

Microphase Separation Produces Interfacial Environment within Diblock Biomolecular Condensates

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Abstract

The phase separation of intrinsically disordered proteins is emerging as an important mechanism for cellular organization. However, efforts to connect protein sequences to the physical properties of condensates, i.e., the molecular grammar, are hampered by a lack of effective approaches for probing high-resolution structural details. Using a combination of multiscale simulations and fluorescence lifetime imaging microscopy experiments, we systematically explored a series of systems consisting of diblock elastin-like polypeptides (ELP). The simulations succeeded in reproducing the variation of condensate stability upon amino acid substitution and revealed different microenvironments within a single condensate, which we verified with environmentally sensitive fluorophores. The interspersed hydrophilic and hydrophobic residues and a lack of secondary structure formation result in an interfacial environment, which explains both the strong correlation between ELP condensate stability and interfacial hydrophobicity scales, as well as the prevalence of protein-water hydrogen bonds. Our study uncovers new mechanisms for condensate stability and organization that may be broadly applicable.

eLife assessment

This **important** study investigates the structural organization of a series of diblock elastin-like polypeptide condensates. The methodology is highly **compelling**, as it combines multiscale simulations and fluorescence lifetime imaging microscopy experiments. The results increase our understanding of model biomolecular condensates.

Introduction

Biological condensates are found in both the cytosol^{1–4} and nucleus^{5–10} playing essential roles in a variety of cellular processes^{11,12} from stress response¹³ to genome organization.^{14,15} Similar to membrane-bound organelles, they assemble a collection of molecules to raise the efficiency of sophisticated tasks. The lack of a membrane barrier allows fast material exchange between condensates and the cellular environment, rendering the molecular composition and stability of condensates more prone to regulations by external signals.^{16–18}

Intrinsically disordered proteins (IDPs) that promote multivalent, promiscuous interactions are key drivers of condensate formation.^{19–21} Multiple mechanisms, including electrostatic, cation- π , π - π , hydrogen bonding, and hydrophobic interactions, contribute to the affinity among various chemical groups.^{22–24} Above a threshold concentration, as predicted by the Flory-Huggins theory,²⁵ interactions among IDPs can drive liquid-liquid phase separation (LLPS) to produce a highly concentrated phase that nevertheless remains dynamic. The simplicity of theory, while insightful, may prove insufficient for a comprehensive understanding of biological condensates.

Much remains to be learned regarding the connection between amino acid sequences and protein phase behaviors, or the so-called “molecular grammar” of protein condensates.^{26–31} These systems often exhibit complex viscoelastic behaviors and substructures with a layered organization,^{32–35} defying the mean-field assumption in the Flory-Huggins theory. Several more advanced theories have been introduced to better account for long-lived structural features that might persist in the polymer network. The sticker and spacer model^{36,37} has been adopted by Pappu and coworkers to account for strong and specific interactions that might form physical cross-links among protein molecules.³⁸ In the meantime, the block copolymer theory may explain the microphase separation that can lead to layered structures.^{39–45} Because of their inherent assumptions, different theories are more appropriate for some systems but not others. High-resolution structural characterizations of condensates could offer further insight into their organizational principles and the applicable theories.

Further decoding the condensate grammar could also benefit from studies of simpler systems. Natural proteins often utilize highly complex amino acid sequences, rendering the attribution of an individual residue’s contribution to collective phenomena challenging. On the other hand, elastin-like polypeptides (ELPs), which are composed of pentapeptide repeats of valine-proline-glycine-X-glycine, serve as excellent models for studying protein phase behaviors. ELPs undergo phase separation upon heating with a lower critical solution temperature (LCST).^{46–50} Importantly, the guest residue X can be modified to any amino acid except proline with modern engineering approaches,⁵¹ enabling systematic characterization of the impact on condensate stability and organization upon introducing specific residues.

We combine multiscale simulations with fluorescence lifetime imaging microscopy (FLIM) to probe condensates formed by diblock ELPs and investigate the contribution of amino acid composition to condensate stability. The simulation approach allows the sampling of largescale conformational rearrangements while providing atomistic resolution to quantify the solvation environment of individual residues. It succeeds in reproducing the stability of condensates, and reveals the formation of tubular network phases similar to gyroids. Such structures deviate from weak micelles that have been proposed for diblock ELPs in solution, and result in heterogeneous microenvironments within the condensate, which we verify *in-vitro* with FLIM. In addition, we find that the condensate stability exhibits a striking correlation with a hydrophobicity scale derived from interfacial transfer free energy, supporting an interfacial microenvironment of the condensate interior. The chemical specificity of the microenvironment is dictated by the peptide

sequence that prevents a complete microphase separation between hydrophobic and hydrophilic groups. As a result, all condensates remain highly solvated after phase separation, producing water molecules that maintain hydrogen bonding with the exposed peptide backbone which persists even in residues with hydrophobic side chains. Our study takes a significant stride at connecting amino acid sequences with the structural and physical properties of biological condensates.

Results

Multiscale simulations of diblock ELP condensates

Decoding the condensate grammar necessitates connecting protein sequences with collective physical properties. ELPs stand out because of their sequence simplicity and amenability for biological engineering, allowing systematic exploration and precise attribution of amino acid contributions. We focus on diblock ELPs of sequence $(V-P-G-V-G)_n(V-P-G-X-G)_n$, which we abbreviate as V_nX_n (**Fig. 1A**). The guest residue in the first block is set as valine to promote phase separation, and we explore 20 systems in which every natural amino acid is substituted into the X position in the second block.

We adopt a multiscale approach to balance accuracy and efficiency for simulating ELP condensates (**Fig. 1B**). The first stage of this strategy is to simulate condensate formation using a coarse-grained force field, MOFF,^{52–55} with one bead per amino acid and implicit solvation. These simulations are highly efficient for system relaxation and promoting largescale conformational rearrangements that occur over slow timescales. We further converted these α -carbon based models to approximately four heavy atoms per bead resolution, and introduced explicit water and ions for simulations with the MARTINI force field.⁵⁶ The latest version of this force field (MARTINI3) provides a more balanced set of parameters for protein-protein interactions and has been applied to study biological condensates.^{57–60} These simulations further relax the protein configurations and the partition of water and counterions in condensed and dilute phases. Finally, we carried out explicit solvent all-atom simulations for 250 ns to produce an accurate characterization of the chemical environment of the condensates.

Utilizing the simulated condensate conformations, we computed various quantities to benchmark against experimental measurements. While the critical temperature has been widely used as a measure for condensate stability, determining it computationally is expensive. As an alternative, we computed the surface tension, τ , using 100- μ s-long MARTINI simulations performed with the NP_NAT ensemble.⁶¹ As detailed in the *Supplemental Theory* in the Supporting information, an inverse relationship is expected between τ and the critical temperature, T_c , for systems exhibiting LCSTs. We further approximate T_c with the transition temperatures (T_t) of ELP sequences,⁴⁶ which are the temperatures at which ELPs undergo an LCST transition at a specified solution condition. T_t was shown to be linearly proportional to T_c .^{62,63} As expected, a negative correlation can be readily seen between computed surface tension and experimental T_t (**Fig. 1C**). This observed negative correlation between T_t and τ supports the simulation approach's accuracy in reproducing the sequence-dependent changes in ELP phase behavior.

We note that the experimental values were determined using different ELP sequences from those simulated here, and the relation between the surface tension and T_c is likely nonlinear,⁶⁴ both contributing to the imperfect agreement. In another study,⁶⁵ we adopted the same multiscale approach to determine the dielectric constant for several ELP condensates. The simulated values agree well with those determined using FLIM experiments.

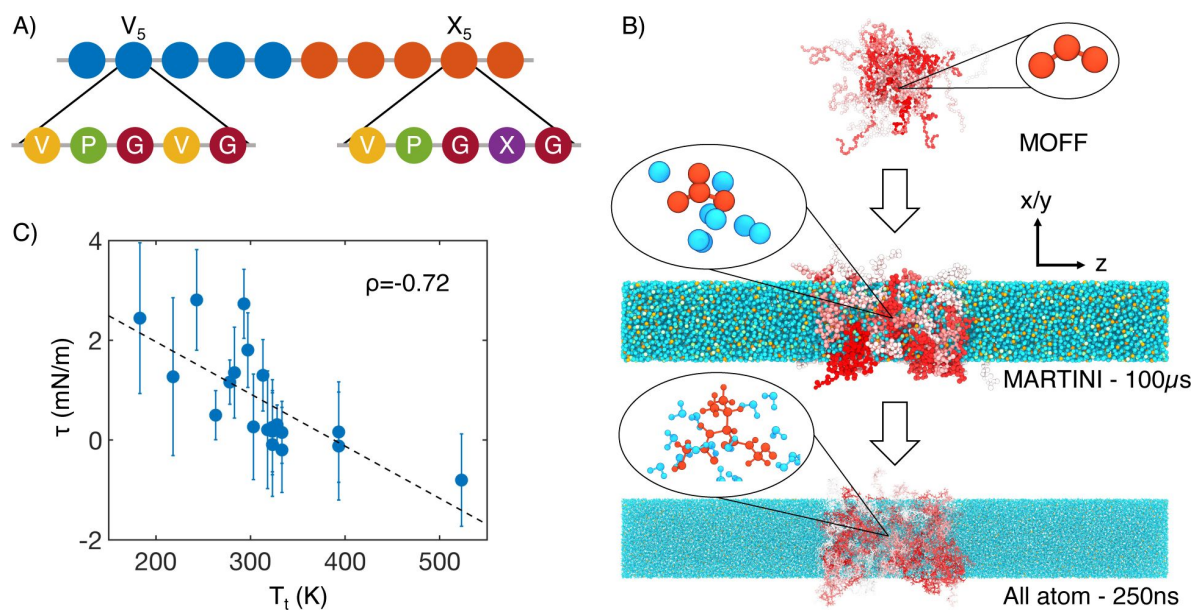


Figure 1

Multiscale simulations enable thermodynamic and structural characterization of ELP condensates. (A) Illustration of the sequence for the simulated diblock ELPs that consist of five-amino acid repeats, where X is substituted with a guest amino acid. (B) Overview of the three-step multiscale simulation approach that gradually increases the model resolution. Simulations of V_5L_5 were used to produce the example configurations at each step. Peptides are shaded red-white, while water molecules, chlorine, and sodium, are colored in blue, green, and orange, respectively. Inserts of a G-V-G repeat and surrounding water molecules are shown to indicate the resolution of each model. (C) Correlation between the simulated surface tension (τ) of 20 ELP condensates and the transition temperatures (T_t) of related systems.⁴⁶ ρ is the Pearson correlation coefficient between the two data sets, and the dashed line is the best fit between simulation and experimental data. Error bars represent the standard deviation of estimates from five independent time windows.

Microphase separation of ELP condensates

Upon validating the accuracy of the simulation protocol for reproducing collective properties of ELP condensates, we examined their structural organization. Hassouneh et al. argued that, in dilute solutions, ELP diblocks self-assemble into the so-called weak spherical micelles with dense cores and almost unstretched coronas.⁶⁶ At phase separation conditions, whether similar structural features are preserved in condensates remains unclear.

We found that most ELP condensates undergo a microphase separation. Representative configurations for four typical systems from MARTINI simulations are shown in the top panels of **Fig. 2**, with the V blocks in blue and X in red. Overall, the microphase separation manages to orient the more hydrophobic blocks towards the condensate interior as opposed to the interface. The relative density of guest blocks near the center of the condensate positively correlates with their hydrophobicity, as can be seen in both MARTINI (Fig. S1 in the Supporting Information) and all-atom (Fig. S2 in the Supporting Information) simulations. Surprisingly, microphase separation did not produce lamellar morphology as expected for block copolymers with equal volume fraction of the two blocks (Fig. S3 in the Supporting Information).^{39–45,67–69} In particular, the condensates appear to form gyroid-like structures, in which the V and X blocks form two interpenetrating networks (Fig. S4 in the Supporting Information). This morphology also differs from micelle-like structures seen in simplified hydrophobic-polar (HP) polymers.^{70,71} It promotes interfacial contacts while maintaining substantial self-interactions as well. Weak interfacial tension between different ELP blocks has also been noted by Hassouneh et al.⁶⁶ These qualitative observations are insensitive to the polymer length and system size in our simulations (Fig. S5 in the Supporting Information). While longer diblock copolymers drive more prominent microphase separation, similar gyroid structures can also be observed. Notably, for more hydrophilic X blocks, the condensates begin to dissolve into a collection of micelle-like structures, consistent with the predictions by Hassouneh et al. in dilute solutions.⁶⁶

Quantitative characterization of the condensate interior supports the presence of interpenetrating networks with substantial interfacial contacts as well. For example, significant contacts between V and X blocks can be readily seen in the inter-chain contact maps (**Fig. 2**, Fig. S6 in the Supporting Information). We further computed the radial distribution functions for amino acids in various blocks (**Fig. 2**, Fig. S7 in the Supporting Information). For all systems, we found that the probability of finding cross-block contacts V-X is often comparable to the intra-block contacts between X-X or V-V, depending on the relative hydrophobicity of V and X. For example, the more hydrophobic F blocks exhibit strong self-clustering, much more prominent than V-V blocks that are comparable to V-F blocks. On the other hand, clustering among V-V blocks and V-A blocks is more substantial than among A-A blocks. These results are again robust with respect to system setups (Fig. S5 in the Supporting Information).

