

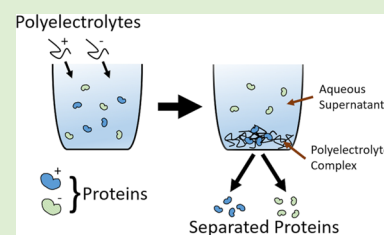
Charge-Based Separation of Proteins Using Polyelectrolyte Complexes as Models for Membraneless Organelles

Jéré J. van Lente,^{†,‡} Mireille M. A. E. Claessens,[†] and Saskia Lindhoud^{*,†}

[†]Department of Nanobiophysics, and [‡]Membrane Science & Technology Cluster, University of Twente, Drienerlolaan 5, 7522 NB Enschede, The Netherlands

Supporting Information

ABSTRACT: Membraneless organelles are liquid compartments within cells with different solvent properties than the surrounding environment. This difference in solvent properties is thought to result in function-related selective partitioning of proteins. Proteins have also been shown to accumulate in polyelectrolyte complexes, but whether the uptake in these complexes is selective has not been ascertained yet. Here, we show the selective partitioning of two structurally similar but oppositely charged proteins into polyelectrolyte complexes. We demonstrate that these proteins can be separated from a mixture by altering the polyelectrolyte complex composition and released from the complex by lowering the pH. Combined, we demonstrate that polyelectrolyte complexes can separate proteins from a mixture based on protein charge. Besides providing deeper insight into the selective partitioning in membraneless organelles, potential applications for selective biomolecule partitioning in polyelectrolyte complexes include drug delivery or extraction processes.



INTRODUCTION

In cells, many (bio)chemical reactions and processes necessary for functioning require environmental conditions that deviate from those in the cytosol. These processes are often performed in specialized compartments called organelles. In some organelles, such as the nucleus and mitochondria, compartmentalization is achieved by membrane encapsulation. Alternatively, cells create microenvironments by inducing liquid–liquid phase separation resulting in the formation of membraneless organelles (MLOs). For several MLOs, the presence of RNA and specific intrinsically disordered RNA-binding proteins (RBPs) with intrinsically disordered regions has been reported to drive phase separation and the formation of MLOs.^{1–6} Both RNA and RBPs are natural polyelectrolytes; polymeric macromolecules consisting of charged monomeric subunits. RNA is a strong polyanion, while RBPs are typically weak polycations.⁷ For these MLOs, phase separation is driven by complex coacervation.^{8–10}

Although the exact function is not known for all MLOs, specific biological functions typically require the controlled accumulation and release of (bio)molecules.^{3,11–14} Additionally, MLOs need to partition specific compounds with a high degree of specificity as the cytosol contains a large variety of different compounds, many of which share structural and physicochemical similarities. MLO malfunction may lead to undesired biological consequences.¹⁵ For example, the hyperphosphorylation of tau observed in several neurodegenerative diseases has been reported to drive liquid–liquid phase separation by coacervation. These tau droplets can serve as an intermediate toward the formation of amyloid deposits of tau found in neurodegenerative diseases.¹⁶

Since the ability to specifically and dynamically accumulate and release compounds is an emergent property of MLOs, it may also be possible to induce this behavior in alternative systems that phase-separate via polyelectrolyte complexation. Oppositely charged polyelectrolytes can phase-separate into polyelectrolyte complexes (PECs) in aqueous solution. The properties of PECs consisting of synthetic polyelectrolytes resemble those of MLOs. Several studies have reported that proteins can accumulate in PECs.^{17–23} However, it is unclear if more complex behavior such as the selective accumulation of compounds also emerges in PECs.

In this study, we investigated the ability of PECs composed of the weak polyelectrolytes poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) to dynamically discriminate between two oppositely charged protein species, lysozyme and succinylated lysozyme. Previous research has focused on two-component systems containing a protein and an (oppositely charged) polyelectrolyte.^{24–29} Such systems have been shown to be able to separate proteins by selective interaction with a polyelectrolyte.^{29–32} In these works, a specific protein in a mixture has a higher affinity to the added polyelectrolyte, allowing the specific protein to complexate with the polyelectrolyte into a coacervate, leaving the other proteins in solution. In our system, the polyelectrolyte complex is formed by two oppositely charged polyelectrolytes, which both interact with the protein, resulting in a three-component system. This allows us to change the ratio between the

Received: May 21, 2019

Revised: August 14, 2019

Published: August 16, 2019

polyelectrolytes and thus gives us an additional parameter by which we can tune partitioning of proteins into the PECs.

Lysozyme is a common antimicrobial enzyme that has been reported to partition in a PEC system.¹⁷ Succinylated lysozyme is chemically modified to hold an equal but opposite charge at physiological pH with a very similar structure³³ to native lysozyme.

PAH and PAA are commonly used polyelectrolytes with known phase behavior. PECs of these polyelectrolytes have been observed previously to enrich proteins.^{21,34} PEC model systems are less complex compared to MLOs and may help provide a better physicochemical understanding of how complex coacervation contributes to intracellular organization. In this study, we find that the partitioning of both lysozyme and succinylated lysozyme strongly depends on the PEC composition with maximal protein partitioning into PECs observed at distinct but different charge ratios. At the charge ratio where maximal partitioning is observed, the partitioning coefficient remains constant for a range of protein concentrations indicating that the PECs behave as a solvent for the protein. Sharp transitions were observed between complete and no protein partitioning, both as functions of the PEC composition and solution pH. We demonstrate that the sharp transitions and difference in PEC composition at which maximal partitioning is observed can be exploited to separate structurally similar proteins of opposite charge from a mixture. We suggest that the mechanism responsible for the composition- and pH-dependent partitioning behavior may be exploited by MLOs.

■ EXPERIMENTAL SECTION (MATERIALS AND METHODS)

Materials. Commercially available materials used were poly(acrylic acid) (PAA) (Polysciences, Cat# 06567, MW = ±6000), poly(allylamine hydrochloride) (PAH) (Sigma-Aldrich, 283215, MW = ±17,500 Da), and lysozyme (Sigma-Aldrich, L6876). Succinylated lysozyme was made as previously described.³³ Stock solutions were adjusted to pH 7–7.4 with HCl (Merck, 1.00317.1000) or NaOH (Merck, 1.06462.1000). Protein concentrations were determined using UV–vis at 281.5 nm on a Shimadzu UV-2401PC spectrophotometer, using a molecular extinction coefficient of 2.635 L g⁻¹ cm⁻¹ for both proteins.³⁵

Charge Concentration and Ratio. To determine the charge ratio, both polyelectrolytes were assumed to be fully charged at pH 7. Under this assumption, the charge of any amount of polyelectrolyte is a function of the molecular weight of the composite monomers. Lysozyme and succinylated lysozyme have charges of +7 and -7 at pH 7–7.4, respectively.^{17,33,36,37} The charge ratio F^- was defined as

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

where $[n^-]$ and $[n^+]$ are the negative (PAA) and positive (PAH) charge concentrations, respectively.^{17,28,38} Different ratios of polyelectrolyte are mixed to result in different F^- charge ratios. The number of charges per polyelectrolyte molecule is a function of monomer weight and remained constant. To change F^- , the concentration of PAA was kept constant while the concentration of PAH was varied. Variation in the order of addition of the polyelectrolytes did not give different results. Lysozyme partitioning into PECs was evaluated for a range of polyelectrolyte and protein concentrations (Supplementary Figure 1). From these experiments, we decided to continue experimentation with concentrations of 1 g/L PAA and 1 g/L protein.

