nature portfolio

Peer Review File

Influence of the Backbone Chemistry and Ionic Functional Groups of Five Pairs of Oppositely Charged Polyelectrolytes on Complex Coacervation

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This paper explores the impact of backbone chemistry and ionic functional groups on complex coacervation in five pairs of oppositely charged polyelectrolytes. These pairs encompass synthetic polymers with aliphatic hydrocarbon backbones, proteins with amide bonds, and carbohydrates with glycosidic linkages. Despite sharing identical charged groups, specific pairs exhibit distinct liquid/liquid and liquid/solid phase separations based on polyelectrolyte mixing ratio, buffer, and ionic strength. The coacervate phase boundary broadens in the order: glycosidic linkages > amide backbone > aliphatic hydrocarbon backbone, and Tris-phosphate > Tris-acetate > Tris-chloride buffers. Coacervates from polyelectrolytes with lower water solubilities resist disassembly at high salt concentrations, displaying a slow merge rate. Observations suggest that hydrophobic segments in polyelectrolytes interfere with complex coacervate formation. However, post-formation, these hydrophobic segments contribute to the stability and elasticity of coacervates. Taken together, I recommend accepting this paper for publication in Communications Chemistry after the authors addressed the following points. A detailed comments are listed as below:
1） FACS is needed for a more comprehensive analysis of the results of mixed polyelectrolyte solutions. While turbidity analysis and microscopic imaging provide insights into droplet formation, FACS flow cytometry can offer additional population-level data, including droplet count and particle size complexity. This approach will contribute to a deeper understanding of the overall characteristics of the mixed polyelectrolyte system, providing more detailed information for the study.
2） Line 426: the author's use of an original solution concentration exceeding the solubility of sodium chloride at room temperature raises a significant concern. Sodium chloride's solubility in water is approximately 36.0g per 100g of water at room temperature [1]. This discrepancy needs clarification from the author to understand the rationale behind utilizing a concentration beyond the solubility limit.

Reviewer #2 (Remarks to the Author):

This manuscript reports the influence of the backbone chemistry and the ionic functional groups of five pairs of oppositely charged polyelectrolytes on complex coacervation. In detail, the study describes the effects of five factors on complex coacervation, including the solubility, the mixing ratio, the buffer specificity, the salt resistance and the coalescence rate. The authors claim that these results demonstrate that the hydrophobic segments render the coacervates stable and elastic. However, the (experimental) results obtained do not allow to make such claims. For example, it is not clear at all from which experimental result any claim about elasticity can be made. In addition, it is not clear whether the effects can be attributed to the hydrophobicity (of the backbones) or rather to the influence of other interactions such as hydrogen bonds or the rigidity of the backbone.
Moreover, several of the polymers in this manuscript (and many other polypeptides, polysaccharides and aliphatic hydrocarbon backbones) have already been reported on and analyzed separately, and it would have been more insightful if the available literature would have been compared.
The manuscript also contains many mistakes, so in conclusion I do not recommend to publish this manuscript.

More detailed comments：
-The title “Hydrophobic segments in polyelectrolytes render complex coacervates stable and elastic” does reflect the content of the manuscript. I advise to retitle the manuscript.
- Page 4, line 99 “Initially, PolyK, Protamine, PolyD, and HA with similar charge densities”
• What do the authors mean by similar charge density? How is it defined and determined?
• Be careful to not mix “charge density” and “ionization degree”.

-Page 4, line 101-102: “with 40 degrees of polymerization (DP) were employed.”
• Replace by “With a degree of polymerization (DP) of 40”.

-Figure S2:
• Why are the GPC chromatograms cut at a retention time of 10.5 min? The peaks are truncated.
• Also, it is not "PMMA" but "PMAA"

-Page 4, line 105: Concerning the solubility measurements, did the authors make sure that the pH is maintained at 7.5 even after addition of such a high amount of polymer? This is very critical to ensure the reliability of these measurements. A section about it should be added in the Experimental section.
-Page 4, line 113: Can the authors elaborate on what they consider as self-coacervation versys what they consider as aggregation/precipitation?
-Page 4, line 117: "This suggests that PolyK and protamine, which both possess peptide backbones, are more hydrophilic than PAEMA, which possesses an aliphatic hydrocarbon backbone." The solubility limit reported for PAEMA is higher than for protamine, then why do the authors state the PAEMA is more hydrophobic than protamine? If self-coacervation is limiting the solubility measurements, protamine seems to be a bad choice to study the effect of solubility on complex coacervation.
-Page 4, line 123-146:
• The authors should cite relevant references in the paragraph about computational DFT to support the claims made.
-Figure 2:
• I'd advise to write clearly in the title of the X-axis that it is wt%.
• what is the mol%? It would be more insightful to report mol% of charged repeat units.
• Line 576 "Schematic representation (top) and phase diagrams (bottom) of phase separation in the presence of increasing NaCl concentrations." There is no change in NaCl concentration in Figure 2.
- Figure 2 and Figure S6-S10:
• I suggest to report the pictures in Figure S6 to S10 in the same order as the graph is made in Figure 2. Currently, the pictures on the right hand side with the polycation to polyanion ratios correspond to the points on the left hand side in Figure 2 (% of PMAA).
- Page 5, line 152-157: "For the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems, narrow phase boundary areas of liquid coacervate formation were observed (marked as filled circles), wherein the majority of charges in the oppositely charged polyelectrolytes were neutralized. For most other mixing ratios, precipitates were observed using an optical microscope."
• It is commonly reported for quite some polyelectrolyte couples that precipitation actually occurs for mixing ratios close to charge neutrality. And liquid coacervates are more dominant where there is charge imbalance (Soft Matter 2015, 11, 44; JCIS 2011, 361, 407; Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2017, 4, e1442). Here the opposite trend is reported. Can the authors elaborate on what could be the explanation?
- Page 5, Lines 161-166: "Given that the tight binding of molecules with the expulsion of water and counterions leads to precipitation, these findings are consistent with the solubility results, wherein all anionic polyelectrolytes displayed higher solubilities than the cationic polyelectrolytes. These results therefore demonstrate that the mixing ratio of oppositely charged polyelectrolytes affects the phase behavior, and that the solubility of the polyelectrolyte plays a crucial role in determining the coacervate phase boundary."
• The explanation is not straightforward. How are these results are coherent with the solubility tests? The authors should elaborate more on it.
- Page 6, Line 184: the authors mention "In contrast, the PAEMA:PolyD and PAEMA:HA pairs exhibited coacervate formation in all the buffers at all mixing ratios (Figs. 2d, 2i, 2n, 2e, 2j, 2o, and supplementary Figs. 9 and 10)", but coacervation region (Gray region) in Fig. 2d is between 50% and 70%.
- Page 6, Lines 186-189: "Given that a broader coacervate phase region is indicative of hydrated and loosely formed complexes, the observed increase in the coacervate phase region according to the order chloride < acetate < phosphate, represents an inversion of the Hofmeister series."
• Similar as before, the explanation of how these different ions affect the extent of coacervation and the transition from solid to liquid coacervate could be improved. It would also be interesting to compare the results to those reported in the following work: Journal of Colloid and Interface Science 2020, 560, 149-160.
- Page 6, Line 194-199: "The critical amount of NaCl required to dissolve the coacervates (referred

to as the critical salt concentration) depends on the interaction strength between the polymers. Thus, the critical salt concentration of each polymer coacervate pair formed at the optimal stoichiometry was determined by adjusting the NaCl concentration until the turbid coacervate suspension (indicated by filled marks) transformed into a uniform liquid phase (indicated by open marks) (Fig. 3)."
• The Critical salt concentration is the highest point of the binodal curve. Here it is more accurate to call it "salt resistance".
• The steps between each NaCl concentrations investigated are quite big. It would be worth narrowing them, to maybe discriminate between the three couples for which a salt resistance of 1 M is reported.
• Also salt resistance should be reported as an interval between two NaCl concentrations. Consequently, error bars should be added in the bar graph.
• Furthermore, did the authors make sure that crashing out of the single polymers at high salt concentrations through salting-out affect did not happen. This should be confirmed and the data should be added in Supporting information.
• The explanation of these results only on the basis of the hydrophobicity of the backbone is too restrictive. As stated earlier, other interactions can be involved in the phase separation process. If the authors want to make such strong statements about the effect of the hydrophobicity of the backbone, they should find a way to actually isolate this effect from other possible ones.
- Page 7, Line 212: "Coalescence behaviors of the complex coacervates"
Did the authors observe any merging of the solid-like phase separated samples?
• Why the authors not choose to investigate the viscoelastic properties of the phase separated samples by rheology?
- What does "polymer residue" mean? Side-groups?
- Page 9, Line 273: "it was expected that the hydration properties of the polyanions would follow this order: HA ≈ PolyD > PMAA." How do the authors make a conclusion that the hydration properties of HA is similar to PolyD? Is there any explanation about the much broader coacervate region of PAEMA:HA pair in Tris-Cl buffer than the PAEMA:PolyD pair?
- Page 11, line 340-360: Check the values for the reactants used and for the conversion. They do not match with the reported DP of 40 reported.

Reviewer #3 (Remarks to the Author):

Based on the abstract, I was very excited to read this paper and see the various comparisons between aliphatic, peptide, and carbohydrate backbones, as well as the different buffer effects. While the manuscript contains a significant amount of interesting data, I feel as though the current discussion of the results is lacking in the depth that is needed to satisfy my incoming excitement. I have detailed out a number of suggestions below and encourage the authors to revise their work.

Specific Comments:
1. In the introduction, the authors state that mechanistically, both the ion pairing and condensation step of coacervation involve substantial entropic contributions. However, that is not my understanding based on a reading of the three papers cited. Indeed, the ion pairing step involves a significant entropic driving force, related to the release of bound counterions. However, the modeling used in the cited papers assumes only an enthalpy of phase change for the second step of condensation. I suggest that the authors modify their text.
2. I found it surprising that epsilon polylysine was used, rather than alpha polylysine given that it appeared as though alpha polyaspartate was used. It would be useful for the authors to clarify the form of the polyaspartate that was used (in the text) and comment on the differences in the two backbones. Indeed, it would also have been very interesting to see a comparison of alpha vs. epsilon polylysine.
3. Would the authors please comment on the polydispersity of their various samples? This information was only listed for the two methacrylate polymers. I mention this because highly polydisperse samples could show apparent higher phase behavior and/or viscosity because of the presence of longer chains.
4. I was unsure how to interpret the discussion of polymer solubility given that the protamine undergoes simple coacervation above 40 mg/mL. How is this evidence that protamine is more hydrophilic than PAEMA? I would suggest that the authors be more conservative and comment that

while they cannot definitively determine the solubility of protamine, the limit is above 40 mg/mL, which is relatively close to the solubility limit of PAEMA, and so they hypothesize that it is more soluble, but cannot prove it.
5. The authors use polyarginine to model their protamine. In reading through the text of the manuscript it was unclear to me how relevant this analogy is, as there was no discussion about the sequence of protamine and the propensity to have multiple arginines next to each other in the sequence. Granted, the figures would not be at the end of the manuscript in final publication, but it might be useful to include a clarification about this specifically in the text.
6. Related to the above point, since the authors purchased their PolyD from Alamanda Polymers, I wonder why they did not also simply purchase PolyK and PolyR from the same source, as these materials are readily available from this supplier in matched chain lengths.
7. Why was an analysis of the solvent accessible surface area performed only for the cations, and not the polyanions? Additionally, I did not find the visual images in Supplementary Figure 3 to be particularly useful. It would be much more useful (and quantitative) if values for the solvent accessible surface area were reported on some equivalent basis, perhaps in Table 1.
8. I found it surprising that DFT results and images were not provided for hyaluronic acid (Supplementary Figure 5). Why was this not included?
9. Relatedly, I found it difficult to parse net charge based on the coloration in Supplementary Figures 4,5, and the choice of red/green means that it would be impossible for someone with red/green colorblindness to do so. The authors might consider modifying their choice of color, and I would suggest the inclusion of text labels in the relevant figures to better communicate this information.
10. Are the various polymers in their acid/base forms or are they salts? From the methods section it seems as though most of the polymers are in their salt form, though it was unclear for PMAA, polyK and hyaluronic acid. Additionally, not all of the salt forms for a given class of polymer appear to be the same (protamine sulfate vs. PAEMA HCl). If in the salt form, I would recommend specifying this when the polymers and their abbreviations are introduced, and perhaps include the information in Figure 1. The authors might also comment on any effect differences in these counterions might have on their materials, particularly that they also discuss ion effects with regards to the buffer.
11. Is the cation/anion mixing ratio described on line 150 on a charge basis or a weight basis? I believe it is on a weight basis, which while easy to work with, does not provide guidance with regards to electrostatic intuition. I would suggest that the authors include an additional axis on their various plots providing the charge basis information, and perhaps include such numbers parenthetically when discussing optimum compositions (see my note below).
12. It would be useful for the authors to discuss what the optimum mixing ratio for complex formation is as a function of the various polymers, and how factors like the distance between charges or the charge density (i.e., protamine) affect these ratios.
13. I do not understand why the data for polyK/HA and protamine/HA were not included at least in the supplementary information. It is one thing to say that they are similar and not include the figures in the main manuscript, but if the authors wish do include this comparison in their manuscript they should at least include the data in the SI.
14. Lines 161-166: The authors state that their turbidity results are consistent with solubility results, particularly citing anionic polyelectrolytes having higher solubility than cationic polyelectrolytes. However, it is unclear to me the basis for this conclusion. In the various datasets shown in Figure 2 we observe turbidity curves that skew to high polycation, high polyanion, and are centered. A much more detailed and clearer discussion of these data and where this conclusion comes from is critical to improve the understandability of this argument.
15. The microscopy data in Supplementary Figure 9 for the Tris-Po samples (and particularly the 9:1 sample) do not appear to match the turbidity data. Would the authors please comment on this?
16. In the discussion of buffer effects, it would be useful to know whether the observed effects only persist if the various ions are present at the same concentration vs. the same ionic strength. Based on the methods, it seemed as though ionic strength might have been taken into consideration for acetate and phosphate, but it was not clear for HCl and should be stated explicitly.
17. The discussion of buffer effects concludes with a statement ordering the ions and a description that this “represents an inversion of the Hofmeister series.” What aspect of the Hofmeister series helps to describe why this ordering exists? Is a phosphate ion expected to bring more water into

the complex and therefore favor coacervation vs. precipitation? I suggest that the authors need to discuss the why behind their observation, rather than limiting their discussion to an eponymous reference to a very complex physicochemical observation.
Building on this point (the above text was largely written with regards to the results section), in the discussion section, the authors talk about the "salting out" effects of ions earlier in the series. However, chloride is situated basically in the middle of the series, so one would likely only expect one half of the behavior (salting in only).
18. While it is true that various reports in the literature have referred to the amount of salt needed to dissolve a complex as the "critical salt concentration," in reality the critical point is a specific defined point in the thermodynamic phase diagram, and a more accurate name for this result would be the "salt resistance" (at a specified polymer concentration), as put forth by Tirrell, Perry, and a number of others working in the field.
19. Would the authors please include error bars on Figure 3b? This should represent the error in sampling from the data shown in Figure 3a.
20. I was surprised to read that the critical salt for PolyK/PMAA, protamine/PMAA and PAEMA/PolyD was "1-2M NaCl," since the data shown in Figure 3b suggest that the value is 1M NaCl for all three (plus or minus sampling error). Please clarify this point.
21. The authors attempt to argue that the higher critical salt value for PAEMA/PMAA compared to the other systems is purely a consequence of hydrophobicity. While I agree that the aliphatic nature of the backbone likely contributes to the difference in phase behavior, I would also point out that there is a significant difference in the charge density of the various systems. The methacrylate polymers have a two-atom backbone, whereas an alpha-polypeptide has a three-atom backbone, and the epsilon-polypeptide has a much longer one. It is difficult to comment on the charge density and hydrophobicity of protamine because it is a more complex protein. However, from an electrostatic perspective alone, these differences in charge density could have an effect, as could the mismatch in charge density along the various chains. It would be useful to caveat this discussion.
22. I feel as though the main comparisons made in this manuscript are between the methacrylate and peptide-adjacent polymers. While data was present (sometimes) for the complexes involving hyaluronic acid, there was very little discussion about the effect of the carbohydrate structure on the various properties. There was also no discussion given to nuances such as the difference in charge density between alpha and epsilon polypeptides and the more complicated arrangement of charges in protamine. The addition of such a discussion would help to improve the depth and impact of the work.
23. Would the authors please include a reference for their mention of the first pioneering work on coacervation (Lines 242-243)?
24. Line 300-301: The authors state that the merging experiments shown in Figure 4 provided evidence of solid-like properties for the methacrylate pair of polymers. However, the images shown in the figure clearly indicate liquid droplets, though their coalescence is slow. I would argue this is evidence for a high viscosity, rather than saying solid-like properties. Similarly, I would not describe the other polymer pairs as having liquid-like properties, but rather a lower viscosity. There are reports describing the effect of hydrophobicity on rheological properties in the literature from groups like Perry, Laaser, Shull, Qin, Tirrell, and Schlenoff which the authors could reference with regards to a discussion of viscosity, if desired.
25. Related to the coalescence experiments, I wonder if the choice of mixing ratio had any effect on this result. A recent paper by Pyo et al. (https://doi.org/10.1016/j.isci.2022.103852) showed that the surface tension of coacervates was decreased at off-stoichiometric conditions, while work by Spruijt et al. (https://doi.org/10.1021/ma301730n) showed that the viscosity of a sample did not change significantly. Such a discussion could provide useful insight into these materials by eliminating potential formulation differences between the samples.
26. Line 311-312: The authors state that "paradoxically, the same hydrophobic segment is able to impede coacervate disassembly at high salt concentrations, likely due to charge screening." Firstly, it does not seem paradoxical to me that complexes which interact strongly and form solid precipitates might have a higher salt stability. Secondly, it is completely unclear to me how hydrophobicity affects charge screening. Dissolving a coacervate back to a single solution phase is effectively akin to a solubility argument. Less soluble polymers will have a higher preference for remaining in the 'less hydrated' coacervate phase, and therefore are able to withstand a higher salt concentration. Please address.

Minor Comments:
1. Lines 65-66: where the authors refer to "lysine ionic groups" I would suggest that they say instead "ammonium groups" and perhaps reference lysine as a comparison (and then arginine in reference to guanidinium groups).
2. Lines 101-102: Rather than saying polymers with "40 degrees of polymerization," the phrasing should be polymers with a degree of polymerization of 40."
3. This is a semantic argument, but the data shown in Figure 2 are not true phase diagrams in the sense that they do not show actual concentrations etc. I would suggest modifying the caption to say something like "plots of turbidity vs. polymer ratio."
4. The middle paragraph of the discussion should be broken up into smaller paragraphs.
5. Line 369: I believe that "PMMA" should be "PMAA."

Response to Reviewers

We would like to thank the reviewers for their time and supportive comments. We have revised the manuscript in accordance with these comments and concerns, as detailed below. The original comments are provided in black, whereas our answers are given in blue. The major revisions are highlighted in yellow in the revised manuscript. The highlighted sections of the manuscript are reproduced in our response below in red font. We hope that the revised manuscript is now acceptable for publication in *Communications Chemistry*.

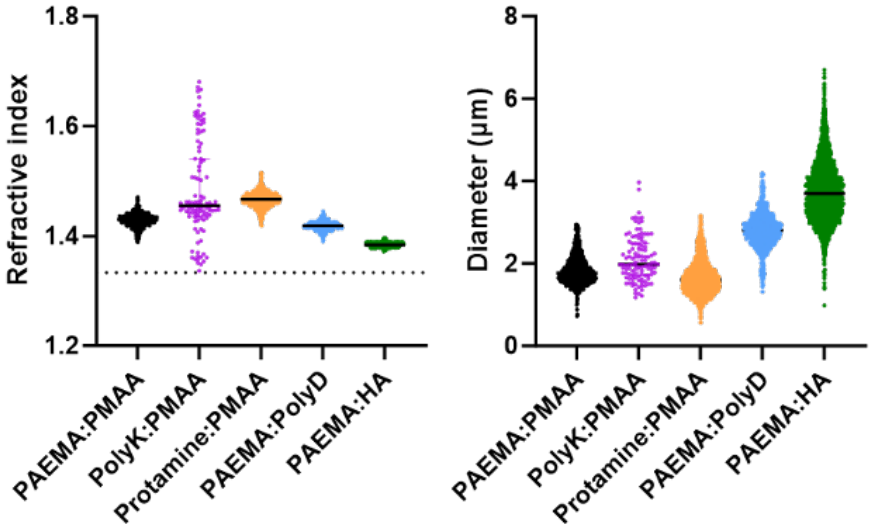
Reviewer #1 (Remarks to the Author):

This paper explores the impact of backbone chemistry and ionic functional groups on complex coacervation in five pairs of oppositely charged polyelectrolytes. These pairs encompass synthetic polymers with aliphatic hydrocarbon backbones, proteins with amide bonds, and carbohydrates with glycosidic linkages. Despite sharing identical charged groups, specific pairs exhibit distinct liquid/liquid and liquid/solid phase separations based on polyelectrolyte mixing ratio, buffer, and ionic strength. The coacervate phase boundary broadens in the order: glycosidic linkages > amide backbone > aliphatic hydrocarbon backbone, and Tris-phosphate > Tris-acetate > Tris-chloride buffers. Coacervates from polyelectrolytes with lower water solubilities resist disassembly at high salt concentrations, displaying a slow merge rate. Observations suggest that hydrophobic segments in polyelectrolytes interfere with complex coacervate formation. However, post-formation, these hydrophobic segments contribute to the stability and elasticity of coacervates. Taken together, I recommend accepting this paper for publication in Communications Chemistry after the authors addressed the following points. A detailed comments are listed as below:

R1Q1. FACS is needed for a more comprehensive analysis of the results of mixed polyelectrolyte solutions. While turbidity analysis and microscopic imaging provide insights into droplet formation, FACS flow cytometry can offer additional population-level data, including droplet count and particle size complexity. This approach will contribute to a deeper understanding of the overall characteristics of the mixed polyelectrolyte system, providing more detailed information for the study.

R1A1. Thank you for these comments. As correctly pointed out by the reviewer, population-level data can contribute to a deeper understanding and provide more detailed information. However, to employ flow cytometry, it is necessary to label the samples with a fluorescence tag, a process that can alter the resulting polymer properties. In coacervation systems, this modification can significantly influence the coacervation tendencies, particularly considering the relatively small molecular weights of the polymers employed in the current study. Thus, we employed xSight (Spheryx, Inc.), an instrument that analyzes suspended particles by measuring holograms and fits them to the Lorenz–Mie Theory. This instrument provides multiple quantitative measurements, including particle size, refractive index, and concentration measurements, in a non-destructive means. The refractive index of individual coacervates can

be correlated to the polymer concentration in the droplet (doi.org/10.1002/adom.202100697). Thus, using this approach, we compared the PAEMA:PMAA, PolyK:PMAA, Protamine:PMAA, PAEMA:PolyD, and PAEMA:HA pairs at their respective maximum coacervation mixing ratios of 7:3, 4:6, 7:3, 6:4, and 4:6. As shown in the figures below, the median refractive indices of these coacervates can be ranked as follows: Protamine:PMAA (1.47), PolyK:PMAA (1.46), PAEMA:PMAA (1.43), PAEMA:PolyD (1.42), and PAEMA:HA (1.38). Correspondingly, their diameters (µm) were measured as PAEMA:HA (3.70), PAEMA:PolyD (2.80), PolyK:PMAA (1.98), PAEMA:PMAA (1.77), and Protamine:PMAA (1.60), indicating a negative correlation between the refractive index and the coacervate diameter. This implies that the coacervates with larger diameters are more hydrated, leading to a lower refractive index.



Refractive index (RI) and diameter of the polymer pairs in 18 mM Tris-Cl buffer.

