CUSJ Article

Protein-Polymer Complex Coacervates as Synthetic Membrane-less Organelles

Hansen Tjo[1]*, Nicholas Zervoudis[2], Allie C. Obermeyer[2]

[1]Department of Chemical Engineering, University of Massachusetts Amherst
[2]Department of Chemical Engineering, Columbia University in the City of New York

COMPLEX COACERVATION, PROTEIN ENGINEERING, SOFT MATTER

ABSTRACT: In solution, oppositely-charged macromolecules undergo charge-mediated liquid-liquid phase separation into a complex coacervate phase – a dense, macromolecule-rich liquid. In nature, the basis for intracellular compartmentalization in the formation of membrane-less organelles has been shown to follow similar complexation principles, where charged proteins represent the ionic species. We seek to capture the spatiotemporal tunability properties of such organelles for enzymatic reactions in vitro. However, the in vitro formation and deformation of protein-based coacervate microenvironments as a nanoreactor is a limiting factor. Here, we prescribe high-precision turbidimetry coupled with optical microscopy, to characterize the phase behavior of binary protein-polymer complexes between the weak anionic enzyme Glucose Oxidase (GOx) and four different synthetic polycations as functions of composition and ionic strength. Establishment of conditions ideal for coacervate formation in each protein-polymer system informed pH titration experiments on characterizing self-assembly regulation. The results from this study will help inform the design of novel coacervate microenvironments for industrial enzyme cascades and elucidate the role of associative phase separation in cellular evolution.

INTRODUCTION

Selectivity and specificity are an enzyme's key characteristics [1, 3, 4]. As biological catalysts, enzymes increase rates of reaction such that greater amounts of biological product may be obtained under less time. They also have been used to improve chemical processes in industries from food, agriculture, and petroleum in addition to reducing energy costs and operation time [4].

It is difficult to synthetically match the efficacy and specificity of enzymes as biochemical systems have had millions of years to evolve [4, 16]. While advents in directed evolution and protein engineering attempt to circumvent the time required for natural evolution, amongst other approaches to improve enzyme activity, there is also particular interest to achieve similar goals by optimizing an enzyme's surroundings [1, 4, 16]. This strategy is inspired by metabolic reactions, such as those that make up cellular respiration, where the spatiotemporal efficacy of enzymes is enhanced by its surrounding biological environment [1, 4]. With recent reports highlighting the complex functions of biological condensates *in vivo* (signaling, reaction networks etc.), we took a biomimetic approach in constructing a stable and responsive enzymatic compartment [1-5]. Using *de novo* liquid-liquid phase separated synthetic organelles, these microenvironments may be the bridge to advance how enzymatic power is harnessed industrially.

Complex coacervation, an example of associative liquid-liquid phase separation, describes how oppositely charged polyelectrolytes phase separate into a coacervate phase – a dense, polyelectrolyte-rich phase with potential applications in biomolecular encapsulation – and a dilute phase, the supernatant [1]. Because the coacervate phase compartmentalizes both enzymes and substrates within the same microenvironment, enzymes can perform their catalytic functions with greater spatiotemporal efficacy [1-4]. These *de novo* systems are especially advantageous due to their tuneability by a variety of parameters: pH, charge stoichiometry, ionic strength, mixing order, and others [1, 2]. The use of proteins or other charged biomacromolecules as coacervating macro-ions allows further structural modulation through ionic tagging and supercharging [6, 7, 9].

Complex coacervate systems typically involve binary mixtures of oppositely charged components. To simplify coacervate formation, a net charged enzyme is chosen such that it constitutes one of the electrostatic components for complexation [10, 11]. In particular, the weakly anionic enzyme Glucose Oxidase (GOx) was used in conjunction with four structurally distinct polycations: poly(4-vinyl *N*-methyl pyridinium iodide) (qP4VP), poly(allylamine hydrochloride) (PAH), poly(ethyleneimine) (PEI), and poly(1-vinyl imidazole methyl iodide) (PVI). In sum, we sought to elucidate the effects of mixing order, salt concentration, and finally pH on the phase behavior of four different GOx-polycation systems: GOx-qP4VP, GOx-PAH, GOx-PEI, GOx-PVI.