The optimal charge ratio F_{opt}^- was defined as the F^- corresponding to the lowest concentration of protein in the supernatant.

Protein Supernatant Measurements. Compounds are mixed as follows: first, mixtures of the like-charged molecule were prepared, and then these mixtures were combined, thoroughly vortexed, and left to equilibrate for 2 days. Protein concentration was set at 0.8–1 g/L unless otherwise specified. Samples were then centrifuged at 12,500 g for 30 min. Protein concentration in the supernatant was then determined by measuring the absorbance spectra of appropriately diluted supernatant on a Shimadzu UV-2401PC spectrophotometer as previously described. If supernatant samples showed an absorbance of over 0.01 AU at 400 nm, this was taken as indicative of the presence of dissolved complexes and the sample was discarded as the presence of dissolved complexes interferes with the protein concentration determination. Protein concentration in the supernatant sample was compared to a control containing only protein, similar to other studies.¹⁹ The presence of PAH or PAA had a negligible influence on the protein concentration measurements (Supplementary Figure 2).

For experiments investigating the supernatant protein as a function of pH, a pH-sensitive electrode (Mettler Toledo, InLab Flex-Micro) was used. Diluted (10 mM) HCl and NaOH were used to adjust the pH to the desired values.

Determination of Partition Coefficient and Partition Free Energy. To determine the partition coefficient and free energies, the supernatant protein concentration was measured as described previously. Additionally, the complex mass was calculated by measuring empty sample tubes and sample tubes with the dilute supernatant phase removed. As an approximation, the PEC density was taken as equal to that of water. From this data, the protein concentration in the complex was calculated, and the partition coefficient and partition free energies for the systems when equilibrated were calculated via:

$$K_{\text{partition}} = \frac{[\text{protein}]_{\text{complex}}}{[\text{protein}]_{\text{supernatant}}} \quad (2)$$

$$\Delta G_{\text{partition}} = -RT \ln(K_{\text{partition}}) \quad (3)$$

Protein Release from PEC. To evaluate whether protein partitioning was reversible, the ability of the PECs to release proteins was investigated using a pH change. First, proteins were partitioned at their optimal charge ratio $F_{\text{opt}}^- = 0.65$ for lysozyme or $F_{\text{opt}}^- = 0.55$ for succinylated lysozyme. The supernatant protein concentration was then measured as previously described, and 1 μL of 1 M HCl was added (resulting in a measured pH of approximately 4) to lower the pH. After 2 more days to equilibrate, supernatant protein concentration was measured again. Supernatant protein concentrations were compared to control samples not containing polyelectrolytes.

Protein Analysis on Polyacrylamide Gel. Polyacrylamide gel electrophoresis was used to qualitatively distinguish between lysozyme and succinylated lysozyme. For the different steps (A–D) of the protocol shown in Figure 3A, supernatant samples were frozen at -80 °C until evaluation. A polyacrylamide gel solution consisting of 65% 0.3 M tris(hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500) adjusted to pH 8.5, 10% acrylamide (Merck, 1.00639.1000), 0.1% ammonium persulfate (Bio-Rad, 1610700), and 0.1% tetramethylethylenediamine (Sigma-Aldrich, T7024) in MilliQ water was prepared. A comb was inserted approximately halfway the gel to create sample slots. The solution was left to polymerize for 45 min under a layer of isopropanol (Merck, 1.09634.1000). Afterward, the isopropanol was decanted, and leftovers were removed by rinsing the gel with demineralized water.

The undiluted supernatant was thawed and mixed 1:1 with sample–buffer consisting of 0.12 M tris(hydroxymethyl)aminomethane (Tris) (Merck, 1.08382.0500), 20% glycerol (Merck, 356350), and 0.02% bromophenol blue (Bio-Rad, 161-0404). Of the sample/sample–buffer mixture, 30 μL was transferred to the individual sample slots on the gel. The electrophoresis was done at 90 V for 3 h in running buffer consisting of 26 mM Tris and 192 mM glycine (Sigma, G8898) in MilliQ water.

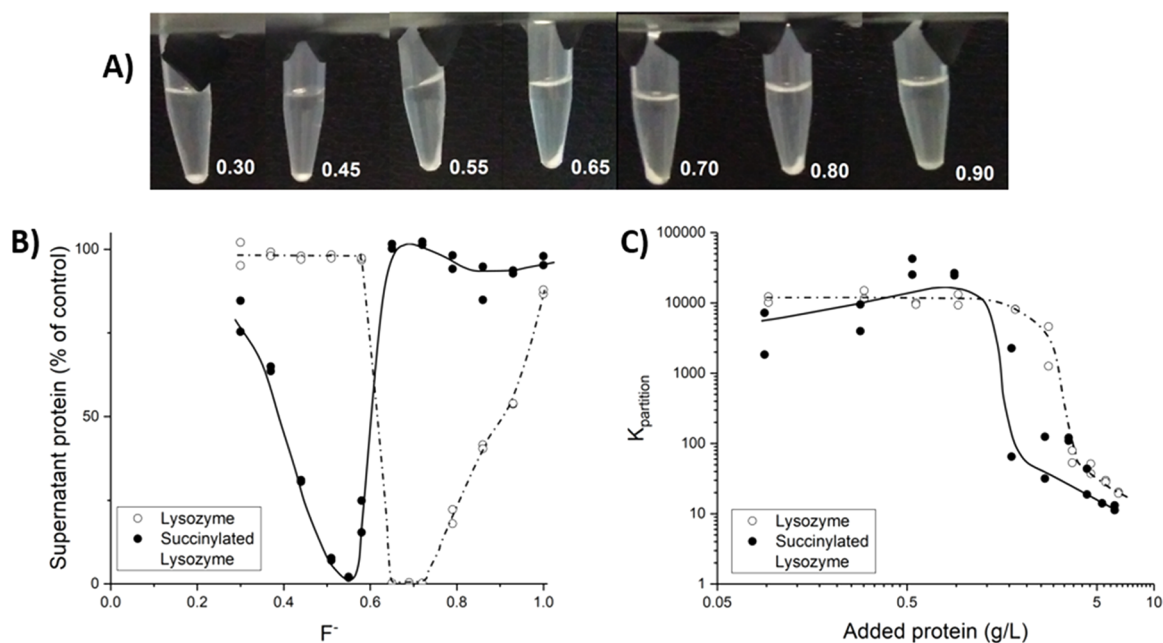


Figure 1. Partitioning of lysozyme (open circle) and succinylated lysozyme (solid circle) in PAH/PAA PECs. Individual measurements are shown as dots, the lines are drawn to guide the eye. (A) Images of samples after centrifugation. The numbers in the images corresponding to the F^- values at which the samples were prepared, indicated by the white numbers. (B) Protein in the supernatant as a function of F^- at a protein concentration of 0.8–1 g/L. Protein concentration in the supernatant is expressed as a percentage of the control system without polyelectrolytes. (C) Partition coefficient of the proteins into the PECs at their F_{opt}^- as a function of added protein.

