while not all of the lysozyme is extracted, everything that is extracted and back-extracted is lysozyme.

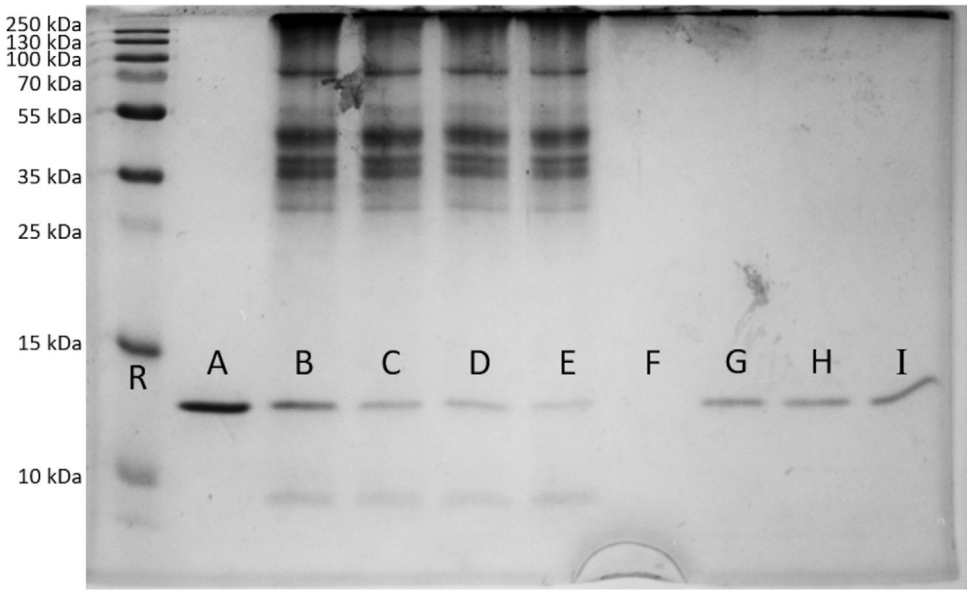

Figure 4. SDS-PAGE gels of the supernatant during extraction and release steps of 1 g L^{-1} albumen solution. R) Reference protein mixture, A) pure lysozyme, B) 1 g L^{-1} albumen solution, C–E) triplicate of supernatant after extraction, F) supernatant of PAH/PAA PECs without proteins, G–I) triplicate of the back-extraction supernatant after lowering the pH.

Columns (C–E) show that not all lysozyme of the albumen is taken up by the PEC. The presence of the (charged) albumen protein could alter $F^-_{optimal}$. The extraction with other PAH/PAA compositions around $F^-_{optimal}$ were investigated but no F^- was found where all lysozyme was clearly extracted (Figure S4, Supporting Information). A plausible explanation for the lack of full lysozyme extraction is the presence of the protein ovomucin; the most common albumen protein after lysozyme.[28] Ovomucin is known to bind to lysozyme via electrostatic interactions.[47,48] As we know that charge plays an important role in the partitioning of lysozyme in PECs,[16,18] the binding to ovomucin could prevent the lysozyme from interacting with the PECs. In SDS-PAGE, the proteins are exposed to SDS which results in denaturation of the proteins and separates ovomucin from lysozyme, resulting in lysozyme being visible as a band on the SDS-PAGE gel. This process is analogous to the known technique of separating ovomucin and lysozyme by increasing the salt concentration.[46,47] From earlier studies, we know that PECs can take-up high concentrations of lysozyme, therefore lysozyme saturation of the PEC is unlikely.[15,16]

Charge plays an important role in the uptake of proteins by PECs. From the release studies presented in Figure 3, screening of the charges on the polyelectrolytes as well as changing the amount of charge by a decrease in pH affects the partitioning of the proteins. In previous work we have shown that by changing the composition of the PECs, lysozyme and the oppositely charged succinylated lysozyme could be separated.[16] Albumen is a significantly more complex mixture than a mixture of lysozyme and succinylated lysozyme. Within the first ten most common proteins in albumen, lysozyme is the only protein with a positive charge at pH 7.[28] The results suggest that also in this study the charge of the protein species is a very important factor in selective extraction of a single protein from a mixture.