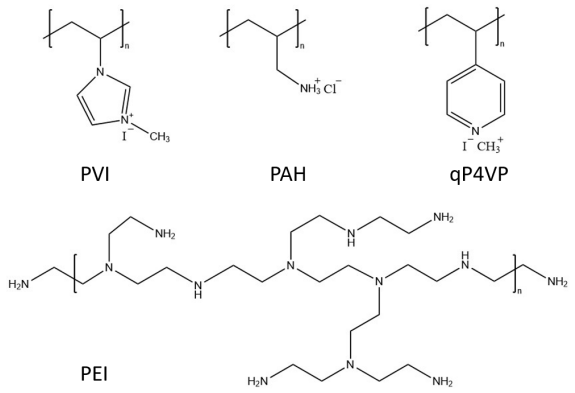

Figure 1. Chemical structures of the four cationic polymers used in this work. Structures were drawn using the ChemDraw Prime software.

METHODS

The phase behavior of four sets of binary GOx-polycation systems were studied. The four sets of polymer-protein mixtures consisted of poly(4-vinyl *N*-methyl pyridinium iodide) (qP4VP), poly(allylamine hydrochloride) (PAH), polyethylenimine (PEI), and poly(1-vinylimidazole methyl iodide) (PVI) as the polycations, with Glucose Oxidase (GOx) as the anionic charged protein. Additionally, we investigated the effects of salt on coacervate formation via the addition of sodium chloride (NaCl). Finally, we utilized pH titrations to explore how complex coacervation can be regulated by solution pH.

Sample Preparation: Glucose Oxidase from Aspergillus Niger was purchased from Sigma Aldrich (G2133). A stock solution of 2 mg/mL GOx was prepared in 10 mM Tris at pH 7.4. Application of Beer's Law was used to determine true concentrations of GOx via its absorbance at 280 nm in a 4 mL quartz cuvette. Polymer solutions were diluted from liquid stocks of 5 mg/mL; relevant stoichiometric calculations using the molar equivalency equation were used to determine the requisite volumes of 10 mM Tris needed to dilute appropriated samples to a mass concentration of 2 mg/mL. Each polycation solution was subsequently adjusted to a pH of 7.4.

Mixing Ratios: Four mixing ratios of GOx/polymer were investigated: 88% GOx/12% polymer, 84% GOx/16% polymer, 80% GOx/20% polymer, 76% GOx/24% polymer. Such values were determined from preliminary data indicating an optimum mixing ratio range for GOx at roughly 80%. Data results for variations in mixing ratios were obtained through turbidimetry analysis and optical microscopy. To exclude external ionic strength contributions in studying mixing ratio effects, salt species were absent in all mixtures.

Salt Effects: The effects of added salt on system phase behavior were examined via turbidimetry analyses and optical microscopy. Sodium chloride (NaCl) concentration was varied from 25 mM to 50 mM on all polymer/protein systems. This salt range was predicted to be conducive for liquid-liquid phase separation in GOx-polymer systems based on preliminary work. For the GOx-PEI system, no form of phase separation was observed at all salt concentrations. Thus, further planned investigations on the GOx-PEI system with salts were abandoned.

pH Titration: The effects of pH on phase behavior reversibility was examined via turbidimetry analysis, based on absorbance readings from an UV-Vis Spectrophotometer, and a pH probe at constant ambient temperature (25 ℃). 1 M Hydrochloric Acid (HCl) was used as the titrant; a 1 cm stirbar at roughly 500 rpm was used to ensure consistent solution mixing throughout the procedure. Each GOx-polycation system was set at its experimentally-determined optimum mixing ratio and salt concentration: 88% mixing ratio, and 50 mM NaCl for all systems with the exception of GOx-PAH, which was set at 175 mM NaCl.