After electrophoresis, the gel was fixed for 1 h in a 30% methanol (ATLAS & ASSINK CHEMIE, 0360.01.210.5) and 10% acetic acid (Merck, 1.00063.1000) solution and then washed with MilliQ water for 30 min and 1 h. The gel was left to stain in Imperial Protein Stain (Thermo Scientific, Prod# 24615) overnight before destaining with MilliQ water twice for 1 hour. The gel was imaged with a ProteinSimple Fluorchem M, and ImageJ was used to evenly remove the background intensity from the images.

RESULTS

Protein Partitioning Depends on PEC Composition.

Intracellular MLOs are able to partition proteins from the cytosol.¹² Polyelectrolyte complexes have been reported to do the same.^{17–21} We previously reported that lysozyme enrichment in PDMAEMA/PAA PECs is a function of the composition of the PEC F^- (eq 1), with maximal partitioning into the PEC at $F^- = \sim 0.63$.¹⁷ To investigate whether enrichment in PECs depends on the protein properties such as the charge of the protein, we investigated the accumulation of lysozyme and chemically modified succinylated lysozyme as functions of F^- . Both proteins are structurally nearly identical but carry a net opposite charge at neutral pH.³³ To investigate the enrichment of both proteins in PAH/PAA PECs, F^- was varied and the amount of protein in the supernatant was measured. In Figure 1A, we show images of the PEC-containing samples after centrifugation within sample tubes. The polyelectrolytes have formed a viscoelastic dense white solid-like precipitate. In a total volume of 250 μL , the PEC volume makes up around 5 μL (2%) with the remaining volume consisting of the dilute supernatant aqueous phase.

Figure 1B shows distinct partitioning profiles for lysozyme and succinylated lysozyme between the PEC and dilute supernatant phase. Both proteins show a minimum in the supernatant protein concentration as a function of F^- . At this minimum, the protein has maximally accumulated in the PEC.

For both proteins, we also observe an F^- region where no partitioning takes place and nearly all protein is found in the supernatant.

Interestingly, the partitioning of lysozyme and succinylated lysozyme follows a mirrored pattern. We defined the optimal partitioning charge ratio F_{opt}^- as the charge ratio with maximum protein partitioning into the PEC. F_{opt}^- was determined to be $F_{\text{opt}}^- = F^- = 0.65$ for lysozyme and $F_{\text{opt}}^- = F^- = 0.55$ for succinylated lysozyme. Note that the optimal partitioning ratio F_{opt}^- for neither lysozyme nor succinylated lysozyme is found at the (calculated) equal net charge of $F^- = 0.50$. The deviation of F_{opt}^- from $F^- = 0.50$ is not explained by the additional charges brought in by the proteins, which, when included, would shift the F_{opt}^- of lysozyme to 0.63 and not affect the F_{opt}^- of succinylated lysozyme. If the partitioning was a solely charge-driven process, we would expect maximum protein partitioning at $F^- = 0.50$. The fact that F_{opt}^- of both proteins deviates from 0.50 indicates that, regardless of the charge of the protein, both polyanions and polycations are required for proteins to accumulate in PECs. This may indicate that the selective partitioning of proteins into PECs is an emergent property of PECs. The necessity for an excess of positive or negative charges compared to positive charges (i.e., F_{opt}^- not equal to 0.5) has been observed previously for protein–polyelectrolyte systems,^{28,38} although no clear mechanism has been established. Charge patchiness of the protein and charge regulation phenomena have been suggested as possible reasons.²²

If the protein enrichment in PECs was solely governed by charge–charge interactions, one would expect the partitioning of lysozyme in PECs to increase with higher values of F^- . However, we observe that the supernatant lysozyme increases at F^- values higher than F_{opt}^- . The total PEC mass decreases at high F^- , as PAA has less PAH available to form PECs. At high F^- , it is likely that smaller soluble PAA–lysozyme complexes

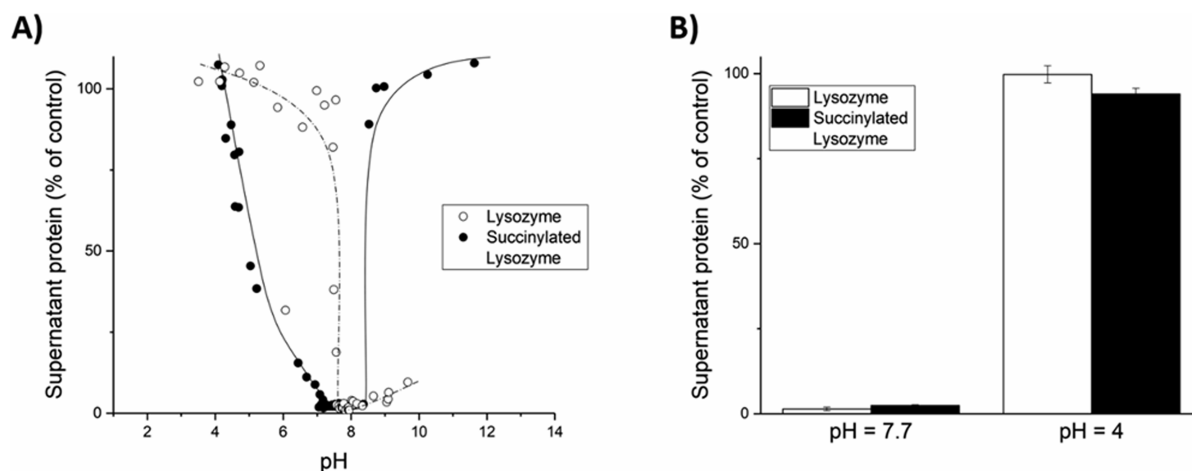


Figure 2. Effect of pH on the partitioning of lysozyme (open circle) and succinylated lysozyme (solid circle) into PECs. (A) PECs are prepared at $F^- = 0.65$ or 0.55 for lysozyme or succinylated lysozyme, respectively, while the pH of the system is varied. Individual measurements are represented by dots. Lines are drawn to guide the eye. (B) Release of proteins from the PECs by lowering the pH from ± 7.7 to 4. $N = 3$.

form instead. At low F^- , the same happens for soluble succinylated lysozyme–PAH complexes.