To experimentally test the microphase separation behavior uncovered in simulations, we studied the micro-physicochemical properties of the V-end and X-end of the peptides. We constructed diblock peptides with the combination of 30 pentameric repeats of V block and X (A or G) block, namely $V_{30}A_{30}$ and $V_{30}G_{30}$ (*Experimental Sequences* Section in the Supporting Information). The amino-termini of $V_{30}A_{30}$ and $V_{30}G_{30}$ sequences were subsequently labeled with environmentally sensitive BODIPY or SBD fluorophores,^{72,73} whose lifetime could be measured to quantify the viscosity or polarity of the V-end (**Fig. 3A**, left panel).⁷⁴ These probes have been reported to be only sensitive to single physicochemical properties.^{65,73} To avoid artifacts induced by fluorophore labeling, we usually used ELPs labeled with a low fraction of dyes. We also constructed $A_{30}V_{30}$ and $G_{30}V_{30}$ diblock peptides, wherein the viscosity or polarity of the A-end or the G-end could be measured by fluorophores that are attached at the amino-terminus (**Fig. 3A**, right panel). Using FLIM, we found that the lifetime of BODIPY for the V-end (5.43 ns) was longer than that for the A-end (4.35 ns), suggesting that the V-end indeed has a higher microviscosity than the A-end ($\eta_V = 2233.54$ cp vs $\eta_A = 969.57$ cp). Accordingly, the lifetime of SBD was longer for the V-

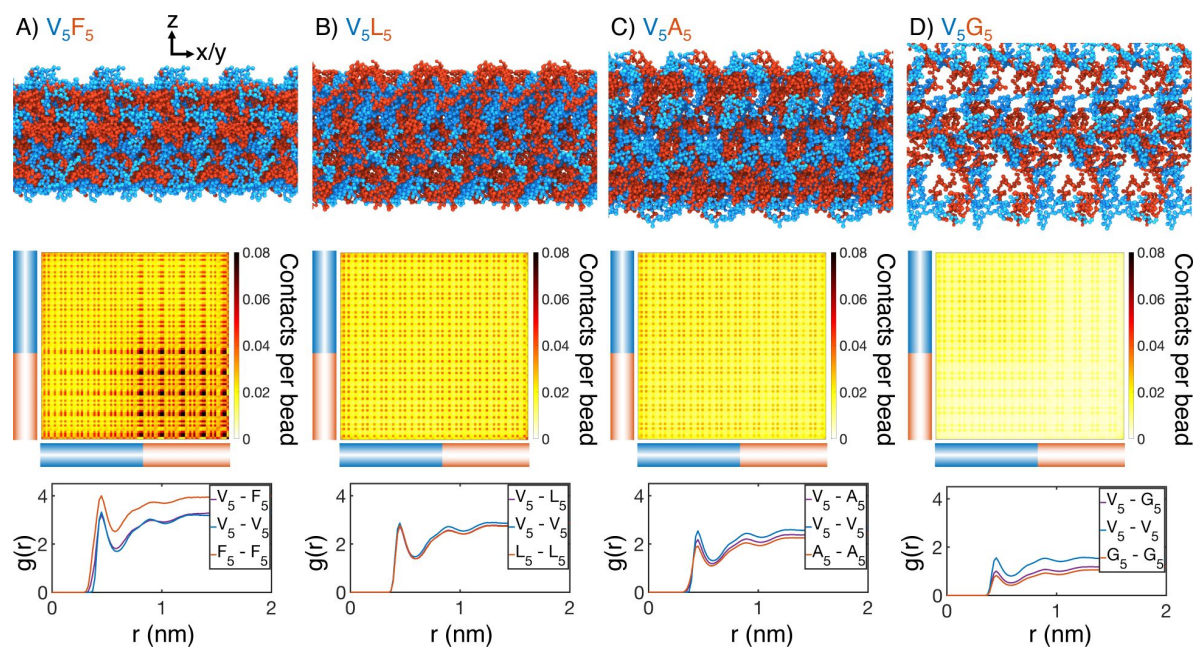


Figure 2

Internal organization of ELP condensates for (A) V_5F_5 , (B) V_5L_5 , (C) V_5A_5 , and (D) V_5G_5 . The uppermost panels present representative configurations from MARTINI simulations for each system. Periodic images along the x and y dimensions are shown for clarity, and the condensate-water interface is perpendicular to the z-axis. Only proteins are shown, with the X and V-substituted halves of the peptides shown in red and blue, respectively. The central panels show contact maps between amino acids from different peptides. The blue and red bars indicate the V- and X-substituted half of the peptides. The lower panels plot the radial distribution functions, $g(r)$, for amino acids only from the V-substituted half of the peptides (V_5-V_5), only from the X-substituted half of the peptides (X_5-X_5), and between the two halves (V_5-X_5). We limited the calculations to amino acid pairs from different peptides.

end (8.75 ns) than the A-end (7.00 ns), indicating that the micropolarity of the V-end was lower than the A-end ($\epsilon_V = 13.25$ vs $\epsilon_A = 18.97$). These observations could be largely attributed to the greater extent of dehydration at the V-end due to its higher local peptide density. We further showed that the observed differences are not results of possible artifacts arising from any subtle distinctions between the two sequences $V_{30}A_{30}$ and $A_{30}V_{30}$ (*Experimental Characterization of ELP Condensates* Section in the Supporting Information, Fig. S8-S9 in the Supporting Information). Similar results were observed using the V-G sequences. FLIM experiments revealed that the V-end was more viscous than the G-end ($\eta_V = 2972.72$ cp vs $\eta_G = 1958.60$ cp) and the V-end was less polar than the G-end ($\epsilon_V = 9.14$ vs $\epsilon_G = 27.50$). These experimental observations provided the first line of evidence to support the microphase separation, as suggested by the simulation results.

To further investigate these phenomena in real-time in motion, optical tweezers were used to stretch the droplet to observe the viscosity changes of different blocks. A single diblock condensate was captured by two optical traps (trap 1 and 2) at the same position, and it was stretched as trap 1 moved away from the original position (**Fig. 3C-D**). We used the peptides $V_{30}A_{30}$ and $A_{30}V_{30}$, with BODIPY labeled at their amino-terminus, to study the microviscosity changes where the fluorescence intensity decreases as the viscosity decrease. We found that the normalized fluorescence intensity of the V-end decreases greater than the A-end, indicating that the viscosity change of the V-end is larger (**Fig. 3E**). We anticipate that during stretching, condensates deform and molecules may deviate from the preferred chemical environment established with microphase separation. Such deviations impact the hydrophobic segments more, leading to more significant reduction in their interactions with surrounding molecules and correspondingly their viscosity.

Frustration produces condensate interior with interfacial properties

The strong dependence of molecular organization on amino acid hydrophobicity suggests that the solvation environment of individual residues might be a determining factor for condensate stability. Indeed, as shown in the *Supplemental Theory* of the Supporting Information, the critical temperature is closely related to the free energy cost of transferring polymer beads from a solution state to a polymer-only environment. This transfer free energy is often used to quantify the hydrophobicity of amino acids.⁷⁵⁻⁸¹ To explore their relationship more quantitatively, we compared the transition temperature for ELP condensates measured by Urry⁴⁶ to several hydrophobicity scales. **Fig. 4A** shows that water-octanol and water-POPC-interface transfer free energies best correlate with the transition temperature. Meanwhile, other hydrophobicity measures, even including those derived specifically for disordered proteins, do not match as well. We note that our conclusion here is not dependent on the limited number of hydrophobicity measures examined in this study, and can be seen in a previous clustering of 98 different hydrophobicity scales.⁸²

The correlation analysis of the Urry transition temperature with various hydrophobicity scales supports a unique chemical environment emerging from the collective behaviors of condensates. To better understand the microenvironment surrounding guest residues in X blocks, we computed the radial distribution functions using MARTINI simulations. As shown in **Fig. 4B-C** and Fig. S10 in the Supporting Information, the guest residues are immediately surrounded with hydrophilic glycine residues as the nearest neighbors. In the second shell, more hydrophobic valine and proline residues appear. These profiles reveal the interfacial nature of the chemical environment surrounding the guest residues consisting of both hydrophobic and hydrophilic amino acids. This interfacial solvation environment is consistent with the strong correlation of the Urry scale with water-POPC-interface transfer free energy. Therefore, ELP condensates do not simply feature a hydrophobic interior that separates from hydrophilic residues as seen for folded proteins, and hence the poor correlation shown in **Fig. 4A** with the burial propensity of amino acids (RSA1, RSA2).

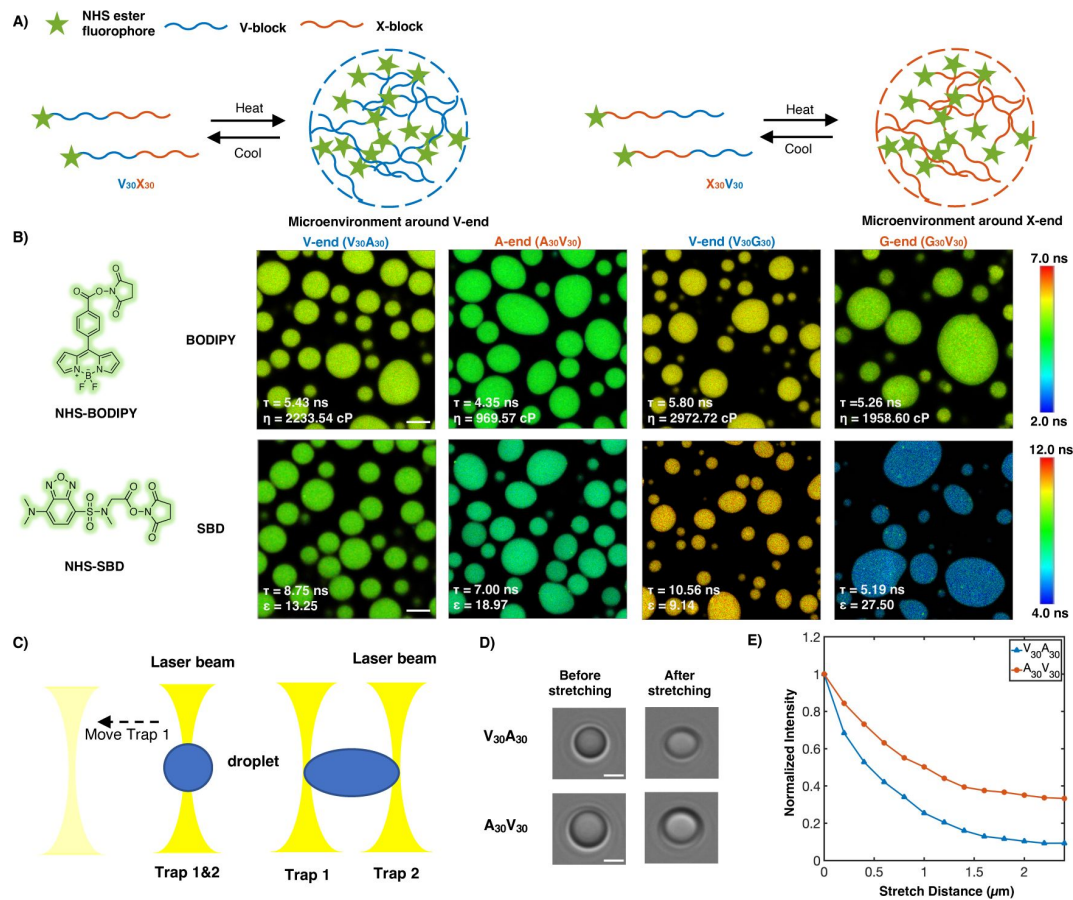


Figure 3

Experimental support of the microphase separation of ELP condensates. (A) Reversible ELP condensates formation via changing temperature. NHS ester fluorophores are attached at the amino-termini of V₃₀X₃₀ and X₃₀V₃₀ to detect the different microphysicochemical properties between the V-end and the X-end. (B) Structures of NHS-BODIPY and NHS-SBD, and FLIM images of V₃₀A₃₀, A₃₀V₃₀, V₃₀G₃₀ and G₃₀V₃₀ labeled with respective fluorophores. The fluorescence lifetime of each image is the average acquired from three independent experiments. Scale bar: 5 μ m. (C) Schematic diagram of optical tweezers stretching experiment. (D) Bright field images of V₃₀A₃₀ and A₃₀V₃₀ in the stretching experiment using the optical tweezer. Scale bar: 2 μ m. (E) Normalized droplets fluorescence intensity changes while stretching (red curve: V₃₀A₃₀; blue curve: A₃₀V₃₀). Fifteen droplets (at size $\sim 4\mu$ m) were imaged and used for statistical analysis.