Turbidimetry Analyses: Turbidimetry analyses were done to investigate the effects of mixing ratios and salt concentration. Each sample was prepared in triplicate in tissue culture-treated polystyrene 96-well half-area plates (Corning), followed by incubation at room temperature for 3 h. Using a plate reader (Tecan Infinite M200 Pro), the absorbance of the mixture was taken at a wavelength ($\lambda = 600\ nm$) to monitor scattering of the phase separated mixture. Each sample had an invariant volume of 50 µL allowing for appropriate absorbance measurements and physical mixing by a Tecan Infinite M200 Pro plate reader (10 s of orbital shaking). Finally, the Absorbance was converted into Turbidity using the following set of relationships:

$$\tau = 100 - \%T \quad (1)$$

$$\%T = 10^{(2-A)} \quad (2)$$

Where:

- τ is the Turbidity of the solution and an indicator of the extent of phase separation present within the sample.
- T is the Transmittance of the solution as a function of mixture Absorbance.
- A is the measured Absorbance of the mixture.

Optical Microscopy: All four protein-polymer samples were prepared in triplicate, followed by individual well examination with optical microscopy using an EVOS FL Auto 2 inverted fluorescence microscope (Invitrogen). Each sample, controlled at a volume of 50 µL, were formulated in an optically clear 384-well plate (Nunc) and then underwent 3 h incubation period at room temperature to maximize the degree of liquid-liquid phase separation taking place in the wells (preliminary data indicated that samples tended to favor

precipitation following initial mixing before eventual transition into a coacervate phase). All optical microscopy images were taken under 20X objectives with transmitted light.

RESULTS AND DISCUSSION

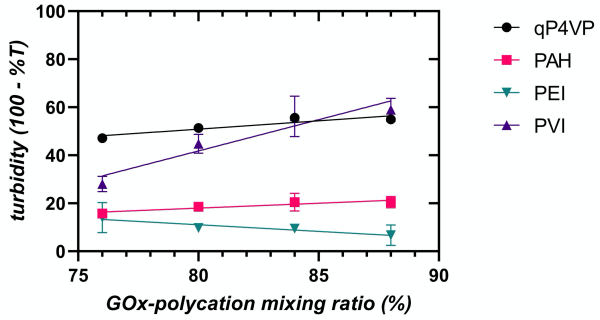

Figure 2. Complex coacervation of Glucose Oxidase (GOx) enzyme with a palette of synthetic polycations. Mixtures were prepared across a select range of macromolecule mixing ratios informed from a more thorough investigation of the protein's phase behavior. Error bars describe the standard deviation of each triplicated data point (n = 3). As a control, all turbidity values shown have had 10 mM Tris turbidity reference values subtracted.

Initial experiments investigated the effects of mixing ratio of protein to polymer using GOx and four different polycations (qP4VP, PAH, PEI, PVI). Preliminary data suggested each binary systems' tendency to phase separate at all four preselected mixing ratios; however, certain mixing ratios resulted in greater extents of phase separation as indicated by their relative turbidity magnitudes. Nevertheless, this initial assumption did not hold as no phase separation was observed with PEI at all mixing ratios. However, we hypothesized that additions of salt may facilitate phase separation through external charge compensations and increasing the total possible conformations of electrostatic associations given the presence of non-polyelectrolyte ions [1, 2, 6]. Thus, PEI was kept for subsequent experiments on the effects of salt addition.