R1Q2. Line 426: the author's use of an original solution concentration exceeding the solubility of sodium chloride at room temperature raises a significant concern. Sodium chloride's solubility in water is approximately 36.0g per 100g of water at room temperature [1]. This discrepancy needs clarification from the author to understand the rationale behind utilizing a concentration beyond the solubility limit.

R1A2. Thank you for this comment. We would like to point out to the reviewer that we employed doubly concentrated samples to cover all NaCl concentrations ranging from 0 mM to 1.5 M through the introduction of 5 M NaCl stock. To achieve higher NaCl concentrations, we formulated samples using Tris-Cl buffer at pH 7.4 (initially containing 5 M NaCl), and subsequently diluted them to specific concentrations. It was observed that at NaCl concentrations >1.5 M, the polymer pairs did not exhibit any discernible differences from those at 1.5 M. Consequently, we have excluded them from the results.

Thus, Figure 3 has been updated and corrected as follows:

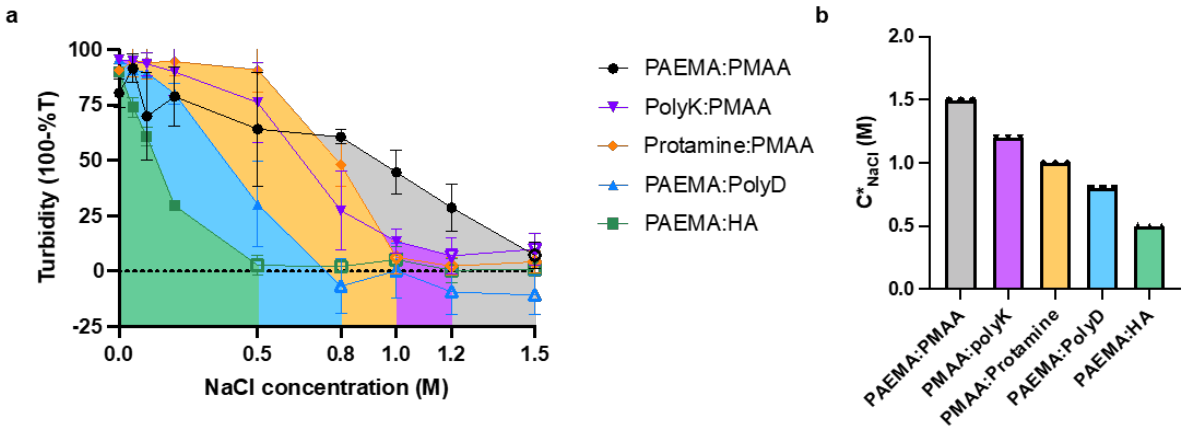


Figure 3. a Turbidity results obtained across various sodium chloride salt concentrations. The areas filled with colors indicate. b Critical NaCl concentrations for the various coacervate pairs. In each case, the polymer concentration (Cp) was 1 mg/mL, and the samples were analyzed immediately after complexation. The error bars indicate the standard deviations determined from three separate measurements.

The Methods section has also been revised as follows (Page 15, Lines 483–493):

"Sample solutions were prepared using the 19 mM Tris-Cl buffer. Complex coacervation was performed at the optimal stoichiometric ratios, as determined above. To achieve various NaCl concentrations, precise volumes of NaCl stock solutions (0.1, 0.2, 0.4, 1, 1.6, 2, 2.4, and 3 M) were added accordingly. All coacervate solutions attained a standardized final concentration of 1 mg/mL."

Reviewer #2 (Remarks to the Author):

This manuscript reports the influence of the backbone chemistry and the ionic functional groups of five pairs of oppositely charged polyelectrolytes on complex coacervation. In detail, the study describes the effects of five factors on complex coacervation, including the solubility, the mixing ratio, the buffer specificity, the salt resistance and the coalescence rate. The authors claim that these results demonstrate that the hydrophobic segments render the coacervates stable and elastic. However, the (experimental) results obtained do not allow to make such claims. For example, it is not clear at all from which experimental result any claim about elasticity can be made. In addition, it is not clear whether the effects can be attributed to the hydrophobicity (of the backbones) or rather to the influence of other interactions such as hydrogen bonds or the rigidity of the backbone.

Moreover, several of the polymers in this manuscript (and many other polypeptides, polysaccharides and aliphatic hydrocarbon backbones) have already been reported on and analyzed separately, and it would have been more insightful if the available literature would have been compared. The manuscript also contains many mistakes, so in conclusion I do not recommend to publish this manuscript.

More detailed comments:

R2Q1. The title "Hydrophobic segments in polyelectrolytes render complex coacervates stable and elastic" does reflect the content of the manuscript. I advise to retitle the manuscript.

R2A1. Thank you for this suggestion. We have revised the title as: "Influence of the Backbone Chemistry and Ionic Functional Groups of Five Pairs of Oppositely Charged Polyelectrolytes on Complex Coacervation." We hope that the reviewer finds this title more appropriate.

R2Q2. Page 4, line 99 "Initially, PolyK, Protamine, PolyD, and HA with similar charge densities"

• What do the authors mean by similar charge density? How is it defined and determined?

• Be careful to not mix "charge density" and "ionization degree".

R2A2. Thank you for pointing out this issue, and we apologize for the original wording. We wished to convey that all polymers are fully charged in the three buffer systems evaluated herein. Consequently, we have revised the term "charge density" as "ionization degree" throughout the article including in the indicated sentence (Page 4, Lines 102–103):

" Initially, PolyK, Protamine, PolyD, and HA with similar ionization degrees and molecular weights (3–7 kDa) were prepared, as outlined in Figure 1."

R2Q3. Page 4, line 101-102: "with 40 degrees of polymerization (DP) were employed."

• Replace by "With a degree of polymerization (DP) of 40".

R2A3. Thank you for this suggestion. The corresponding sentence has now been revised accordingly (Page 4, Lines 103–105):

"More specifically, to prepare synthetic polymers with the same number of charged moieties, PMAA and PAEMA with a degree of polymerization (DP) of 40 were employed."

R2Q4. Figure S2:

• Why are the GPC chromatograms cut at a retention time of 10.5 min? The peaks are truncated.

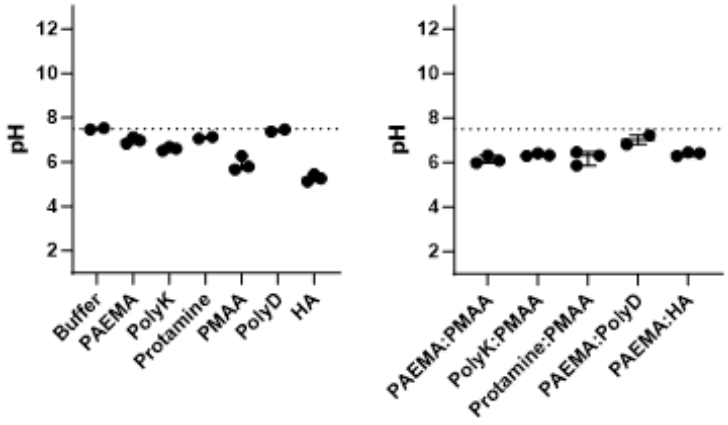
• Also, it is not "PMMA" but "PMAA"

R2A4. Thank you for these comments. We have now corrected Supplementary Fig. 2 accordingly, and ensured that PMMA was replaced with PMAA in Supplementary Fig. 2.

R2Q5. Page 4, line 105: Concerning the solubility measurements, did the authors make sure that the pH is maintained at 7.5 even after addition of such a high amount of polymer? This is

very critical to ensure the reliability of these measurements. A section about it should be added in the Experimental section.

R2A5. Thank you for this comment. We note that some pH changes were observed after the addition of polymers (1 mg/mL) to the 10 mM Tris-HCl buffer pH 7.5. However, the pKa values of 2-aminoethyl methacrylate (~9), lysine (~10), arginine (~12), methacrylic acid (~4), aspartic acid (~3.9), and hyaluronic acid (~3.0) differ from the pH values of the polymers and polymer pairs by >1.0. This indicates that the polymer degrees of ionization are close to 99% in this buffer. Therefore, we believe that our system is reliable. The Methods section has now been revised to include information regarding the average pH values of the various polymers and polymer pairs. In addition, Supplementary Fig. 16 has been added as follows:



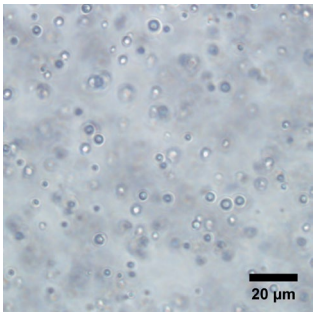
Supplementary Figure 16. pH values of the polymers and polymer pairs. For the polymer pairs, the optimal stoichiometric ratios determined by turbidity measurements were used.

Revision to the Methods section (Page 15, Lines 475–477):

"To confirm complete polymer ionization, the pH values of the polymers and polymer pairs at their optimal stoichiometric ratios (as determined by turbidity measurements) were measured (Supplementary Fig. 16)."

R2Q6. Page 4, line 113: Can the authors elaborate on what they consider as self-coacervation versus what they consider as aggregation/precipitation?

R2A6. Self-coacervation, or simple coacervation, occurs when a single polymer species undergoes phase separation. This process leads to the formation of well-defined spherical structures characterized by fluidity and subsequent growth through fusion. We checked the spherical-shaped self-coacervates of protamine at high protein concentrations using optical microscopy as shown in the following image (70 mg/mL protamine):



An optical microscopy image of protamine at the concentration of 70 mg/mL. The sample was analyzed immediately after complexation.

In addition, the sentence on page 4, lines 120–122 has been revised as follows:

"In the case of protamine, at concentrations >40 mg/mL, simple coacervation occurred, characterized by a spherical shape, indicating that phase separation driven by hydrophobic arginine–arginine stacking had taken place[21]."

R2Q7. Page 4, line 117: "This suggests that PolyK and protamine, which both possess peptide backbones, are more hydrophilic than PAEMA, which possesses an aliphatic hydrocarbon backbone." The solubility limit reported for PAEMA is higher than for protamine, then why do the authors state the PAEMA is more hydrophobic than protamine? If self-coacervation is limiting the solubility measurements, protamine seems to be a bad choice to study the effect of solubility on complex coacervation.

R2A7. Thank you for this comment, which we agree with. In contrast to PAEMA, which precipitates at concentrations >63 mg/mL, protamine forms coacervates at concentrations >40 mg/mL, reaching concentrations as high as 70 mg/mL, and surpassing the solubility of PAEMA. We interpreted this behavior as indicative of protamine being more hydrophilic, given that coacervates are inherently more hydrated than the precipitates. However, considering the challenge in comparing the solubilities when the substances are in different phase states (i.e., coacervates and precipitates), we have revised the text to remove the reference to protamine, as follows (Page 4, Line 122–124):

"The occurrence of simple coacervation in protamine, rather than precipitation, suggests that protamine exhibits a greater degree of hydration[24]."

[24] Liu, X. et al. The correlation between thermally induced precipitate-to-coacervate transition and glass transition in a polyelectrolyte-bolaamphiphile complex. Aggregate 4, (2023).

R2Q8. Page 4, line 123-146:

• The authors should cite relevant references in the paragraph about computational DFT to support the claims made.

R2A8. Thank you for this suggestion. Accordingly, we have updated the Results section to include some additional references that highlight the amphiphilic and quasi-aromatic properties of the guanidine group of arginine, which promote π-stacking. Furthermore, we have expanded the Methods section (DFT calculations) with additional references that detail our computational approach (Page 5, Line 148–150):

"Moreover, the amphiphilic and quasi-aromatic properties of the arginine guanidine group likely promote π stacking, and lead to the formation and stabilization of simple coacervation[21,25,26]."

[21] Hong, Y. *et al.* Hydrophobicity of arginine leads to reentrant liquid-liquid phase separation behaviors of arginine-rich proteins. *Nat. Commun.* **13**, 7326 (2022).

[25] Vazdar, M. et al. Arginine 'magic': Guanidinium Like-Charge Ion Pairing from Aqueous Salts to Cell Penetrating Peptides. Acc Chem Res 51, 1455–1464 (2018).

[26] Gund, P. Guanidine, trimethylenemethane, and 'Y-delocalization.' Can acyclic compounds have 'aromatic' stability? J Chem Educ 49, 100–103 (1972).

R2Q9. Figure 2:

- *I'd advise to write clearly in the title of the X-axis that it is wt%.*
- *what is the mol%? It would be more insightful to report mol% of charged repeat units.*
- *Line 576 "Schematic representation (top) and phase diagrams (bottom) of phase separation in the presence of increasing NaCl concentrations." There is no change in NaCl concentration in Figure 2.*

R2A9. Thank you for pointing out these issues. The manuscript and figures have been revised as outlined below. Additionally, we would like to point out that we found that the Tris-Cl buffer (10 mM Tris base + 8.89 mM HCl, pH 7.5) exhibited a different ionic concentration than the Tris-Ac (6.6 mM Tris base + 3.3 mM glacial acetic acid, pH 7.5) and Tris-Po (7.67 mM Tris base + 2.33 mM phosphoric acid, pH 7.5) buffers. Thus, we performed the measurements again using the Tris-Cl buffer (5.34 mM Tris base + 4.66 mM HCl, pH 7.5) and corrected Figure 2 along with the related supplementary figures.

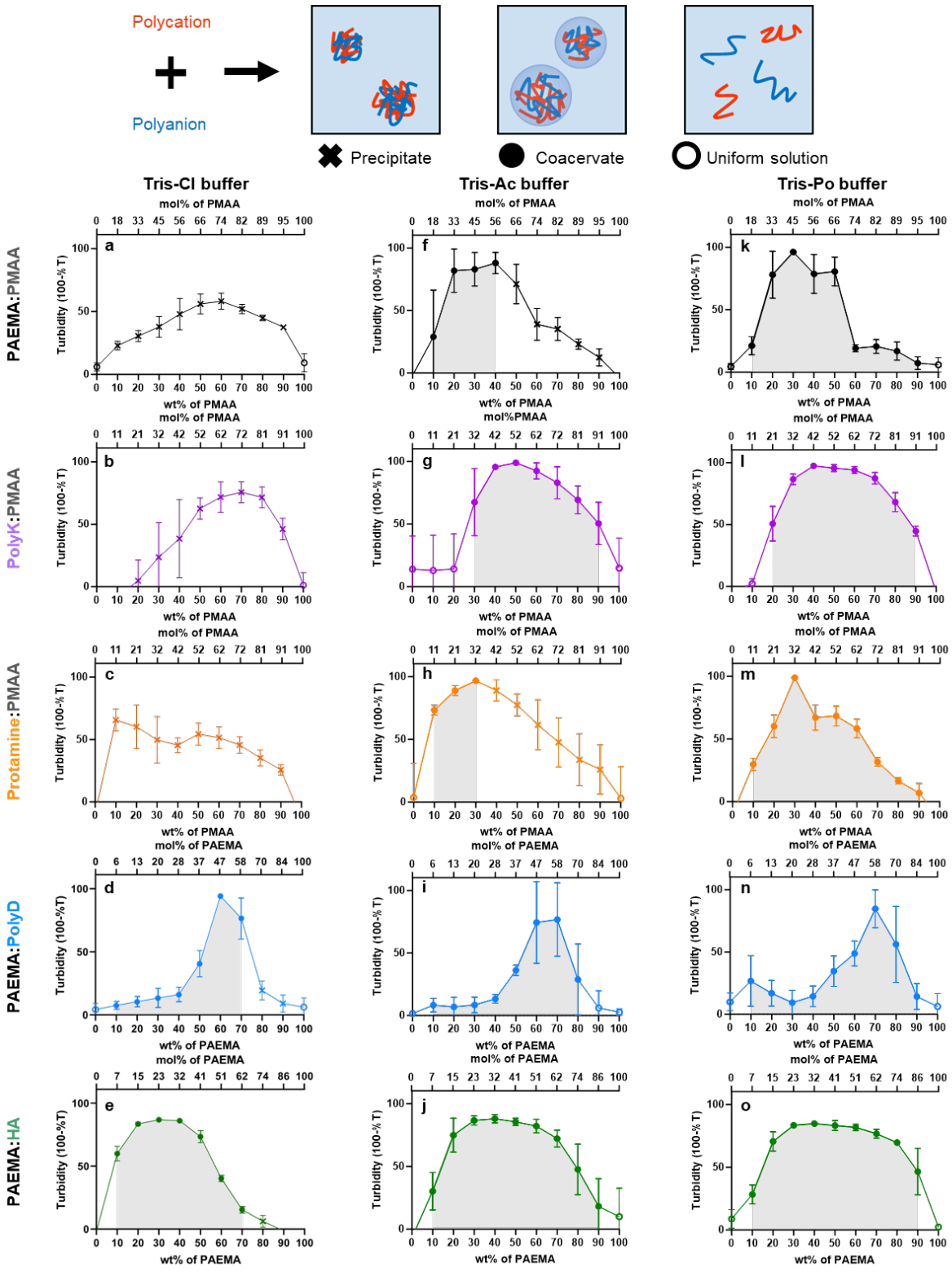
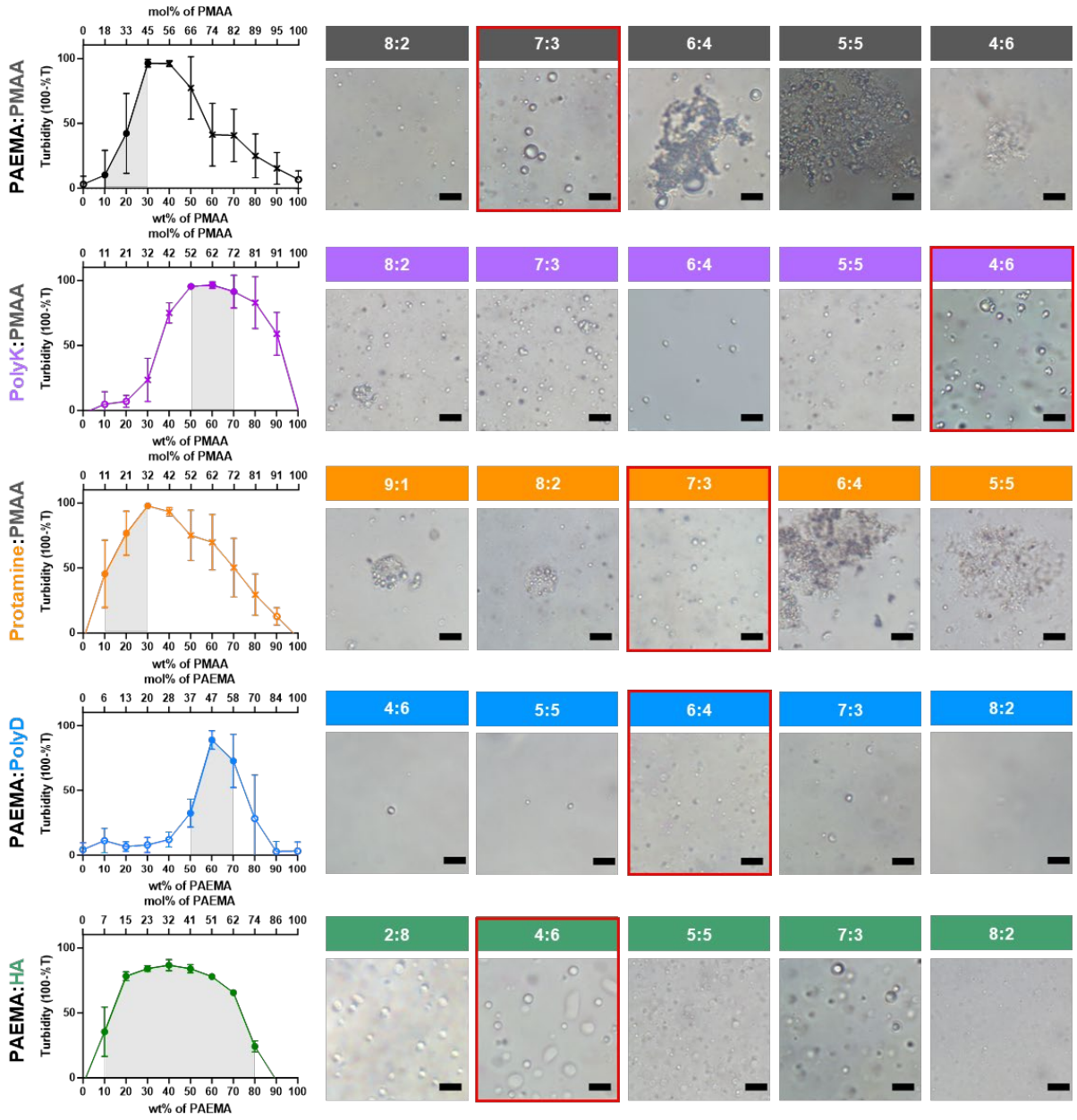


Figure 2. Complex coacervation behaviors in different buffers. Schematic representation (top) and plots of turbidity vs. polymer mixing ratio (bottom) for phase separation. a, b, c, d, and e: In Tris-Chloride (Tris-Cl) buffer; f, g, h, i, and j: In Tris-Acetate (Tris-Ac) buffer; k, l, m, n, and o: In Tris-Phosphate (Tris-Po) buffer. Black plots = PAEMA:PMAA pairs; purple plots = PolyK:PMAA pairs; orange plots = Protamine:PMAA pairs; blue plots = PAEMA:PolyD pairs; green plots = PAEMA:HA pairs. In the phase diagrams, closed markers indicate the mixing ratios wherein phase separation was observed using an optical microscope. The open markers indicate the mixing ratios wherein phase separation did not occur. The X marks indicate the mixing ratios wherein aggregation was observed. The gray region in each phase diagram represents the

coacervation region where phase separation of the polyelectrolytes occurs. Each data is based on at least three replicate experiments carried out for each respective polyelectrolyte.

Supplementary Figure 12 has also been added as follows:



Supplementary Figure 12. Turbidity and representative optical microscopy images of the coacervation assemblies of the various pairs obtained by varying the mixing ratios in 10 mM Tris base containing 8.89 mM HCl at pH 7.5. Polymer concentrations (Cp) were 1 mg/mL for all coacervate pairs, and the samples were analyzed immediately after complexation.

The manuscript text has been revised as follows:

(Page 4, Lines 12–13): "...a 19 mM Tris-chloride (Tris-Cl) buffer at pH 7.5 was employed due to its ability to dissolve all polyelectrolytes at room temperature."

(Page 6, Lines 173–175): "To investigate the formation of coacervates in a higher-ionic-strength buffer for the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems,

identical experiments were conducted using 19 mM Tris-Cl buffer (pH 7.5, Supplementary Fig. 12)."

(Page 7, Lines 233–234): "From this point onward, the experiments were conducted using 19 mM Tris-Cl buffer at a mixing ratio favoring coacervate formation rather than precipitation."

(Page 14, Lines 461–464): "The Tris-chloride buffer (Tris-Cl, 10×10^{-3} M, pH 7.57) was prepared by combining 5.34 mM Tris base with 4.66 mM HCl in distilled water. To achieve a Tris-Cl buffer with higher ionic strength (Tris-Cl, 19×10^{-3} M, pH 7.5), 10 mM Tris base was mixed with 8.89 mM HCl in distilled water."

(Page 15, Line 489): "Sample solutions were prepared using the 19 mM Tris-Cl buffer."

R2Q10. Figure 2 and Figure S6-S10:

• I suggest to report the pictures in Figure S6 to S10 in the same order as the graph is made in Figure 2. Currently, the pictures on the right hand side with the polycation to polyanion ratios correspond to the points on the left hand side in Figure 2 (% of PMAA).

R2A10. Thank you for this suggestion. We revised the supplementary figures as suggested.

R2Q11. Page 5, line 152-157: "For the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems, narrow phase boundary areas of liquid coacervate formation were observed (marked as filled circles), wherein the majority of charges in the oppositely charged polyelectrolytes were neutralized. For most other mixing ratios, precipitates were observed using an optical microscope."