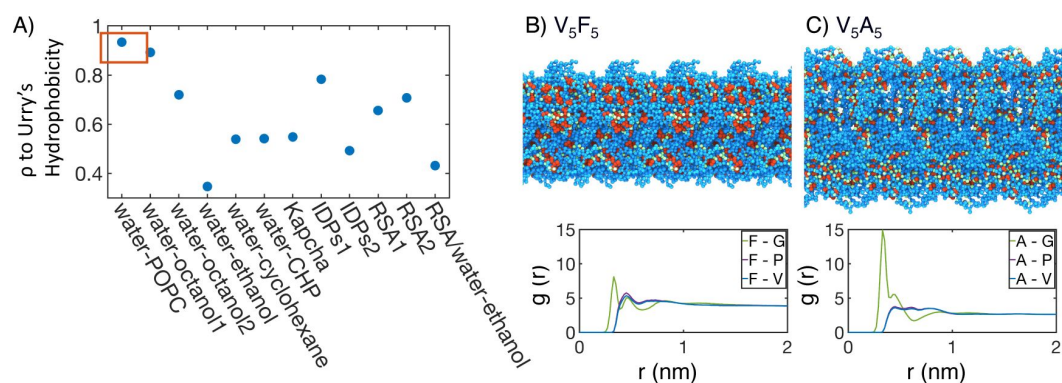


Figure 4

Interior of ELP condensates exhibits interfacial properties as a result of microphase separation. (A) Pearson correlation coefficient, ρ , between the stated measure of hydrophobicity and the condensate transition temperature, T_t , measured by Urry.⁴⁶ To remove discrepancies in which end of the scale is hydrophobic and which end is hydrophilic, all hydrophobicity scales, including the Urry scale, are first normalized such that 1 corresponds to the most hydrophobic residue and 0 corresponds to the least hydrophobic residue. Hydrophobicity measures considered include experimental measures of water-solvent transfer free energies (water-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine[POPC]-interface,⁷⁶ water-octanol1,⁷⁷ water-octanol2,⁷⁸ water-ethanol,^{78,79} water-cyclohexane,⁸⁰ and water-Ncyclohexyl-2-pyrrolidone[CHP]⁸¹), atomic level analysis of moieties within amino acids (Kapcha),⁸³ computational approaches to estimate hydrophobicity within disordered proteins (IDPs1⁸⁴ and IDPs2⁸⁵), bioinformatics techniques that approximate the burial propensity, or relative solvent accessibility (RSA) of amino acids (RSA1⁸⁶ and RSA2⁸⁷), and a method that mixed protein burial fraction with water-vapor transfer free energy (RSA/watervapor⁸⁸). The red box highlights the high correlation between T_t and water-octanol or water-POPC-interface transfer free energies. (B, C) Condensate organization from MARTINI simulations for V₅F₅ (B) and V₅A₅ (C). The top panels provide protein-only views for simulated condensates, with the guest residue X, glycine adjacent to the guest residue, and the remaining residues colored red, green, and blue, respectively. Condensate images are repeated periodically in the x/y plane. The bottom panel shows the overall radial distribution function from the guest amino acid to those amino acids native to the ELP sequence.

The ELP peptide sequences partly dictate the interfacial environment. For example, the guest residues are juxtaposed between two glycine residues in the pentamer (**Fig. 1A**), creating the immediate shell of hydrophilic residues. However, the presence of valine in the pentamer prevents the movement of GXG motifs to the interface due to the clustering of hydrophobic residues that prefer the condensate interior. Therefore, while microphase separation occurs in ELP condensates, frustration remains in the system. Hydrophilic residues cannot completely separate from hydrophobic ones due to constraints imposed by the amino acid sequence, creating unique microenvironments. Such a microenvironment arises from the collective behavior of many proteins and can deviate from individual chains,⁸⁹ explaining the less-than-ideal correlation between the Urry scale and the hydrophobicity scales optimized to reproduce the conformational ensemble of IDP monomers.

Solvation environment from atomistic simulations support interfacial properties

The interfacial environment uncovered in the previous section suggests the presence of significant solvation in the condensate interior. For condensates with lower surface tension, such as V_5G_5 , the protein density near the center of the condensate is only around $\sim 30\%$. Even for the stable condensates with high surface tension, such as V_5F_5 , the protein density near the center only reaches $\sim 70\%$. The relative density of protein, water, and ions in different systems generally follows the trend observed for the transition temperature (Fig. S11-S12 in the Supporting Information).

The large water mass fractions in the condensates prompt the question of the extent to which water is engaged in meeting the protein's hydrogen bonding requirements. We determined the average number of hydrogen bonds per residue with ELP residues or water from extensive atomistic simulations. As shown in **Fig. 5A**, over 75% of the hydrogen bonds are between protein and water, in all of the condensates. Prior work on a different ELP condensate⁴⁷ similarly indicated that water-protein hydrogen bonds constitute most of the hydrogen bonds found in the system. This finding highlights that ELPs, lacking noticeable secondary structures (Fig. S13 in the Supporting Information), are less capable of meeting its own hydrogen bonding needs, as compared to folded proteins.⁹⁰ Therefore, high water concentration within the condensate is crucial to fulfilling the protein's hydrogen bonding requirements.

Strikingly, given the importance of water molecules, the average number of hydrogen bonds per residue remained relatively constant across condensates, even when the amount of water inside the condensate varied by almost two folds. Therefore, we hypothesize that each water molecule in more hydrophobic condensates must engage more in hydrogen bonding than each water in hydrophilic condensates. To test this hypothesis, we determined the water hydrogen bond density, defined as the ratio of the number of protein-water hydrogen bonds divided by the total number of water molecules near the condensate (**Fig. 5D** and **5E**). As shown in **Fig. 5B** and **5C**, the water hydrogen bond density indeed negatively correlates with the condensate transition temperature, T_t , supporting our hypothesis that more hydrophobic condensates would have a higher hydrogen bond density.

The water hydrogen bond density also highlights an interfacial environment of blended hydrophobic and hydrophilic regions. For example, when we limit the analysis to water molecules near the guest residues, X, the hydrogen bond density now becomes positively correlated with T_t . Therefore, locally, protein-water interactions are strongly influenced by the chemistry of side chains, and water molecules have fewer hydrogen bonding opportunities with more hydrophobic residues. However, the opposite trend is observed for the hydrogen bond density near glycine residues that immediately surround the guest residues. Therefore, the exposed backbones retain

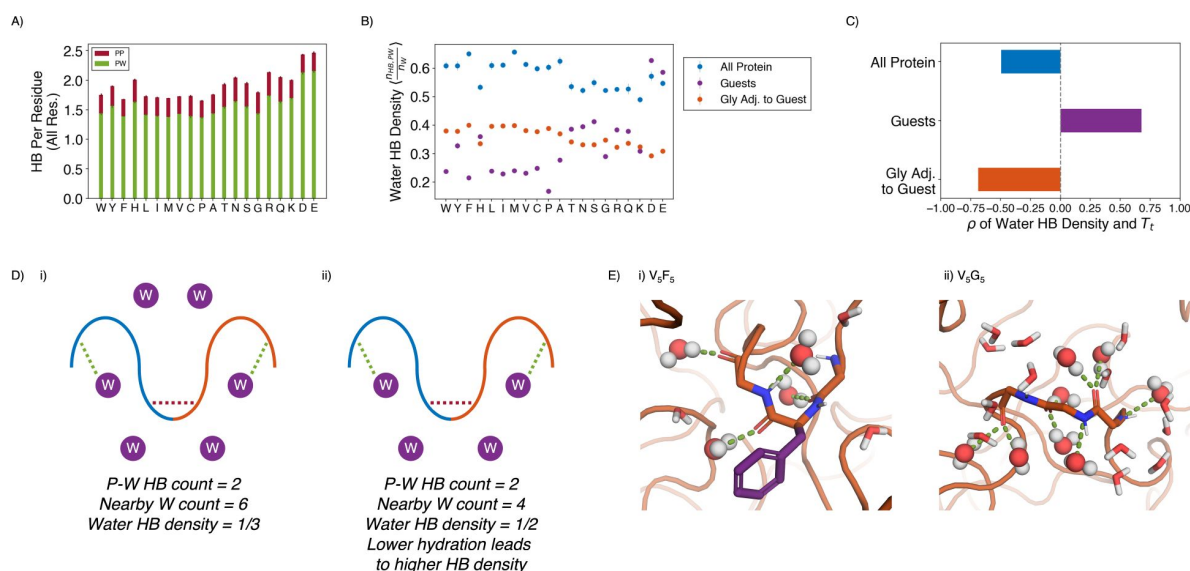


Figure 5

Water hydrogen bonding environment of condensates from all-atom simulations. (A) Bar chart depicting the average number of protein-water (PW, in green) and protein-protein (PP, in red) hydrogen bond (HB) per residue for each condensate system. The x-axis presents the guest residue of the system and is ordered by the Urry scale. Error bars represent standard deviations of four independent estimates. (B) Water HB density for each of the 20 condensates, for three different residue selections. The metric is shown for all residues in the system (blue), guest residues (purple), and glycine residues adjacent to the guest residue in the sequence (orange). The x-axis presents the guest residue of the system and is ordered by the Urry scale. Error bars represent standard deviations of four independent estimates. (C) Correlation coefficients of the water HB density shown in part B with Urry T_t . (D) Schematics depicting two systems with low (i) and high (ii) water hydrogen bond density. The protein is depicted as blue and orange lines, and the water molecules are depicted as purple circles. Protein-protein and protein-water hydrogen bonds are drawn as red and green dashed lines respectively. (E) Visualizing hydrogen bond density with illustrative snapshots for V_5F_5 (i) and V_5G_5 (ii) condensate. Side chain carbon atoms are shown in purple. Water molecules near the protein are explicitly depicted, with oxygen and hydrogen atoms colored in red and white. Water molecules hydrogen bonded to the protein are depicted as spheres (with hydrogen bonds shown in green lines).

water molecules even when hydrophobic side chains do not provide hydrogen bonding opportunities, and the inseparability between the two further contributes to the observed interfacial property of condensates.

As a complementary measure of the solvation environment of individual residues, we computed the relative solvent accessibility (RSA) for the guest amino acids using atomistic simulations (**Fig. 6A-B**). The RSA measures the solvent-accessible surface area normalized by the maximum possible solvent exposure. RSA values in ELP condensates are much lower than those computed from all-atom simulations of ELP monomers, consistent with the increase of polymer density upon phase separation. However, they are almost always higher than the values estimated for folded proteins due to many water molecules roaming inside the condensates.⁸⁷ Therefore, while ELP condensates provide a mechanism to shield hydrophobic amino acids from solvent, they are less effective in doing so than folded proteins.

While the amino acid RSA values increase upon phase separation, unlike protein folding, the increase does not result from polymer collapse. We observe a significant expansion for almost all the studied peptides upon condensation, supporting the notion that water is a poor solvent for ELP (**Fig. 6C**). The expansion is most evident for more hydrophobic ELPs, the radii of gyration of which are now comparable to that of an ideal Gaussian chain. Notably, the sizes of the two separate peptide blocks are similar in most systems, supporting an interfacial environment that can accommodate both hydrophobic and hydrophilic residues (Fig. S14 in the Supporting Information).

Conclusions and discussion

We carried out multiscale simulations to elucidate the connection between protein sequences and emergent properties of ELP condensates. The multiscale approach overcomes challenges in sampling slow conformational rearrangements to produce equilibrium atomistic condensate configurations. The simulations successfully reproduced condensate stability variation upon amino acid substitution. While our study is performed at set salt concentration and temperature to isolate the contributions of amino acid hydrophobicity to condensate organization, future studies may consider implementing temperature⁵⁰ or salt⁹³ dependent models to explore how solution conditions affect the organization of ELP condensates.

We found that diblock ELPs undergo microphase separation, with the more hydrophobic blocks localizing towards the condensate interior. The more hydrophobic blocks increase the local protein density, resulting in higher viscosity and lower dielectric constants, as determined in FLIM experiments. In contrast to the interior of folded proteins, these blocks are significantly solvated with water occupying over 30% of mass density. Unlike the weak micelles formed in solution,⁶⁶ ELP condensates exhibit gyroid-like morphologies that promotes contacts between blocks. Further studies on the thermodynamic stability of these morphologies and comparing with predictions from the self-consistent field theory shall provide more insights into the driving forces for their emergence.