The concentration of the extracted lysozyme can be determined with UV–vis spectroscopy. Out of the initial concentration of 1 g L^{-1} albumen powder, the extracted concentration of lysozyme is 41.4 ± 2.8 mg L^{-1} (average ± standard deviation, n = 5). This is similar to the reported concentration of 34–35 mg L^{-1}.[28,29] It is possible that the lyophilized powder solution is more concentrated than native albumen or that the exact protein composition per egg depends on biological and external factors. Recently, a study has shown that the lysozyme content of hen eggs has notable variation depending on chicken breed as well as a large spread within breeds.[49]

Protein function is intrinsically linked to protein structure. Uptake and release of lysozyme in and from PECs could have a detrimental effect on the lysozyme structure either by the protein's temporary presence in a different environment or by structural disruption via direct interactions between either polyelectrolyte and the lysozyme. To confirm that extraction via PECs does not disrupt the structure (and thus function) of proteins, we evaluated the activity of lysozyme extracted and back-extracted from the albumen solution as shown in Figure 4. The assay we use is based on the turbidity of a bacteria suspension. A decrease of turbidity over time indicates the presence of active antibacterial enzymes like lysozyme.

In **Figure 5** the activity of lysozyme extracted from albumen solution is compared to that of a negative control (substrate only) or positive control (lysozyme solution). We observe a decrease of absorbance at 450 nm for both the positive control as well as the extracted lysozyme over time indicating that both solutions contain active enzymes. The concentration of the lysozyme in the positive control was 7.5 mg L^{-1} and the measured concentration of the extracted lysozyme was 41.4 ± 2.8 mg L^{-1}.

The enzymatic activity in units mL^{-1} was determined from the decrease in absorbance using Equation 2. The activity of the extracted lysozyme was found to be 929 ± 85 units mL^{-1}.

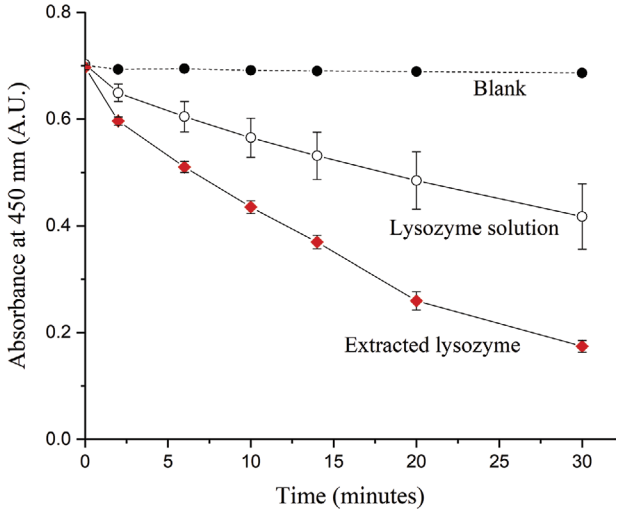

Figure 5. Enzymatic activity of the lysozyme extracted from albumen solution. A decrease in absorbance at 450 nm over time is associated with enzymatic activity. Filled circles (●) are substrate only. Empty circles (○) are 7.5 mg L^{-1} commercially bought purified lysozyme. Red diamonds (♦) are lysozyme extracted and back-extracted with PAH/PAA PECs. Symbols represent averages and the error bars represent standard deviations ($n = 3$). Lines connect the averages.

The activity of the commercial purified lysozyme was 457 ± 146 units mL^{-1}.

The activity of the extracted lysozyme seems approximately two times higher than commercial purified lysozyme. However, the concentration of the extracted lysozyme was ≈5.5 times higher than the commercial lysozyme. Effectively, this is a decrease in activity per amount of lysozyme of a factor 2.7 compared to commercial purified lysozyme. It is known that lysozyme activity varies between chicken populations as well as individual chickens.[50,51] The commercially available purified lysozyme is a composite of many albumen sources, whereas our extraction was done on one egg.

The activity of the complete albumen solution was 1490 ± 289 units mL^{-1} (Figure S5, Supporting Information). The extraction process takes ≈48 h and occurs at room temperature. In this time the activity of the enzymes could have decreased due to naturally occurring enzyme denaturation. For the back-extraction a decrease in pH is used. This could also have an effect on the enzymatic activity. No significant effect of these factors on the activity of albumen solution was observed (Figure S6, Supporting Information). The difference in activity between the extracted lysozyme and the complete albumen solution can be explained by a combination of two factors. First, other albumen proteins such as ovotransferrin have been reported with similar antibacterial properties as lysozyme,[52,53] so the extracted lysozyme would be expected to show decreased antibacterial enzymatic activity even with a perfect back-extraction efficiency. Second, we know from Figure 4 that we do not extract all lysozyme.

It has previously been shown that the structure of BSA as determined by circular dichroism remained intact after a pH-induced release from liquid-like polypeptide PECs. In this work we show that the selectively extracted and back-extracted lysozyme (Figure 4) also retains its enzymatic properties (Figure 5). Our results show the potential of PECs as extraction media for the recovery and isolation of proteins from complex protein mixtures.