• It is commonly reported for quite some polyelectrolyte couples that precipitation actually occurs for mixing ratios close to charge neutrality. And liquid coacervates are more dominant where there is charge imbalance (Soft Matter 2015, 11, 44; JCIS 2011, 361, 407; Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2017, 4, e1442). Here the opposite trend is reported. Can the authors elaborate on what could be the explanation?

R2A11. Thank you for your insightful comment and reference. Firstly, we have revised the manuscript based on additional experiments conducted with varying ionic strengths for the buffers. Based on our observations, coacervate formation seemed to dominate at a charge-balanced state. [ref]

[ref] YS Jho, HY Yoo, Y Lin, S Han, DS Hwang, Advances in Colloid and Interface Science 2017, 239, 61-73

In the current study, we examined and compared coacervation behaviors in a biological system, and it appeared that coacervation was evident at mixing ratios corresponding to the maximum turbidity values. The final reference provided by the reviewer demonstrates that coacervation occurs at its maximum point and net charge, aligning with our findings. In terms of the second reference, strong polyelectrolytes (e.g., sulfate) appeared to induce aggregation. In our initial

experiments, we also observed a similar phenomenon wherein aggregation occurred at all mixing ratios with polymers containing sulfonic acid residues. However, we would like to point out that the first reference, which focuses on polymer brushes, is difficult to compare with our system because of their different formulations. Based on these considerations, the text has been revised as follows (Page 6, Lines 168–187):

"In the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems, precipitates were observed by optical microscopy at all mixing ratios evaluated herein (marked with an "X" in Figs. 2a–2c, see also Supplementary Figs. 7–9). However, for the PAEMA:PolyD and PAEMA:HA systems, broader coacervate ranges were observed (marked as filled circles in Figs. 2d and 2e, see also Supplementary Figs.10 and 11).

To investigate the formation of coacervates in a higher-ionic-strength buffer for the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems, identical experiments were conducted using 19 mM Tris-Cl buffer (pH 7.5, Supplementary Fig. 12). In these systems, narrow phase boundary areas were observed, corresponding to liquid coacervate formation (marked as filled circles in Supplementary Figs. 12a, 12b, and 12c). In these cases, coacervates were formed at narrow regions where the turbidity reached a maximum value, aligning with a previous study showing that coacervates are dominant at their maximum and net charge points[27]. For the majority of other mixing ratios, precipitates were noticeable under optical microscopy observations (marked with an "X" in Supplementary Figs. 12a, 12b, and 12c). Interestingly, the α-poly-L-lysine:PMAA pair formed coacervates at all phase-separated mixing ratios, highlighting the substantial influence of the backbone hydrophobicity on the phase behaviors of such polyelectrolyte complexes (Supplementary Figs. 13e and 13h). In the case of the PAEMA:PolyD and PAEMA:HA pairs, coacervates were observed at almost all phase-separated mixing ratios; however, their phase separation propensities decreased compared to those observed in the 10 mM buffer, and this was attributed to the increased ionic strength."

[27] Blocher, W. C. & Perry, S. L. Complex coacervate-based materials for biomedicine. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology vol. 9 Preprint at https://doi.org/10.1002/wnan.1442 (2017).

R2Q12. Page 5, Lines 161-166: "Given that the tight binding of molecules with the expulsion of water and counterions leads to precipitation, these findings are consistent with the solubility results, wherein all anionic polyelectrolytes displayed higher solubilities than the cationic polyelectrolytes. These results therefore demonstrate that the mixing ratio of oppositely charged polyelectrolytes affects the phase behavior, and that the solubility of the polyelectrolyte plays a crucial role in determining the coacervate phase boundary."

• The explanation is not straightforward. How are these results are coherent with the solubility tests? The authors should elaborate more on it.

R2A12. Thank you for this comment. For clarity purposes, the manuscript has now been revised as follows (Page 6, Lines 188–194):

"Given that the tight binding of molecules and the corresponding expulsion of water and counterions leads to precipitation, the coacervate phase region can provide insights into the hydrophobicity of a polyelectrolyte. While a direct comparison of the solubilities of PMAA, PolyD, and HA poses challenges due to their high solubilities > 1000 mg/mL, the observed coacervate phase regions suggest that both PolyD (with an amide backbone) and HA (with a carbohydrate backbone) are more hydrophilic than PMAA (with an aliphatic chain). Notably, this observation correlates with the DFT calculation results."

R2Q13. Page 6, Line 184: the authors mention "In contrast, the PAEMA:PolyD and PAEMA:HA pairs exhibited coacervate formation in all the buffers at all mixing ratios (Figs. 2d, 2i, 2n, 2e, 2j, 2o, and supplementary Figs. 9 and 10)", but coacervation region (Gray region) in Fig. 2d is between 50% and 70%.

R2A13. Thank you for this comment. We agree that the original sentence was unclear and confusing. We defined the coacervate region as a non-precipitated region, and in that regard, all paired regions showed coacervation. As can be seen from Fig. 2, the PAEMA:PolyD and PAEMA:HA pairs did not form precipitation, but instead, assembled coacervates when paired. The corresponding sentence has been revised as indicated below. In addition, we conducted additional experiments by varying the ionic strength of the buffer and found minimal changes in the results, although the coacervation tendency remains consistent.

(Page 7, Lines 211–216): "Moreover, the PAEMA:PolyD and PAEMA:HA pairs predominantly formed coacervates in the Tris-Cl buffer, although precipitates were observed at certain mixing ratios. Similar to the behavior of the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA pairs, in both the Tris-Ac and Tris-Po buffers, the PAEMA:PolyD and PAEMA:HA pairs exclusively formed a coacervate phase (Figs. 2d, 2i, 2n, 2e, 2j, 2o, and Supplementary Figs. 10 and 11)."

R2Q14. Page 6, Lines 186-189: "*Given that a broader coacervate phase region is indicative of hydrated and loosely formed complexes, the observed increase in the coacervate phase region according to the order chloride < acetate < phosphate, represents an inversion of the Hofmeister series."*

• *Similar as before, the explanation of how these different ions affect the extent of coacervation and the transition from solid to liquid coacervate could be improved. It would also be interesting to compare the results to those reported in the following work: Journal of Colloid and Interface Science 2020, 560, 149-160.*

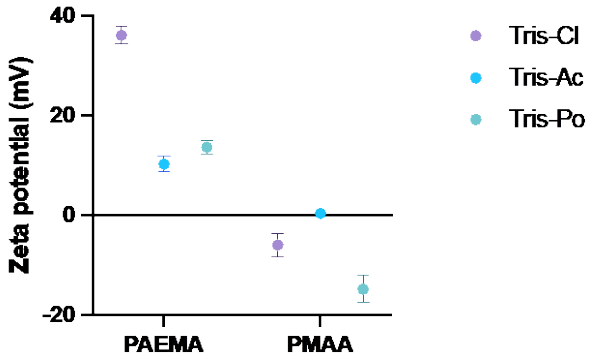
R2A14. Thank you for these suggestions. We note that phosphate is more kosmotropic than chloride, resulting in the salting-out of proteins through a reduction in the accessible surface area of the bulk water. However, in the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA pairs, the liquid coacervates were dominant in the Tris-Po buffer, indicating that the complexes were more hydrated (c.f., precipitation in the Tris-Cl buffer). This ion-specific effect is related

to ion adsorption or depletion from the solute/water interface, which is dependent on the surface properties of the coacervates, such as surface charge and hydrophobicity [ref 1, 2].

In this study, polymers were prepared in the buffers before forming coacervates, suggesting that the Hofmeister ion effect was already reflected in the polymers. We measured the zeta potential of PAEMA and PMAA in 10 mM Tris-Cl, Tris-Ac, and Tris-Po buffers to assess ion absorption levels in the buffers. The data presented below shows that phosphate species are more hydrated than others (ionic radii: $H_2PO_4^-$: 2.13, CH_3COO^-: 1.94, and Cl^-: 1.81), indicating more absorbed ions on the polymers. This implies that relatively more phosphate species are present in the formed coacervates than other species, inducing charge screening between polymer strands and resulting in preferred coacervate assembly. Likewise, relatively fewer chloride species are expected to be found in the formed coacervates, inducing relatively stronger electrostatic interactions between polymers and resulting in precipitation.

[Ref 1] Kunz, W. Specific ion effects (1st ed.), Wiley & Sons, Chichester (2007).

[Ref 2] Schwierz, N., Horinek, D., Sivan, U., & Netz, R.R. Reversed Hofmeister series—The rule rather than the exception, *Current Opinion in Colloid & Interface Science*, **23**, 10–18 (2016).



Zeta potential values of the polymers in three different buffers. The polymer concentrations were 1 mg/mL for all samples.

R2Q15. Page 6, Line 194-199: "The critical amount of NaCl required to dissolve the coacervates (referred to as the critical salt concentration) depends on the interaction strength between the polymers. Thus, the critical salt concentration of each polymer coacervate pair formed at the optimal stoichiometry was determined by adjusting the NaCl concentration until the turbid coacervate suspension (indicated by filled marks) transformed into a uniform liquid phase (indicated by open marks) (Fig. 3)."

• *The Critical salt concentration is the highest point of the binodal curve. Here it is more accurate to call it "salt resistance".*

• *The steps between each NaCl concentrations investigated are quite big. It would be worth narrowing them, to maybe discriminate between the three couples for which a salt resistance of 1 M is reported.*

• Also salt resistance should be reported as an interval between two NaCl concentrations. Consequently, error bars should be added in the bar graph.

• Furthermore, did the authors make sure that crashing out of the single polymers at high salt concentrations through salting-out affect did not happen. This should be confirmed and the data should be added in Supporting information.

• The explanation of these results only on the basis of the hydrophobicity of the backbone is too restrictive. As stated earlier, other interactions can be involved in the phase separation process. If the authors want to make such strong statements about the effect of the hydrophobicity of the backbone, they should find a way to actually isolate this effect from other possible ones.

R2A15. Thank you for these comments. We have now updated the manuscript to use the term 'salt resistance', as follows:

(Page 7, Line 227–235): "The amount of NaCl required to dissolve the coacervates (referred to as the salt resistance) depends on the interaction strength between the polymers. Thus, the salt resistance of each polymer coacervate pair formed at the optimal stoichiometry was determined by adjusting the NaCl concentration until the turbid coacervate suspension (indicated by filled marks) transformed into a uniform liquid phase (indicated by open marks) (Fig. 3). From this point onward, the experiments were conducted using 19 mM Tris-Cl buffer at a mixing ratio favoring coacervate formation rather than precipitation. The complex coacervation of the PAEMA:PMAA pair exhibited the highest salt resistance, remaining stable up to a NaCl concentration of 1.5 M."

(Page 11, Line 356–357): "…including variations in the coacervate phase region, the salt resistance, and the interface instability."

In addition, we conducted additional experiments using NaCl concentrations of 0, 0.5, 0.8, 1.0, 1.2, and 1.5 mM to narrow down the precise concentration range. The overall tendency was consistent; however, we were able to differentiate the precise NaCl concentrations at which each polymer coacervate pair was effectively dissolved. Additionally, error bars have been added to the bar graph in Figure 3.

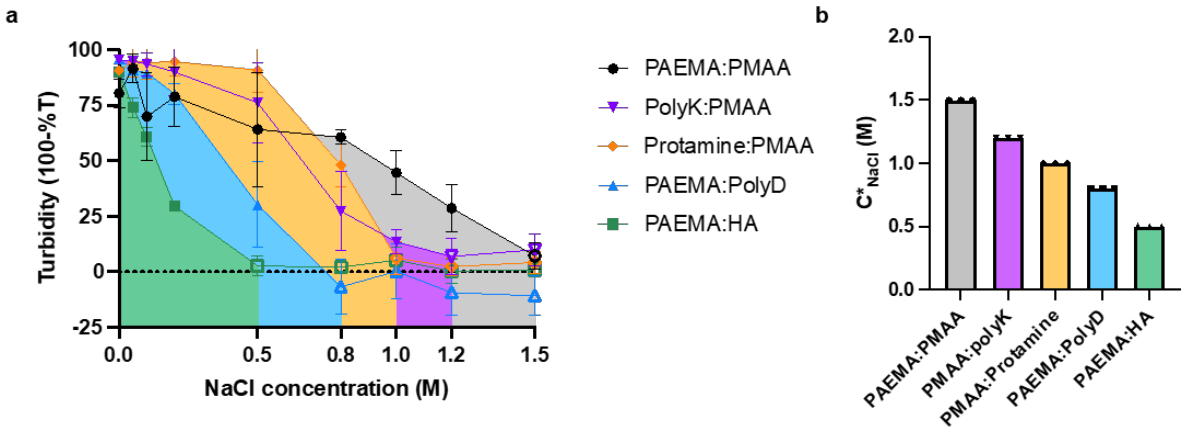
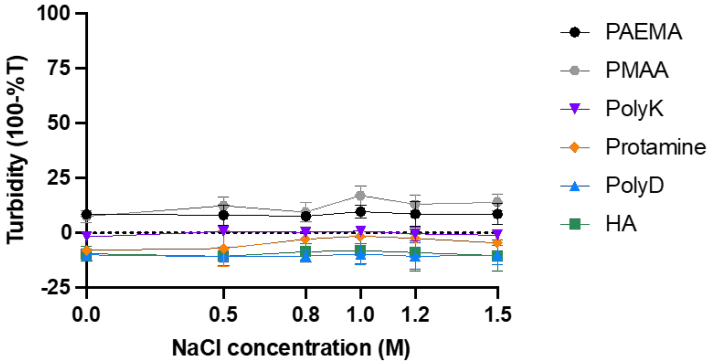


Figure 3. a Turbidity results obtained across various sodium chloride salt concentrations. The areas filled with colors indicate their coacervate regions. b NaCl salt resistance for the various coacervate pairs. In each case, the polymer concentration (C_p)

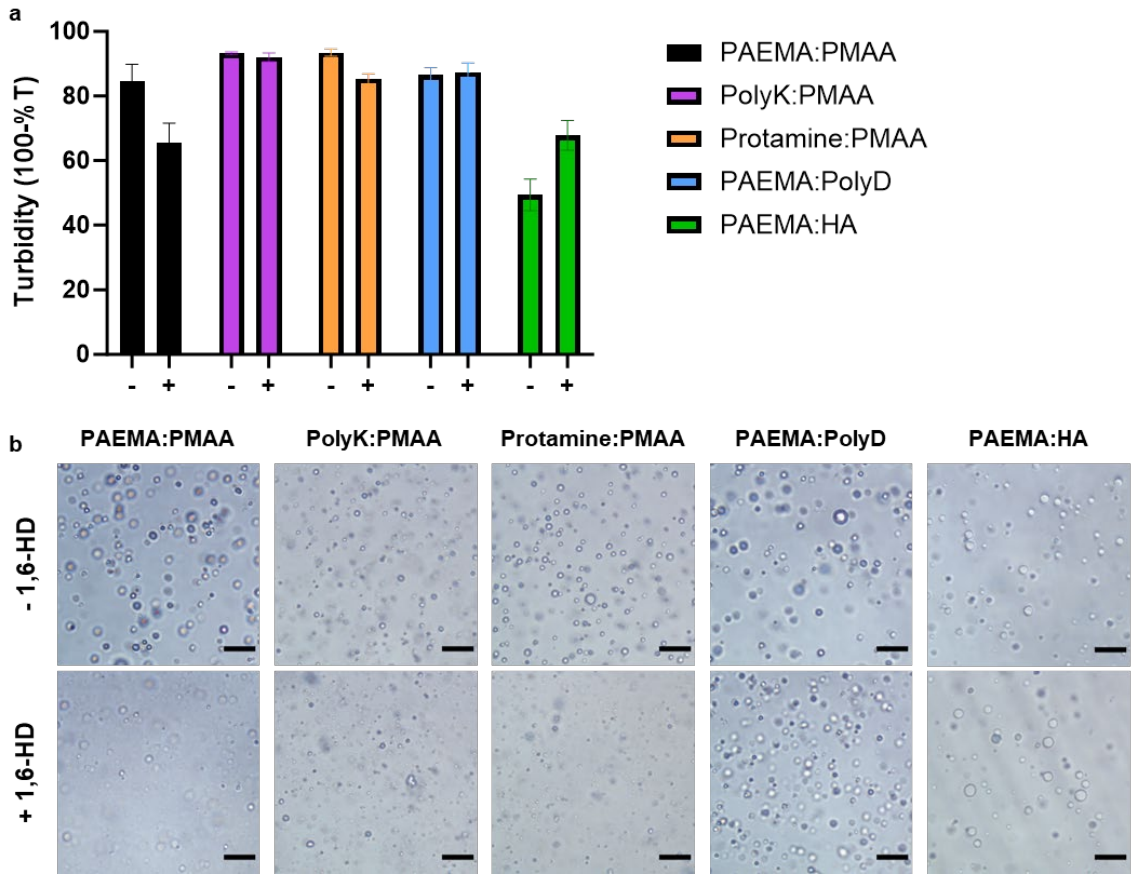
was 1 mg/mL, and the samples were analyzed immediately after complexation. The error bars indicate the standard deviations determined from three separate measurements.

To ascertain the unaffected state of each polymer following salting-out, we mixed the polymers with varying concentrations of NaCl. It was evident from the obtained data that there was no substantial turbidity difference after the addition of NaCl at the concentrations employed in our system.



Turbidity results obtained across various sodium chloride salt concentrations. The polymer concentration was 1 mg/mL. The error bars indicate the standard deviations determined from three separate measurements.

Furthermore, as correctly highlighted by the reviewer, additional interactions, such as hydrogen bonding, may have played a role in influencing phase separation. Thus, to explore the impact of the backbone hydrophobicity, we introduced 10% 1,6-hexanediol into the 19 mM Tris-Cl buffer containing 100 mM NaCl. The reduction in ionic strength due to the presence of the salt was expected to facilitate the observation of hydrophobic-driven coacervates. Notably, the PAEMA:HA pair exhibited more pronounced coacervate formation, suggesting the pivotal role of the hydrophobic interactions in this system containing 1,6-hexanediol, which in turn influenced the polymer solubility. Supplementary Figure 15 has now been added as follows:



Supplementary Figure 15. Effect of 1,6-hexanediol on the coacervates of the polymer pairs. To observe the effect of 1,6-hexanediol, 10% 1,6-hexanediol was added to a buffer containing 100 mM NaCl (+). The blank buffer contained 100 mM NaCl (−). Scale bars are 20 μM in all images.

R2Q16. Page 7, Line 212: "Coalescence behaviors of the complex coacervates"

Did the authors observe any merging of the solid-like phase separated samples?

• Why the authors not choose to investigate the viscoelastic properties of the phase separated samples by rheology?

R2A16. Throughout the study, we did not differentiate between solid- and liquid-like coacervates. During the observation of coalescence, variations in the merging rates were evident among different polymer pairs. While determining the viscoelastic properties is crucial for identifying solid or liquid coacervates, the challenge in our system lies in the difficulty of conducting bulk rheology experiments due to not enough polymer quantities. Consequently, we opted for investigating the passive particle rheology. However, due to the high viscosities of these systems, the particle movement and background noise were hard to differentiate. Nevertheless, the point raised by the reviewer is of interest to us, and so in future work, we may incorporate an active rheology device in our methodology to allow the measurement of highly viscous samples.

R2Q17. What does "polymer residue" mean? Side-groups?

R2A17. That is correct. The "polymer residue," which is also known as the "monomer residue," is defined as a chemical group that is attached to a core part of the molecule known as the "main chain" or the "backbone."

R2Q18. Page 9, Line 273: "it was expected that the hydration properties of the polyanions would follow this order: HA ≈ PolyD > PMAA." How do the authors make a conclusion that the hydration properties of HA is similar to PolyD? Is there any explanation about the much broader coacervate region of PAEMA:HA pair in Tris-Cl buffer than the PAEMA:PolyD pair?

R2A18. As stated in R2A13, we defined the coacervate region as a non-precipitated region, and in that regard, it was considered that both the PAEMA:HA and PAEMA:PolyD pairs both showed coacervation in the three buffer regimes, unlike in the case of the PAEMA:PMAA pair. Furthermore, we showed that the PolyK:HA and Protamine:HA pairs form coacervate phase at all phase-separated ratios, unlike the PolyK:PMAA and Protamine:PMAA pairs. Hence, it was concluded that the hydration properties of HA are similar to those of PolyD.

R2Q19. Page 11, line 340-360: Check the values for the reactants used and for the conversion. They do not match with the reported DP of 40 reported.

R2A19. Thank you for this comment. We note that the total amount of chemicals did not change, and we recalculated the concentration considering the content percentages in the chemicals. Thus, the Methods section has been updated as follows (Pages 12–13, Lines 396–417):

"**Synthesis of poly(methacrylic acid).** Phosphate buffer solution (6 mL, 10 mM, pH 7), 4-((((2-carboxyethyl)thio)carbonothioyl)thio)-4-cyanopentanoic acid (CETCPA) (95%, 17.9 mg, 9.2 mM), methacrylic acid (99%, 0.187 mL, 0.367 M), and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (V-50, 97%, 3.0 mg, 1.79 mM) were introduced into a 100 mL flask equipped with a magnetic stirrer bar and sealed with a rubber septum. The mixture was then deoxygenated by freeze–pump–thaw cycling for a minimum of 3 cycles, after which polymerization was performed at 70 °C using a temperature-controlled heating mantle, followed by stirring at 60 rpm for 2 h to reach nearly full conversion. A sample was extracted from the polymerization medium using a degassed syringe for analysis by ^{1}H nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC) to determine the monomer conversion, the experimental molar mass ($M_{n,GPC}$), and the dispersity ($Đ$) values.

Synthesis of poly(2-aminoethyl methacrylate hydrochloride). Phosphate buffer solution (36 mL, 10 mM, pH 7), CETCPA (95%, 17.9 mg, 1.5 mM), 2-aminoethyl methacrylate hydrochloride (90%, 0.642 g, 0.09 M), and V-50 (97%, 3.0 mg, 0.3 mM) were introduced into a 100 mL flask equipped with a magnetic stirrer bar and sealed with a rubber septum. The mixture was then deoxygenated by freeze–pump–thaw cycling for a minimum of 3 cycles, after which polymerization was performed at 70 °C using a temperature-controlled heating mantle,

followed by stirring at 60 rpm for 2 h to reach nearly 67% conversion. A sample was extracted from the polymerization medium using a degassed syringe for analysis by ^{1}H NMR spectroscopy and GPC to determine the monomer conversion, the experimental molar mass, and the dispersity values."

Reviewer #3 (Remarks to the Author):

Based on the abstract, I was very excited to read this paper and see the various comparisons between aliphatic, peptide, and carbohydrate backbones, as well as the different buffer effects. While the manuscript contains a significant amount of interesting data, I feel as though the current discussion of the results is lacking in the depth that is needed to satisfy my incoming excitement. I have detailed out a number of suggestions below and encourage the authors to revise their work.

Specific Comments:

R3Q1. In the introduction, the authors state that mechanistically, both the ion pairing and condensation step of coacervation involve substantial entropic contributions. However, that is not my understanding based on a reading of the three papers cited. Indeed, the ion pairing step involves a significant entropic driving force, related to the release of bound counterions. However, the modeling used in the cited papers assumes only an enthalpy of phase change for the second step of condensation. I suggest that the authors modify their text.

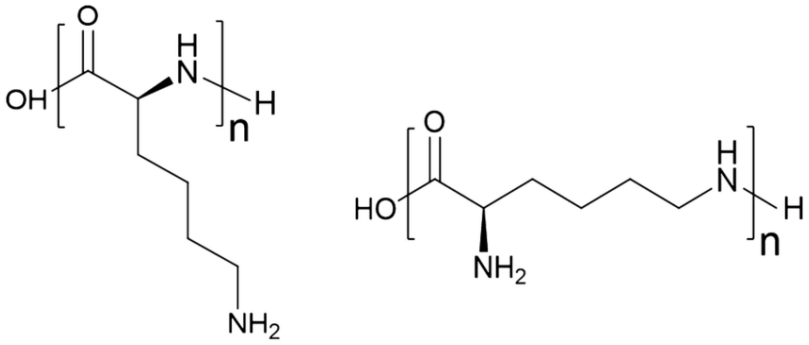
R3A1. Thank you for addressing this matter. Your observation is accurate, and we value your thoroughness. The sentence has been appropriately revised in the introduction as follows: (Page 2, Lines 50–53):

"Notably, the first stage corresponding to ion pairing is characterized by substantial entropic contributions in their free energy changes, which are commonly believed to be due to counterion release upon complexation between the oppositely charged polyelectrolyte chains[12–14]."