Our study uncovered a remarkably simple grammar for determining the stability of ELP condensates. A single quantity specific to the guest residue suffices to predict the emergent properties of the condensate, as evidenced by the strong correlation of the transition temperatures with the transfer free energies. The lack of higher-order effects is striking, supporting a mean field approximation to account for the contribution of individual residues in the system. However, the hydrophobicity scale is itself sensitive to the local microenvironment that is sequence specific. Such a microenvironment arises from the collective behavior of many proteins, can deviate from that of individual chains, and is likely sensitive to the solution conditions,⁹⁴ which are held

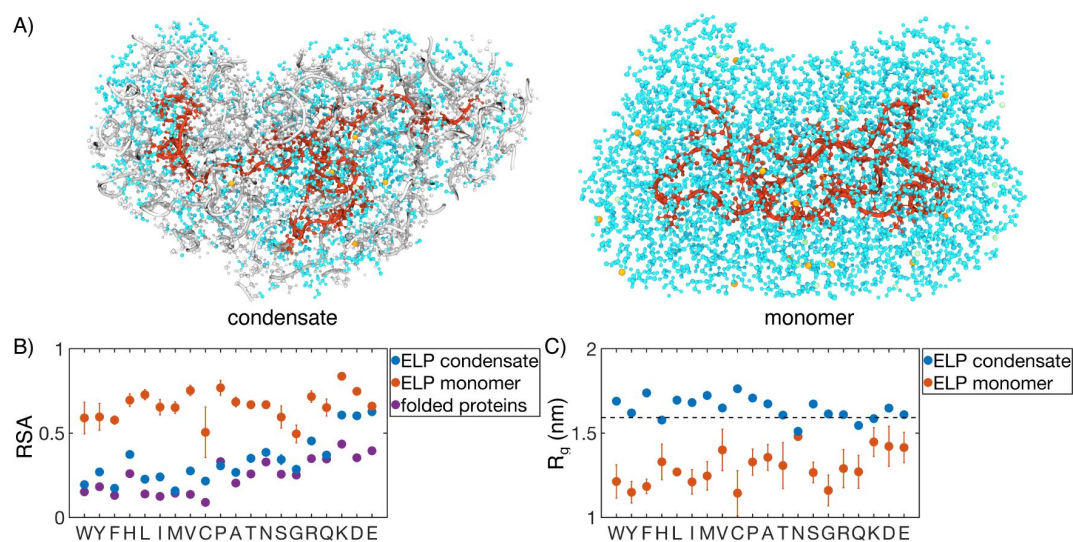


Figure 6

Comparison of protein conformation and solvation between ELP condensates and monomers. (A) Zoom in view of a single peptide chain (red) from atomistic simulations of V₅A₅ condensate (left) and monomer (right). Atoms within one nm of the peptide are shown, including water (cyan), chlorine (green), sodium (orange), and protein atoms from other chains (gray). (B) RSA of guest residues estimated from atomistic simulations of condensates (blue) and monomers (orange). For comparison, the corresponding values estimated using folded proteins are included as purple dots.⁸⁷ Error bars represent the standard deviation of four independent time estimates. (C) Comparison of the radius of gyration (R_g) for peptides estimated from atomistic simulations of condensates (blue) and monomers (orange). The dashed line represents the expected R_g of an ideal chain, utilizing values that have been previously suggested for IDPs.^{91,92} Error bars represent the standard deviation of four independent time estimates.

constant in our study. Future work on systems with double amino acid substitutions or changes to salt concentration or temperature could elucidate the generality of the mean field interpretation and the additivity of individual contributions.

While the transferability of the extracted rule for condensate stability to other systems remains to be shown, there are reasons to be hopeful. For instance, we anticipate that the microphase separation that produces the interfacial properties is a general principle for condensate organization. For most IDPs, the hydrophobic and hydrophilic residues are often interspersed throughout the chain to prevent the collapse of individual proteins⁹⁵ and, correspondingly, the complete phase separation as in the lamellar phase. In addition, the hydrophilic backbones are expected to be exposed due to a lack of secondary structure formation, contributing to the interfacial property in the presence of hydrophobic side chains. As such, we anticipate that future research will discover the broad presence of interfacial environments within microphase separated motifs, as observed here.

Methods

Molecular dynamics simulation details

To better understand the properties of biological condensates, we performed simulations on 40 diblock ELPs with the sequence $(V-P-G-V-G)_n-(V-P-G-X-G)_n$. We set $n = 5$ to balance computational efficiency and polymer topological effects. As shown in Fig. S5 in the Supporting Information, the results presented in the main text are qualitatively robust with respect to the system size and peptide length.

The multiscale simulations of ELP condensates started with an implicit solvent model, MOFF,⁵² and a similar homopolymer model, to generate initial configurations for each system. Next, we converted the α -carbon only configurations into those consistent with the MARTINI force field⁵⁷ for coarse-grained, explicit solvent simulations. These simulations measured the coexistence between the condensate and solvent, lasted for 100 μ s, and were conducted in the NP_NAT ensemble. Finally, we converted the MARTINI configurations into atomistic structures for simulations with CHARMM36m⁹⁷ force field, with protein-water interactions shifted to better characterize IDPs. Atomistic simulations were conducted in the NP_NAT ensemble and lasted 250 ns. All simulations were conducted using the software GROMACS⁹⁸ and are described in detail in the *Condensate simulation details* section of the *Supporting Information*.

In addition to the condensates, we performed atomistic simulations of ELP monomers. These simulations began from RoseTTAFold predictions of the peptide structure^{99,100} and lasted for 1 μ s in the NPT ensemble. Full details are available in the *Monomer simulation details* section of the *Supporting Information*.

Analysis of condensate simulations

Surface tension

In explicit solvent simulations, two coexisting phases are present, in which proteins form a dense slab in the x-y plane, and the solvent creates a dilute phase in the z-dimension.^{101,102} We controlled the pressure normal to the protein-solvent interface by performing simulations in the NP_NAT ensemble.⁶¹ The surface tension (τ) was calculated from these simulations as

$$\tau = \frac{L_z}{2N} * (P_{ZZ} - \frac{1}{2}(P_{XX} + P_{YY})), \quad (1)$$

where L_z is the length of the Z-dimension and N is the number of protein droplets. P_{XX} , P_{YY} , and P_{ZZ} are the components of the pressure tensor along the three axes.

Clustering

Since the condensate can diffuse along the z-axis, i.e., the direction perpendicular to the interface, we aligned the simulated configurations using the position of the largest cluster of peptide molecules. To find the largest cluster, we first computed a center of mass contact matrix between peptide molecules with a distance cutoff of 4 nm. Then, using a depth first search algorithm,¹⁰³ we identified the size and location of the largest protein cluster. The system was then shifted such that the z coordinate of the center of mass of the largest cluster is at zero. We implemented the alignment using the software MDAnalysis.¹⁰⁴,¹⁰⁵

Contact map

Protein contact maps were calculated using a cutoff distance of 0.6 nm. Specifically, two residues were defined as in contact if any pair of their coarse-grained beads were within 0.6 nm.

Radial distribution function

Radial distribution functions were calculated using the InterRDF function of MDAnalysis between 0.00001 nm and 2 nm. For analyzing the microphase separation of each condensate, we divided each protein into the V-substituted blocks and X-substituted blocks. This radial distribution function excluded intra-chain contacts to better capture how inter-chain contacts determine condensate organization. To analyze the origin of chain frustration within diblock phase separation, we computed the radial distribution function of each guest amino acid to the amino acids native to the sequence. Intra-chain contacts were included to fully capture the local environment experienced by the guest amino acid.

Relative solvent accessibility

The relative solvent accessibility (RSA) was measured based on the solvent accessible surface area (SASA) of guest amino acids determined from all atom simulations. We first calculated the SASA for each residue using GROMACS tools sasa, with a probe radius of 0.14 nm.¹⁰⁶ We then averaged across all repeats of guest amino acids, and normalized the SASA by the maximum possible value for a given amino acid to compute RSA⁸⁷ (see Table S1).

Radius of gyration

The estimated R_g from atomistic simulations were compared to the following analytical expression⁹¹

$$R_g = \sqrt{\frac{2l_p b}{(2\nu + 1)(2\nu + 2)}} N^\nu, \quad (2)$$

where N is the length of the polymer, $b = 0.38\text{nm}$ is the bond length, and $l_p = 0.40\text{nm}$ is the persistence length.

Secondary structure

Secondary structure for protein condensates was calculated from atomistic simulations using the GROMACS do dssp command.¹⁰⁷,¹⁰⁸ Fig. S13 in the Supporting Information displays the ordered fraction of secondary structures, which includes α -helices, β -sheets, β -bridges, and turns.

Hydrogen bonding

Protein-protein and protein-water hydrogen bonds were determined using the hydrogenbonds package within MDAnalysis.¹⁰⁴¹⁰⁵¹⁰⁹ A hydrogen bond was defined as having a donor heavy atom within 3.30 Å of an acceptor heavy atom and a donor heavy atom-hydrogen atom-acceptor heavy atom angle of 135.0° or larger. The nearby water count for each analysis selection was determined by counting the water molecules whose oxygen atoms were within 4.0 Å of the heavy atoms of this selection. Water hydrogen bond density for each residue selection for each frame was computed as the ratio of protein-water hydrogen bonds to the number of nearby water molecules (Fig. S15 in the Supporting Information).

Experimental methods

Fluorescence lifetime imaging microscopy (FLIM)

The proteins of interest were labeled with NHS ester fluorophore. We used ELPs with 1% BODIPY labels or 2.5% SBD labels to form condensates, which avoid the artifacts induced by fluorophores. Droplets were formed with the final concentration of 70 μM ELP in 2 M NaCl for V-A and 1.5 M NH₄SO₄ for V-G diblock, respectively. A drop of droplets containing solution was placed on a 0.17 mm coverslip with a 500 μm spacer. Images were acquired by Leica Falcon Fluorescence Microscope equipped with WIL pulse laser and 63X/0.12 oilimmersion objective. The BODIPY was excited at 488 nm and the SBD was excited at 448 nm. The fluorescence lifetime fitting and image analysis were performed in LAS X and Image J.

Optical tweezer experiments

The protocol describes the preparation and manipulation of a sample containing a BODIPYlabeled ELP protein using an optical tweezer, specifically the C-Trap from LULICKS. The goal is to form a single large condensate from small droplets and then stretch it using the optical tweezers while monitoring its fluorescence intensity. The first step is to mix the BODIPY-labeled ELP protein with NaCl solution to obtain a final concentration of 20 μM protein in 2 M NaCl. The sample is then loaded into the flow channel of the optical tweezer. The dual trap mode of the C-Trap is then used to move the two traps to the same position. The sample is flushed until a single large condensate of about 4 μm is caught by the dual trap via the fusion of small droplets. The traps are then moved along with the droplet to another flow channel containing 2 M NaCl buffer. The channel and pressure are turned off to avoid any additional force on the droplet. The next step is to stretch the droplet by moving trap 1 0.2 μm each time. Fluorescent images of each stretch are taken using a 488 nm excitation laser. The mean fluorescence intensity of the whole droplet is then quantified using Image J.

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Reviewer #1 (Public Review):

This is an interesting, informative, and well-designed study that combines theoretical and experimental methodologies to tackle the phenomenon of higher-resolution structures/substructures in model biomolecular condensates. However, there is significant room for improvement in the presentation and interpretation of the results. As it stands, the precise definition of "frustration," which is a main theme of this manuscript (as emphasized in the title), is not sufficiently well articulated. This situation should be rectified to avoid "frustration" becoming a "catch-all" term without a clear perimeter of applicability rather than a precise, informative description of the physical state of affairs. There are also a few

other concerns, e.g., regarding interpretation of correlation of phase-separation critical temperature and transfer free energy of amino acid residues as well as the difference between critical temperature and onset temperature, and the way the simulated configurations are similar to that of gyroids. Accordingly, the manuscript should be revised to address the following:

(1) It is accurately pointed out on p.4 that elastin-like polypeptides (ELPs) undergo heat-induced phase separation and therefore exhibit lower critical solution temperatures (LCSTs). But it is not entirely clear how this feature is reproduced by the authors' simulation. A relationship between simulated surface tension and "transition temperature" is provided in Fig.1C; but is the "transition temperature" (authors cited ref.41 by Urry) the same as critical temperature? Apparently, Urry's T_t is "critical onset temperature", the temperature when phase separation happens at a given polymer concentration. This is different from the (global) critical temperature LCST - though the two may be correlated-or not-depending on the shape of the phase boundary. Moreover, is the MOFF coarse-grained forcefield (first step in the multi-scale simulation), by itself, capable of reproducing heat-induced phase separation in a way similar to the forcefield of Dignon et al., ACS Cent Sci 5, 821-230 (2019)? Or, is this temperature-dependent effect appearing only subsequently, after the implementation of the MARTINI and/or all-atom steps? Clarification is needed. To afford a more informative context for the authors' introductory discussion, the aforementioned Dignon et al. work and the review by Cinar et al. [Chem Eur J 25, 13049-13069 (2019)], both touching upon the physical underpinning of the LCST feature of elastin, should also be cited along with refs.41-43.