In a previous study, we have enriched lysozyme in a PDMAEMA/PAA complex coacervate system and observed a 90–95% decrease of the protein in the supernatant phase and concomitant accumulation in the PEC phase.¹⁷ For the PAH/PAA system investigated here, we report a decrease of 99.8% of lysozyme in the supernatant at a comparable F^- (0.65 vs 0.63). Interestingly, Zhao and Zacharia used a similar PAH/PAA system to partition bovine serum albumin (BSA) but only saw a decrease of 50% of the supernatant protein concentration.²¹ Our experimental findings and the literature combined suggests that the partitioning behavior of proteins in polyelectrolyte complexes is likely dependent on the structural and physicochemical properties of the polyelectrolytes and the partitioned protein. Future research in which multiple polyelectrolyte and protein systems with distinctly different properties are evaluated is necessary to elucidate the exact nature of the responsible interactions and mechanisms.

Protein Partitioning Coefficient Are Protein Concentration-Dependent. The PAH/PAA PECs studied here form a separate aqueous phase in which proteins can be localized. The partitioning between the dilute phase and the PEC phase can be quantified by the partitioning coefficient $K_{\text{partition}}$ (eq 2), which we show as a function of the protein concentration (c_{protein}) in Figure 1C. In this figure, two regimes of $K_{\text{partition}}$ as a function of c_{protein} are visible. For low c_{protein} up to 2–3 g/L, $K_{\text{partition}} > 1000$ was found. At higher c_{protein} , the $K_{\text{partition}}$ decreases presumably because the PEC becomes saturated with proteins. In this regard, PAH/PAA PECs behave as normal solvents despite being in a solid-like phase.

The $K_{\text{partition}}$ values for (succinylated) lysozyme in PEC/water systems are within the range of reported $K_{\text{partition}}$ values for small molecules such as heptane in octanol/water systems.^{39–41} Comparable or lower $K_{\text{partition}}$ values are reported for proteins in polypeptide coacervates^{18,19,22} or in other synthetic polyelectrolyte systems.²³ Interestingly, BSA completely partitioned into polypeptide coacervates,¹⁹ whereas only half of BSA was partitioned in PAH/PAA PECs.²¹ In one study where multiple proteins were evaluated in the polypeptide coacervate system, lysozyme was found to have a noticeable higher maximum $K_{\text{partition}}$ (~ 1000) compared to

other proteins,²² although this $K_{\text{partition}}$ was still lower than that for lysozyme in the PAH/PAA PECs. It is important to note that different quantities of PECs and protein concentrations can give an inaccurate partition coefficient if the experimental conditions are not below that of the saturation of the PEC.

Like polyelectrolytes, the intrinsically disordered regions of some proteins have been shown to undergo liquid–liquid phase separation.^{42,43} Schuster et al. prepared model MLOs from such proteins and investigated the partitioning of fluorescent proteins into the protein-rich phase. In these phases, partition coefficients up to 27 were found, depending on the type of fluorescent protein and any additional protein modification.²⁰ The differences in partitioning of proteins between the dilute and coacervate phases of different polyelectrolytes suggest that the exact partitioning properties of systems depend on the polyelectrolyte and protein species.

The protein partitioning between the PAH/PAA PECs and the dilute supernatant is a passive equilibration process; no active energy-consuming biological mechanism is required to enrich the proteins in the PECs. As such, the accumulation of protein in the PECs is associated with a gain in free energy. At their F_{opt}^- , we report a partition free energy of -20.2 ± 0.3 kJ/mol (mean \pm standard deviation, $n = 4$) for lysozyme and -19.5 ± 0.5 kJ/mol ($n = 5$) for succinylated lysozyme at a protein concentration of 0.8–1 g/L (eq 3). In comparison, for a system of phase-separated complexes consisting of disordered regions of proteins, partition free energies of -8 kJ/mol for single-stranded DNA and 2 kJ/mol for double-stranded DNA were reported.⁴⁴

Protein Partitioning Is pH-Dependent. In Figure 1B, we modulated the partitioning of lysozyme and succinylated lysozyme in the PECs by changing the composition in terms of F^- . An alternative method to effectively alter F^- is by changing the pH of the solution. At low pH values, polyanionic PAA will become less negatively charged while the charge of the polycationic PAH remains unaffected. At high pH values, PAA charge remains unaffected while PAH becomes less positively charged. As a consequence, a pH decrease increases the total negative charge in the complex and is equivalent to lowering the F^- via compositional changes and vice versa. Additionally, lysozyme remains positively charged at $\text{pH} < 10$ ($\text{pI} = 11.35$), while succinylated lysozyme undergoes a net charge shift in the