R3Q2. I found it surprising that epsilon polylysine was used, rather than alpha polylysine given that it appeared as though alpha polyaspartate was used. It would be useful for the authors to clarify the form of the polyaspartate that was used (in the text) and comment on the differences in the two backbones. Indeed, it would also have been very interesting to see a comparison of alpha vs. epsilon polylysine.liquid

R3A2. Initially, we utilized εPolyK due to its availability; however, acknowledging your valuable insight, we recognized the significance of exploring the differences between εPolyK and αPolyK in coacervates. Deeming this question worthy of investigation, we conducted an additional experiment with αPolyK. Regarding the impact of the polycation:polyanion ratio on determining the phase behavior, it was found that the εPolyK:PMAA pair exhibited a narrow

phase boundary area conducive to liquid coacervate formation due to its hydrophobic aliphatic backbone. In contrast, the αPolyK:PMAA system exclusively displayed the coacervate phase across all mixing ratios evaluated herein.

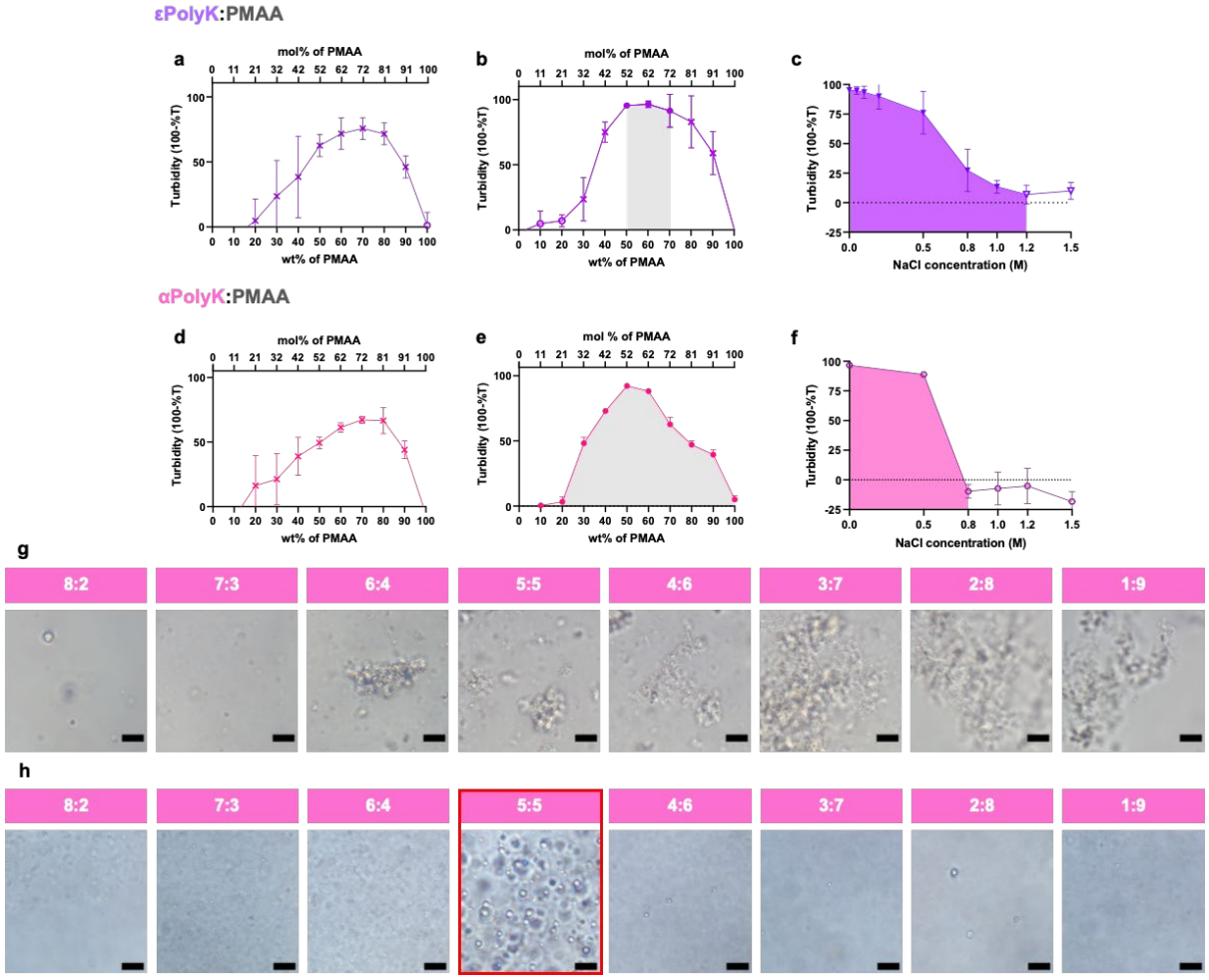


Structures of alpha-poly-L-lysine (αPolyK) and epsilon-poly-L-lysine (εPolyK).

Furthermore, the salt resistance test revealed a consistent trend. More specifically, the εPolyK:PMAA pair bearing a more hydrophobic backbone exhibited a higher NaCl resistance compared to the αPolyK:PMAA pair. This suggests that complexation and coacervation contribute not only to electrostatic attractions, but also to hydrophobic interactions, thereby sustaining liquid–liquid phase separation at elevated salt concentrations.

These observations align with our previously reported results [21], indicating that the tight binding of molecules, accompanied by water expulsion and counterion release, leads to precipitation. Thus, Supplementary Figure 13 has been added as follows:

[21] Hong, Y. *et al.* Hydrophobicity of arginine leads to reentrant liquid-liquid phase separation behaviors of arginine-rich proteins. *Nat. Commun.* **13**, 7326 (2022).



Supplementary Figure 13. Variations in the mixing ratio and salt resistance between the εPolyK:PMAA and αPolyK:PMAA coacervates. a, d, and g Using a 10 mM Tris-Cl buffer; and b, e, h, c, and f Using a 19 mM Tris-Cl buffer.

Additionally, the results section has been revised as follows:

(Page 6, Line 181–184): "Interestingly, the α-poly-L-lysine:PMAA pair formed coacervates at all phase-separated mixing ratios, highlighting the substantial influence of the backbone hydrophobicity on the phase behaviors of such polyelectrolyte complexes (Supplementary Figs. 13e and 13h)."

(Page 6, Line 244–247): "Additionally, it was observed that α-poly-L-lysine:PMAA coacervates bearing a shorter backbone chain disappeared at a lower NaCl concentration than the ε-poly-L-lysine(PolyK):PMAA coacervates (Supplementary Fig. 13), indicating that a more hydrophobic backbone contributes to an improved salt resistance."

R3Q3. Would the authors please comment on the polydispersity of their various samples? This information was only listed for the two methacrylate polymers. I mention this because highly polydisperse samples could show apparent higher phase behavior and/or viscosity because of the presence of longer chains.

R3A3. Thank you for this suggestion. We have now included the appropriate information for the source materials as follows: PAEMA (DP 40), PMAA (DP 40), PolyK (Mw/Mn 1.14, DP 25–35), protamine (DP 31) PolyD (Mw/Mn 1.00–1.20, Mn 3700–4500, DP 27–33), hyaluronic acid (0.5–10.1 kDa). In addition, Figure 1 has been updated as follows:

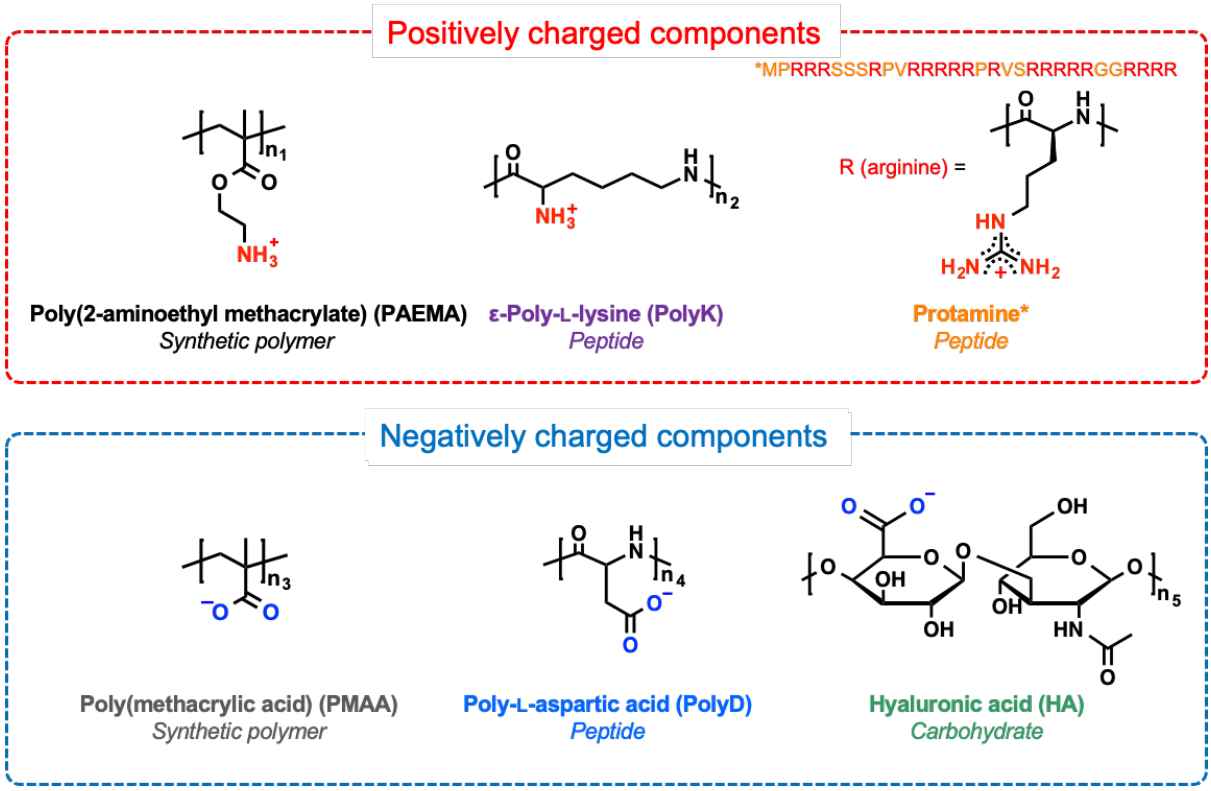


Figure 1. Chemical structures of selected polyelectrolytes. The asterisk(*) indicates the primary sequence of protamine. The values n_1 = 40, n_2 = 25–35, n_3 = 40, n_4 = 30, and n_5 = 1.2-25 correspond to the respective polymer repeating units.

R3Q4. I was unsure how to interpret the discussion of polymer solubility given that the protamine undergoes simple coacervation above 40 mg/mL. How is this evidence that protamine is more hydrophilic than PAEMA? I would suggest that the authors be more conservative and comment that while they cannot definitively determine the solubility of protamine, the limit is above 40 mg/mL, which is relatively close to the solubility limit of PAEMA, and so they hypothesize that it is more soluble, but cannot prove it.

R3A4. Thank you for these comments, which we agree with. In contrast to PAEMA, which precipitates at concentrations >63 mg/mL, protamine forms coacervates at concentrations >40 mg/mL, reaching concentrations as high as 70 mg/mL, and surpassing the solubility of PAEMA. We interpreted this behavior as indicative of protamine being more hydrophilic, given that coacervates are inherently more hydrated than the precipitates. However, considering the challenge in comparing the solubilities when the substances are in different

states (i.e., coacervates and precipitates), we have revised the text to remove the reference to protamine, as follows (Page 4, Line 122–124):

"The occurrence of simple coacervation in protamine, rather than precipitation, suggests that protamine exhibits a greater degree of hydration[24]."

[24] Liu, X. et al. The correlation between thermally induced precipitate-to-coacervate transition and glass transition in a polyelectrolyte-bolaamphiphile complex. Aggregate 4, (2023).

R3Q5. The authors use polyarginine to model their protamine. In reading through the text of the manuscript it was unclear to me how relevant this analogy is, as there was no discussion about the sequence of protamine and the propensity to have multiple arginines next to each other in the sequence. Granted, the figures would not be at the end of the manuscript in final publication, but it might be useful to include a clarification about this specifically in the text.

R3A5. In one of our previous studies [21], we conducted a comparison between polylysine, polyarginine, protamine, and a pseudo-protamine-RtoK chain (where all the Arg residues of protamine are substituted by Lys to give a poly-Lys chain) through computational simulations. The results indicated that protamine exhibited a similar outcome to polyarginine. Consequently, we utilized polyarginine to model protamine. As suggested by the reviewer, the protamine sequence has been included in the revised introduction (Page 3, Lines 81–83):

"…protamine, an arginine-rich protein (~65 mol% arginine; MPRRRSSSRPVRRRRRPRVSRRRRRGGRRRR) possessing numerous guanidinium groups."

In addition, Page 16, Lines 514–518 have also been updated as follows:

"For Protamine, poly-arginine with a β-sheet structure was employed as a simplified model, referencing the work by Morga et al.[38] and based on a previous study that used molecular dynamics simulations to demonstrate similar hydrophobic and conformational properties between poly-arginine and protamine[21]."

[21] Hong, Y. *et al.* Hydrophobicity of arginine leads to reentrant liquid-liquid phase separation behaviors of arginine-rich proteins. *Nat. Commun.* **13**, 7326 (2022).

R3Q6. Related to the above point, since the authors purchased their PolyD from Alamanda Polymers, I wonder why they did not also simply purchase PolyK and PolyR from the same source, as these materials are readily available from this supplier in matched chain lengths.

R3A6. In our previous study [21], we demonstrated that protamine exhibits similar conformational properties to polyR using molecular dynamics simulations. Given the advantages of protamine in biomedical applications, such as drug delivery, we have continued to investigate the coacervation of protamine as an arginine-rich protein. While εPolyK and protamine were readily available and chosen for the current study, we acknowledge that

αPolyK and polyR from Alamandra would be valuable for a more straightforward comparison of their side-chain effects.

As recommended, α-poly-L-lysine (αPolyK) was purchased from Alamanda, and experiments were conducted with it, presenting the additional data (please refer to our response to your comment #4 and supporting figure 13). Additionally, We plan to conduct a comparative study between Protamine and *PolyR from Alamanda* in a future study, as the guanidine moiety is one of the main interests of my group. Please excuse us for not including *PolyR* in the current study.

[21] Hong, Y. *et al.* Hydrophobicity of arginine leads to reentrant liquid-liquid phase separation behaviors of arginine-rich proteins. *Nat. Commun.* **13**, 7326 (2022).

R3Q7. *Why was an analysis of the solvent accessible surface area performed only for the cations, and not the polyanions? Additionally, I did not find the visual images in Supplementary Figure 3 to be particularly useful. It would be much more useful (and quantitative) if values for the solvent accessible surface area were reported on some equivalent basis, perhaps in Table 1.*

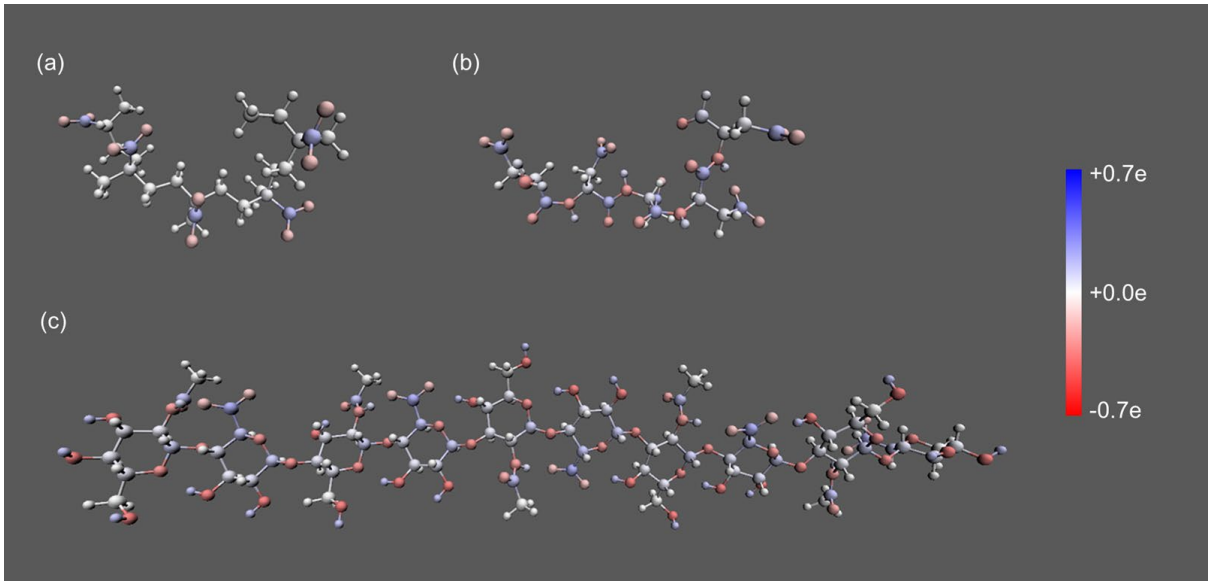
R3A7. Thank you for your valuable feedback which has greatly contributed to improving the quality of our manuscript. Our initial manuscript did not include the DFT calculations for the HA polyanions because these calculations had not been completed at the time of submission. We have since completed these calculations and incorporated the results into the revised manuscript. Additionally, to address your request for more quantitative data, we have added the solvent accessible surface area (SASA) information for both cations and polyanions in Table 1.

Table 1. Average degrees of polymerization, molecular weights, solubilities, and SASA values of the polyelectrolytes. All experiments were conducted in triplicate, and the data are presented as mean values ± SD. *For PAEMA and PMAA, the values are determined using eq (2) in Methods. For protamine, the value indicates the number of amino acids in the primary sequence. ** For PAEMA and PMAA, the average molecular weight was determined using eq (1) in Methods. The average molecular weight was determined as per the manufacturer's specifications. ***SASA calculations were performed on a pentamer, with polyarginine serving as the basis for protamine analysis, as detailed in the Methods section. ****The solubility of protamine was determined through turbidity measurements, which marked the transition from a two-phase to a one-phase solution

Polyelectrolyte	Degree of Polymerization*	Molecular weight** (g/mol)	Solubility (mg/mL)	SASA(Å2)***
PAEMA	40	7098	63.33 ± 9.43	954
PolyK	30	4000	326.67 ± 81.34	1309
Protamine****	31	4068	<40	1383
PMAA	40	3750	>1000	757
PolyD	30	4100	>1000	816
HA	12	5000	>1000	2196

R3Q8. I found it surprising that DFT results and images were not provided for hyaluronic acid (Supplementary Figure 5). Why was this not included?

R3A8. As mentioned above, the DFT calculations for HA were not included in the initial submission because they had not been completed at the time. We recognized the importance of these results and have since completed the necessary calculations. The revised manuscript now includes both the DFT results and corresponding images for HA in Supplementary Figure 5:



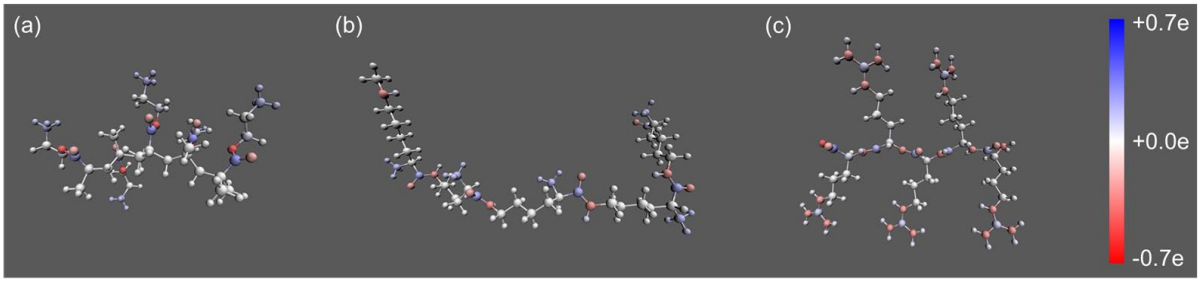
Supplementary Figure 5. Snapshots of the DFT-optimized structures of the anionic poly(penta)-electrolytes. Each atom is colored based on its partial atomic charge. The color scale runs from −0.7e (red) to +0.7e (blue). a PMAA, b poly-aspartic acid, and c hyaluronic acid.

In addition, the text has been revised as follows (Page 5, Line 157–161):

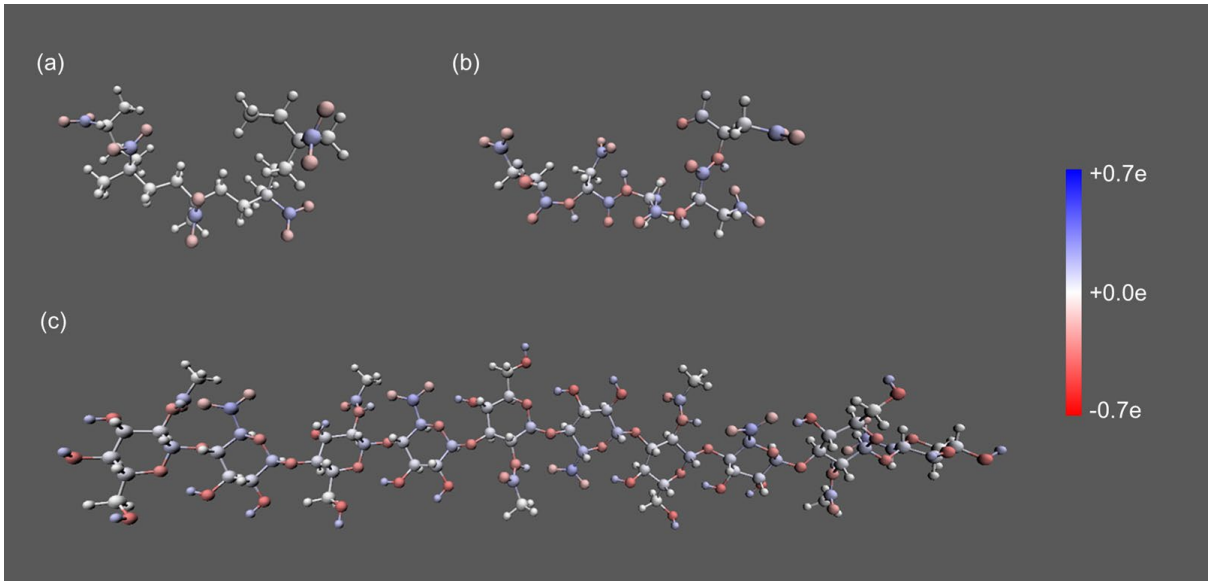
"It was also found that the optimized structure of hyaluronic acid (HA) was linear, and that the SASA value of this species was exceptionally large (Supplementary Figs. 5c and 6b). Thus, the large accessible area of HA, along with its abundant hydroxyl groups, should promote interactions with water molecules and ions to render HA highly soluble."

R3Q9. Relatedly, I found it difficult to parse net charge based on the coloration in Supplementary Figures 4,5, and the choice of red/green means that it would be impossible for someone with red/green colorblindness to do so. The authors might consider modifying their choice of color, and I would suggest the inclusion of text labels in the relevant figures to better communicate this information.