The observed protein partitioning behavior of the PECs used in our study has implications for the greater understanding of MLOs. In this study it is shown that PECs respond to changes in pH, salt concentration, and the PEC composition. These three factors can fluctuate in the intracellular environment: cells can produce more or less of IDPs or RNA and thereby alter the MLO composition, and the pH[54–56] as well as the concentration of salt ions[57] are known to vary within cells and as function of the cell cycle.

Two of the PEC systems in this study are expected to have a similar pH response as cellular MLOs. The weak/weak PAH/PAA PECs are analogous to MLOs consisting of two oppositely charged IDPs.[58] The PAH/PAA PECs will readily release its lysozyme due to a decrease in pH or due to increasing salt concentration, this behavior could also apply to IDP/IDP MLOs. The weak/strong PAH/PSS PECs can be compared to IDP/RNA MLOs. PAH/PSS PECs are very resistant to releasing lysozyme despite a decrease in pH or an increase in salt concentrations. Therefore IDP/RNA MLOs are expected to be relatively robust with respect to fluctuations in pH or salt concentration. Direct comparison between PECs and MLOs is difficult due to the complexity of the cellular environment and the unknown composition of MLOs. However, direct comparison between in vitro PECs and in vivo MLOs is difficult due to the complexity of the cellular environment and the unknown composition of MLOs.

3. Conclusion

Here we have shown that lysozyme can be back-extracted from a chicken albumen solution using PECs while retaining its enzymatic function. Although all the PECs studied display similar partitioning profiles of lysozyme as function of the complex composition, lysozyme release strongly depended on the type of polyelectrolytes and the release method used. For the lysozyme back-extraction, charge plays a dominant role, this will not necessarily be the case for all proteins. A systematic study of different types of proteins is required to make this method widely applicable for industrial applications.

4. Experimental Section

Materials: PAH solution with MW of 150 kDa (monomer mass 94 Da) was purchased from Nittobo. PAA acidic form solution (MW of 100 kDa, monomer mass 72 Da product number 523925), PDADMAC solution (MW of 200–350 kDa, monomer mass 162 Da, product number 409022), PSS sodium salt solution (MW 200 kDa, monomer mass 206 Da, product number 561967), hen-egg lysozyme (MW of 14.3 kDa, isoelectric point of 11.35, activity of 40000 U mg^{-1}, product number L6876), HCl solution, NaOH, NaCl, tris(hydroxymethyl)aminomethane (Tris), tetramethylethylenediamine, sodium dodecyl sulfate (SDS), glycerol, glycine, methanol, acetic acid, isopropanol, and lyophilized *Micrococcus lysodeikticus* bacteria were purchased from Sigma-Aldrich/Merck. Ammonium persulfate, Coomassie brilliant blue R-250, and bromophenol blue were purchased from Bio-Rad. PageRuler Plus prestained protein ladder was purchased from ThermoFischer Scientific. Lyophilized albumen powder was purchased from "De Zuidmolen"

baking store. According to the manufacturer, albumen powder was made by freeze-drying the albumen directly after separation from the egg yolk. Unless otherwise specified, solutions were made with ultrapure water (mQ) filtered by an Advantage A10 water purification system (Millipore).

PEC Composition, Formation, and Lysozyme Incorporation: Polyelectrolyte stock solutions were diluted from purchased solutions to working solutions (80 g L^{-1}). The pH of the working solutions was set to 7 (within the range of 6.8 to 7.2) with NaOH or HCl solutions. PECs are formed by mixing specific amounts of polycationic and polyanionic solutions. The composition, in term of the charge fraction, of the PEC was quantified in F^-, which was defined as:[15–18]

$$F^- = \frac{[n^-]}{[n^-]+[n^+]} \quad (1)$$

where $[n^-]$ is the concentration of negative monomers and $[n^+]$ is the concentration of positive monomers upon mixing of polyelectrolyte solutions under the assumption that all monomers are fully charged.[15–17,33,59]

Unless otherwise specified, solutions were mixed so that the final total concentration of polyelectrolytes was 2 g L^{-1} and the concentration of lysozyme was 1 g L^{-1}. These concentrations were chosen based on earlier reports that for PAH/PAA PEC systems, there would likely be a PEC composition F^- with complete lysozyme partitioning in the PEC.[16] Where possible, like-charged compounds were mixed prior to addition of an oppositely charged compound and then thoroughly mixed. The total volume was set to 250 µL. Unless specified, no salt ions were added beyond those brought into the system as counterions to the polyelectrolytes are as a result of the setting of PH with NaOH and HCl.