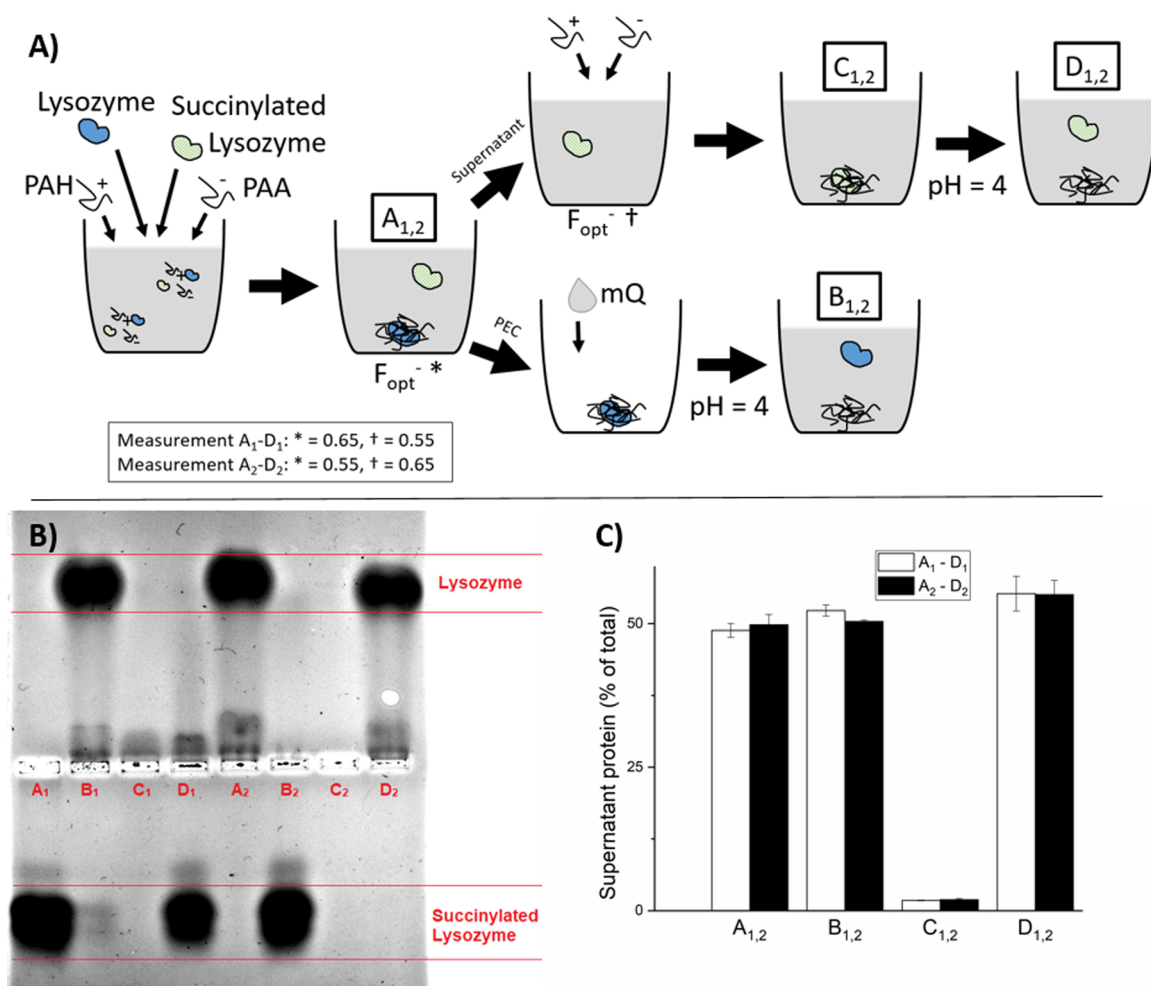


Figure 3. Separation of lysozyme and succinylated lysozyme from a protein mixture containing 1 g/L both lysozyme and succinylated lysozyme. (A) Schematic representation of the experimental procedure. The protein species were qualitatively and quantitatively measured at the points indicated as $A_{1,2}$ – $D_{1,2}$. (B) Qualitative analysis of the protein species present in the supernatant using SDS-PAGE electrophoresis. (C) Quantitative UV–vis analysis to determine total supernatant protein concentrations.

evaluated pH range ($pI = 4.5$) from negative to positive.³⁷ Lysozyme and succinylated lysozyme remain stable at room temperature for pH values as low as 3 and 3.5, respectively.^{33,45} Earlier studies also suggest that proteins recovered from PECs remain functional.⁴⁶

To evaluate the effect of pH on the partitioning of the proteins, PAH/PAA PECs were prepared at $F^- = 0.65$ and 0.55 for lysozyme and succinylated lysozyme, respectively, at a pH between 4 and 12. In Figure 2A, we show that the shape of the partitioning curve of the proteins as a function of the pH is similar to the F^- dependence shown in Figure 1B: for both proteins, a region in which none to very little partitioning and a region of maximum partitioning into the PECs is observed. In the presence of lysozyme, at $pH > 10$, the presence of soluble complexes resulted in light scattering, which obscured the measurements and the protein concentration could therefore not be accurately determined.

The pH-dependent partitioning of proteins in PECs and the sharp transitions in partitioning as a function of pH and composition offer an interesting strategy to recover proteins from the PECs. This approach was previously shown to work for BSA in polypeptide complexes.¹⁹ To investigate whether changes in pH also shift the equilibrium distribution and result in the release of lysozyme and succinylated lysozyme from

PECs, the systems were first equilibrated at F_{opt}^- . Subsequently, the pH was lowered from ± 7.7 to 4, where according to Figure 2A, the proteins are found in the dilute supernatant phase. Indeed, we show in Figure 2B that lowering of the pH recovers all lysozyme and nearly all succinylated lysozyme from the PAH/PAA PECs.

PECs are also known to be sensitive to ionic strength. An increase in salt concentration is known to disrupt polyelectrolyte complexes and recover partitioned protein.⁴⁷ Protein release using changes in ionic strength was, however, found to be a less efficient as lowering the pH (Supplementary Figure 3). Additionally, the disruption of the complex via salt addition leads to soluble complexes, which interfered with the spectroscopic determination of the protein concentration.

Protein Separation Using PECs. The ability to selectively partition proteins based on F^- composition (Figure 1B) and release proteins by adjusting the pH (Figure 2B) opens up the possibility to separate lysozyme or succinylated lysozyme from a mixture of the two in PECs. Figure 1B shows that at the F^- for which maximal partitioning into PECs is observed for one protein, the other protein remains in the dilute supernatant phase. We therefore hypothesized that if we start with a mixture of lysozyme and succinylated lysozyme and add polyelectrolytes at F_{opt}^- for one of the proteins, it will

selectively partition that protein, while the other protein remains in the supernatant.

Following this strategy, we separated a 1:1 mixture of lysozyme and succinylated lysozyme using PAH/PAA PECs via the procedure illustrated in Figure 3A. After each step, the total protein concentration and composition of the dilute phase were quantitatively and qualitatively investigated by UV-vis (Figure 3C) and gel electrophoreses (Figure 3B), respectively. The gel electrophoresis experiments (Figure 3B) verified that for each measurement, only one of the proteins was dominantly present in the supernatant, and thus, only one of the proteins was present in the PEC. Quantification by UV-vis spectroscopy (Figure 3C) shows that the total relative concentration of supernatant protein is either approximately half of the total protein concentration or nearly zero. Taken together, the results show that PAH/PAA PECs can be used to selectively separate either lysozyme or succinylated lysozyme from a mixture of the two proteins.

DISCUSSION

Previously, single polyelectrolytes have been used to selectively form complexes with proteins from mixtures, resulting in the polyelectrolyte-protein complex forming a separate phase.^{29–32} We have demonstrated that PECs consisting of oppositely charged polyelectrolytes PAH and PAA can also separate proteins based on charge. The protein partitioned by the PAH/PAA PEC was found to be dependent on the PEC composition F^- , which is a tunable factor. Depending on F^- , PAH/PAA PECs can act as selective solvents with high partitioning coefficients for either lysozyme or succinylated lysozyme. From Figure 1B and Figure 2A, we observe that PAH/PAA PECs have very steep transitions between no partitioning and full partitioning of proteins with very high partition coefficients as a functions of PEC composition and solution pH. The exact region of the transitions depended on the charge of the protein, and we hypothesize that this region is also dependent on other physicochemical properties of the protein and the constituent polyelectrolytes. We suggest that MLOs in biological systems may have similar steep transitions that can be manipulated by the cell via composition changes or variations in pH. Interestingly, we observed for lysozyme and succinylated lysozyme that maximum partitioning did not occur at $F^- = 0.5$.

Cells might be able to alter their MLO composition by manipulating the RNA or RBP concentrations by production, recruitment from other cellular components, or degradation mechanisms. An early model suggests that cells could make such adjustments.⁴⁸ Protein modifications via phosphorylation, sumoylation, and methylation are also known to influence phase separation, providing an additional mechanism for the cells to control MLO solvent properties.^{44,48–50} In line with this, it has recently been shown that cells are able to regulate the dissolution and formation of specific MLOs during and after mitosis by regulating the presence of certain kinase enzymes.⁵¹ Additionally, changes in the primary structure of RBPs may have drastic effects on complex coacervation and solvent properties as they affect the RBP's charge and isoelectric point. Minor protein modifications may thus result in a steep transition between maximum and no partitioning of proteins. One study where artificial membraneless compartments consisting of customized RNA and synthetic polycations were made showed that enzymes can indeed be partitioned

and retain a level of activity in at least partially synthetic complexes.⁵²

The cytosolic pH is generally very tightly regulated to a slightly alkaline (7–7.4) value.⁵³ However, Figure 2A shows that for PECs, only very slight variations in pH are required to make proteins switch from full to no partitioning in PAH/PAA PECs. Similar steep transitions might be found in MLOs, allowing changes in intracellular pH to influence protein partitioning behavior. Variation in intracellular pH has been reported to vary depending on the cell's phase in the cell cycle and exact intracellular location.^{53,54} Most notably, a consistent drop in cytosolic pH from physiological conditions to 5.5 has been observed for proliferating yeast.⁵⁵ Variations in both more alkaline and acidic directions occur at different phases during mitosis.^{56,57} Interestingly, several MLOs have been observed to disappear during mitosis and reappear afterward, while the centrosome and spindle assemblies are MLOs that play key roles in cell division.^{51,58} Additionally, pH gradients are present within migrating cells when different functionalities are required within the cell depending on the distance from the migrating leading edge.⁵⁹