R3A9. In response to your suggestions, we have revised the figures by changing the color scheme from red/green to red/blue, offering greater clarity for those with color vision deficiencies. Additionally, we have introduced a color bar in these figures to aid in interpreting the atom colors. Furthermore, to provide a more comprehensive understanding of the calculated atomic charges, we have added two new figures (Supplementary Figures 3 and 5). These figures illustrate the ranges of atomic charges for the various polymer species, along with their minimum and maximum values. We believe that these additional graphs and tables will greatly enhance the clarity and offer a more quantitative representation of the differences in atomic charge distribution across different polymer types.



Supplementary Figure 3. Snapshots of the DFT-optimized structures of the cationic poly(penta)-electrolytes. Each atom is colored by its partial atomic charges. Each atom is colored based on its partial atomic charge. The color scale runs from −0.7e (red) to +0.7e (blue). a PAEMA, b poly-lysine, and c poly-arginine.



Supplementary Figure 5. Snapshots of the DFT-optimized structures of the anionic poly(penta)-electrolytes. Each atom is colored based on its partial atomic charge. The color scale runs from −0.7e (red) to +0.7e (blue). a PMAA, b poly-aspartic acid, and c hyaluronic acid.

R3Q10. Are the various polymers in their acid/base forms or are they salts? From the methods section it seems as though most of the polymers are in their salt form, though it was unclear for PMAA, polyK and hyaluronic acid. Additionally, not all of the salt forms for a given class of polymer appear to be the same (protamine sulfate vs. PAEMA HCl). If in the salt form, I would recommend specifying this when the polymers and their abbreviations are introduced, and perhaps include the information in Figure 1. The authors might also comment on any effect differences in these counterions might have on their materials, particularly that they also discuss ion effects with regards to the buffer.

R3A10. Thank you for these comments. We acknowledge that different salt forms can influence coacervate formation. However, we did not investigate this in the current study due to its challenging controllability.

R3Q11. Is the cation/anion mixing ratio described on line 150 on a charge basis or a weight basis? I believe it is on a weight basis, which while easy to work with, does not provide guidance with regards to electrostatic intuition. I would suggest that the authors include an additional axis on their various plots providing the charge basis information, and perhaps include such numbers parenthetically when discussing optimum compositions (see my note below).

R3A11. Thank you for pointing out this issue. In the initial manuscript, the polymer mixing ratios were presented using weight basis calculations. Following your suggestion, we have now incorporated molar basis values, which offer additional information on a charge basis. Figure 2 has also been revised as follows:

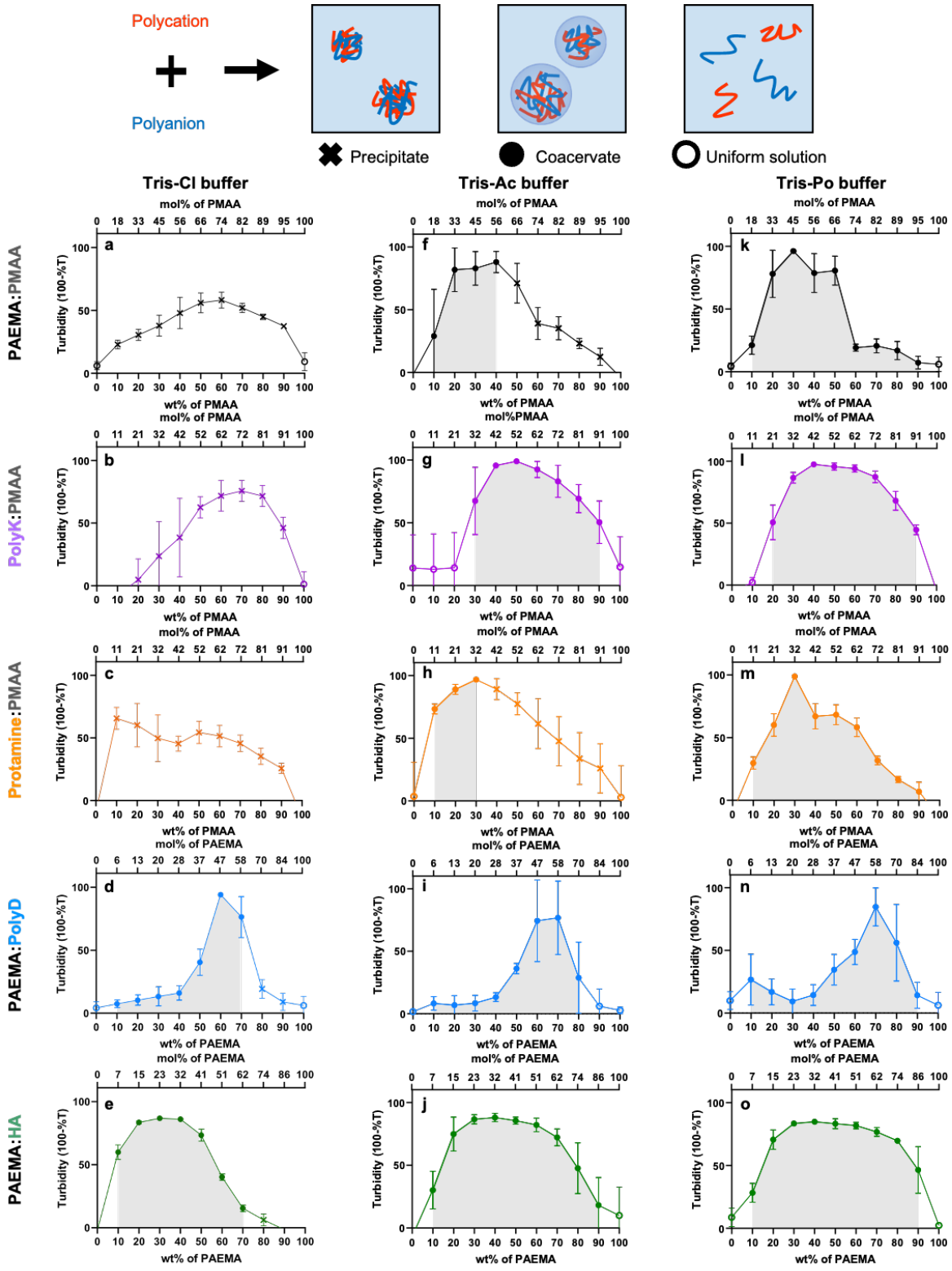


Figure 2. Complex coacervation behaviors in different buffers. Schematic representation (top) and plots of turbidity vs. polymer mixing ratio (bottom) for phase separation. a, b, c, d, and e: In Tris-Chloride (Tris-Cl) buffer; f, g, h, i, and j: In Tris-Acetate (Tris-Ac) buffer; k, l, m, n, and o: In Tris-Phosphate (Tris-Po) buffer. Black plots = PAEMA:PMAA pairs; purple plots = PolyK:PMAA pairs; orange plots = Protamine:PMAA pairs; blue plots = PAEMA:PolyD pairs; green plots = PAEMA:HA pairs. In the phase diagrams, closed markers indicate the mixing ratios wherein phase separation was observed using an optical microscope. The open markers indicate the mixing ratios wherein phase separation did not occur. The X marks indicate the mixing ratios wherein aggregation was observed. The gray region in each phase diagram represents the

coacervation region where phase separation of the polyelectrolytes occurs. Each data is based on at least three replicate experiments carried out for each respective polyelectrolyte.

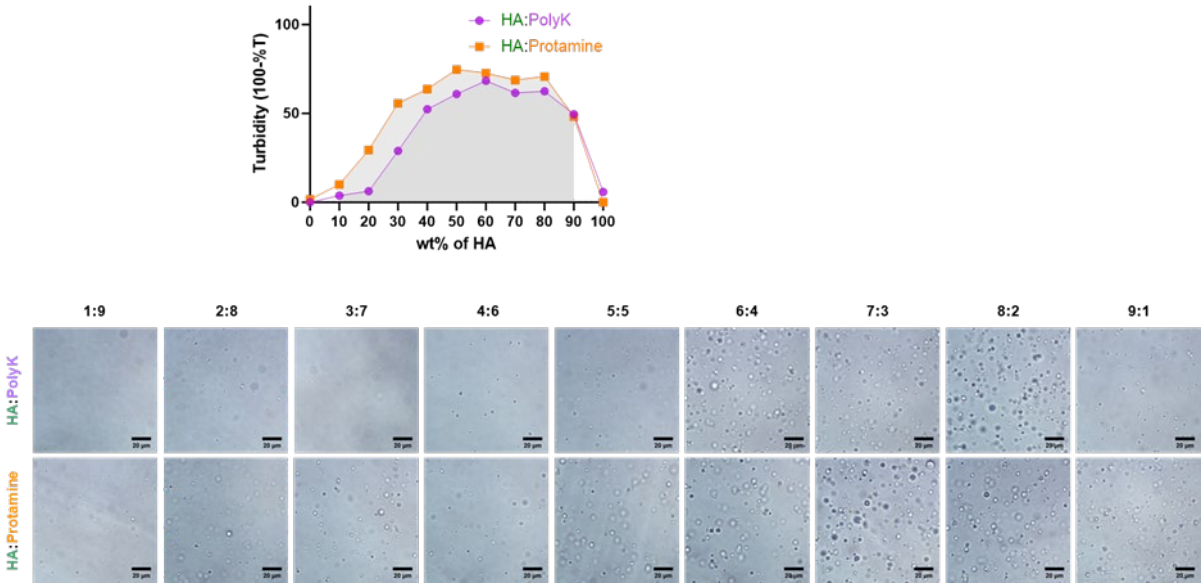
R3Q12. It would be useful for the authors to discuss what the optimum mixing ratio for complex formation is as a function of the various polymers, and how factors like the distance between charges or the charge density (i.e., protamine) affect these ratios.

R3A12. Thank you for this useful suggestion. We have incorporated the molar ratio as an additional axis in Fig. 2 as presented in R3Q11. Notably, in the current study, we did not consider the charge density as a variable due to the difficulties in controlling this aspect. However, your suggestion raises an important question that we plan to address in a follow-up study. For example, we intend to explore this further using a simpler system, such as a comparison of alpha-PolyK and epsilon-PolyK. The Discussion has now been revised to include the following details: (Page 11, Line 367–372):

"Additionally, while hydrophobic segments can contribute to differences in the phase behavior, it is essential to note that the disparity in the charge density among different polymers may also play a role, particularly when electrostatic interactions are favored. Although the charge density was not considered as a variable in this study due to control difficulties, it remains an important factor. A follow-up study is therefore required to explore this aspect further."

R3Q13. I do not understand why the data for polyK/HA and protamine/HA were not included at least in the supplementary information. It is one thing to say that they are similar and not include the figures in the main manuscript, but if the authors wish do include this comparison in their manuscript they should at least include the data in the SI.

R3A13. Thank you for this suggestion. We have included the data for the PolyK:HA and Protamine:HA systems in the revised Supplementary Information. In addition, Supplementary Figure 14 has been added:



Supplementary Figure 14. Turbidity and representative optical microscopy images of coacervation assembly in the PolyK:HA and Protamine:HA pairs at different mixing ratios. The polymer concentrations (Cp) were 1 mg/mL for all coacervate pairs, and the samples were analyzed immediately after complexation.

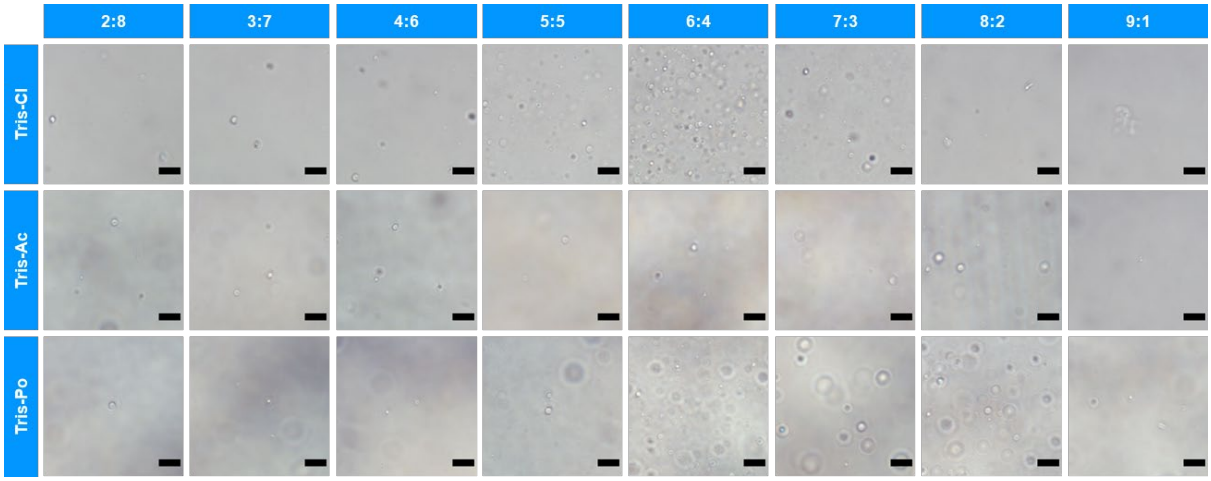
R3Q14. Lines 161-166: The authors state that their turbidity results are consistent with solubility results, particularly citing anionic polyelectrolytes having higher solubility than cationic polyelectrolytes. However, it is unclear to me the basis for this conclusion. In the various datasets shown in Figure 2 we observe turbidity curves that skew to high polycation, high polyanion, and are centered. A much more detailed and clearer discussion of these data and where this conclusion comes from is critical to improve the understandability of this argument.

R3A14. To clarify the means by which our conclusion was derived, we have revised the manuscript with updated results as follows (Page 5, Lines 188–197):

"Given that the tight binding of molecules and the corresponding expulsion of water and counterions leads to precipitation, the coacervate phase region can provide insights into the hydrophobicity of a polyelectrolyte. While a direct comparison of the solubilities of PMAA, PolyD, and HA poses challenges due to their high solubilities > 1000 mg/mL, the observed coacervate phase regions suggest that both PolyD (with an amide backbone) and HA (with a carbohydrate backbone) are more hydrophilic than PMAA (with an aliphatic chain). Notably, this observation correlates with the DFT calculation results. Moreover, when comparing the coacervate phase regions of PolyK:PMAA and PolyK:HA, in addition to those of Protamine:PMAA and Protamine:HA, it was clear that coacervate formation is more favored in the HA combinations. This preference is likely due to the higher hydrophilicity of HA (Supplementary Fig. 14)."

R3Q15. The microscopy data in Supplementary Figure 9 for the Tris-Po samples (and particularly the 9:1 sample) do not appear to match the turbidity data. Would the authors please comment on this?

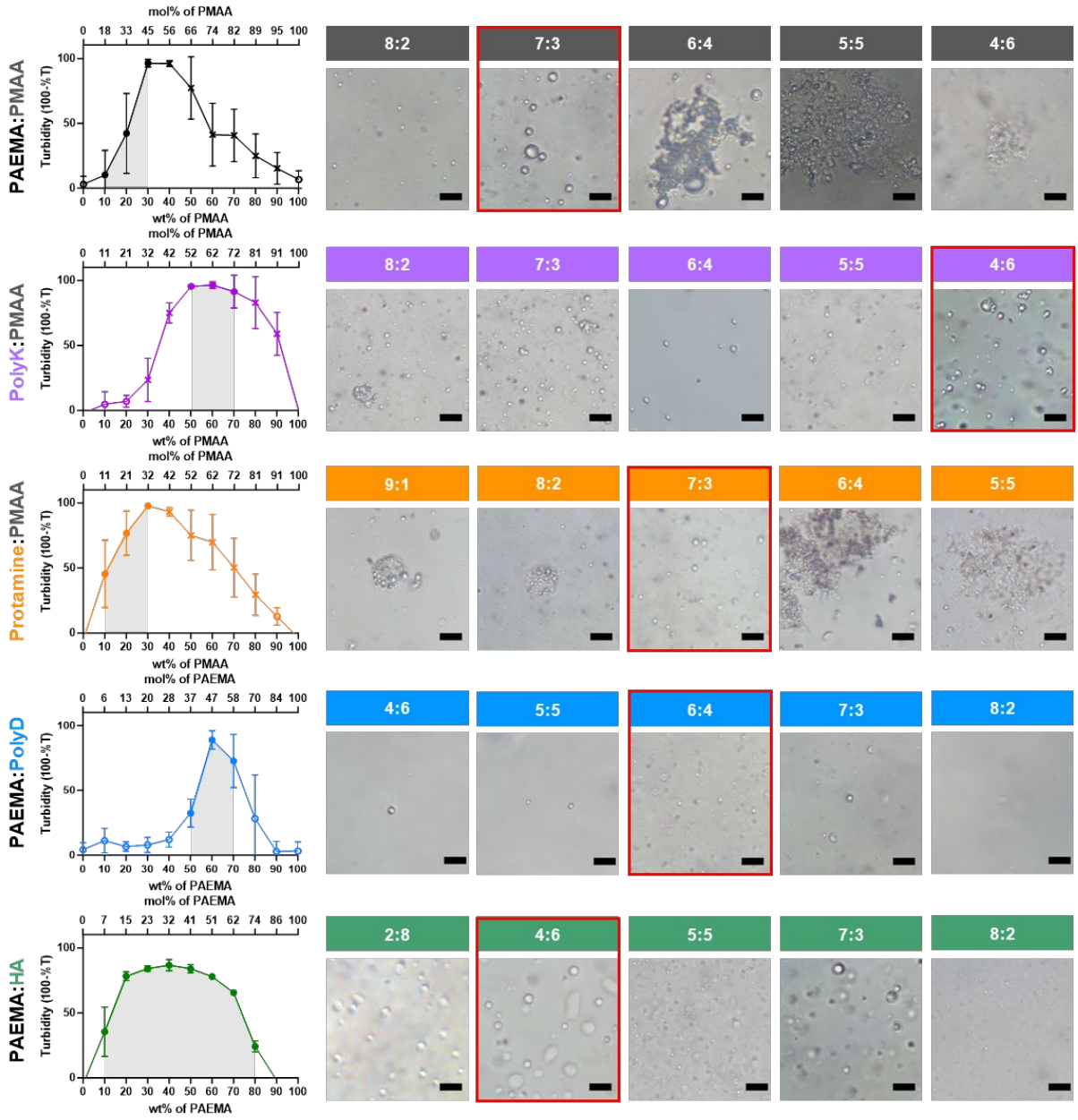
R3A15. Thank you for bringing this to our attention. To ensure the accuracy of the experiment, we conducted a repeat trial, and the figure has been modified accordingly. Please note that Supplementary Figure 9 has been renamed as Supplementary Figure 10:



Supplementary Figure 10. Representative optical microscopy images of coacervation assembly for the PAEMA:PolyD pair at different mixing ratios and in the presence of various buffers. The polymer concentrations (Cp) were 1 mg/mL for all coacervate pairs, and the samples were analyzed immediately after complexation.

R3Q16. In the discussion of buffer effects, it would be useful to know whether the observed effects only persist if the various ions are present at the same concentration vs. the same ionic strength. Based on the methods, it seemed as though ionic strength might have been taken into consideration for acetate and phosphate, but it was not clear for HCl and should be stated explicitly.

R3A16. Thank you for pointing out these issues. The manuscript and figures have been revised as outlined below. Additionally, we would like to point out that we found that the Tris-Cl buffer (10 mM Tris base + 8.89 mM HCl, pH 7.5) exhibited a different ionic concentration than the Tris-Ac (6.6 mM Tris base + 3.3 mM glacial acetic acid, pH 7.5) and Tris-Po (7.67 mM Tris base + 2.33 mM phosphoric acid, pH 7.5) buffers. Thus, we performed the measurements again using the Tris-Cl buffer (5.34 mM Tris base + 4.66 mM HCl, pH 7.5) and corrected Figure 2 along with the related supplementary figures. See R3Q11. In addition, Supplementary Figure 12 has also been added as follows:



Supplementary Figure 12. Turbidity and representative optical microscopy images of the coacervation assemblies of the various pairs obtained by varying the mixing ratios in 10 mM Tris base containing 8.89 mM HCl at pH 7.5. Polymer concentrations (Cp) were 1 mg/mL for all coacervate pairs, and the samples were analyzed immediately after complexation.

The manuscript text has been revised as follows:

(Page 4, Lines 12–13): "...a 19 mM Tris-chloride (Tris-Cl) buffer at pH 7.5 was employed due to its ability to dissolve all polyelectrolytes at room temperature."

(Page 6, Lines 173–175): "To investigate the formation of coacervates in a higher-ionic-strength buffer for the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA systems, identical experiments were conducted using 19 mM Tris-Cl buffer (pH 7.5, Supplementary Fig. 12)."

(Page 7, Lines 233–234): "From this point onward, the experiments were conducted using 19 mM Tris-Cl buffer at a mixing ratio favoring coacervate formation rather than precipitation."

(Page 14, Lines 461–464): "The Tris-chloride buffer (Tris-Cl, 10×10^{-3} M, pH 7.57) was prepared by combining 5.34 mM Tris base with 4.66 mM HCl in distilled water. To achieve a Tris-Cl buffer with higher ionic strength (Tris-Cl, 19×10^{-3} M, pH 7.5), 10 mM Tris base was mixed with 8.89 mM HCl in distilled water."

(Page 15, Line 489): "Sample solutions were prepared using the 19 mM Tris-Cl buffer."

R3Q17. The discussion of buffer effects concludes with a statement ordering the ions and a description that this "represents an inversion of the Hofmeister series." What aspect of the Hofmeister series helps to describe why this ordering exists? Is a phosphate ion expected to bring more water into the complex and therefore favor coacervation vs. precipitation? I suggest that the authors need to discuss the why behind their observation, rather than limiting their discussion to an eponymous reference to a very complex physicochemical observation.

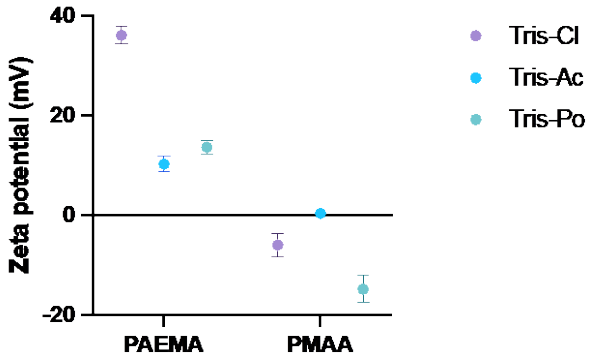
Building on this point (the above text was largely written with regards to the results section), in the discussion section, the authors talk about the "salting out" effects of ions earlier in the series. However, chloride is situated basically in the middle of the series, so one would likely only expect one half of the behavior (salting in only).

R3A17. Thank you for these suggestions. We note that phosphate is more kosmotropic than chloride, resulting in the salting-out of proteins through a reduction in the accessible surface area of the bulk water. However, in the PAEMA:PMAA, PolyK:PMAA, and Protamine:PMAA pairs, the liquid coacervates were dominant in the Tris-Po buffer, indicating that the complexes were more hydrated (c.f., precipitation in the Tris-Cl buffer). This ion-specific effect is related to ion adsorption or depletion from the solute/water interface, which is dependent on the surface properties of the coacervates, such as surface charge and hydrophobicity [ref 1, 2].

In this study, polymers were prepared in the buffers before forming coacervates, suggesting that the Hofmeister ion effect was already reflected in the polymers. We measured the zeta potential of PAEMA and PMAA in 10 mM Tris-Cl, Tris-Ac, and Tris-Po buffers to assess ion absorption levels in the buffers. The data presented below shows that phosphate species are more hydrated than others (ionic radii: $H_2PO_4^-$: 2.13, CH_3COO^-: 1.94, and Cl^-: 1.81), indicating more absorbed ions on the polymers. This implies that relatively more phosphate species are present in the formed coacervates than other species, inducing charge screening between polymer strands and resulting in preferred coacervate assembly. Likewise, relatively fewer chloride species are expected to be found in the formed coacervates, inducing relatively stronger electrostatic interactions between polymers and resulting in precipitation.

[Ref 1] Kunz, W. Specific ion effects (1st ed.), Wiley & Sons, Chichester (2007).