Turbidity data as a function of mixing ratio was plotted in such a way that optimum mixing ratios for each GOx-polycation system can be determined. Although ranging in value from 0 – 100%, turbidity is typically employed as a qualitative indicator in characterizing phase behavior: for example, turbidity values above 20% usually suggest (but do not guarantee) the presence of phase separation whereas values nearing 0% imply the absence of phase separation. However, the presence and exact nature of phase separation, be it liquid-liquid or liquid-solid phase separation, can only be verified through optical microscopy. Nevertheless, turbidity is useful as a continuous measure for when a system exhibits the same morphology under different conditions, and therefore can indicate the conditions most conducive to the desired phase behavior. In this paper, for example, turbidity is used to determine mixing ratios that best drives phase separation. Out of the four mixing ratios investigated, the two mixing ratios most favoring liquid-liquid phase separation will be selected for subsequent investigations involving salt and pH.

It was expected that maximum complexation, as determined by peaks in relative turbidity, for a given GOx-polycation system would lie around mixing ratios of 84% and 88% (although turbidity values were generally higher at a mixing ratio of 92%, optical microscopy showed greater tendencies for precipitation). Indeed, all GOx-polycation systems except for GOx-PEI exhibited coacervate formation at such mixing ratios as determined via optical microscopy (data not shown). Phase separation absence in GOx-PEI also explains the low turbidity magnitudes (below 20% at all mixing ratios). We predicted the absence of phase separation in the GOx-PEI system to be due to the lack of entropic gains from bound counter-ion release in electrostatic interactions within the system as a result of PEI being too weakly charged. The larger relative sample standard deviations with GOx-PVI and GOx-PEI suggests possible systematic inaccuracies: e.g., bubble formation interference at select mixing ratios, but the consistency of turbidity maximums coupled with optical microscopy images (data not shown) at mixing ratios of 84% and 88% supported the selection of these particular mixing ratios for subsequent experiments. While the quantitative limitations of turbidity must be considered, this may also suggest the dominance of (charged) protein-polymers system's electrostatic interactions driving macro-phase separation on turbidity readings as opposed to the strengths of individual components.

Salt Effects

Based on the two selected mixing ratios (88% and 84%) from the previous experimental section, the effects of salt (NaCl) concentration on GOx-polycation phase behavior were investigated (Fig. 3).

Fig. 3c illustrates the characteristic binodal curve phase diagram in phase separating polyelectrolyte systems [1, 8, 12, 14]. In this investigation, however, we are more concerned with determining salt concentrations that promoted liquid-liquid phase separation for the two mixing ratios: 84% and 88%. Turbidimetry analysis was used to provide a relative measure for the degree of phase separation across all GOx-polycation systems and facilitate comparison across the two different salt concentrations. The two chosen salt concentrations were 25 mM and 50 mM based on preliminary data suggesting that this resulted in liquid-liquid phase separation in the GOx-polycation systems being studied.

At both mixing ratios of 84% and 88%, all GOx-polycations that exhibited liquid-liquid phase separation in the absence of salt (Fig. 2) continued to do so with increasing salt concentration. Although GOx-PVI and GOx-qP4VP systems underwent a decrease in turbidity with the addition of salt from 25 mM to 50 mM at constant mixing ratio of 84% (Fig. 3b), optical microscopy still showed liquid-like morphologies. Given that there were still no indicators of phase separation in GOx-PEI with the addition of salt, as suggested by low turbidity magnitudes below 20% (Figs. 3a,