Beyond gaining insight into the discrimination of coacervate phases between proteins based on charge and into mechanisms by which MLOs can regulate protein partitioning in the cell, we suggest possible applications. For these applications, it is important to realize that PECs behave as solvents. Understanding the factors that influence the partitioning behavior of these tunable aqueous solvents may open new directions for the extraction and concentration of molecules from wastewater streams. Partitioning for various small molecules from solution has been reported.^{60–62} The same principle is worth investigating for other compounds.

Another field where PECs might be promising is controlled drug delivery,^{27,63,64} especially with the possibility of a triggered release system.⁶⁵ Early-stage experimentation has suggested that PECs can show reduced cytotoxicity compared to free drug⁶⁶ and can have a tunable drug release rate based on environmental pH.⁶⁷

CONCLUSIONS

Membraneless organelles have the ability to partition intracellular proteins and act as an additional organizing mechanism for the regulation of intracellular processes.^{3,11–14} The ability to selectively partition the desired protein(s) while excluding other cytosolic compounds is essential for MLO functioning. Polyelectrolyte complexes have been shown previously to be able to enrich a variety of proteins from solution into PECs,^{17–21} but the ability to selectively partition proteins starting from a mixture using tunable PECs consisting of oppositely charged polyelectrolytes had not yet been shown. In this study, we showed that a high degree of selectivity is possible based on protein net charge, even when the proteins are otherwise structurally very similar.

Finally, beyond insight into MLOs, intracellular regulation, and potential new avenues to explore diseases, more direct applications of the ability of PECs to selectively and tunably partition proteins, biomolecules, or other organic compounds can be found in waste- or surface water treatment and in drug delivery systems.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.biomac.9b00701](https://doi.org/10.1021/acs.biomac.9b00701).

Figure S1: Influence of PAA and PAH on absorbance spectra; Figure S2: Effect of total polyelectrolyte and protein concentration; Figure S3: Release of Proteins from PECs via salt addition (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: s.lindhoud@utwente.nl.

ORCID

Jéré J. van Lente: [0000-0001-5896-9269](https://orcid.org/0000-0001-5896-9269)

Mireille M. A. E. Claessens: [0000-0002-2206-4422](https://orcid.org/0000-0002-2206-4422)

Saskia Lindhoud: [0000-0002-4164-0763](https://orcid.org/0000-0002-4164-0763)

Author Contributions

J.J.v.L. conducted experiments and processed results. M.M.A.E.C. and S.L. were responsible for supervision. S.L. directed the study. All authors contributed to experimental design, manuscript writing, and editing.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge funding from the Netherlands Organization for Scientific Research (NWO) VENI (# 722.013.013) and VIDI (# 723.015.003) grants.