[Ref 2] Schwierz, N., Horinek, D., Sivan, U., & Netz, R.R. Reversed Hofmeister series—The rule rather than the exception, *Current Opinion in Colloid & Interface Science*, **23**, 10–18 (2016).



Zeta potential values of the polymers in three different buffers. The polymer concentrations were 1 mg/mL for all samples.

R3Q18. While it is true that various reports in the literature have referred to the amount of salt needed to dissolve a complex as the "critical salt concentration," in reality the critical point is a specific defined point in the thermodynamic phase diagram, and a more accurate name for this result would be the "salt resistance" (at a specified polymer concentration), as put forth by Tirrell, Perry, and a number of others working in the field.

R3A18. Thank you for these comments. We have now updated the manuscript to use the term 'salt resistance', as follows:

(Page 7, Line 227–235): "The amount of NaCl required to dissolve the coacervates (referred to as the salt resistance) depends on the interaction strength between the polymers. Thus, the salt resistance of each polymer coacervate pair formed at the optimal stoichiometry was determined by adjusting the NaCl concentration until the turbid coacervate suspension (indicated by filled marks) transformed into a uniform liquid phase (indicated by open marks) (Fig. 3). From this point onward, the experiments were conducted using 19 mM Tris-Cl buffer at a mixing ratio favoring coacervate formation rather than precipitation. The complex coacervation of the PAEMA:PMAA pair exhibited the highest salt resistance, remaining stable up to a NaCl concentration of 1.5 M."

(Page 11, Line 356–357): "…including variations in the coacervate phase region, the salt resistance, and the interface instability."

R3Q19. Would the authors please include error bars on Figure 3b? This should represent the error in sampling from the data shown in Figure 3a.

R3A19. Thank you for this suggestion. We note that the data in Figure 3b was derived from the data presented in Figure 3a based on experiments carried out in triplicate. The figure has now been revised to represent the corresponding errors.

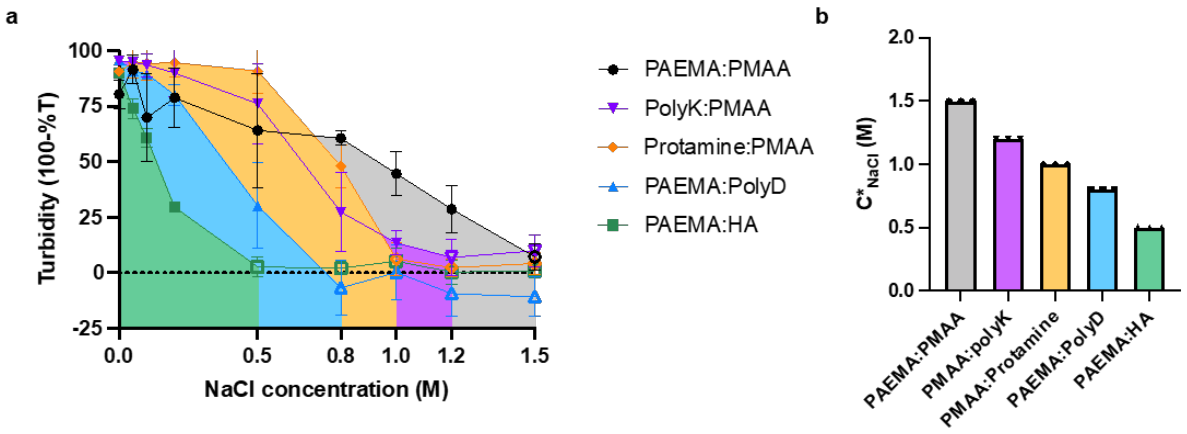


Figure 3. a Turbidity results obtained across various sodium chloride salt concentrations. The areas filled with colors indicate their coacervate regions. b NaCl salt resistance for the various coacervate pairs. In each case, the polymer concentration (Cp) was 1 mg/mL, and the samples were analyzed immediately after complexation. The error bars indicate the standard deviations determined from three separate measurements.

R3Q20. I was surprised to read that the critical salt for PolyK/PMAA, protamine/PMAA and PAEMA/PolyD was "1-2M NaCl," since the data shown in Figure 3b suggest that the value is 1M NaCl for all three (plus or minus sampling error). Please clarify this point.

R3A20. Thank you for these comments. We note that experiments were carried out using NaCl concentrations of 0, 0.5, 0.8, 1.0, 1.2, and 1.5 mM to narrow down the precise concentration range. The overall tendency was consistent; however, we were able to differentiate the precise NaCl concentrations at which each polymer coacervate pair was effectively dissolved. Additionally, error bars have been added to the bar graph in Figure 3, as shown in the previous response.

R3Q21. The authors attempt to argue that the higher critical salt value for PAEMA/PMAA compared to the other systems is purely a consequence of hydrophobicity. While I agree that the aliphatic nature of the backbone likely contributes to the difference in phase behavior, I would also point out that there is a significant difference in the charge density of the various systems. The methacrylate polymers have a two-atom backbone, whereas an alpha-polypeptide has a three-atom backbone, and the epsilon-polypeptide has a much longer one. It is difficult to comment on the charge density and hydrophobicity of protamine because it is a more complex protein. However, from an electrostatic perspective alone, these differences in charge density could have an effect, as could the mismatch in charge density along the various chains. It would be useful to caveat this discussion.

R3A21. As previously mentioned in our response to R3Q12, we did not consider the charge density as a variable in this study. Thus, the discussion has been revised as follows (Page 1, Line 367–372):

"Additionally, while hydrophobic segments can contribute to differences in the phase behavior, it is essential to note that the disparity in the charge density among different polymers may also

play a role, particularly when electrostatic interactions are favored. Although the charge density was not considered as a variable in this study due to control difficulties, it remains an important factor. A follow-up study is therefore required to explore this aspect further."

R3Q22. I feel as though the main comparisons made in this manuscript are between the methacrylate and peptide-adjacent polymers. While data was present (sometimes) for the complexes involving hyaluronic acid, there was very little discussion about the effect of the carbohydrate structure on the various properties. There was also no discussion given to nuances such as the difference in charge density between alpha and epsilon polypeptides and the more complicated arrangement of charges in protamine. The addition of such a discussion would help to improve the depth and impact of the work.

R3A22. In terms of the effect of the carbohydrate structure of HA, comparing the backbone effect itself proved challenging due to its relatively unique structure. Therefore, we attempted to assess this by conducting a solvent-accessible surface area (SASA) analysis. The significantly higher SASA value of HA indicates that HA is highly hydrated compared to the other polymers. Moreover, in the cases of the εPolyK:PMAA and αPolyK:PMAA pairs, the mixing ratios corresponding to their highest turbidity would be expected to vary significantly if their charge density played a significant role. However, the optimal mixing ratios showed very little variation, and if anything, the presence of coacervates or precipitates was influenced to a greater degree. This suggests that the hydrophobicity could have a more significant impact on this system than the charge density. The text has been revised to reflect this (Page 10, Lines 319–324):

"Moreover, for the εPolyK:PMAA and αPolyK:PMAA pairs, the mixing ratios at their highest turbidity values (i.e., the optimal mixing ratios) exhibited minimal variations. Instead, their tendencies to form coacervates or precipitations were significantly different (Supplementary Fig. 13). This observation suggests that hydrophobicity may have a more significant impact on this system than the charge density. This inference is drawn from the fact that significant variations in the optimal mixing ratios would be anticipated if the charge density played a substantial role."

R3Q23. Would the authors please include a reference for their mention of the first pioneering work on coacervation (Lines 242-243)?

R3A23. Thank you for this suggestion. Reference 30 has been added accordingly:

[30] Bungenberg de Jong, H. G. (Kruyt, H.R. ed.) *Colloid Scinece* **2**, Reversible systems. Elsevier Publ. Co., New York, p 335 (1949).

R3Q24. Line 300-301: The authors state that the merging experiments shown in Figure 4 provided evidence of solid-like properties for the methacrylate pair of polymers. However, the images shown in the figure clearly indicate liquid droplets, though their coalescence is slow. I

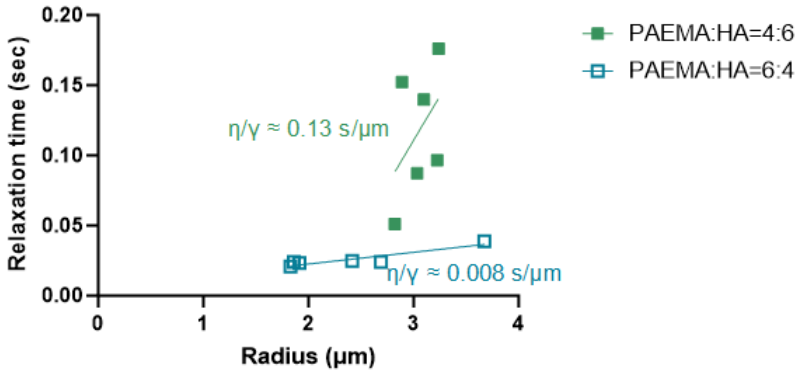
would argue this is evidence for a high viscosity, rather than saying solid-like properties. Similarly, I would not describe the other polymer pairs as having liquid-like properties, but rather a lower viscosity. There are reports describing the effect of hydrophobicity on rheological properties in the literature from groups like Perry, Laaser, Shull, Qin, Tirrell, and Schlenoff which the authors could reference with regards to a discussion of viscosity, if desired.

R3A24. Thank you for raising these points. We have now revised the text as follows to reflect this (Page 11, Lines 347–349):

"Furthermore, during the merging event (Fig. 4), it was found that the coacervates formed by the synthetic polymer pairs containing hydrophobic aliphatic backbones exhibited slower merging, indicating a higher viscosity."

R3Q25. Related to the coalescence experiments, I wonder if the choice of mixing ratio had any effect on this result. A recent paper by Pyo et al. (https://doi.org/10.1016/j.isci.2022.103852) showed that the surface tension of coacervates was decreased at off-stoichiometric conditions, while work by Spruijt et al. (https://doi.org/10.1021/ma301730n) showed that the viscosity of a sample did not change significantly. Such a discussion could provide useful insight into these materials by eliminating potential formulation differences between the samples.

R3A25. Thank you for these valuable comments. As suggested, we intended to conduct coalescence experiments at various mixing ratios. However, we lack the necessary equipment (e.g., optical tweezers) that can use lasers to closely position the coacervates, and so we had to rely on observing the passive fusion events of coacervates using confocal microscopy. While we attempted to observe merging events at other mixing ratios, unfortunately, the coacervates were too sparsely distributed to observe any merging. Interestingly, for the PAMEA:HA pair, we were able to observe merging events even under the 6:4 ratio, which is an inversion of our experimented 4:6 ratio. This result was imaged and analyzed. Notably, at a 6:4 ratio, we observed significantly faster coalescence compared to that at a 4:6 ratio, as indicated in the figure below. The changes in rheological properties within the same sample at different mixing ratios therefore offer critical insight into the surface properties and structures of such coacervates. However, although we know the polymer mixing ratios employed, we do not know the component stoichiometries of the resulting coacervates. Understanding these component stoichiometries and rheological properties of the coacervates as a function of the mixing ratio, as correctly pointed out by the reviewer, is an important area of research and will be our future direction.



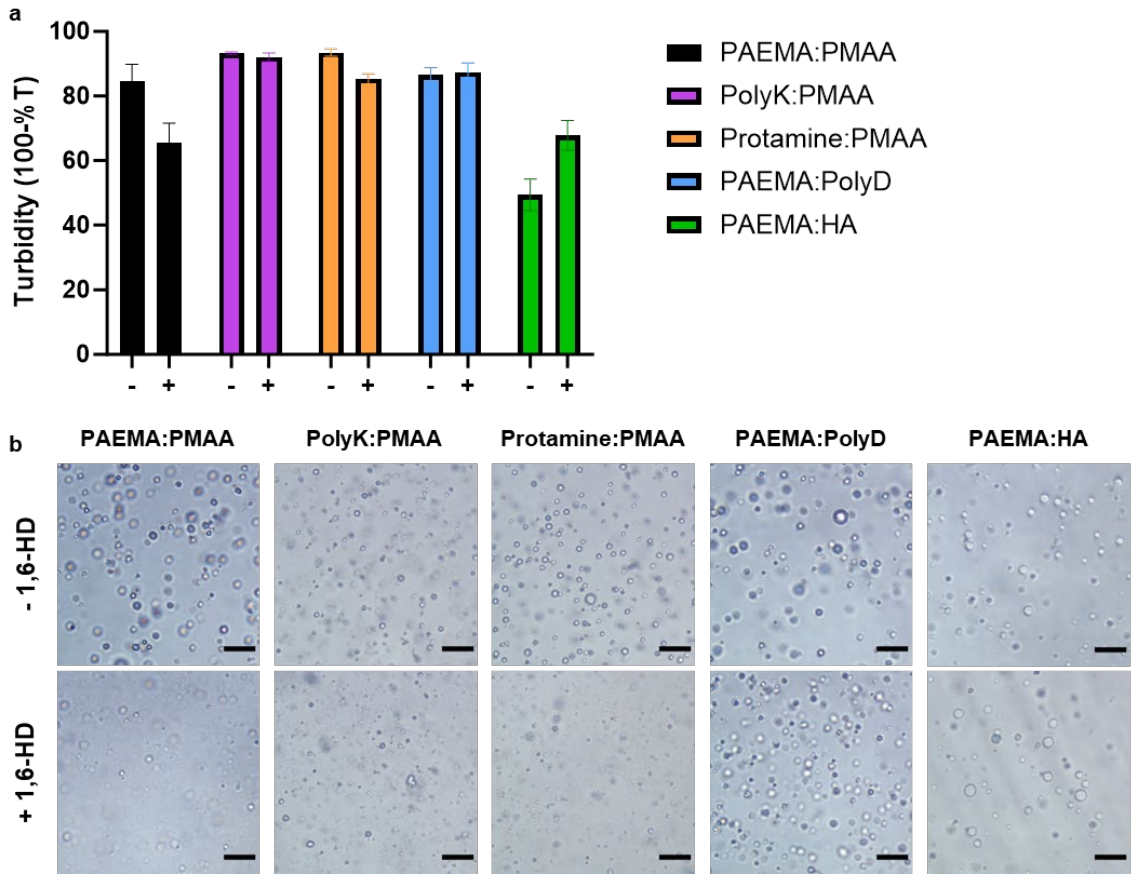
Relaxation time vs. length scale for each polymer pair formed in the 19 mM Tris-Cl buffer. For the measurements, all liquid-condensate pairs were prepared at the maximum of fixed total polymer concentration (Cp = 1 mg/mL). The inverse capillary velocity values (s/μm) are inserted. All samples were prepared at their optimal stoichiometry.

R3Q26. Line 311-312: The authors state that "paradoxically, the same hydrophobic segment is able to impede coacervate disassembly at high salt concentrations, likely due to charge screening." Firstly, it does not seem paradoxical to me that complexes which interact strongly and form solid precipitates might have a higher salt stability. Secondly, it is completely unclear to me how hydrophobicity affects charge screening. Dissolving a coacervate back to a single solution phase is effectively akin to a solubility argument. Less soluble polymers will have a higher preference for remaining in the 'less hydrated' coacervate phase, and therefore are able to withstand a higher salt concentration. Please address.

R3A26. Thank you for this feedback. We acknowledge that these sentences were unclear and so we have revised as follows (Page 11, Lines 357–362):

"In addition, the hydrophobic segment of the polyelectrolyte plays a crucial role in determining the solubility of the polyelectrolyte and significantly influences whether the polyelectrolyte forms a coacervate or undergoes precipitation. This implies that the conditions for coacervate formation must be meticulously controlled to avoid precipitation. However, once a coacervate is formed, its stability is enhanced even at high salt concentrations, possibly due to enhanced hydrophobic interactions in a charge-screened environment."

Regarding the second point, as correctly highlighted, additional interactions, such as hydrogen bonding, may have played a role in influencing phase separation. Thus, to explore the impact of the backbone hydrophobicity, we introduced 10% 1,6-hexanediol into the 19 mM Tris-Cl buffer containing 100 mM NaCl. The reduction in ionic strength due to the presence of the salt was expected to facilitate the observation of hydrophobic-driven coacervates. Notably, the PAEMA:HA pair exhibited more pronounced coacervate formation, suggesting the pivotal role of the hydrophobic interactions in this system containing 1,6-hexanediol, which in turn influenced the polymer solubility. Supplementary Figure 15 has now been added as follows:



Supplementary Figure 15. Effect of 1,6-hexanediol on the coacervates of the polymer pairs. To observe the effect of 1,6-hexanediol, 10% 1,6-hexanediol was added to a buffer containing 100 mM NaCl (+). The blank buffer contained 100 mM NaCl (−). Scale bars are 20 μM in all images.

Minor Comments:

R3Q27. Lines 65-66: where the authors refer to "lysine ionic groups" I would suggest that they say instead "ammonium groups" and perhaps reference lysine as a comparison (and then arginine in reference to guanidinium groups).

R3A27. Thank you for your suggestion. We have revised as suggested (Page 3, Lines 65–67):

"In addition, the guanidinium ionic groups of arginine have been reported to contribute to a larger two-phase region and a more viscous coacervate phase than the ionic ammonium groups of lysine[17,21]."

R3Q28. Lines 101-102: Rather than saying polymers with "40 degrees of polymerization," the phrasing should be polymers with a degree of polymerization of 40."

R3A28. Thank you for this suggestion. The corresponding sentence has now been revised accordingly (Page 4, Lines 103–105):

"More specifically, to prepare synthetic polymers with the same number of charged moieties, PMAA and PAEMA with a degree of polymerization (DP) of 40 were employed."

R3Q29. This is a semantic argument, but the data shown in Figure 2 are not true phase diagrams in the sense that they do not show actual concentrations etc. I would suggest modifying the caption to say something like "plots of turbidity vs. polymer ratio."

R3A29. As suggested, the caption of Figure 2 has been revised as follows:

Figure 2. Complex coacervation behaviors in different buffers. Schematic representation (top) and plots of turbidity vs. polymer mixing ratio (bottom) for phase separation. a, b, c, d, and e: In Tris-Chloride (Tris-Cl) buffer; f, g, h, i, and j: In Tris-Acetate (Tris-Ac) buffer; k, l, m, n, and o: In Tris-Phosphate (Tris-Po) buffer. Black plots = PAEMA:PMAA pairs; purple plots = PolyK:PMAA pairs; orange plots = Protamine:PMAA pairs; blue plots = PAEMA:PolyD pairs; green plots = PAEMA:HA pairs. In the phase diagrams, closed markers indicate the mixing ratios wherein phase separation was observed using an optical microscope. The open markers indicate the mixing ratios wherein phase separation did not occur. The X marks indicate the mixing ratios wherein aggregation was observed. The gray region in each phase diagram represents the coacervation region where phase separation of the polyelectrolytes occurs. Each data is based on at least three replicate experiments carried out for each respective polyelectrolyte.

R3Q30. The middle paragraph of the discussion should be broken up into smaller paragraphs.

R3A30. Thank you for pointing this out. We have now restructured as suggested.

R3Q31. Line 369: I believe that "PMMA" should be "PMAA."

R3A31. Thank you for pointing this out. We have now ensured that PMMA was replaced with PMAA throughout.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

In this paper, the authors explored the influence of the backbone chemistry and the ionic functional groups of five pairs of oppositely charged polyelectrolytes on complex coacervation. The research details described the effects of five factors on complex coacervation, including the solubility, the mixing ratio, the buffer specificity, the salt resistance and the coalescence rate. However, Reviewer # 2 raised a question, indicating that the current experimental results do not fully support the claims made in the article, which are about the stability and elasticity of the coacervates.

Reviewer 2's detailed comments pointed out multiple key issues, including inconsistency between title and content, inadequate experimental design and data interpretation, and missing method descriptions. Although the authors responded to the questions raised by Reviewer 2, in many cases, their answers were either insufficient or did not directly address the questions raised by Reviewer 2. For example, in R2Q2, the reviewer expressed concern about measuring "charge density", but the author's response did not clearly explain the experimental measurement method and only made terminology modifications. In R2Q4 and R2Q5, the author was unable to provide supplementary experiments or data on GPC chromatogram truncation and pH change testing.

More importantly, the reviewer questioned the author's claims regarding the stability and elastic properties of condensed matter, and raised potential other interactions (such as hydrogen bonding and main chain rigidity). The author still did not provide sufficient evidence or experiments in the R2A11 and R2A15 responses to evaluate the impact of main chain hydrophobicity in isolation, and did not compare and discuss the literature mentioned by the reviewer.

In addition, in the revised manuscript the author applied xSight (Spheryx, Inc.) to explore coacervate suspension. They showed a negative correlation between the refractive index and the coacervate diameter, suggesting that the coacervates with larger diameters are more hydrated, leading to a lower refractive index. This is an interesting observation, but not necessarily related to this paper. FACS can provide additional population-level data, including droplet count and particle size complexity, which cannot obtain from turbidity analysis and microscopic imaging. While the use of xSight (Spheryx, Inc.) did not provide such detailed information. FACS can obtain forward scatter (FCS) and side scatter (SCS) data, which not necessarily need fluoresce labeling the microdroplet. So, it is not necessary to label the samples with a fluorescence tag. So, I do not understand the author’s argument on the labelling coacervate microdroplet. Even if we need to label the microdroplet, there are many different ways to label coacervate microdroplet, and many papers have employed FACS to obtain more quantitative data. For instance, small dye molecules could be sequestered within the microdroplet for the FACS measurements.

After comprehensive consideration, although the author has made some modifications, there are still many key issues that have not been resolved. Especially, the author did not fully explain the differences between the observed phenomena and existing literature reports, nor did they conduct supplementary experiments to verify the hypothesis. Therefore, based on the above reasons, I suggest that the author revise the manuscript more thoroughly to present the experimental data more clearly, explain the results more accurately, and provide more evidence to support its conclusion. Before this, I do not recommend accepting this manuscript.

Reviewer #3 (Remarks to the Author):

I very much appreciate the effort that the authors undertook to modify their manuscript and also perform additional experiments. I believe that the concerns raised by myself and the other reviewers have been addressed. I would note that there are a couple of places where these new edits/additions of information do not flow well. I have included suggestions of ways in which the text could be further refined before publication, and I look forward to seeing this article in print.

Specific Comments:

1. Starting at line 173, the authors describe experiments that were done at higher buffer concentrations. However, it was not clear why they performed this analysis (though the discussion from the rebuttal document makes it clear). I would suggest that the authors add in some context.

2. On line 182 the authors reference data for poly-alpha-lysine. While I am very excited about this data and the potential comparisons between alpha and epsilon polylysine, this line represents the first time that this polymer is mentioned. I would suggest including it in Figure 1 and in the prior discussion, as well as one of the listed systems in the actual Discussion. This is not to say that I am suggesting they do all of the experiments with alpha-polylysine, but I think this comparison is worth highlighting more than in the current document. Any particular suggestion I might make about the writing is complicated by the separate nature of the Results and Discussion sections, but I could imagine using the comparison of the two lysine systems as a way of introducing things, before then transitioning to discuss the larger systematic study of all of the parameters. This allows introducing these results in a meaningful way and highlighting the significant difference that can be obtained even when two different isomers are used. Furthermore, this comparison helps to support the argument that the authors make about charge density not affecting the optimum mixing ratio in a clear and unconvoluted way.
The only request for additional material that I would make is to also add in the SASA for alpha-polylysine. It would also be nice to have solubility data, but this might be more challenging.

3. It would be useful for the authors to explain near line 244 and/or 342 that 1,6-hexanediol was used to test for hydrophobic effects and how to understand the results. The current presentation assumes that the reader would know this information.

Minor Comments:

1. On page 5, lines 136-137 the authors use the term “branches” to describe the side-chains of their various polymers. I would advise against this since branched polymers are something quite different. “Side-chain” would be a potentially safer term.

2. Line 327, I suggest a new paragraph when transitioning to the discussion of the different buffers.

3. I suggest that the authors check through the paper for instances where they say “protein” but are really referring to “polypeptides.”