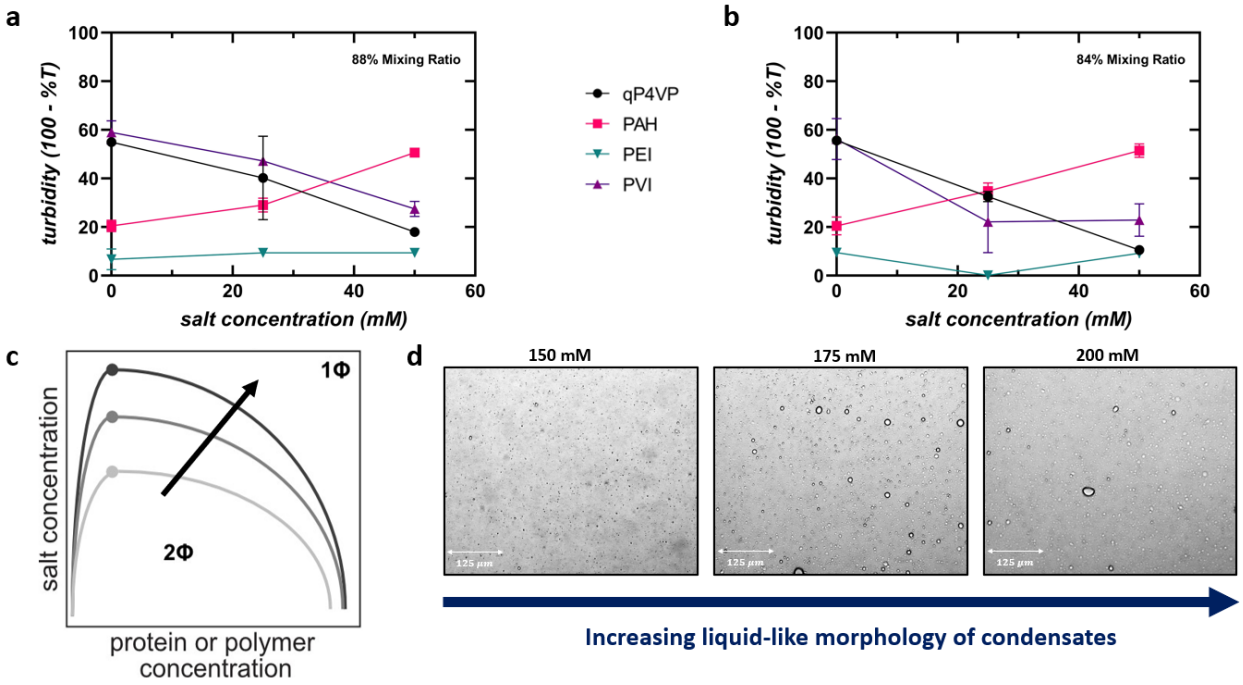

Figure 3. The phase behaviors of multiple GOx-polycation mixtures as functions of salt (NaCl) concentration. GOx-polycation mixtures were prepared using the two optimum mixing ratios for complex coacervation from Fig. 2 (84% and 88%). Both turbidity (λ = 600 nm) and optical microscopy were used to confirm liquid-liquid phase separation. Error bar values represent sample standard deviation; n = 3 for all data points. **a,** Turbidity versus salt concentration at constant mixing ratio of 88%. **b,** Turbidity versus salt concentration at constant mixing ratio of 84%. All turbidity values shown in Figs. 3a and 3b have had 10 mM Tris turbidity reference values subtracted; results from Fig. 2 were also included as no-salt controls. **c,** Binodal phase boundary of complex coacervate systems where both charged polymers and charged proteins facilitate phase separation; Φ denotes 'phase'. Arrow points to increasing two-phase region with increasing macromolecular charge density or patterning, demonstrating phasic tuneability with charge-associated parameters. **d,** Optical microscopy images of GOx-PAH phase behavior (88% mixing ratio) provide sufficient qualitative evidence of liquid-liquid phase separation at salt concentration ranges beyond those in Figs. 3a and 3b.

3b), and confirmed by optical microscopy, the system was excluded from subsequent experiments on the effects of pH on coacervate formation.