(2) "Frustration" and "frustrated" are used prominently in the manuscript to characterize certain observed molecular configurations (11 times total, in both the title and in the abstract). Apparently, it is the most significant conceptual pronouncement of this work, hence its precise meaning is of central importance to the authors' thesis. Whereas one should recognize that the theoretical and experimental observations are striking without invocation of the "frustration" terminology, usage of the term can be useful if it offers a unifying conceptual framework. However, as it stands, a clear definition of the term "frustration" is lacking, leaving readers to wonder what molecular configurations are considered "frustrated" and what are not (i.e., is the claim of observation of frustration falsifiable?). For instance, "frustrated microphase separation" appears in both the title and abstract. A logical question one may ask is: "Are all microphase separations frustrated"? If the answer is in the affirmative, does invocation of the term "frustration" add anything to our physical insight? If the answer is not in the affirmative, then how does one distinguish between microphase separations that are frustrated from those that are not frustrated? Presumably all simulated and experimental molecular configurations in the present study are those of lowest free energy for the given temperature. In other words, they are what they are. In the discussion about frustrated phase separation on p.13, for example, the authors appear to refer to the fact that chain connectivity is preventing hydrophobic residues to come together in a way to achieve the most favorable interactions as if there were no chain connectivity (one may imagine in that case all the hydrophobic residues will form a large cluster without microphase separation). Is this what the authors mean by "frustration"? If that's true, isn't that merely stating the obvious, at least for the observed microphase separation? In general, does "frustration" always mean deviation of actual, physical molecular configurations from certain imagined/hypothetical/reference molecular configurations, and therefore dependent upon the choice of the imagined reference configuration? If this is how the authors apply the term "frustration" in the present work, what is the zero-frustration reference state/configuration for microphase separation? And, similarly, what is the zero-frustration reference state/configuration when frustrated EPS-water interactions are discussed (~p.14-p.15, Fig.5)? How do non-frustrated water-protein interactions look like? Is the classic clathrate-like organization of water hydrogen bonds around small nonpolar solute "frustrated"?

(3) In the discussion about the correlation of various transfer free energy scales for amino acids and Urry's critical onset temperature (ref.41) on p.11 and Fig.4, is there any theoretical relationship to be expected between the interactions among amino acids of ELPs and their critical onset temperatures? While a certain correlation may be intuitively expected if the free energy scale "is working", is there any theoretical insight into the mathematical form of this relationship? A clarifying discussion is needed because it bears logically on whether the observed correlation or lack thereof for different transfer energy scales is a good indication of the adequacy of the energy scales in describing the actual physical interactions at play. This question requires some prior knowledge of the expected mathematical relationship between interaction parameters and onset temperature.

(4) To provide a more comprehensive context for the present study, it is useful to compare the microphase separation seen in the authors' simulation with the micelle-like structures observed in recent simulated condensed/aggregated states of hydrophobic-polar (HP) model sequences in Statt et al., J Chem Phys 152, 075101 (2020) [see esp. Fig.6] and Wessén et al., J Phys Chem B 126, 9222-9245 (2022) [see, e.g., Fig.10].

(5) "Gyroid-like morphology" is mentioned several times in the manuscript (p.4, p.8, p.17, Fig.S3). This is apparently an interesting observation but a clear explanation is lacking. A more detailed and specific discussion, perhaps with additional graphical presentations, should be provided to demonstrate why the simulated condensed-phase ELP configurations are similar to the classical description of gyroid as in, e.g., Terrones & Mackay, Chem Phys Lett 207, 45-50 (1993) and Lambert et al., Phil Trans R Soc A 354, 2009-2023 (1996).

Comments on the revised manuscript:

The authors have adequately addressed my previous concerns.

<https://doi.org/10.7554/eLife.90750.2.sa1>

Reviewer #2 (Public Review):

Summary:

Latham A.P. et al. apply simulations and FLIM to analyse several di-block elastin-like polypeptides and connect their sequence to the micro-structure of coacervates resulting from their phase-separation.

Strengths:

Understanding the molecular grammar of phase separating proteins and the connection with mesoscale properties of the coacervates is highly relevant. This work provides insights into micro-structures of coacervates resulting from di-block polypeptides.

Weaknesses:

The results apply to a very specific architecture (di-block polypeptides) with specific sequences.

<https://doi.org/10.7554/eLife.90750.2.sa0>

Author response:

The following is the authors' response to the original reviews.

In this letter, we respond to each of the reviewers' comments. We support responses by referring to the revised manuscript and, where necessary, by including additional descriptions and analyses that we consider extrinsic to the manuscript itself. In this letter, all changes to the manuscript are shown in blue. As noted, the displayed figures have been added to the manuscript or the SI. We believe that we have successfully addressed all comments and that the quality of our paper has improved significantly.

Comment 1: In addition to the technical comments by the reviewers, I would encourage the authors to discuss the dependency of their observations, e.g. emergence of microphase separation, not only on the sequence of the polypeptides, but also on the solution conditions. Similarly, the distributions of ions in the condensate bulk, interphase, and diluted phase, and hence the interfacial free energy, are significantly affected both by the chemical composition of the condensate and the salt concentration itself, see: <https://pubs.acs.org/doi/10.1021/acs.nanolett.1c03138>

We thank the editor for this suggestion. Here, we have focused on the effect of sequence on condensate organization. We agree that how changes in solution condition affect condensate, including microphase separation of ELPs, is potentially interesting as well. We note this as a possible future direction at multiple places in the revised Conclusions and Discussion:

“The simulations successfully reproduced condensate stability variation upon amino acid substitution. While our study is performed at set salt concentration and temperature to isolate the contributions of amino acid hydrophobicity to condensate organization, future studies may consider implementing temperature [cite] or salt [cite] dependent models to explore how solution conditions affect the organization of ELP condensates.”

“Such a microenvironment arises from the collective behavior of many proteins, can deviate from that of individual chains, and is likely sensitive to the solution conditions,[cite] which are held constant in our study. Future work on systems with double amino acid substitutions or changes to salt concentration or temperature could elucidate the generality of the mean field interpretation and the additivity of individual contributions.”

Response to referee 1

Comment 0: This is an interesting, informative, and well-designed study that combines theoretical and experimental methodologies to tackle the phenomenon of higher-resolution structures/substructures in model biomolecular condensates. The results should be published. However, there is significant room for improvement in the presentation and interpretation of the results. As it stands, the precise definition of “frustration,” which is a main theme of this manuscript (as emphasized in the title), is not sufficiently well articulated. This situation should be rectified to avoid “frustration” becoming a “catch-all” term without a clear perimeter of applicability rather than a precise, informative description of the physical state of affairs. There are also a few other concerns, e.g., regarding interpretation of correlation of phase-separation critical temperature and transfer free energy of amino acid residues as well as the difference between critical temperature and onset temperature, and the way the simulated configurations are similar to that of gyroids.

We want to thank the reviewers for their insightful comments. We revised the manuscript extensively to improve its clarity and to address the reviewers' concerns. In the following, we provide point-to-point responses to all the comments.

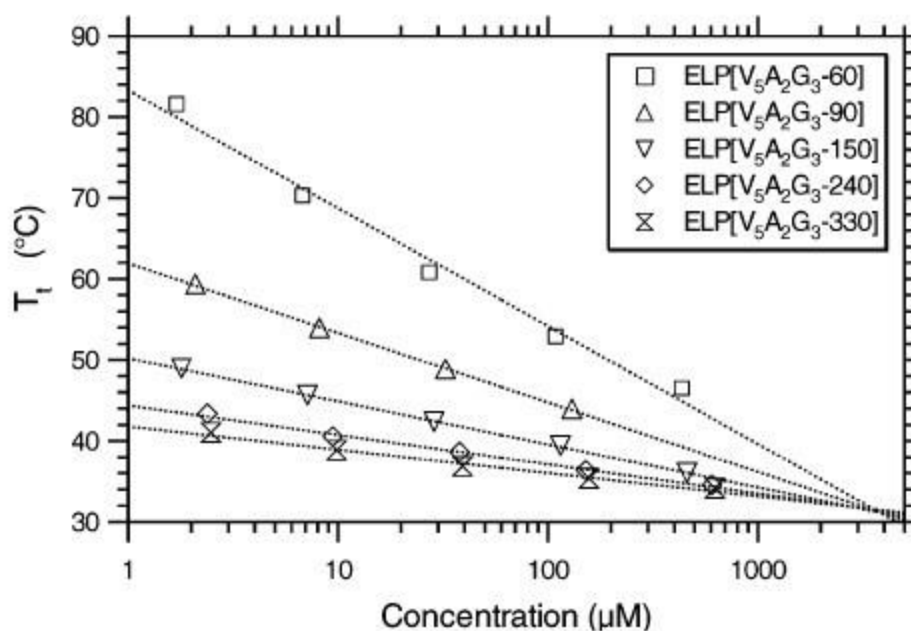
Comment 1: It is accurately pointed out on p.4 that elastin-like polypeptides (ELPs) undergo heat-induced phase separation and therefore exhibit lower critical solution

temperatures (LCSTs). But it is not entirely clear how this feature is reproduced by the authors' simulation. A relationship between simulated surface tension and "transition temperature" is provided in Fig.1C; but is the "transition temperature" (authors cited ref.41 by Urry) the same as critical temperature? Apparently, Urry's T_t is "critical onset temperature", the temperature when phase separation happens at a given polymer concentration. This is different from the (global) critical temperature LCST - though the two may be correlated-or not-depending on the shape of the phase boundary. Moreover, is the MOFF coarse-grained forcefield (first step in the multi-scale simulation), by itself, capable of reproducing heat-induced phase separation in a way similar to the forcefield of Dignon et al., ACS Cent Sci 5, 821-230 (2019)? Or is this temperature-dependent effect appearing only subsequently, after the implementation of the MARTINI and/or all-atom steps? Clarification is needed. To afford a more informative context for the authors' introductory discussion, the aforementioned Dignon et al. work and the review by Cinar et al. [Chem Eur J 25, 13049-13069 (2019)], both touching upon the physical underpinning of the LCST feature of elastin, should also be cited along with refs.41-43.

We thank the reviewer for their comment. First, we apologize for the lack of clarity between the global lower critical solution temperature, T_c , and the transition temperature, T_t . We have modified the manuscript to be more explicit that the transition temperature we utilize is dependent on the solution conditions, instead of the global lower critical solution temperature.

Author response image 1.

T_t as a function of concentration for ELP[V₅A₂G₃] constructs of different chain lengths. Logarithmic fits to the data for each construct using Eq. 1 are also shown. It is evident that the different curves converge to the critical temperature T_c at the critical concentration C_c . Figure reproduced from ref.[2] CC BY 4.0.



However, as shown by Chilkoti and coworkers [1, 2] and in Author response image 1, the critical temperature of ELPs T_c is indeed linearly related to T_t with the following relationship

$$T_t = T_c + \frac{k}{\text{length}} \ln \frac{C_c}{\text{conc}}. \quad (1)$$

The above equation highlights the dependence of T_t on the chain length (length) and polymer concentration (conc). The parameter C_c is the corresponding theoretical polypeptide concentration that would be required to achieve T_c , and k is the proportionality constant. Instead of making computationally expensive predictions of condensate critical temperatures, we focused on the surface tension, which can be more readily determined from single constant temperature simulations as detailed in the Methods section. This decision was made so to make it computationally feasible to systematically probe the properties of all 20 amino acids in diblock ELPs in our multiscale model. Furthermore, an expected relationship between the critical temperature and the surface tension can be inferred based on the Flory Huggins theory. In particular, relationships between the Flory Huggins parameter, χ , and interfacial tension (τ) have been investigated, and the relationship can be approximated as

$$\tau \propto \chi^\alpha, \quad (2)$$

where α is a positive constant, whose exact value depends on the proximity of χ to the critical value of χ necessary for phase separation (χ_C). [3, 4] As detailed in new Supplemental Theory of the Supporting Information, for systems undergoing LCST,

$$\chi = \frac{T_C}{T} \left(\frac{1}{2} - \frac{z\Delta s}{k_B} \right) + \frac{z\Delta s}{k_B}, \quad (3)$$

with $\frac{z\Delta s}{k_B} > \frac{1}{2}$. Therefore, we have

$$\tau \propto \left[\frac{T_C}{T} \left(\frac{1}{2} - \frac{z\Delta s}{k_B} \right) + \frac{z\Delta s}{k_B} \right]^\alpha. \quad (4)$$

Several conclusions can be drawn from Eq. 4. First, for $\alpha = 1$, τ is linearly proportional to T_c .

Secondly, τ decreases at larger values for T_c since $\frac{z\Delta s}{k_B} > \frac{1}{2}$, trend that is consistent with

results presented in Figure 1 of the main text. Finally, as detailed in the Supplemental Theory, the inverse relationship between τ and T_c is only expected for systems exhibiting LCSTs. For systems with UCST, τ increases at larger T_c . Therefore, reproducing the correct trend supports the model's ability to capture the temperature-dependent effect specific to the ELP system.

We modified the text to define the physical meaning of T_t more explicitly. Furthermore, we added a new section in the Supporting Information titled Supplemental Theory to detail the relationship between T_t , T_c , the Flory-Huggins parameter χ , and the surface tension τ . The updated text now reads:

“Utilizing the simulated condensate conformations, we computed various quantities to benchmark against experimental measurements. While the critical temperature has been

widely used as a measure for condensate stability, determining it computationally is expensive. As an alternative, we computed the surface tension, τ , using 100- μ s-long MARTINI simulations performed with the NPNAT ensemble.[cite] As detailed in the Supplemental Theory in the Supporting information, an inverse relationship is expected between τ and the critical temperature, T_c , for systems exhibiting LCSTs. We further approximate T_c with the transition temperatures (T_t) of ELP sequences,[cite] which are the temperatures at which ELPs undergo an LCST transition at a specified solution condition. T_t was shown to be linearly proportional to T_c [cite]. As expected, a negative correlation can be readily seen between computed surface tension and experimental T_t (Fig. 1C). This observed negative correlation between T_t and τ supports the simulation approach's accuracy in reproducing the sequence-dependent changes in ELP phase behavior."