4. The “Supplementary Information” title of the SI is misspelled.

5. The caption for Supplemental Figure 12 should say “varying” rather than “varing.” Also, what is the meaning behind the red outlines on some of the micrographs? A similar red outline is also present in Supplemental Figure 13 and is not explained. I would also ask that the definitions of the X and open/closed symbols used in these figures be repeated in the captions (they are currently only in the main text).

6. At the bottom of page 6 the authors compare the size of the coacervate regions of PolyK/PMAA, PolyK/HA and Protamine/PMAA, Protamine/HA. This presumably involves a comparison of the data present in Figure 2 and Supplemental Figure 14. However, it is unclear what buffer was used for the data in Supplemental Figure 14. One assumes based on the text in the main document that it is Tris HCl, but it is useful to not require the reader to remember something that was mentioned a page earlier when flipping back and forth between figures that could easily be labeled. Please address.

7. A minor point, but in the main paper the authors refer to the polymer systems as cation:anion. However, in the legend for Supplemental Figure 14 this order is reversed.

Response Letter to Reviewers

The authors would like to thank the reviewers for their time and supportive comments. We have revised the manuscript in accordance with these comments and concerns, as detailed below. The original comments are provided in black, whereas our answers are given in blue. The major revisions are highlighted in yellow in the revised manuscript, and are reproduced in our response below in red font. We hope that the revised manuscript is now acceptable for publication in *Communications Chemistry*.

Editorial Requests

ER1. *We ask that you address all of Reviewer #3's additional comments.*

ER1A1. Thank you for this comment. We have now addressed all the comments raised by Reviewer #3.

ER2. *With regards to Reviewer #1's report, the Guest Editor team have provided the following comments:*

** "In R2Q2, the reviewer expressed concern about measuring "charge density", but the author's response did not clearly explain the experimental measurement method and only made terminology modifications."*

> Guest Editor comment: It seems this was an English mistake rather than a scientific one, terminology modifications is exactly what was needed. No further action required.

** "In R2Q4 and R2Q5, the author was unable to provide supplementary experiments or data on GPC chromatogram truncation and pH change testing."*

> Guest Editor comment: The authors provided new data on both GPC and pH. For clarity, the authors should provide the exact conditions (polymer concentration, buffer concentration) in the caption of Figure S16.

** "The reviewer questioned the author's claims regarding the stability and elastic properties of condensed matter, and raised potential other interactions (such as hydrogen bonding and main chain rigidity). The author still did not provide sufficient evidence or experiments in the R2A11 and R2A15 responses to evaluate the impact of main chain hydrophobicity in isolation, and did not compare and discuss the literature mentioned by the reviewer."*

> Guest Editor comment: The authors should provide an explicit comparison with the literature and discuss observed differences.

** "FACS can provide additional population-level data, including droplet count and particle size complexity, which cannot obtain from turbidity analysis and microscopic imaging."*

> Guest Editor comment: I do not think FACS will add anything here, and I do not recommend adding it. FACS will mostly give information about the size of the coacervates, but that is not very useful in systems that are not in equilibrium yet - as the coacervates continuously fuse and coarsen. Such measurement would heavily depend on the mixing order, speed and time between preparation and measurement. I do not agree with reviewer 1 that "population" information is useful. The authors have reported average droplet sizes.

ER1A2. Thank you for these suggestions. We have addressed all the comments raised by Reviewer #1, as per the guidance of the Guest Editor.

ER3. *The Guest Editors additionally provided the following comment and request:*

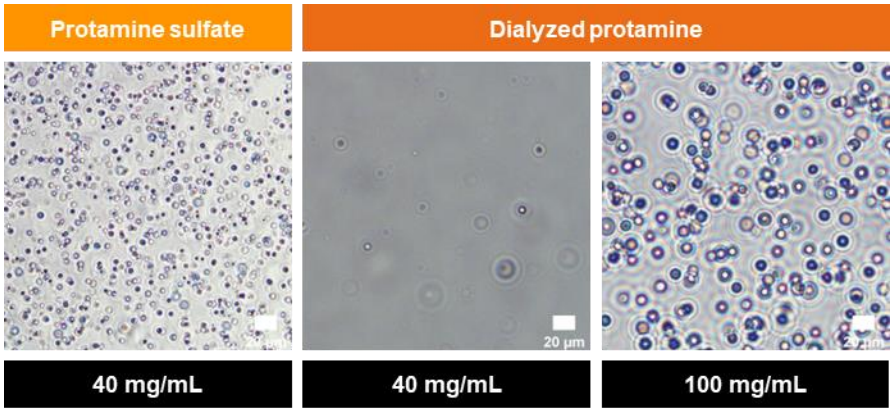
** Regarding simple coacervation of protamine (raised by both Reviewers #2 and #3 in the previous round), we do not think that protamine undergoes simple coacervation above 40 mg/mL as the authors write. Most likely, this is still complex coacervation, but with sulfate as the oppositely charged species. In fact, protamine has a much higher solubility, as can be verified with protamine chloride, which is also available from Sigma. This puts the discussion about solubility and hydrophobicity in a slightly different perspective and we would recommend that you follow the advice given in R3Q4 and be more conservative about your statements here.*

ER1A3. Thank you for bringing this to our attention. To address whether the simple coacervation phenomenon of protamine is ascribed to the sulfate salt, we dialyzed protamine sulfate against water and observed coacervation assembly in 19 mM Tris-HCl buffer at pH 7.4 using a microscope. At 40 mg/mL, the dialyzed protamine still underwent coacervation, although the quantity of coacervates was smaller than that observed in the case of protamine sulfate. We also confirmed that coacervation is more favored at a higher concentration of 100 mg/mL. However, considering the above points, we agree with the advice given by Reviewer 3 and the Guest Editors that this statement should be presented more conservatively.

Text has been added as follows to reflect this (Page 4, Lines 125–138):

In the case of protamine, at concentrations >40 mg/mL, simple coacervation occurred, characterized by a spherical shape, indicating that phase separation driven by hydrophobic arginine–arginine stacking had taken place[21]. The occurrence of simple coacervation in protamine, rather than precipitation, suggests that protamine exhibits a greater degree of hydration[24]. Nonetheless, it should be noted that coacervation is more favored in its salt form (i.e., protamine sulfate), as shown in Supplementary Fig. 3. In the case of dialyzed protamine (protamine sulfate thoroughly dialyzed against deionized water), its coacervation assembly in the 19 mM Tris-HCl buffer pH 7.4 was also observed using a microscope. At 40 mg/mL, the dialyzed protamine still underwent coacervation, although the yield of coacervates was smaller than that observed in the case of protamine sulfate. We also confirmed that coacervation is more dominant at a higher concentration of 100 mg/mL. This demonstrates that although protamine on its own undergoes simple coacervation above 40 mg/mL, the impact of counter ions (i.e., sulfate) should also be considered.

In addition, Supplementary Fig. 3 has been added:



Supplementary Figure 3. Optical microscopy images of the protamine sulfate and dialyzed protamine at concentrations of 40 and 100 mg/mL (scale bar = 20 μm). Protamine sulfate was thoroughly dialyzed against water, and its coacervation assembly in the 19 mM Tris-HCl buffer pH 7.4 was observed using a microscope. The samples were analyzed immediately after complexation.

ER4. *We ask that you edit your manuscript to comply with our journal policies and formatting style in order to maximise the accessibility and therefore the impact of your work.*

ER1A4. Thank you for this feedback. We have revised our manuscript and supplementary file to align with the journal's policies and recommended formatting style.

Reviewer #1 (Remarks to the Author):

In this paper, the authors explored the influence of the backbone chemistry and the ionic functional groups of five pairs of oppositely charged polyelectrolytes on complex coacervation. The research details described the effects of five factors on complex coacervation, including the solubility, the mixing ratio, the buffer specificity, the salt resistance and the coalescence rate. However, Reviewer # 2 raised a question, indicating that the current experimental results do not fully support the claims made in the article, which are about the stability and elasticity of the coacervates. Reviewer 2's detailed comments pointed out multiple key issues, including inconsistency between title and content, inadequate experimental design and data interpretation, and missing method descriptions. Although the authors responded to the questions raised by Reviewer 2, in many cases, their answers were either insufficient or did not directly address the questions raised by Reviewer 2.

R1Q1. *For example, in R2Q2, the reviewer expressed concern about measuring "charge density", but the author's response did not clearly explain the experimental measurement method and only made terminology modifications.*

> ***Guest Editor comment:*** *It seems this was an English mistake rather than a scientific one, terminology modifications is exactly what was needed. No further action required.*

R1A1. Thank you for these comments. As per the Guest Editor's suggestion, we will not be making any changes or taking further action regarding this question.

R1Q2. *In R2Q4 and R2Q5, the author was unable to provide supplementary experiments or data on GPC chromatogram truncation and pH change testing.*

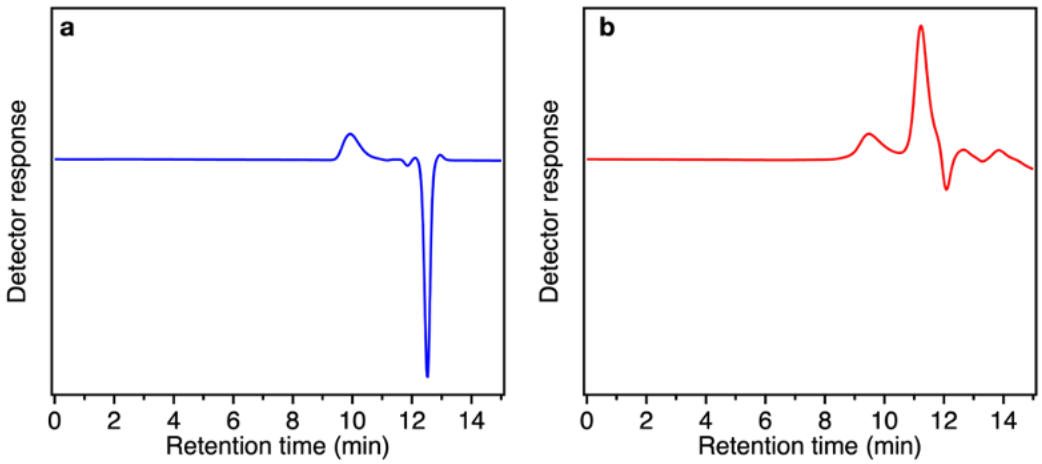
> ***Guest Editor comment:*** *The authors provided new data on both GPC and pH. For clarity, the authors should provide the exact conditions (polymer concentration, buffer concentration) in the caption of Figure S16.*

R2A2. Thank you for bringing this to our attention. We have indeed provided new data on both the GPC and pH experiments in the revised manuscript, as indicated below. Additionally, we have revised the caption of Figure S16 and the Method sections for clarity purposes.

To confirm the molecular weights and polydispersity indices of the polymers, GPC was employed. Cationic and anionic polymers were dissolved at a concentration of 1 mg/mL in a 0.3 M aqueous $NaNO_3$ solution at pH 3 (adjusted using 95% sulfuric acid) and 90% aqueous 0.15 M $NaNO_3$ with 10% methanol, respectively. Both solutions were filtered through and were filtered through a polyvinylidene fluoride membrane (0.2 µm pore size) before injection.

Considering the potential impact of polymer dissolution on the solubility and charge properties, we measured the pH changes after dissolution to confirm complete polymer ionization. All polymers and polymer pairs were dissolved at a final concentration (Cp) of 1 mg/mL in 10 mM Tris-HCl buffer at pH 7.5. Consequently, some pH changes were observed, as presented in Figure S16. However, it should be noted that the pKa values of all polymers differed from the resulting pH values by >1.0, indicating that they were ionized to a degree of ~99%. This demonstrates that our coacervation conditions are reliable, with the buffer system having little to no effect on the solubility changes.

The GPC data presented in the previously revised Supplementary Fig. 2 are as follows:

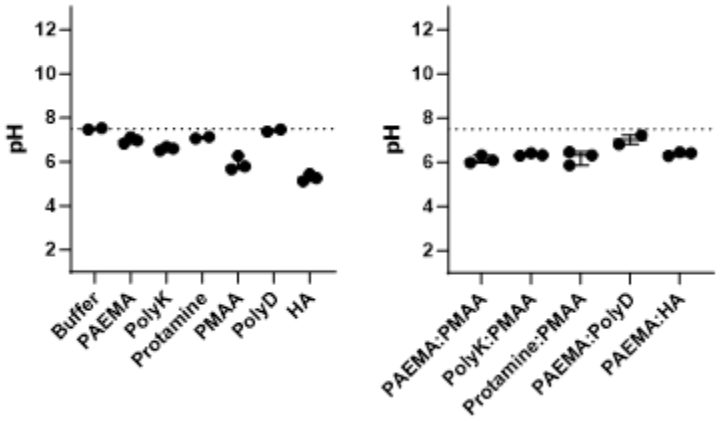


c

Polymer	Monomer conversion[a] (%)	$M_{n,th}$[b] (g mol^{-1})	$M_{n,GPC}$[c] (g mol^{-1})	$Đ$[c]
$PMAA_{40}$	> 99	3,750	3,365	1.16
$PAEMA_{40}$	67	7,098	26,330	1.22

Supplementary Figure 2. GPC analyses of the PMAA and PAEMA specimens synthesized by RAFT polymerization. GPC was performed to confirm the molecular weights and polydispersity indices of the polymers. The cationic and anionic polymers were dissolved at a concentration of 1 mg/mL in a 0.3 M aqueous $NaNO_3$ solution at pH 3 (adjusted using 95% sulfuric acid) and 90% aqueous 0.15 M $NaNO_3$ with 10% methanol, respectively. Both solutions were filtered through a polyvinylidene fluoride membrane (0.2 µm pore size) before injection. **a** GPC spectrum of PMAA. **b** GPC spectrum of PAEMA. **c** Final conversions, number-average molar masses, and dispersity values of the polymers. [a] Determined by ^{1}H NMR spectroscopy. [b] Determined using eq (1). [c] Determined using GPC.

Supplementary Fig. 18 has been updated as follows:



Supplementary Figure 18. pH values of the polymers and polymer pairs. For the polymer pairs, the optimal stoichiometric ratios determined through the turbidity measurements were used. The polymers and polymer pairs

were dissolved in 10 mM Tris-HCl buffer at pH 7.5 to give a concentration of 1 mg/mL. The pH values of all samples differ from the pKa values of the polymers by >1, indicating ~99% ionization of the polymers in the buffer.

Thus, the Methods section has been updated as follows (Pages 16, Lines 521–528):

Sample solutions (1 mg/mL) were prepared and dissolved in the desired Tris-Cl, Tris-Ac, or Tris-Po buffer in a tube. Complex coacervation was performed at weight ratios ranging from 1:9 to 9:1 for each polyelectrolyte pair, and the solutions were mixed by gentle pipetting. The formation of coacervates was confirmed using optical microscopy (BX63, Olympus, Tokyo, Japan). The corresponding images are provided in the Supplementary Information. To confirm complete polymer ionization, the pH values of the polymers and polymer pairs at their optimal stoichiometric ratios (as determined by turbidity measurements) were measured at a final concentration of 1 mg/mL in 10 mM Tris-HCl buffer at pH 7.5 (Supplementary Fig. 18).

R1Q3. *More importantly, the reviewer questioned the author's claims regarding the stability and elastic properties of condensed matter, and raised potential other interactions (such as hydrogen bonding and main chain rigidity). The author still did not provide sufficient evidence or experiments in the R2A11 and R2A15 responses to evaluate the impact of main chain hydrophobicity in isolation, and did not compare and discuss the literature mentioned by the reviewer.*

> ***Guest Editor comment:*** *The authors should provide an explicit comparison with the literature and discuss observed differences.*

R1A3. Thank you for this comment. The experiments related to R2A11 were included on page 5, lines 152–157 of the original manuscript. The revisions can now be found on page 6, lines 189–196 as follows:

In the cases of the PAEMA:PMAA, εPolyK:PMAA, and Protamine:PMAA systems in 10 mM Tris-Cl buffer, precipitation occurred at all mixing ratios, rendering it difficult to observe any coacervation tendencies. To address this, a higher-ionic-strength buffer was employed (19 mM Tris-Cl buffer, pH 7.5) to investigate the formation of coacervates through charge screening (Supplementary Fig. 13). Under these conditions, coacervates were formed at narrow regions where, in most cases, the turbidity reached a maximum value (marked as filled circles in Supplementary Figs. 13a, 13b, and 13c). This aligns with a previous study showing that coacervates are dominant at their maximum and net charge points[27,28].

Furthermore, we would like to provide a comparison of our observed results with the literature provided by Reviewer 2:

The first reference provided by Reviewer 2 (Soft Matter 2015, 11, 44) investigated polymer brushes (PBs), also known as bottle brush polymers, which are defined as polymer chains densely grafted to a backbone, attaining a brush-type configuration. This study demonstrated

that the exceptions of PB behaviors can be ascribed to the solvent quality (i.e., solvophobic interactions between the solvent and the polymer), the nature of the substrate, and other effects, such as the chain stiffness and homogeneity. According to the report, achieving a brush-like configuration requires the grafting density ($\boldsymbol{\sigma}$) to be above a critical value ($\boldsymbol{\sigma}^*$). This critical value is determined by the condition that the separation distance ($\boldsymbol{\sigma}^{1/2}$) should be smaller than the chain radius (R). This polyelectrolyte brush system differs fundamentally from our polyelectrolyte system, making direct comparisons challenging. Hence, we would like to kindly enquire regarding the specific aspects in which the reviewer thinks this reference could help improve our paper.

Another reference provided (JCIS 2011, 361, 407) offers an insight into polyelectrolytes (i.e., macroions–complexation phase behavior), focusing mainly on linear polyelectrolyte chains and block copolymers. According to this review, from a thermodynamic perspective, a two-step process can be introduced to account for the influence of smaller soluble complexes on the phase diagram. This process involves the spontaneous aggregation of oppositely charged polyelectrolyte chains, which then rearrange into a concentrated coacervate phase driven by an increase in configurational entropy. When one of the two polyelectrolytes is in excess, this rearrangement is hindered, favoring soluble complexes over phase separation and remaining as aggregates. This review also demonstrated that while polyelectrolyte complexation at stoichiometric charge conditions usually leads to macroscopic phase separation, the stoichiometric charge compositions (1:1) can vary at different salt concentrations. Smaller complexes can be prepared at non-stoichiometric mixing ratios, particularly with significantly different molar masses or pairs of strong polyelectrolytes. Given that our system is based on polyelectrolytes with a similar range of molar masses and relatively weak charged groups, this report aligns with the majority of our observed results in which coacervation was observed at stoichiometric mixing ratios.

The last reference provided (Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2017, 4, e1442) presents an overview of complex coacervate phase behavior influenced by various parameters, such as the stoichiometric charge ratio, pH, and salt content (ionic strength). In terms of this stoichiometry, the reference states that "coacervation occurs in an extremely narrow range, centered around 'net neutrality'; however, it is also possible to broaden the range through salt addition due to intrinsic charge compensation". This review also mentions that for proteins, the driving force for coacervation does not necessarily originate from the overall charge of the macromolecules, but from specific charge patches on the protein surface, due to their zwitterionic nature and structural complexity. Hence, coacervation can be observed on the 'wrong' side of the isoelectric point (pI), where a molecule carries no net charge. This report aligns with our findings, where the coacervation was observed at approximately stoichiometric mixing ratios in most cases, with the exception of the Protamine:PMAA pair. This exception is likely attributed to the conformational properties of protamine, as well as the differences in molecular weight and charge density, as discussed in the literature.

Based on these considerations, the text has been revised as follows (Page 11, Lines 350–362):

Similar to the hydration propensities of the polyelectrolytes, the coacervate phase regions, which vary depending on the mixing ratio and choice of buffer ion, exhibit the following order: PAEMA:HA ≈ PAEMA:PolyD ≈ αPolyK:PMAA > εPolyK:PMAA > Protamine:PMAA ≈ PAEMA:PMAA (Fig. 2 and Supplementary Figs. 13 and 14). In line with previous studies demonstrating that coacervation occurs within a range centered around stoichiometric charge compositions, which can be adjusted through salt addition and is influenced by surface charge patches (i.e., particularly in proteins due to their zwitterionic nature and structural complexity), it was observed that coacervation predominantly occurred around the stoichiometric molar ratios. However, an exception was noted in the case of Protamine:PMAA. This deviation was attributed to the unique conformational properties of protamine, as well as the differences in molecular weight and charge density as discussed in the literature[27,28]. Moreover, for the εPolyK:PMAA and αPolyK:PMAA pairs, the mixing ratios at their highest turbidity values (i.e., the optimal mixing ratios) exhibited minimal variations.

[27] Blocher, W. C. & Perry, S. L. Complex coacervate-based materials for biomedicine. Wiley Interdisciplinary Reviews: *Nanomedicine and Nanobiotechnology* vol. 9 Preprint at https://doi.org/10.1002/wnan.1442 (2017).

[28] Gucht, J. van der, Spruijt, E., Lemmers, M. & Cohen Stuart, M. A. Polyelectrolyte complexes: Bulk phases and colloidal systems. *J Colloid Interface Sci* **361**, 407–422 (2011).

R1Q4. *In addition, in the revised manuscript the author applied xSight (Spheryx, Inc.) to explore coacervate suspension. They showed a negative correlation between the refractive index and the coacervate diameter, suggesting that the coacervates with larger diameters are more hydrated, leading to a lower refractive index. This is an interesting observation, but not necessarily related to this paper. FACS can provide additional population-level data, including droplet count and particle size complexity, which cannot obtain from turbidity analysis and microscopic imaging. While the use of xSight (Spheryx, Inc.) did not provide such detailed information. FACS can obtain forward scatter (FCS) and side scatter (SCS) data, which not necessarily need fluoresce labeling the microdroplet. So, it is not necessary to label the samples with a fluorescence tag. So, I do not understand the author's argument on the labelling coacervate microdroplet. Even if we need to label the microdroplet, there are many different ways to label coacervate microdroplet, and many papers have employed FACS to obtain more quantitative data. For instance, small dye molecules could be sequestered within the microdroplet for the FACS measurements.*

*> **Guest Editor comment:** I do not think FACS will add anything here, and I do not recommend adding it. FACS will mostly give information about the size of the coacervates, but that is not very useful in systems that are not in equilibrium yet - as the coacervates continuously fuse and coarsen. Such measurement would heavily depend on the mixing order, speed and time between preparation and measurement. I do not agree with reviewer 1 that "population" information is useful. The authors have reported average droplet sizes.*

R1A4. Thank you for this comment. As is correctly mentioned, we employed xSight (Spheryx, Inc.), to analyze the characteristics of our coacervation system in more depth, expanding on the turbidity analysis and microscopic imaging. xSight is an instrument that provides additional population-level data, including the droplet size, count, refractive index, and concentration in a non-destructive manner. This is achieved by measuring holograms of suspended particles and fitting them with the Lorenz–Mie Theory.

In our previous response letter, we reported the average droplet sizes and refractive indices, and we compared PAEMA:PMAA, PolyK:PMAA, Protamine:PMAA, PAEMA:PolyD, and PAEMA:HA at their respective maximum coacervation mixing ratios of 7:3, 4:6, 7:3, 6:4, and 4:6. As presented in the figure below, the particle sizes (diameters in μm) were determined to be as follows: PAEMA:HA (3.70), PAEMA:PolyD (2.80), PolyK:PMAA (1.98), PAEMA:PMAA (1.77), and Protamine:PMAA (1.60). Correspondingly, the median refractive indices of these coacervates were ranked as follows: Protamine:PMAA (1.47), PolyK:PMAA (1.46), PAEMA:PMAA (1.43), PAEMA:PolyD (1.42), and PAEMA:HA (1.38), showing a tendency towards a negative correlation between the size and the refractive index. This implies that the larger coacervates are more hydrated, resulting in a lower refractive index[29].