As an exception amongst the phase-separating systems, PAH did not demonstrate the desired formation of biomolecular condensates at both salt concentrations of 25 mM and 50 mM based on optical microscopy (data not shown). Instead, its turbidity values consistently above 20% for 25 mM and 50 mM salt concentrations (Figs. 3a, 3b) were shown to be a result of precipitate formation, which furthers evidence for the GOx-PAH system's greater relative propensity for liquid-solid phase separation at the current salt concentration range. For GOx-PAH, low concentrations of salt ions may not provide sufficient charge compensations to favor liquid-liquid phase separation, so much as it is strengthening electrostatic interactions via increasing possible Coulombic-driven conformations. Conversely, at higher salt concentrations, its charge screening effects would work towards dampening such electrostatic interactions to reduce the entropic gains from bound counter-ion release and drive coacervation [2, 6, 12]. An alternative explanation as to the GOx-PAH system's propensity for liquid-solid phase separation at low salt concentrations could be due to kinetic trapping effects as is prevalent in solid phases [2, 8, 12].

Next, experiments with the GOx-PAH system involving higher salt concentrations were necessary to identify a "minimum" salt concentration at which the system undergoes complex coacervation instead of precipitation. Thus, we conducted an additional salt titration exploring GOx-PAH phase behavior at higher salt concentrations: 150 mM to 200 mM NaCl at 25 mM intervals. Samples were analyzed via turbidimetry (data not shown) and optical microscopy (Fig. 3d). While the possibility to utilize a different salt as per the Hofmeister series e.g. KBr was considered, ultimately liquid-liquid phase separation was observed at a NaCl concentration of 150 mM, with the optimum salt concentration for GOx-PAH determined to be 175 mM (Fig. 3d), thereby negating the need to use a different salt species and maintaining consistency across all GOx-polycation systems.

pH Effects

Building on preceding work determining ideal mixing ratio and salt compositions conducive to coacervate formation, the goal of this section is to effectively explore coacervate

self-regulation by pH. All GOx-polycation systems were set at an initial pH of ~9.5 in addition to their optimum

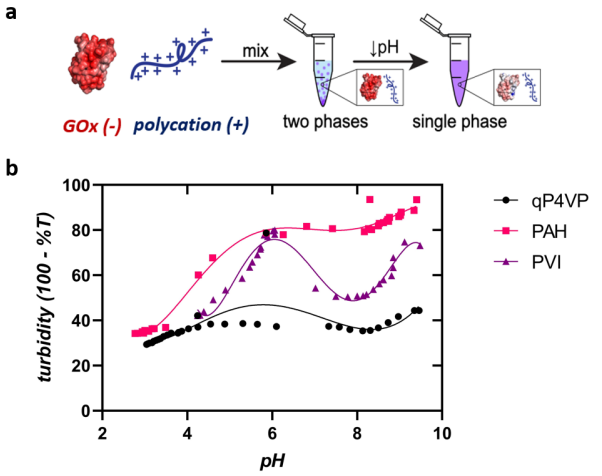

Figure 4. The effects of pH on complex coacervation in multiple GOx-polycation systems. Each GOx-polycation mixture was set at pH of roughly 9.5, followed by titration of hydrochloric acid (HCl) until dissolution of complexation. Turbidity (λ = 600 nm) was used to indicate absence of phase separation upon convergence towards a minimum value after multiple pH titrates. **a,** Schematic depicting pH-induced dissolution of complex coacervation via protonation of GOx, which reduces its net negative charge. **b,** Measured turbidity of GOx-polycation systems as a function of pH. 5th order polynomials were plotted to visualize turbidity trends with pH and to guide the eye (a 5th order polynomial represented the lowest-degree polynomial containing the local extremas that track the data)