Supernatant Lysozyme Concentration Determination: After mixing, the samples were left to equilibrate for one day. Prior to measurement, samples were centrifuged at 12400 g for 30 min.[15,16,20] The protein concentration of diluted supernatant was determined on a 2401PC spectrophotometer (Shimadzu) at 281.5 nm. The supernatant lysozyme sample concentration was expressed relative to a lysozyme control without polyelectrolytes. A lower amount of lysozyme in the supernatant corresponds with a larger amount of lysozyme in the PEC. Finally, the absorbance value of a PEC control containing the same polyelectrolytes but not containing lysozyme was subtracted from the measured absorbance to compensate for absorbance caused by only the PECs or polyelectrolytes.

Lysozyme Back-Extraction from PECs: To determine the back-extraction of lysozyme from PECs as a result of added NaCl or HCl, PECs containing lysozyme were first formed at the F^- composition found to be optimal for lysozyme partitioning as previously described. The supernatant was then removed and replaced with mQ water containing either NaCl solution (0.5 M) or HCl solution (4 mM, corresponding to a pH of ≈4 in the presence of the polyelectrolytes). After another day to equilibrate, the supernatant lysozyme concentration was determined as previously described.

Lysozyme Extraction from Lysozyme and Back-Extraction from PECs: Lyophilized chicken albumen powder was dissolved to prepare a stock solution (10 g L^{-1}), which was then diluted to 1 g L^{-1} for extraction. Extraction was done by adding PAH and PAA (in that order) at $F^- = 0.53$ at a total concentration of 4 g L^{-1} albumen solution. After one day to equilibrate, PECs were centrifuged at 10000 g for 30 min (Figure S1, Supporting Information). Supernatant samples were taken for evaluation of protein content via SDS-PAGE after which the remaining supernatant was aspirated. New mQ (250 µL) was added as new supernatant with 4 mM HCl. After an additional day to equilibrate, supernatant samples were taken again for evaluation via SDS-PAGE and for evaluation of the enzymatic activity via an activity assay.

Protein Analysis via SDS-PAGE: The protein composition of the albumen solution, supernatant during the extraction step, and supernatant after the back-extraction were determined via SDS-PAGE. Samples were stored at −20 °C before evaluation. Samples were thawed and mixed with an equal volume of loading buffer consisting of Tris (0.12 M), glycerol (20%), SDS (4%), and bromophenol blue (0.02%) before electrophoresis on a polyacrylamide gel (15%) in a Mini-PROTEAN vertical electrophoresis cell (Bio-Rad) for 30 min at 90 V, 30 min at 120 V, and 45–60 min at 150 V until the bromophenol blue indicator had left the gel. The gel was then transferred to and stained in a methanol (30%), acetic acid (10%), and Coomassie brilliant blue R-250 (0.05%) for 1 h. Destaining of the gel was done by submerging in flushes of methanol (30%) and acetic acid (10%) solution until background staining was removed. The gel was imaged with a Fluorchem M (ProteinSimple).

Lysozyme Activity Assay: Lysozyme has anti-bacterial properties by cleaving the cell walls of gram-positive bacteria. Enzymatic activity can be determined and quantified by adding lysozyme solution to a suspension of lyophilized *M. lysodeikticus* bacteria (150 mg L^{-1}). The bacteria results in a turbidity determined by optical absorbance at 450 nm,[60] which will decrease in the presence of active lysozyme or remain mostly constant in the absence of active lysozyme. The activity of lysozyme as determined via this protocol can be determined by:

$$units\ ml^{-1} = \frac{A(T) - A(B)}{0.001 * 0.05} \quad (2)$$

The amount of units mL^{-1} is derived by the decrease in absorbance at 450 nm (A(T)) relative to the decrease in absorbance at 450 nm of the bacteria suspension without the addition of active component (A(B)). The factor 0.001 is part of the unit definition, and the factor 0.05 is to translate the tested volume (50 µL) to units mL^{-1}. In this study, lyophilized bacteria suspension (2 mL) was used as the substrate, and solution containing active enzyme (back-extracted lysozyme solution) (50 µL) or purified commercially bought lysoyzme (7.5 mg L^{-1}, 50 µL) or additional substrate (50 µL) was added.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge funding from the Netherlands Organization for Scientific Research (NWO) VIDI grant (# 723.015.003).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

egg whites, extraction, lysozyme, membrane-less organelles, polyelectrolyte complexes, polyelectrolytes, proteins

Received: August 26, 2021
Revised: October 18, 2021
Published online:

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