■ REFERENCES

- Banani, S. F.; Lee, H. O.; Hyman, A. A.; Rosen, M. K. Biomolecular Condensates: Organizers of Cellular Biochemistry. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 285–298.
- Elbaum-Garfinkle, S.; Kim, Y.; Szczepaniak, K.; Chen, C. C.-H.; Eckmann, C. R.; Myong, S.; Brangwynne, C. P. The Disordered P Granule Protein LAF-1 Drives Phase Separation into Droplets with Tunable Viscosity and Dynamics. *Proc. Natl. Acad. Sci.* **2015**, *112*, 7189–7194.
- Fay, M. M.; Anderson, P. J. The Role of RNA in Biological Phase Separations. *J. Mol. Biol.* **2018**, *430*, 4685–4701.
- Protter, D. S. W.; Parker, R. Principles and Properties of Stress Granules. *Trends Cell Biol.* **2016**, *26*, 668–679.
- Wright, P. E.; Dyson, H. J. Intrinsically Disordered Proteins in Cellular Signalling and Regulation. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 18–29.
- Hentze, M. W.; Castello, A.; Schwarzl, T.; Preiss, T. A Brave New World of RNA-Binding Proteins. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 327–341.
- Ahmad, S.; Sarai, A. Analysis of Electric Moments of RNA-Binding Proteins: Implications for Mechanism and Prediction. *BMC Struct. Biol.* **2011**, *11*, 8.
- Uversky, V. N.; Kuznetsova, I. M.; Turoverov, K. K.; Zaslavsky, B. Intrinsically Disordered Proteins as Crucial Constituents of Cellular Aqueous Two Phase Systems and Coacervates. *FEBS Lett.* **2015**, *589*, 15–22.
- Eghbal, N.; Choudhary, R. Complex Coacervation: Encapsulation and Controlled Release of Active Agents in Food Systems. *LWT* **2018**, *90*, 254–264.
- Pak, C. W.; Kosno, M.; Holehouse, A. S.; Padrick, S. B.; Mittal, A.; Ali, R.; Yunus, A. A.; Liu, D. R.; Pappu, R. V.; Rosen, M. K. Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. *Mol. Cell* **2016**, *63*, 72–85.
- Aguilera-Gomez, A.; Rabouille, C. Membrane-Bound Organelles versus Membrane-Less Compartments and Their Control of Anabolic Pathways in *Drosophila*. *Dev. Biol.* **2017**, *428*, 310–317.
- Boeynaems, S.; Alberti, S.; Fawzi, N. L.; Mittag, T.; Polymenidou, M.; Rousseau, F.; Schymkowitz, J.; Shorter, J.; Wolozin, B.; Van Den Bosch, L.; Tompa, P.; Fuxreiter, M. Protein Phase Separation: A New Phase in Cell Biology. *Trends Cell Biol.* **2018**, *28*, 420–435.
- Gomes, E.; Shorter, J. The Molecular Language of Membraneless Organelles. *Journal of Biological Chemistry* **2019**, *294*, 7115.
- Ambadipudi, S.; Biernat, J.; Riedel, D.; Mandelkow, E.; Zweckstetter, M. Liquid–Liquid Phase Separation of the Microtubule-Binding Repeats of the Alzheimer-Related Protein Tau. *Nat. Commun.* **2017**, *8*, 275.
- Li, Y. R.; King, O. D.; Shorter, J.; Gitler, A. D. Stress Granules as Crucibles of ALS Pathogenesis. *J. Cell Biol.* **2013**, *201*, 361–372.
- Wegmann, S.; Eftekhazadeh, B.; Tepper, K.; Zoltowska, K. M.; Bennett, R. E.; Dujardin, S.; Laskowski, P. R.; MacKenzie, D.; Kamath, T.; Commins, C.; Vanderburg, C.; Roe, A. D.; Fan, Z.; Molliex, A. M.; Hernandez-Vega, A.; Muller, D.; Hyman, A. A.; Mandelkow, E.; Taylor, J. P.; Hyman, B. T. Tau Protein Liquid–Liquid Phase Separation Can Initiate Tau Aggregation. *EMBO J.* **2018**, *37*, No. e98049.
- Lindhoud, S.; Claessens, M. M. A. E. Accumulation of Small Protein Molecules in a Macroscopic Complex Coacervate. *Soft Matter* **2016**, *12*, 408–413.
- McCall, P. M.; Srivastava, S.; Perry, S. L.; Kovar, D. R.; Gardel, M. L.; Tirrell, M. V. Partitioning and Enhanced Self-Assembly of Actin in Polypeptide Coacervates. *Biophys. J.* **2018**, *114*, 1636–1645.
- Black, K. A.; Priftis, D.; Perry, S. L.; Yip, J.; Byun, W. Y.; Tirrell, M. Protein Encapsulation via Polypeptide Complex Coacervation. *ACS Macro Lett.* **2014**, *3*, 1088–1091.
- Schuster, B. S.; Reed, E. H.; Parthasarathy, R.; Jahnke, C. N.; Caldwell, R. M.; Bermudez, J. G.; Ramage, H.; Good, M. C.; Hammer, D. A. Controllable Protein Phase Separation and Modular Recruitment to Form Responsive Membraneless Organelles. *Nat. Commun.* **2018**, *9*, 2985.
- Zhao, M.; Zacharia, N. S. Protein Encapsulation via Polyelectrolyte Complex Coacervation: Protection against Protein Denaturation. *J. Chem. Phys.* **2018**, *149*, 163326.
- Blocher McTigue, W. C.; Perry, S. L. Design Rules for Encapsulating Proteins into Complex Coacervates. *Soft Matter* **2019**, *15*, 3089–3103.
- Martin, N.; Li, M.; Mann, S. Selective Uptake and Refolding of Globular Proteins in Coacervate Microdroplets. *Langmuir* **2016**, *32*, 5881–5889.
- Cooper, C. L.; Dubin, P. L.; Kayitmazer, A. B.; Turksen, S. Polyelectrolyte–Protein Complexes. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 52–78.
- Horn, J.; Kapelner, R.; Obermeyer, A. Macro- and Microphase Separated Protein–Polyelectrolyte Complexes: Design Parameters and Current Progress. *Polymer* **2019**, *11*, 578.
- Gao, S.; Holkar, A.; Srivastava, S. Protein–Polyelectrolyte Complexes and Micellar Assemblies. *Polymer* **2019**, *11*, 1097.
- Kayitmazer, A. B.; Seeman, D.; Minsky, B. B.; Dubin, P. L.; Xu, Y. Protein–Polyelectrolyte Interactions. *Soft Matter* **2013**, *9*, 2553.
- Cummings, C. S.; Obermeyer, A. C. Phase Separation Behavior of Supercharged Proteins and Polyelectrolytes. *Biochemistry* **2018**, *57*, 314–323.
- Dubin, P. L.; Gao, J.; Mattison, K. Protein Purification by Selective Phase Separation with Polyelectrolytes. *Sep. Purif. Methods* **2006**, *23*, 1–16.
- Du, X.; Dubin, P. L.; Hoagland, D. A.; Sun, L. Protein-Selective Coacervation with Hyaluronic Acid. *Biomacromolecules* **2014**, *15*, 726–734.
- Xu, Y.; Mazzawi, M.; Chen, K.; Sun, L.; Dubin, P. L. Protein Purification by Polyelectrolyte Coacervation: Influence of Protein

Charge Anisotropy on Selectivity. *Biomacromolecules* **2011**, *12*, 1512–1522.

(32) Wang, Y. F.; Gao, J. Y.; Dubin, P. L. Protein Separation via Polyelectrolyte Coacervation: Selectivity and Efficiency. *Biotechnol. Prog.* **1996**, *12*, 356–362.

(33) van der Veen, M.; Norde, W.; Stuart, M. C. Effects of Succinylation on the Structure and Thermostability of Lysozyme. *J. Agric. Food Chem.* **2005**, *53*, 5702–5707.

(34) Chollakup, R.; Smitthipong, W.; Eisenbach, C. D.; Tirrell, M. Phase Behavior and Coacervation of Aqueous Poly(Acrylic Acid)–Poly(Allylamine) Solutions. *Macromolecules* **2010**, *43*, 2518–2528.

(35) Chalikian, T. V.; Totrov, M.; Abagyan, R.; Breslauer, K. J. The Hydration of Globular Proteins as Derived from Volume and Compressibility Measurements: Cross Correlating Thermodynamic and Structural Data. *J. Mol. Biol.* **1996**, *260*, 588–603.

(36) Kuehner, D. E.; Engmann, J.; Fergg, F.; Wernick, M.; Blanch, H. W.; Prausnitz, J. M. Lysozyme Net Charge and Ion Binding in Concentrated Aqueous Electrolyte Solutions. *J. Phys. Chem. B* **1999**, *103*, 1368–1374.

(37) van der Veen, M.; Norde, W.; Stuart, M. C. Electrostatic Interactions in Protein Adsorption Probed by Comparing Lysozyme and Succinylated Lysozyme. *Colloids Surf., B* **2004**, *35*, 33–40.

(38) Obermeyer, A. C.; Mills, C. E.; Dong, X.-H.; Flores, R. J.; Olsen, B. D. Complex Coacervation of Supercharged Proteins with Polyelectrolytes. *Soft Matter* **2016**, *12*, 3570–3581.

(39) Bannan, C. C.; Calabró, G.; Kyu, D. Y.; Mobley, D. L. Calculating Partition Coefficients of Small Molecules in Octanol/Water and Cyclohexane/Water. *J. Chem. Theory Comput.* **2016**, *12*, 4015–4024.

(40) Berau, T.; Kremer, K. Automated Parametrization of the Coarse-Grained Martini Force Field for Small Organic Molecules. *J. Chem. Theory Comput.* **2015**, *11*, 2783–2791.

(41) Sangster, J. Octanol-Water Partition Coefficients of Simple Organic Compounds. *J. Phys. Chem. Ref. Data* **1989**, *18*, 1111–1229.

(42) van der Lee, R.; Buljan, M.; Lang, B.; Weatheritt, R. J.; Daughdrill, G. W.; Dunker, A. K.; Fuxreiter, M.; Gough, J.; Gsponer, J.; Jones, D. T.; Kim, P. M.; Kriwacki, R. W.; Oldfield, C. J.; Pappu, R. V.; Tompa, P.; Uversky, V. N.; Wright, P. E.; Babu, M. M. Classification of Intrinsically Disordered Regions and Proteins. *Chem. Rev.* **2013**, *114*, 6589–6631.