compositions (specified in the Experimental Section), and a fixed volume of acid was titrated into the solution to slowly decrease the solution pH (Fig. 4b). Reductions in turbidity as pH decreases marks the dissolution of the coacervate microenvironment. Since GOx is characterized as a weak polyelectrolyte and has an isoelectric point (PI) of roughly ~4.2, protonation of GOx below its PI reduces its net negative charge. Thus, the coacervate microenvironment formed by GOx and a polycation would deform as liquid-liquid phase separation dissipates from weakening electrostatic interactions. This is evident from the turbidity of each system being lowest at pH values below 4.2, the isoelectric point of GOx (Fig. 4b). However, the periodicity of the trends, notably with the GOx-PAH and GOx-PVI systems, was unexpected (Fig. 4b). We hypothesize possible induced charging effects on prolonging liquid-liquid phase separation given the uneven anisotropy and charge patchiness of GOx [5, 8]. It is possible the coacervate microenvironment may persist at some of the lower pH ranges due to such effects, which may explain why each system undergoes a transition towards a turbidity maximum at the same pH value of 6 (indicating GOx as the limiting factor) following a local minimum at pH 8~ due to the initial decrease in pH.

Ultimately, we hope to demonstrate pH-mediated regulation of our coacervate microenvironments. Throughout the experimental section, we have shown how coacervate microcompartments can be formed with polymer-enzyme complexes. Further, we can tune the interaction strength and morphology via mixing ratio, salt, and pH. Due to many enzymatic reactions being affected by system pH, demonstrating the microenvironment's ability to form and dissolve reversibly in response to pH represents the primary goal of our work on protein-polymer synthetic nanoreactors [4, 16]. It is important to stress that these results are building blocks towards this objective.

CONCLUSION

An enzyme-polymer complex coacervate system was investigated on the basis of its ability to capture the complexities seen in biological condensates. The phase behaviors of multiple GOx-polycation systems as functions of mixing ratios and ionic strength were investigated. We were interested in each GOx-polycation system's propensity to undergo liquid-liquid phase separation, and the ionic stability of their formed coacervate microenvironments. GOx phase separated upon mixing with the polycations qP4VP, PAH, and PVI; but did not phase separate with PEI. For the GOx-PAH system, the addition of salt was needed to screen existing charge such that the entropic gains favoring liquid-solid phase separation may be suppressed. The GOx-PEI system did not undergo any type of phase separation both with and without the addition of salt. It is also worth remarking that the same species of salt, NaCl, may be used to induce liquid-liquid phase separation for all phase separating GOx-polycation systems, suggesting the salt species' versatility for driving complexation. pH titrations were used to investigate the dynamics of the formed coacervate microenvironments for each GOx-polycation system; turbidimetry suggests that all GOx-polycation systems that phase separated at a pH of ~9.5 no longer phase separated upon reaching pH < PI of GOx. We suggested the possibility of induced-charging effects as an explanation for the periodicity in phase behavior of GOx-PVI as pH decreases, where the anisotropy of GOx and presence of charge patches prolonged phase separation despite non-ideal pH [5, 10, 11]. Nevertheless, while the exact phase behavior of GOx-polycation systems at low pH were not determined as with optical microscopy, due to methodology limitations, the global minimums in turbidity at low pH compared to relative maximums at high pH strongly suggested dissolution of coacervates and dissipation of liquid-liquid phase separation. This crucial finding will inform future work on demonstrating the reversibility of coacervate formation in GOx-polycation systems such that these microenvironments can be made smart and self-regulated to mimic the complexity of condensates found in cells. The establishment of ideal parameters at which various GOx-polycation systems undergo liquid-liquid phase separation provides a foundation for coacervate microenvironment formation for use in advanced synthetic nanoreactor design and elucidation of cellular compartmentalization phenomena.

AUTHOR INFORMATION

Corresponding Author

*htjo@umass.edu

Author Contributions

Hansen Tjo performed the experiments, analyzed data, and produced the figures and the manuscript. Nick Zervoudis devised the experimental plan and reviewed the manuscript. Dr. Allie Obermeyer reviewed the manuscript.

Funding Sources

This work was supported by the Fu Foundation School of Engineering and Applied Sciences at Columbia University and funding from the Columbia Chemical Engineering REU program.

ACKNOWLEDGMENTS

The authors would like to acknowledge members of the Obermeyer Group for helpful discussions.