The reviewer is correct that MOFF does not explicitly account for temperature-dependent effects in its interaction parameters. But as mentioned above and indicated by the reviewer, the following steps with explicit solvent simulations in the multiscale strategy succeed in capturing sequence-dependent differences in ELP systems, which are evident in both transition temperature and surface tension.

We cited the two references suggested by the reviewer in the introduction. We further added the following text in the discussion section to suggest explicitly exploring temperature-dependent effects as an interesting future direction.

"While our study is performed at set salt concentration and temperature to isolate the contributions of amino acid hydrophobicity to condensate organization, future studies may consider implementing temperature[cite] or salt[cite] dependent models to explore how solution conditions effect the organization of ELP condensates."

Comment 2: "Frustration" and "frustrated" are used prominently in the manuscript to characterize certain observed molecular configurations (11 times total, in both the title and in the abstract). Apparently, it is the most significant conceptual pronouncement of this work, hence its precise meaning is of central importance to the authors' thesis. Whereas one should recognize that the theoretical and experimental observations are striking without invocation of the "frustration" terminology, usage of the term can be useful if it offers a unifying conceptual framework. However, as it stands, a clear definition of the term "frustration" is lacking, leaving readers to wonder what molecular configurations are considered "frustrated" and what are not (i.e., is the claim of observation of frustration falsifiable?). For instance, "frustrated microphase separation" appears in both the title and abstract. A logical question one may ask is: "Are all microphase separations frustrated"? If the answer is in the affirmative, does invocation of the term "frustration" add anything to our physical insight? If the answer is not in the affirmative, then how does one distinguish between microphase separations that are frustrated from those that are not frustrated? Presumably all simulated and experimental molecular configurations in the present study are those of lowest free energy for the given temperature. In other words, they are what they are. In the discussion about frustrated phase separation on p.13, for example, the authors appear to refer to the fact that chain connectivity is preventing hydrophobic residues to come together in a way to achieve the most favorable interactions as if there were no chain connectivity (one may imagine in that case all the hydrophobic residues will form a large cluster without microphase separation). Is this what the authors mean by "frustration"? If that's true, isn't that merely stating the obvious, at least for the observed microphase separation? In general, does "frustration" always mean deviation of actual, physical molecular configurations from certain imagined/hypothetical/reference molecular configurations, and therefore dependent upon the choice of the imagined reference configuration? If this is how the authors apply the term "frustration" in the present work, what is the zero-frustration reference state/configuration for microphase separation?

And, similarly, what is the zero-frustration reference state/configuration when frustrated EPS-water interactions are discussed (p.14-p.15, Fig.5)? How do non-frustrated water-protein interactions look like? Is the classic clathrate-like organization of water hydrogen bonds around small nonpolar solute “frustrated”?

We thank the reviewer for their insightful comment, and agree that the concept of “frustration” is both important to our conclusions and, upon review, is too vague in our previous draft of the manuscript.

For conceptual simplicity and to maximize transferability to real biological systems, we will focus our discussion of frustration on one specific type, which we term “chain frustration.” Chain frustration occurs in states where tertiary interactions between chemically distinct polymer blocks favor phase separation, while chain connectivity prevents macroscopic phase separation from occurring.[5] This frustration leads to microphase separation with microdomains of different monomers.

We agree with the reviewer that “all microphase separations” are frustrated, and have revised the title to

“Microphase Separation Produces Interfacial Environment within Diblock Biomolecular Condensates”

Furthermore, we also removed frustration from the abstract to read

“The interspersions of hydrophilic and hydrophobic residues and a lack of secondary structure formation result in an interfacial environment, which explains both the strong correlation between ELP condensate stability and interfacial hydrophobicity scales, as well as the prevalence of protein-water hydrogen bonds.”

We have limited our discussion of the frustration to the incomplete separation of hydrophobic and hydrophilic groups. As pointed out by the reviewer, in this case, frustration refers to the fact that chain connectivity is preventing hydrophobic residues from coming together in a way to achieve the most favorable interactions as if there were no chain connectivity. The reference would be a perfectly macroscopic phase separation that partitions hydrophobic from hydrophilic groups.

While the frustration from chain connectivity is well understood for block copolymers[5], its effect on producing the interfacial solvation environment, to the best of our knowledge, has not been emphasized before. We have revised the text at the point where we mention frustration to clearly define its meaning.

“Therefore, while microphase separation occurs in ELP condensates, frustration remains in the system. Hydrophilic residues cannot completely separate from hydrophobic ones due to constraints imposed by the acid sequence, creating unique microenvironments.”

When discussing the interactions between ELP and water, we used the hydrogen bond analysis to emphasize the interfacial environment. For example, the hydrophobic residues tend to “repel” water molecules, reducing the hydrogen bond density; on the other hand, hydrophilic residues and backbone retain water molecules. This difference resulted in the positive and negative correlation with Tt shown in Fig 5C. The behavior of water molecules is, therefore, inhomogeneous inside the condensate. We expect water molecules to become frustrated due to the simultaneous contact with both hydrophobic and hydrophilic chemical groups, and a perfect reference state would be the pure water environment. However, since this point is not central to our study, to avoid confusion, we have avoided mentioning frustration and revised the text to read amino acid sequence, creating unique microenvironments.”

“The water hydrogen bond density also highlights an interfacial environment of blended hydrophobic and hydrophilic regions.”

After revising the text, frustration only appears three times in the manuscript.

Comment 3: In the discussion about the correlation of various transfer free energy scales for amino acids and Urry's critical onset temperature (ref.41) on p.11 and Fig.4, is there any theoretical relationship to be expected between the interactions among amino acids of ELPs and their critical onset temperatures? While a certain correlation may be intuitively expected if the free energy scale "is working", is there any theoretical insight into the mathematical form of this relationship? A clarifying discussion is needed because it bears logically on whether the observed correlation or lack thereof for different transfer energy scales is a good indication of the adequacy of the energy scales in describing the actual physical interactions at play. This question requires some prior knowledge of the expected mathematical relationship between interaction parameters and onset temperature.

We thank the reviewer for their comment. The exact relationship between the interactions between amino acids and their transition temperature can be understood in terms of the Flory-Huggins theory, which describes the thermodynamics of polymer mixtures using a lattice model. The chemical composition of the mixture is built into the polymer-solvent interaction parameter

$$\chi = \frac{z}{2k_B T} [\epsilon_{PP} + \epsilon_{SS} - 2\epsilon_{PS}] = \frac{z\Delta\epsilon}{k_B T}, \quad (5)$$

Where $\Delta\epsilon = \frac{1}{2} [\epsilon_{PP} + \epsilon_{SS} - 2\epsilon_{PS}]$. z is the coordination number, T is the temperature, k_B is the Boltzmann constant, and $\{\epsilon_{PP}, \epsilon_{SS}, \epsilon_{PS}\}$ are the strength of polymer-polymer, solventsolvent, and polymer-solvent interactions respectively.[6]

From the original derivation of Flory-Huggins theory, it can be shown that phase separation occurs when χ is greater than its critical value, or $\chi > \chi_C \approx \frac{1}{2}$. χ_C , we can derive the critical temperature as

$$T_c = \frac{2z\Delta\epsilon}{k_B}. \quad (6)$$

$\Delta\epsilon$ can indeed be interpreted as the free energy cost of transferring a polymer bead from a solution phase to a polymer phase. It corresponds to the change of energy from a mixed state, with contacts between polymer and solvent (ϵ_{PS}), to the demixed state with only polymer-polymer (ϵ_{PP}) and solvent-solvent (ϵ_{SS}) contacts.

Therefore, the transfer free energy, and the interactions among amino acids of ELPs, are expected to correlate with the critical temperature. The above discussion has been incorporated into the new section Supplemental Theory in the Supporting Information. There, we also discuss the more general scenario where $\Delta\epsilon$ is temperature dependent, which is essential for giving rise to LCST.

We have modified the main text in the discussions of Figure 4 to better explain these mathematical relationships and their necessary assumptions in order to help interpret our simulations. Here is an expert from where we discuss Figure 4:

“The strong dependence of molecular organization on amino acid hydrophobicity suggests that the solvation environment of individual residues might be a determining factor for condensate stability. Indeed, as shown in the Supplemental Theory of the Supporting Information, the critical temperature is closely related to the free energy cost of transferring polymer beads from a solution state to a polymer-only environment. This transfer free energy is often used to quantify the hydrophobicity of amino acids [cite]. To explore their relationship more quantitatively, we compared the transition temperature for ELP condensates measured by Urry [cite] to several hydrophobicity scales.”

Comment 4: To provide a more comprehensive context for the present study, it is useful to compare the microphase separation seen in the authors' simulation with the micelle-like structures observed in recent simulated condensed/aggregated states of hydrophobic-polar (HP) model sequences in Statt et al., J Chem Phys 152, 075101 (2020) [see esp. Fig.6] and Wesseń et al., J Phys Chem B 126, 9222-9245 (2022) [see, e.g., Fig.10].

We thank the reviewer for this suggestion. The results of Statt et al. and Wessen et al. indeed provide a nice comparison to our results. While we capture some of the same behavior they observe, the full array of chemical space in our model seems to give some additional morphologies as well.

First, as predicted by the self-consistent field theory, block copolymers are expected to form primarily lamellar like micelles that clearly separate the dense and dilute phase when the volume fraction, f , is 0.5 (Response to Comment 5). This prediction is indeed consistent with results from simulations with the HP model, and is consistent with our simulations when the substituted amino acid, X , is sufficiently polar.

However, this observation is only one of several behaviors we observe. In particular, our simulations also produce gyroid-like structures, which are predicted to emerge at small volume differences, i.e. $f \approx 0.4$ or $f \approx 0.6$. These different configurations likely emerge due to the more realistic representation of amino acids in our model, which presents more frustration than the HP model. In particular, the backbone atoms are inherently hydrophilic and cannot separate from the hydrophobic side chains. Therefore, under microphase separation, it is inherently difficult to separate the different chemical groups to form lamellar or micelle-like structures. This produces a condensate interior with interfacial properties that may not be captured by the HP model.

We make note of the micelle-like topologies predicted by HP models in the revised text, citing both Statt et al. and Wessen et al.:

“Surprisingly, microphase separation did not produce lamellar morphology as expected for block copolymers with equal volume fraction of the two blocks (Fig. S3 in the Supporting Information) [cite]. In particular, the condensates appear to form gyroid-like structures (Fig. S4 in the Supporting Information), in which the V and X blocks form two interpenetrating networks. This morphology also differs from micelle-like structures seen in simplified hydrophobicpolar (HP) polymers [cite]. It promotes interfacial contacts while maintaining substantial self-interactions as well. Weak interfacial tension between different ELP blocks has also been noted by Hassounah et al.[cite]”

Comment 5: “Gyroid-like morphology” is mentioned several times in the manuscript (p.4, p.8, p.17, Fig.S3). This is apparently an interesting observation, but a clear explanation is lacking. A more detailed and specific discussion, perhaps with additional graphical

presentations, should be provided to demonstrate why the simulated condensed-phase ELP configurations are similar to the classical description of gyroid as in, e.g., Terrones & Mackay, Chem Phys Lett 207, 45-50 (1993) and Lambert et al., Phil Trans R Soc A 354, 2009-2023 (1996).

We thank the reviewer for their comment. Gyroids are canonical structures for diblock copolymers.[5, 7, 8, 9] Their stability is predicted using self-consistent field theory (SCFT), and occurs due to the balance of the volume fraction of polymer block A (f_A), the length of the polymer (N), and the Flory-Huggins interaction parameter (χ).[8, 9] The prediction from SCFT suggests that gyroids occur at smaller values of χN and values f_A near, but not equal to 0.5 (Author response image 2).[10] We hypothesize that these configurations emerge at equal molar fraction of V and X amino acids due to small differences in solvation volume between each half of the polymer chain.

Our support for gyroid-like structures is mainly from observations of two interpenetrating networks formed by the two ELP blocks. We have revised Figure S4 to clearly highlight the two networks as shown in Author response image 3.

We have revised the main text to clearly define the gyroid-like structures as interpenetrating networks, and added the theoretical phase diagram of diblock copolymers predicted by SCFT as Figure S3 in the Supporting Information.