(43) Das, R. K.; Pappu, R. V. Conformations of Intrinsically Disordered Proteins Are Influenced by Linear Sequence Distributions of Oppositely Charged Residues. *Proc. Natl. Acad. Sci.* **2013**, *110*, 13392–13397.

(44) Nott, T. J.; Petsalaki, E.; Farber, P.; Jarvis, D.; Fussner, E.; Plochowitz, A.; Craggs, T. D.; Bazett-Jones, D. P.; Pawson, T.; Forman-Kay, J. D.; Baldwin, A. J. Phase Transition of a Disordered Nuage Protein Generates Environmentally Responsive Membraneless Organelles. *Mol. Cell* **2015**, *57*, 936–947.

(45) Venkataramani, S.; Truntzer, J.; Coleman, D. Thermal Stability of High Concentration Lysozyme across Varying PH: A Fourier Transform Infrared Study. *J. Pharm. BioAllied Sci.* **2013**, *5*, 148.

(46) Lindhoud, S.; Norde, W.; Cohen Stuart, M. A. Effects of Polyelectrolyte Complex Micelles and Their Components on the Enzymatic Activity of Lipase. *Langmuir* **2010**, *26*, 9802–9808.

(47) Lindhoud, S.; de Vries, R.; Schweins, R.; Cohen Stuart, M. A.; Norde, W. Salt-Induced Release of Lipase from Polyelectrolyte Complex Micelles. *Soft Matter* **2009**, *5*, 242–250.

(48) Banani, S. F.; Rice, A. M.; Peeples, W. B.; Lin, Y.; Jain, S.; Parker, R.; Rosen, M. K. Compositional Control of Phase-Separated Cellular Bodies. *Cell* **2016**, *166*, 651–663.

(49) Shen, T. H.; Lin, H.-K.; Scaglioni, P. P.; Yung, T. M.; Pandolfi, P. P. The Mechanisms of PML-Nuclear Body Formation. *Mol. Cell* **2006**, *24*, 331–339.

(50) Monahan, Z.; Ryan, V. H.; Janke, A. M.; Burke, K. A.; Rhoads, S. N.; Zerze, G. H.; O'Meally, R.; Dignon, G. L.; Conicella, A. E.; Zheng, W.; Best, R. B.; Cole, R. N.; Mittal, J.; Shewmaker, F.; Fawzi, N. L. Phosphorylation of the FUS Low-complexity Domain Disrupts

Phase Separation, Aggregation, and Toxicity. *EMBO J.* **2017**, *36*, 2951–2967.

(51) Rai, A. K.; Chen, J.-X.; Selbach, M.; Pelkmans, L. Kinase-Controlled Phase Transition of Membraneless Organelles in Mitosis. *Nature* **2018**, *559*, 211–216.

(52) Poudyal, R. R.; Guth-Metzler, R. M.; Veenis, A. J.; Frankel, E. A.; Keating, C. D.; Bevilacqua, P. C. Template-Directed RNA Polymerization and Enhanced Ribozyme Catalysis inside Membraneless Compartments Formed by Coacervates. *Nat. Commun.* **2019**, *10*, 490.

(53) Madhus, I. H. Regulation of Intracellular PH in Eukaryotic Cells. *Biochem. J.* **1988**, *250*, 1–8.

(54) Casey, J. R.; Grinstein, S.; Orlowski, J. Sensors and Regulators of Intracellular PH. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 50–61.

(55) Orii, R.; Urbanus, M. L.; Vizeacoumar, F. J.; Giaever, G.; Boone, C.; Nislow, C.; Brul, S.; Smits, G. J. Genome-Wide Analysis of Intracellular PH Reveals Quantitative Control of Cell Division Rate by PHc in *Saccharomyces Cerevisiae*. *Genome Biol.* **2012**, *13*, R80.

(56) Gagliardi, L. J.; Shain, D. H. Is Intracellular PH a Clock for Mitosis? *Theor. Biol. Med. Modell.* **2013**, *10*, 8.

(57) Shain, D. H.; Gagliardi, L. J. Intracellular PH as an Electrostatic Regulator of the Spindle Assembly Checkpoint. *Med. Res. Arch.* **2015**, *2* (), DOI: 10.18103/mra.v2i7.368.

(58) Brangwynne, C. P. Phase Transitions and Size Scaling of Membrane-Less Organelles. *J. Cell Biol.* **2013**, *203*, 875–881.

(59) Martin, C.; Pedersen, S. F.; Schwab, A.; Stock, C. Intracellular PH Gradients in Migrating Cells. *Am. J. Physiol.* **2011**, *300*, C490–C495.

(60) Huang, S.; Zhao, M.; Dawadi, M. B.; Cai, Y.; Lapitsky, Y.; Modarelli, D. A.; Zacharia, N. S. Effect of Small Molecules on the Phase Behavior and Coacervation of Aqueous Solutions of Poly-(Diallyldimethylammonium Chloride) and Poly(Sodium 4-Styrene Sulfonate). *J. Colloid Interface Sci.* **2018**, *518*, 216–224.

(61) de Silva, U. K.; Weik, B. E.; Lapitsky, Y. Simple Preparation of Polyelectrolyte Complex Beads for the Long-Term Release of Small Molecules. *Langmuir* **2014**, *30*, 8915–8922.

(62) Zhao, M.; Eghtesadi, S. A.; Dawadi, M. B.; Wang, C.; Huang, S.; Seymore, A. E.; Vogt, B. D.; Modarelli, D. A.; Liu, T.; Zacharia, N. S. Partitioning of Small Molecules in Hydrogen-Bonding Complex Coacervates of Poly(Acrylic Acid) and Poly(Ethylene Glycol) or Pluronic Block Copolymer. *Macromolecules* **2017**, *50*, 3818–3830.

(63) Lankalapalli, S.; Kolapalli, V. R. M. Polyelectrolyte Complexes: A Review of Their Applicability in Drug Delivery Technology. *Indian J. Pharm. Sci.* **2009**, *71*, 481.

(64) Taber, L.; Umerska, A. Polyelectrolyte Complexes as Nanoparticulate Drug Delivery Systems. *Eur. Biopharm. Rev.* **2015**, *20*, 36–40.

(65) Mitragotri, S.; Lahann, J. Materials for Drug Delivery: Innovative Solutions to Address Complex Biological Hurdles. *Adv. Mater.* **2012**, *24*, 3717–3723.

(66) Vehlou, D.; Schmidt, R.; Gebert, A.; Siebert, M.; Lips, K.; Müller, M. Polyelectrolyte Complex Based Interfacial Drug Delivery System with Controlled Loading and Improved Release Performance for Bone Therapeutics. *Nanomaterials* **2016**, *6*, 53.

(67) Quadrado, R. F.; Fajardo, A. R. Microparticles Based on Carboxymethyl Starch/Chitosan Polyelectrolyte Complex as Vehicles for Drug Delivery Systems. *Arabian J. Chem.* **2018**, DOI: 10.1016/j.arabjc.2018.04.004.