ABBREVIATIONS

GOx – Glucose Oxidase
HCl – Hydrochloric Acid
NaCl – Sodium Chloride
PAH – Poly(allylamine hydrocholoride)
PEI – Poly(ethylenimine)
PVI – Poly(1-vinyl imidazole methyl iodide)
PI – Isoelectric Point
qP4VP – Poly(4-vinyl N-methyl pyridinium iodide)

REFERENCES

[1] L.P. Bergeron-Sandoval, N. Safaee, S.W. Michnick, Mechanisms and Consequences of Macromolecular Phase Separation. *Cell* **165(5)**,1067–1079 (2016).

[2] W.C. Blocher, S.L. Perry, Complex coacervate-based materials for biomedicine. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **9(4)**, doi:10.1002/wnan.1442 (2016).

[3] M. Castellana, M. Z. Wilson, Y. Xu, P. Joshi, I. M. Cristea, J. D. Rabinowitz, Z. Gitai, N. S. Wingreen, Enzyme clustering accelerates processing of intermediates through metabolic channeling. *Nature Biotechnology* **32(10)**, 1011–1018 (2014).

[4] J.-M. Choi, S.-S. Han, H.-S. Kim, Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnology Advances* **33(7)**,1443–1454 (2015).

[5] F. Comert, P.L. Dubin, Liquid-liquid and liquid-solid phase separation in protein-polyelectrolyte systems. *Advances in Colloid and Interface Science* **239**, 213–217 (2017).

[6] C.S. Cummings, A.C. Obermeyer, Phase Separation Behavior of Supercharged Proteins and Polyelectrolytes. *Biochemistry* **57(3)**, 314–323 (2017).

[7] J. Horn, R. A. Kapelner, A.C. Obermeyer, Macro- and Microphase Separated Protein-Polyelectrolyte Complexes: Design Parameters and Current Progress. *Polymers* **11(4)**, 578 (2019).

[8] P. Jha, P. Desai, J. Li, R. Larson, pH and Salt Effects on the Associative Phase Separation of Oppositely Charged Polyelectrolytes. *Polymers* 6(5):1414–1436 (2014).

[9] R. A. Kapelner, A.C. Obermeyer, Ionic polypeptide tags for protein phase separation. *Chemical Science* **10(9)**, 2700–2707 (2019).

[10] L. Li, S. Srivastava, M. Andreev, A.B. Marciel, J. J. D. Pablo, M. Tirrell, Phase Behavior and Salt Partitioning in Polyelectrolyte Complex Coacervates. *Macromolecules* **51(8)**, 2988–2995 (2018).

[11] A.C. Obermeyer, C.E. Mills, X.-H. Dong, R. J. Flores, B.D. Olsen, Complex coacervation of supercharged proteins with polyelectrolytes. *Soft Matter* **12(15)**, 3570–3581 (2016).

[12] S.L. Perry, Phase separation: Bridging polymer physics and biology. *Current Opinion in Colloid & Interface Science* **39**, 86–97 (2019).

[13] S.L. Perry, Y. Li, D. Priftis, L. Leon, M. Tirrell, The Effect of Salt on the Complex Coacervation of Vinyl Polyelectrolytes. *Polymers* **6(6)**, 1756–1772 (2014).

[14] D. Priftis, M. Tirrell, Phase behaviour and complex coacervation of aqueous polypeptide solutions. *Soft Matter* **8(36)**, 9396–9405 (2012).

[15] D. Priftis, N. Laugel, M. Tirrell, Thermodynamic Characterization of Polypeptide Complex Coacervation. *Langmuir* **28(45)**, 15947–15957 (2012).

[16] E. Ricca, B. Brucher, J.H. Schrittwieser, Multi-Enzymatic Cascade Reactions: Overview and Perspectives. *Advanced Synthesis & Catalysis* **353(13)**, 2239–2262 (2011).