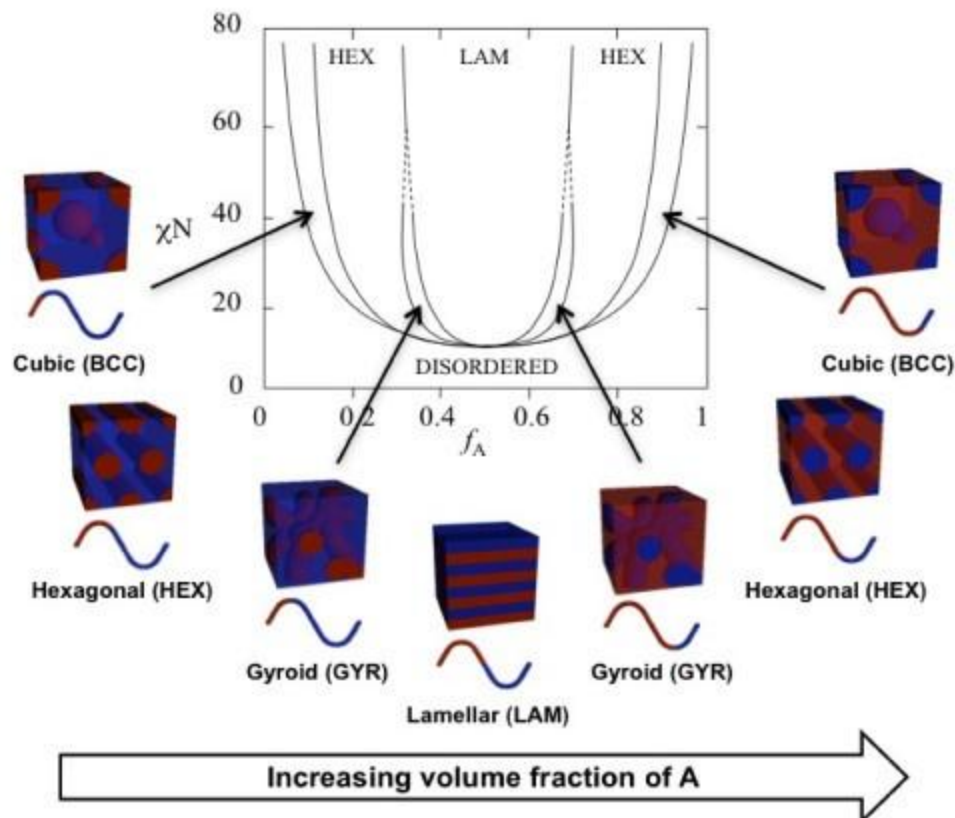
“In particular, the condensates appear to form gyroid-like structures (Fig. S4 in the Supporting Information), in which the V and X blocks form two interpenetrating networks. This morphology also differs from micelle-like structures seen in simplified hydrophobic-polar (HP) polymers [cite]. It promotes interfacial contacts while maintaining substantial self-interactions as well. Weak interfacial tension between different ELP blocks has also been noted by Hassounah et al.[cite]”

We note, however, that proving that our observations are indeed gyroid structures requires more sophisticated mathematical analysis that is beyond the scope of the study. It is also possible that these structures are metastable in our simulations. We emphasize these caveats in the updated Discussion Section.

“Further studies on the thermodynamic stability of these morphologies and comparing them with predictions from the self-consistent field theory shall provide more insights into the driving forces for their emergence [cite].”

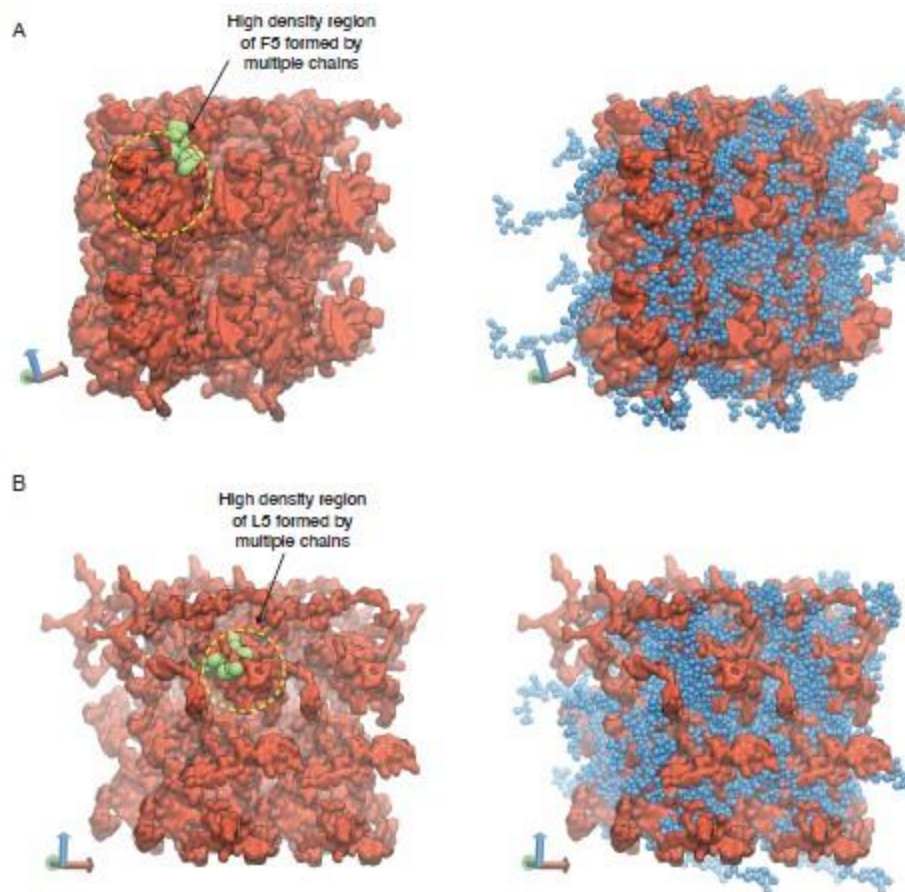
Author response image 2.

Theoretical phase diagram[8] and corresponding morphologies for diblock copolymers. The phases are labeled as: body centered cubic (BCC), hexagonal cylinders (HEX), gyroid (GYR), and lamellar (LAM). f_A is the volume fraction of a single polymer block, denoted A, χ is the Flory-Huggins interaction parameter, and N is the total degree of polymerisation. Figure reproduced from ref.[10] CC BY 4.0.



Author response image 3.

Representative configurations of (A) V5F5 and (B) V5L5 condensates from MARTINI simulations. The valine substituted half of the chain is colored blue (V5) and the X substituted half of the chain is colored red (X5). To highlight the interpenetrating networks formed by the two halves, only the X substituted half of the chain is shown on the left. Simulation interfaces are once repeated periodically in the positive x and positive y dimensions for clarity. High density regions formed by the multiple X substituted half of the chains are highlighted in yellow circles, with one of the chain shown in green.



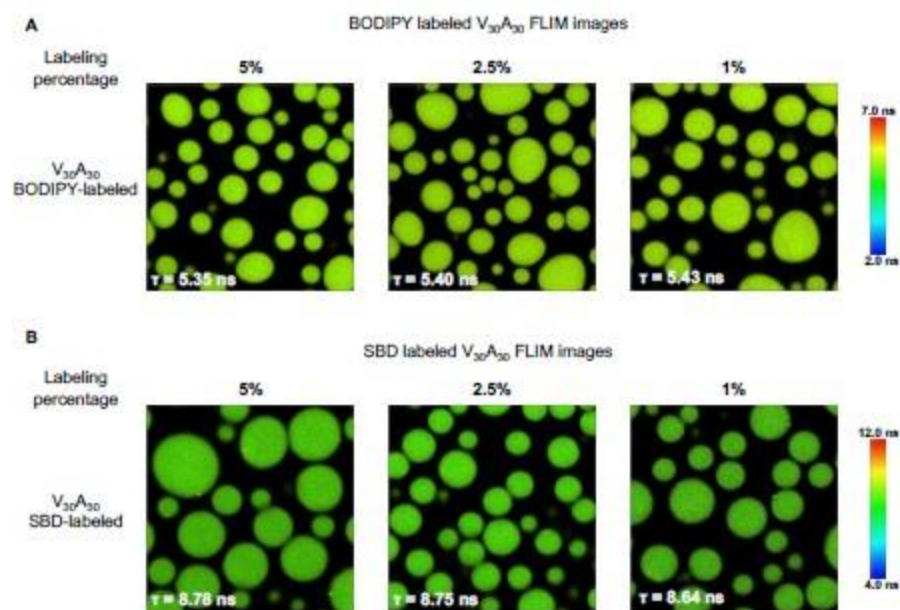
Response to referee 2

Comment 1: The experimental characterization relies on BODIPY and SBD reporting, respectively, on viscosity and polarity. The fluorescent signal of these dyes can possibly depend on many other factors, including quenching. Additional controls are required, or a more extensive discussion with additional references, and a mention to potential limitations of this approach.

We agree with the reviewer that the fluorescence lifetime signal will be affected by many factors. Compared with the fluorescence intensity, the fluorescence lifetime mainly depends on the dyes' self properties and environmental factors. BODIPY and SBD have been used in biological systems to detect the microviscosity and micropolarity of condensates. Our group published the same SBD and BODIPY fluorophores in previous work to quantify the microenvironment of protein aggregation and condensations. The extended data (ChemBioChem 20:1078–1087. doi: 10.1002/cbic.201800782; Aggregate 4:e301. doi:10.1002/agt2.301; Nat Chem Biol 1–9. doi:10.1038/s41589-023-01477-1) shows evidences that the BODIPY is only sensitive to the viscosity while SBD is only sensitive to the polarity, but nonsensitive to other environmental factors. As for the quenched issue, the fluorophores with extended pi-rich structure display aggregation-caused quenching (ACQ) effect in high probe concentration, which will lower the fluorescence lifetime and intensity. We usually labeled the 20% molar ratio of the ELPs using NHS-ester fluorophores to get stock solutions. Due to the labeling efficiency, the exact labeling ratio is much lower than 20%. The labeled ELP stock solution will be further mixed with unlabeled ELP to get ELP solutions with low labeling fractions. We measured the ELPs labeled with a different fraction of dyes. The result shows that only BODIPY performs slight ACQ phenomena at a high

Author response image 4.

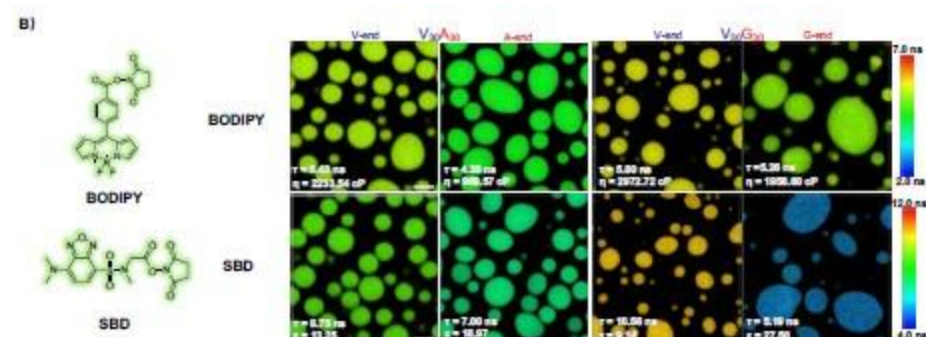
FLIM images of ELP condensates labeled with different fractions of dyes. A) FLIM images of V30A30 condensates with 5%, 2.5%, and 1% BODIPY labels. B) FLIM images of V30A30 condensates with 5%, 2.5%, and 1% fraction of SBD. Droplets were formed with a final concentration of 70 μ M ELP labeled with different fractions of BODIPY or SBD in 2 M NaCl solution. Scale bar: 5 μ m.



To mostly avoid the potential ACQ effect and achieve enough fluorescence signals, we finally use the ELP labeled with a lower fraction of dyes, 1% of BODIPY and 2.5 % of SBD, to perform the FLIM experiments. The data in Figure 3 will be corrected with the following data.

Author response image 5.

Structures of NHS-BODIPY and NHS-SBD, and representative FLIM images of V30A30, A30V30, V30G30 and G30V30 labeled with respective fluorophores. The fluorescence lifetime of each image is the average acquired from three independent experiments. Scale bar: 5 μ m.



We revised the text in the section Microphase separation of ELP condensates as follows “To experimentally test the microphase separation behavior uncovered in simulations, we studied the micro-physicochemical properties of the V-end and X-end of the peptides. We

constructed diblock peptides with the combination of 30 pentameric repeats of V block and X (A or G) block, namely V30A30 and V30G30 (Experimental Sequences Section in the Supporting Information). The amino-termini of V30A30 and V30G30 sequences were subsequently labeled with environmentally sensitive BODIPY or SBD fluorophores [cite], whose lifetime could be measured to quantify the viscosity or polarity of the V-end (Fig. 3A, left panel) [cite]. These probes have been reported to be only sensitive to single physicochemical properties.[cite] To avoid artifacts induced by fluorophore labeling, we usually used ELPs labeled with a low fraction of dyes. We also constructed A30V30 and G30V30 diblock peptides, wherein the viscosity or polarity of the A-end or the G-end could be measured by fluorophores that are attached at the amino-terminus (Fig. 3A, right panel). Using FLIM, we found that the lifetime of BODIPY for the V-end (5.43 ns) was longer than that for the A-end (4.35 ns), suggesting that the V-end indeed has a higher microviscosity than the A-end ($\eta_V = 2233.54$ cp vs $\eta_A = 969.57$ cp). Accordingly, the lifetime of SBD was longer for the V-end (8.75 ns) than the A-end (7.00 ns), indicating that the micropolarity of the V-end was lower than the A-end ($\epsilon_V = 13.25$ vs $\epsilon_A = 18.97$). These observations could be largely attributed to the greater extent of dehydration at the V-end due to its higher local peptide density. We further showed that the observed differences are not results of possible artifacts arising from any subtle distinctions between the two sequences V30A30 and A30V30 (Experimental Characterization of ELP Condensates Section in the Supporting Information, Fig. S8-S9 in the Supporting Information). Similar results were observed using the V-G sequences. FLIM experiments revealed that the V-end was more viscous than the G-end ($\eta_V = 2972.72$ cp vs $\eta_G = 1958.60$ cp) and the V-end was less polar than the G-end ($\epsilon_V = 9.14$ vs $\epsilon_G = 27.50$). These experimental observations provided the first line of evidence to support the microphase separation, as suggested by the simulation results.”