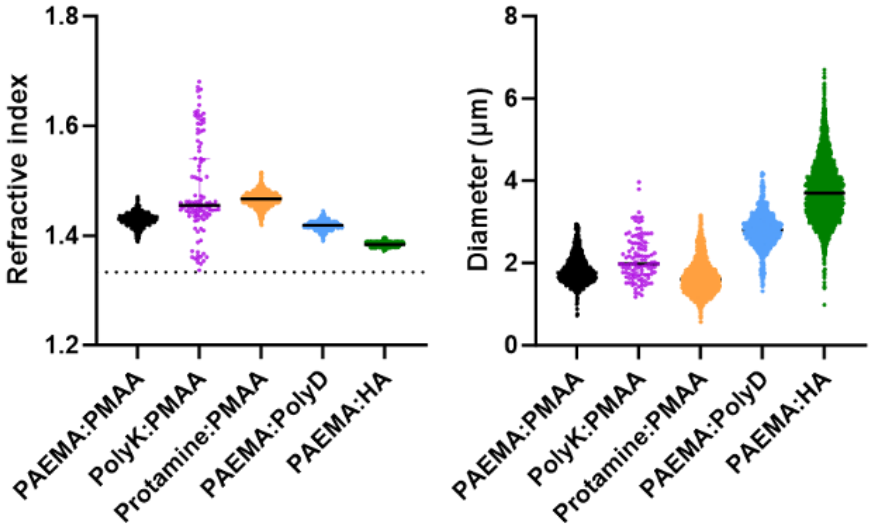
Regarding the droplet counts, these were determined to be as follows: Protamine:PMAA (7118), PolyK:PMAA (111), PAEMA:PMAA (2352), PAEMA:PolyD (836), and PAEMA:HA (3804). Although it was possible to obtain the droplet count data from xSight, we agree with the Guest Editor's comment that measurement of the droplet count heavily depends on the sample preparation conditions, rather than providing reliable quantitative data.

[29] Hong, Y. et al. Label-Free Quantitative Analysis of Coacervates via 3D Phase Imaging. Adv Opt Mater 9, 2100697 (2021).

To clarify the means by which our conclusion was derived, we have revised the manuscript as follows (Page 7, Lines 216–219):

This can be further supported by the refractive index and average droplet size (Supplementary Fig. 16), which showed a tendency toward a negative correlation. This implies that coacervates with larger sizes, such as the PAEMA:HA and PAEMA:PolyD pairs, are more hydrated, resulting in a lower refractive index.

In addition, Supplementary Fig. 16 has been added:



Supplementary Figure 16. Refractive indices and diameters of the polymer pairs measured using xSight. For the polymer pairs, the optimal stoichiometric ratios determined from the turbidity measurements were used.

R1Q5. *After comprehensive consideration, although the author has made some modifications, there are still many key issues that have not been resolved. Especially, the author did not fully explain the differences between the observed phenomena and existing literature reports, nor did they conduct supplementary experiments to verify the hypothesis. Therefore, based on the above reasons, I suggest that the author revise the manuscript more thoroughly to present the experimental data more clearly, explain the results more accurately, and provide more evidence to support its conclusion. Before this, I do not recommend accepting this manuscript.*

R1A5. Thank you for addressing this matter. We have revised the manuscript according to the guidance provided by the Reviewers and the Guest Editor, including comparisons between our observed results and the literature reports mentioned by Reviewer 2, as addressed in R1Q3.

Reviewer #3 (Remarks to the Author):

I very much appreciate the effort that the authors undertook to modify their manuscript and also perform additional experiments. I believe that the concerns raised by myself and the other reviewers have been addressed. I would note that there are a couple of places where these new edits/additions of information do not flow well. I have included suggestions of ways in which the text could be further refined before publication, and I look forward to seeing this article in print.

Specific Comments:

R3Q1. *Starting at line 173, the authors describe experiments that were done at higher buffer concentrations. However, it was not clear why they performed this analysis (though the discussion from the rebuttal document makes it clear). I would suggest that the authors add in some context.*

R3A1. Thank you for pointing out this issue. We have now included additional explanation for the buffer choices as follows (Page 6, Lines 184–196):

In the PAEMA:PMAA, εPolyK:PMAA, and Protamine:PMAA systems, precipitates were observed by optical microscopy at all mixing ratios evaluated herein (marked with an "X" in Figs. 2a–2c, see also Supplementary Figs. 8–10). However, for the PAEMA:PolyD and PAEMA:HA systems, broader coacervate ranges were observed (marked as filled circles in Figs. 2d and 2e, see also Supplementary Figs. 11 and 12).

In the cases of the PAEMA:PMAA, εPolyK:PMAA, and Protamine:PMAA systems in 10 mM Tris-Cl buffer, precipitation occurred at all mixing ratios, rendering it difficult to observe any coacervation tendencies. To address this, a higher-ionic-strength buffer was employed (19 mM Tris-Cl buffer, pH 7.5) to investigate the formation of coacervates through charge screening (Supplementary Fig. 13). Under these conditions, coacervates were formed at narrow regions where, in most cases, the turbidity reached a maximum value (marked as filled circles in Supplementary Figs. 13a, 13b, and 13c). This aligns with a previous study showing that coacervates are dominant at their maximum and net charge points[27,28].

R3Q2. *On line 182 the authors reference data for poly-alpha-lysine. While I am very excited about this data and the potential comparisons between alpha and epsilon polylysine, this line represents the first time that this polymer is mentioned. I would suggest including it in Figure 1 and in the prior discussion, as well as one of the listed systems in the actual Discussion. This is not to say that I am suggesting they do all of the experiments with alpha-polylysine, but I think this comparison is worth highlighting more than in the current document. Any particular suggestion I might make about the writing is complicated by the separate nature of the Results and Discussion sections, but I could imagine using the comparison of the two lysine systems as a way of introducing things, before then transitioning to discuss the larger systematic study of all of the parameters. This allows introducing these results in a meaningful way and highlighting the significant difference that can be obtained even when two different isomers are used. Furthermore, this comparison helps to support the argument that the authors make about charge density not affecting the optimum mixing ratio in a clear and unconvoluted way.*

The only request for additional material that I would make is to also add in the SASA for alpha-polylysine. It would also be nice to have solubility data, but this might be more challenging.

R3A2. Thank you for this comment. To further explore differences between the two isomers, we conducted SASA and solubility tests on α-polylysine. As a result, no significant difference

in SASA value of α-polylysine (1219) was observed compared with that of ε-polylysine (1309). This may be due to the number of monomers used for the simulation, suggesting that five monomers were insufficient to closely represent the comparison between the two systems, especially considering the significantly higher polymerization degree of 30 used in the experimental conditions. However, the experimental results supported our claims regarding the influence of the backbone on coacervation. The solubility of α-polylysine (> 1000 mg/mL) far exceeded that of ε-polylysine (326.67 ± 81.34 mg/mL), suggesting that the isomer with a more hydrophilic backbone is significantly more soluble than that with a relatively more hydrophobic aliphatic backbone. This was also supported by the broader coacervation region (Supplementary Fig. 14e) and the lower salt resistance (Supplementary Fig. 14f), as discussed in the previously revised manuscript.

Considering the reviewer's suggestion, we have included the corresponding data for α-polylysine data the appropriate discussion sections, as well as in Figure 1 and Table 1, to underscore our claims regarding the influence of the backbone and charge density. We have also revised the term "PolyK" as "εPolyK" and "αPolyK" throughout the article, as appropriate.

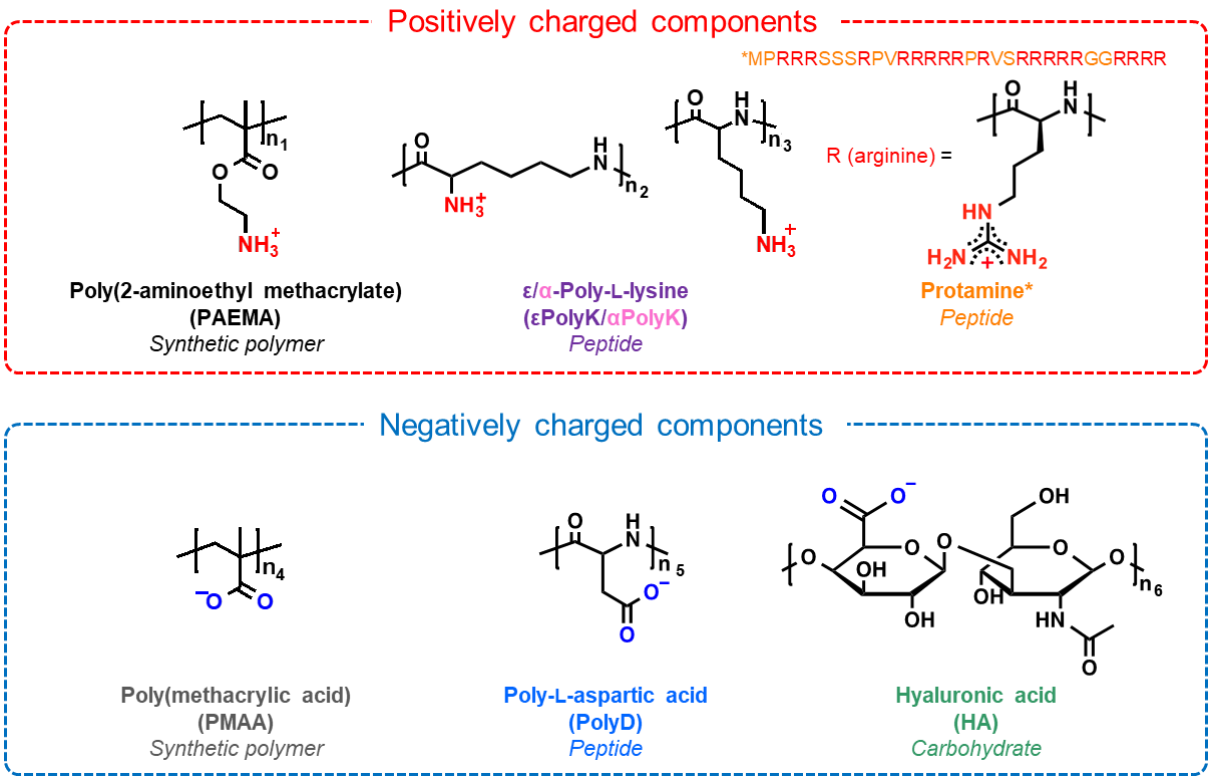


Figure 1. Chemical structures of the selected polyelectrolytes. The asterisk (*) indicates the primary sequence of protamine. The values $n_1 = 40$, $n_2 = 25–35$, $n_3 = 30$, $n_4 = 40$, $n_5 = 30$, and $n_6 = 1.2\text{-}25$ correspond to the respective polymer repeating units.

Table 1. Average degrees of polymerization, molecular weights, solubilities, and SASA values of the polyelectrolytes. All experiments were conducted in triplicate, and the data are presented as mean values ± SD. *For PAEMA and PMAA, the values are determined using eq (2) in the Methods section. For protamine, the value indicates the number of amino acids in the primary sequence. **For PAEMA and PMAA, the average molecular weights were determined using eq (1) in the Methods section. The average molecular weight was determined as

per the manufacturer's specifications. ***SASA calculations were performed on a pentamer, with polyarginine serving as the basis for protamine analysis, as detailed in the Methods section. ****The solubility of protamine was determined through turbidity measurements, which marked the transition from a two-phase to a one-phase solution

Polyelectrolyte	Degree of Polymerization*	Molecular weight** (g/mol)	Solubility (mg/mL)	SASA(Å^2)***
PAEMA	40	7098	63.33 ± 9.43	954
αPolyK	30	4900	>1000	1219
εPolyK	30	4000	326.67 ± 81.34	1309
Protamine****	31	4068	<40	1383
PMAA	40	3750	>1000	757
PolyD	30	4100	>1000	816
HA	12	5000	>1000	2196

Additionally, text has been added in the Results section as follows (Page 5, Lines 163–166):

In the cases of εPolyK (1309 Å^2) and αPolyK (1219 Å^2), only a minor difference was observed between their SASA values. This was likely due to the limitations of the pentamer system, since discernable differences were observed in the experimental results (as detailed in the subsequent sections).

R3Q3. *It would be useful for the authors to explain near line 244 and/or 342 that 1,6-hexanediol was used to test for hydrophobic effects and how to understand the results. The current presentation assumes that the reader would know this information.*

R3A3. Thank you for this comment. We have now added the requested explanation as follows (Pages 8–9, Lines 262–282):

Overall, the obtained results demonstrated a negative correlation between the solubility and simulation outcomes. The maintenance of coacervates at higher salt concentrations indicates that complexation and coacervation contributed not only to the electrostatic attractions, but also to other non-ionic interactions. To investigate the impact of hydrophobic interactions, 10% 1,6-hexanediol was introduced into each coacervate pair dissolved in 19 mM Tris-Cl buffer (pH 7.4) containing 100 mM NaCl (Supplementary Fig. 17). The reduction in ionic strength caused by the presence of NaCl was expected to facilitate the observation of hydrophobic-driven coacervates. In addition, the presence of 1,6-hexanediol, a weak hydrophobic interaction disruptor[31,32], was anticipated to provide insights into the role of hydrophobic interactions in coacervate formation. In the case of the PAEMA:PMAA pair, coacervate deformation was observed after the addition of 1,6-hexanediol. This indicates that hydrophobic interactions stemming from their hydrophobic aliphatic backbones played a significant role in driving

coacervate formation. Conversely, in the case of the highly hydrated PAEMA:HA pair, the contribution of the hydrophobic interactions to maintaining LLPS seemed to be weaker. Taking the above results into account, the PAEMA:HA pair was considered to be mainly dependent on electrostatic interactions. Additionally, it was observed that αPolyK:PMAA coacervates bearing a shorter backbone chain disappeared at a lower NaCl concentration than the εPolyK:PMAA coacervates (Supplementary Fig. 14), indicating that a more hydrophobic backbone contributes to an improved salt resistance. This agrees with previous findings, in which the phase separation of complex coacervates is also dependent on the hydrophobic interactions present in a high-salt regime[33].

[31] Lin, Y. X. et al. Liquid-liquid phase separation of tau driven by hydrophobic interaction facilitates fibrillization of tau. J. Mol. Biol. **433**, 166731 (2021).

[32] Ribbeck, K. & Görlich, D. The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion. EMBO J. **21**, 2664–2671 (2002).

[33] Li, L. et al. Effect of Solvent Quality on the Phase Behavior of Polyelectrolyte Complexes. Macromolecules **54**, 105–114 (2021).

Furthermore, the Discussion have been revised as follows (Page 12, Lines 381–388):

The higher salt resistance observed in the PAEMA:PMAA coacervates and the difference found between the εPolyK:PMAA and αPolyK:PMAA pairs indicate that their coacervation is primarily driven by electrostatic interactions, and that additional contributions from non-ionic interactions are also involved. These non-ionic interactions likely stem from the hydrophobic aliphatic hydrocarbon backbones of the synthetic polymers, as supported by the deformation of coacervation observed when adding 1,6-hexanediol as a hydrophobic interaction disruptor (Supplementary Fig. 17).

Minor Comments:

R3Q4. *On page 5, lines 136-137 the authors use the term "branches" to describe the side-chains of their various polymers. I would advise against this since branched polymers are something quite different. "Side-chain" would be a potentially safer term.*

R3A4. Thank you for pointing out this issue. We agree that the original sentence was unclear and confusing. We have revised the term "branches" as "side-chains" throughout the article, including in the indicated sentence (Page 5, Lines 148–158):

More specifically, PAEMA exhibits a short backbone with long, radiating side chains, while εPolyK features a long backbone and no side chains, resulting in a linear elongated shape (Supplementary Figs. 4a and 4b). This leads to different levels of accessibility in a solvated environment, wherein εPolyK exposes nearly all atoms, while PAEMA, which contains many overlapping areas, exposes relatively fewer atoms, as verified by the computed SASA values

(Table 1). More specifically, the larger SASA value of εPolyK (1309 $Å^2$) compared to that of PAEMA (953 $Å^2$) indicates that εPolyK is more hydrated. Thus, despite their identical charged groups, the different backbone shapes render εPolyK more soluble than PAEMA. Similar to PAEMA, poly-arginine, a simplified model that was used herein to represent protamine, possesses a short backbone bearing long side chains (Supplementary Fig. 4c).

R3Q5. *Line 327, I suggest a new paragraph when transitioning to the discussion of the different buffers.*

R3A5. Thank you for pointing this out. We have now restructured as suggested.

R3Q6. *I suggest that the authors check through the paper for instances where they say "protein" but are really referring to "polypeptides."*

R3A6. Thank you for this comment. We apologize for the original wording. We have revised the term "protein" as "polypeptides" throughout the article and highlighted the changes in the manuscript document.

R3Q7. *The "Supplementary Information" title of the SI is misspelled.*

R3A7. Thank you for bringing this to our attention. We have corrected the spelling of the title in the supplementary information.

R3Q8. *The caption for Supplemental Figure 12 should say "varying" rather than "varing." Also, what is the meaning behind the red outlines on some of the micrographs? A similar red outline is also present in Supplemental Figure 13 and is not explained. I would also ask that the definitions of the X and open/closed symbols used in these figures be repeated in the captions (they are currently only in the main text).*

R3A8. Thank you for pointing out this issue. We have now corrected and revised the caption for Supplementary Fig. 13 as follows. Please note that Supplementary Figure 12 has been renamed as Supplementary Figure 13:

Supplementary Figure 13. Turbidity and representative optical microscopy images of the coacervation assemblies of various pairs obtained by varying the mixing ratios in 10 mM Tris base containing 8.89 mM HCl at pH 7.5. Polymer concentrations (Cp) were 1 mg/mL for all coacervate pairs, and the samples were analyzed immediately after complexation. In the phase diagrams, the filled markers indicate the mixing ratios wherein phase separation was observed using an optical microscope. The empty markers indicate the mixing ratios wherein phase

separation did not occur. The X marks indicate the mixing ratios wherein aggregation was observed. The gray region in each phase diagram represents the coacervation region where phase separation of the polyelectrolytes occurred. The representative images with red outlines refer to the mixing ratios at the highest turbidity values (i.e., the optimal mixing ratios). Each data point is based on at least three replicate experiments carried out for each respective polyelectrolyte.

The caption for Supplementary Fig. 14 has also been revised as follows. Please note that Supplementary Figure 13 has been renamed as Supplementary Figure 14:

Supplementary Figure 14. Variations in the mixing ratios and salt resistances between the εPolyK:PMAA and αPolyK:PMAA coacervates: **a**, **d**, and **g** Using a 10 mM Tris-Cl buffer; and **b**, **e**, **h**, **c**, and **f** Using a 19 mM Tris-Cl buffer. In the phase diagrams, the filled markers indicate the mixing ratios wherein phase separation was observed using an optical microscope. The empty markers indicate the mixing ratios wherein phase separation did not occur. The X marks indicate the mixing ratios wherein aggregation was observed. The gray region in each phase diagram represents the coacervation region where phase separation of the polyelectrolytes occurred. The representative images with red outlines refer to the mixing ratios at their highest turbidity values (i.e., the optimal mixing ratios). Each data point is based on at least three replicate experiments carried out for each respective polyelectrolyte.

R3Q9. *At the bottom of page 6 the authors compare the size of the coacervate regions of PolyK/PMAA, PolyK/HA and Protamine/PMAA, Protamine/HA. This presumably involves a comparison of the data present in Figure 2 and Supplemental Figure 14. However, it is unclear* ***what buffer was used for the data in Supplemental Figure 14.*** *One assumes based on the text in the main document that it is Tris HCl, but it is useful to not require the reader to remember something that was mentioned a page earlier when flipping back and forth between figures that could easily be labeled. Please address.*

R3A9. Thank you for raising these points. We acknowledge that these sentences were unclear, and so we have now revised the text as follows to reflect this (Page 7, Lines 211–216):

Moreover, when comparing the coacervate phase regions of εPolyK:PMAA and εPolyK:HA, in addition to those of Protamine:PMAA and Protamine:HA, in 19 mM Tris-HCl buffer pH 7.4 (Figs. 2b and 2c, see also Supplementary Fig. 15), it was clear that coacervate formation was more favored in the HA combinations. This preference is likely due to the higher hydrophilicity of HA (Supplementary Fig. 15).

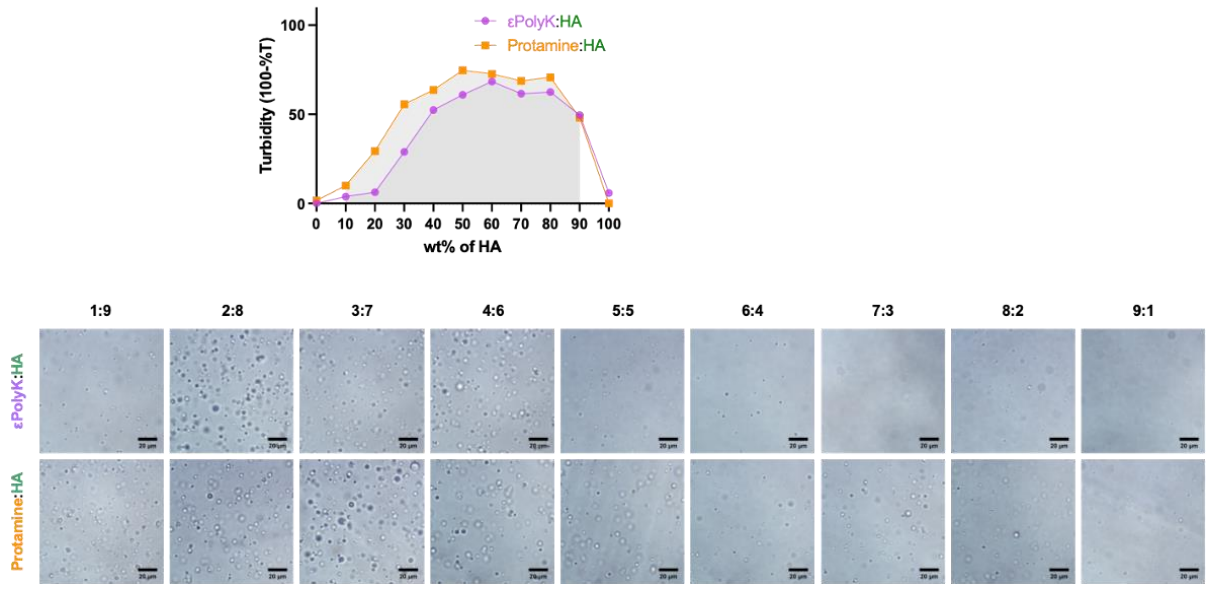
In addition, the caption for Supplementary Fig. 15 has been revised as follows. Please note that Supplementary Figure 14 has been renamed as Supplementary Figure 15:

Supplementary Figure 15. Turbidity and representative optical microscopy images of coacervation assembly in the PolyK:HA and Protamine:HA pairs at different mixing ratios. The polymer concentrations (Cp) were 1 mg/mL in 19 mM Tris-HCl buffer at pH 7.4 for all

coacervate pairs, and the samples were analyzed immediately after complexation. The gray region in each phase diagram represents the coacervation region where phase separation of the polyelectrolytes occurred.

R3Q10. *A minor point, but in the main paper the authors refer to the polymer systems as cation:anion. However, in the legend for Supplemental Figure 14 this order is reversed.*

R3A10. Thank you for bringing this to our attention. The corresponding legend in Supplementary Fig. 15 has now been revised accordingly. Please note that Supplementary Figure 14 has been renamed as Supplementary Figure 15:



Supplementary Figure 15. Turbidity and representative optical microscopy images of coacervation assembly in the PolyK:HA and Protamine:HA pairs at different mixing ratios. The polymer concentrations (Cp) were 1 mg/mL in 19 mM Tris-HCl buffer at pH 7.4 for all coacervate pairs, and the samples were analyzed immediately after complexation. The gray region in each phase diagram represents the coacervation region where phase separation of the polyelectrolytes occurred.