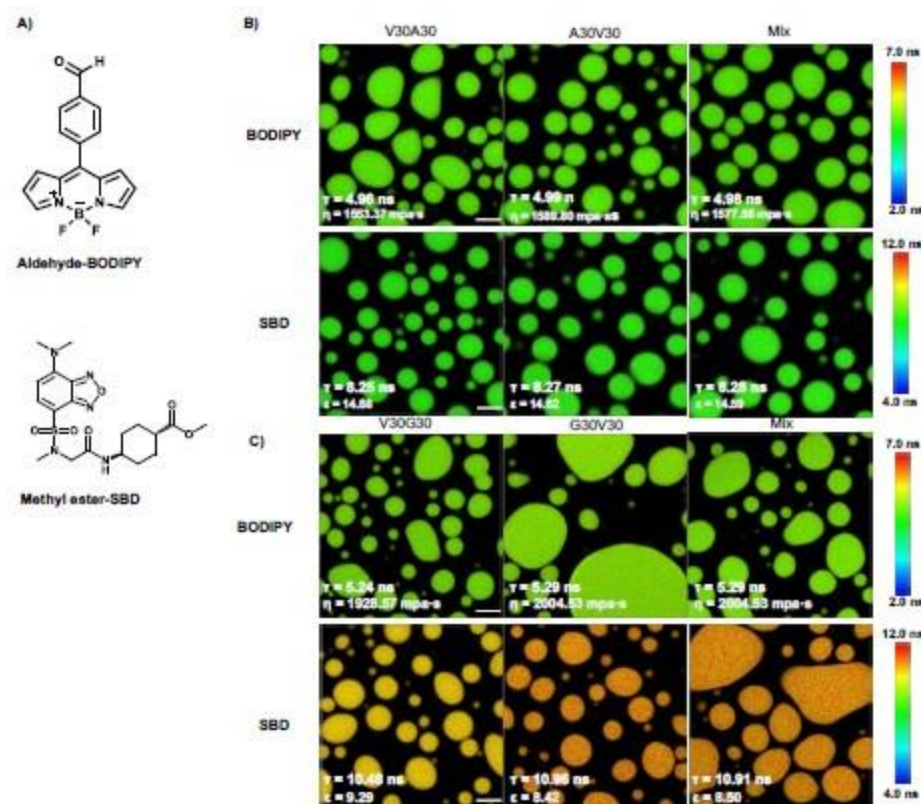
We revised the text in the section Experimental methods as follows

“The proteins of interest were labeled with NHS ester fluorophore. We used ELPs with 1% BODIPY labels or 2.5% SBD labels to form condensates, which avoid the artifacts induced by fluorophores. Droplets were formed with the final concentration of 70 μ M ELP in 2 M NaCl for V-A and 1.5 M NH_4SO_4 for V-G diblock, respectively. A drop of droplets containing solution was placed on a 0.17 mm coverslip with a 500 μ m spacer. Images were acquired by Leica Falcon Fluorescence Microscope equipped with WIL pulse laser and 63X/0.12 oil-immersion objective. The BODIPY was excited at 488 nm and the SBD was excited at 448 nm. The fluorescence lifetime fitting and image analysis were performed in LAS X and Image J.”

We also used a lower concentration of free dyes to remeasure the properties of the ELP condensates. The Figure S9 data are corrected as follows. The slight differences between the results are caused by experimental errors, which don't affect the conclusion.

Author response image 6.

FLIM image of unlabeled ELP condensates. A) Chemical structure of free fluorophore, which can measure the physicochemical properties of condensates without labeling. B) Representative FLIM images of V30A30 and A30V30. The mix is the mixture of V30A30 (35 μ M) and A30V30 (35 μ M). Droplets were formed with a final concentration of 70 μ M ELP in 2 M NaCl solution with 1 μ M fluorophore. C) Representative FLIM images of V30G30 and G30V30. Droplets were formed with a final concentration of 70 μ M ELP in 1.5 M $(\text{NH}_4)_2\text{SO}_4$ solution with 1 μ M fluorophore. The mix is the mixture of V30G30(35 μ M) and G30V30 (35 μ M). Scale bar, 5 μ m. The fluorescence lifetime of each image is the average from three independent measurements.



We also revised the Sequence dependence of micro-viscosity and polarity section of the Supporting Information as follows

“Since we used V30X30 and X30V30 to quantify the V- and X-end of the V-X blocks, it is possible that the observed differences arose from the innate property of the V30X30 and X30V30 sequences. To rule out this artifact, we formed the ELP condensates with sequences of V30X30, X30V30, or the V30X30 and X30V30 mixture. The condensates were subsequently treated with the aldehydeBODIPY and methyl-ester SBD fluorophores without the NHS ester reactive warhead (Fig. S9A in the Supporting Information). After brief incubation, aldehyde-BODIPY and methyl-ester SBD fluorophores were recruited into and homogeneously distributed in the ELP condensates. The fluorescence lifetime of aldehyde-BODIPY was the same for V30A30 (4.96 ns), A30V30 (4.99 ns), and their mixture (4.98 ns) (Fig. S9B in the Supporting Information, upper panel). Interestingly, this value is around the average (4.89 ns) of the A-end (4.35 ns) and the V-end (5.43 ns) labeled NHS-BODIPY. For the SBD measurement, methyl-ester SBD resulted in almost identical lifetime values of V30A30 (8.25 ns), A30V30 (8.27 ns), and their mixture (8.28 ns) (Fig. S9B in the Supporting Information, lower panel), again around the average values (7.88 ns) of the A-end (7.00 ns) and the V-end (8.75 ns) labeled NHS-SBD. In addition to the V-A blocks, similar observations were made for the V-G blocks as V30G30 and G30V30 sequences (Fig. S9C in the Supporting Information). The slight difference between the results is attributed to the experiment errors. Because the fluorophores did not covalently label the amino-terminus of the ELP peptides, their lifetime reports closer to the averaged property of the condensates instead of the microscopic property of the V-end or the X-end when the number of molecules is sufficient and the molecular distribution has no preference.

Our results reveal that the V30X30 and X30V30 condensates exhibited similar macroscopic viscosity or polarity, suggesting that the previously observed different viscosity or polarity of V30X30 and X30V30 could be attributed to the microscopic property of the V-end or X-end.”

The FLIM technique combined with environment-sensitive fluorophores is a powerful tool for us to investigate the physicochemical properties of the microenvironment within the condensates. However, there are some limitations to this method. As the fluorophore is labeled in the protein, we can only detect the microenvironment surrounding the surface of the probe(the distance may be angstrom level). The fluorescence signal values we got are the statistical average of the fluorescence signals from the complex microenvironments. The signal from the probes is determined by the sampling position, orientation, and number of fluorescent probes. So the quantified values can be compared relatively, but these values can not accurately describe the physical or chemical states in different systems. In addition, the resolution in FLIM experiments is not enough to directly distinguish the microstructure in condensates.

Comment 2: It is unclear if, after the application of stretching, the micro-structure will eventually return to the original configuration or not. Overall, the point of this experiment remains somewhat unclear.

We thank the reviewer for this comment. The ELP condensates are actually viscous fluids and they could coalesce into larger droplets within seconds. Due to the high viscosity, ELP condensates show slow fluorescence recovery after photobleaching. As stretching the condensates, the micro-structure of condensates changes to show a response to the outer force. The fluorophores may be pulled out from the microenvironment. For such a dynamic system, we speculate that the microstructure will return to the original after the condensation system equilibrium, which may be a long process. However, it is hard to characterize whether these microstructures have completely returned to their original positions. The purpose of this experiment is to show the microenvironment properties of each terminal in another aspect. The experiment also shows evidence that the microenvironment around the V terminus is more dense than the A terminus.

Comment 3: The title is too generic and does not reflect the content of the work. There is no analysis of biological condensates. The results are specific to di-block polypeptides with specific sequences. This should be clearly specified in text and title.

We have revised the title to "Microphase Separation Produces Interfacial Environment within Diblock Biomolecular Condensates"

Comment 4: MD is out of the expertise of this reviewer. However, when looking at the density profiles (Figure S2), the simulation does not seem to be fully converged. The densities fluctuate inconsistently along the Z direction. The authors should comment on assessing simulation convergence. In many cases, the section used for the density values in the plot (i.e., below 0.06 box lengths away from the condensate center) does not seem representative of the dense phase. It should be justified, why these simulations can still be used for density/hydrogen bonding analysis.

We thank the reviewer for their comment, and agree that convergence of MD simulations is simultaneously important and difficult to control for. To demonstrate the convergence of our simulations, we have taken an example system (V5F5) and reproduced the density profile in 4 unique time windows of 50 ns each (Author response image 7A-D). We find that all distributions are nearly identical, indicating that further extending these simulations is unlikely to change our findings.

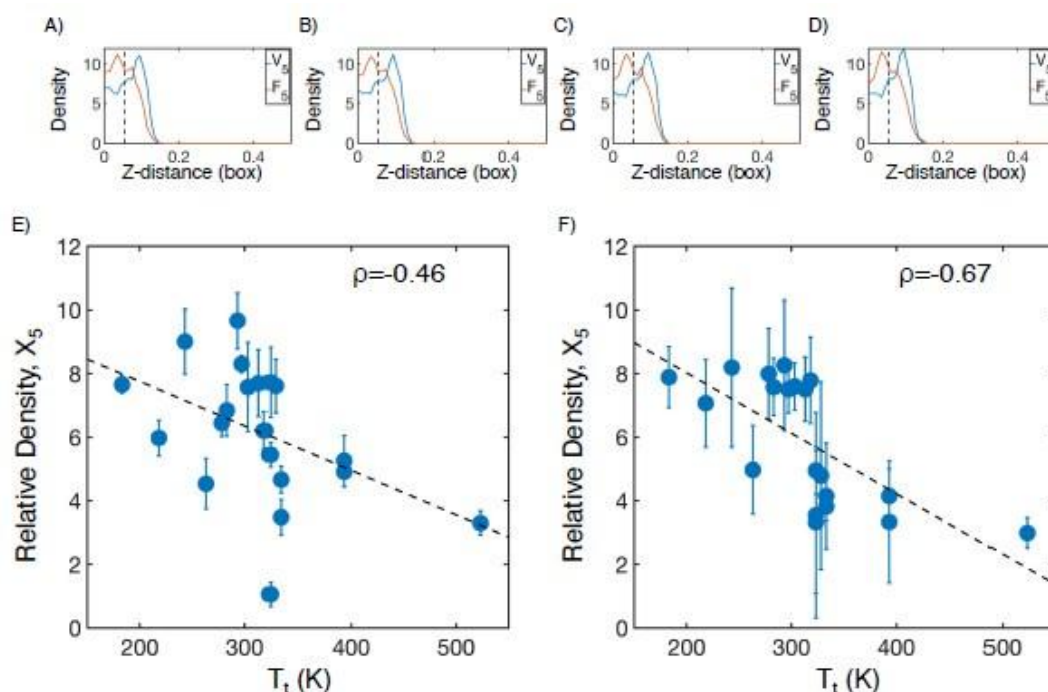
While we agree that the choice of 0.06 box lengths is arbitrary, it was chosen as an approximation for the interior of the condensate, where the more hydrophobic half of the protein chain tends to be at higher concentration. However, this choice is not important to our overall conclusion. Halving (Author response image 7E) or doubling (Author response

image 7F) the cutoff maintains the inverse correlation between the protein density of the X5 half of the condensate and experimental transition temperature.

Finally, in our multiscale simulation approach, the all-atom portion of the simulation is mostly used to examine water structure and protein solvation. We can see that dividing the simulation into four independent time estimates does not substantially change these properties, resulting in low standard deviations in Figure 5 and Figure 6. Similarly, our previous work on the dielectric of ELP condensates has shown that choosing different starting structures from MARTINI simulations is unlikely to effect the estimate of similar quantities.[11]

Author response image 7.

Checking convergence of all-atom simulations of ELP condensates. (A-D) The relative mass density along the Z-distance from the condensate center is shown for the V-substituted and X-substituted halves of V5F5 in four independent time windows of 50 ns each. The Z-axis is defined as the direction perpendicular to the condensate-water interface. The dashed line represents a Z-distance of 0.06 box lengths away from the condensate center, which was the original cutoff for correlation analysis. E-F) Correlation between the mass fraction of the X5 half of the condensate and transition temperature (T_t) from Urry.[12] The condensate is defined as having a Z-distance of 0.03 box lengths (E) or 0.12 box lengths (F) away from the condensate center. ρ is the Pearson correlation coefficient between the two data sets, and the dashed diagonal line is the best fit line. Error bars represent standard deviations of the mean taken over box length intervals of 0.01.



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