

1 **Amphiphilic proteins coassemble into multiphasic condensates** 2 **and act as biomolecular surfactants**

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11 **Abstract**

13 Cells contain membraneless compartments that assemble due to liquid-liquid phase separation,
14 including biomolecular condensates with complex morphologies. For instance, certain condensates are
15 surrounded by a film of distinct composition, such as Ape1 condensates coated by a layer of Atg19, required
16 for selective autophagy in yeast. Other condensates are multiphasic, with nested liquid phases of distinct
17 compositions and functions, such as in the case of ribosome biogenesis in the nucleolus. The size and
18 structure of such condensates must be regulated for proper biological function. We leveraged a bio-inspired
19 approach to discover how amphiphilic, surfactant-like proteins may contribute to the structure and size
20 regulation of biomolecular condensates. We designed and examined families of amphiphilic proteins
21 comprising one phase-separating domain and one non-phase separating domain. In particular, these
22 proteins contain the soluble structured domain glutathione S-transferase (GST) or maltose binding protein
23 (MBP), fused to the intrinsically disordered RGG domain from P granule protein LAF-1. When one
24 amphiphilic protein is mixed in vitro with RGG-RGG, the proteins assemble into enveloped condensates,
25 with RGG-RGG at the core, and the amphiphilic protein forming the surface film layer. Importantly, we found
26 that MBP-based amphiphiles are surfactants and control droplet size, with increasing surfactant
27 concentration resulting in smaller droplet radii. In contrast, GST-based amphiphiles at increased
28 concentrations co-assemble with RGG-RGG into multiphasic structures. We propose a mechanism for
29 these experimental observations, supported by molecular simulations of a minimalist model. We speculate
30 that surfactant proteins may play a significant role in regulating the structure and function of biomolecular
31 condensates.

32

33 Introduction

34 The intracellular environment is like a complex emulsion. This paradigm originated more than a
35 century ago but is enjoying a renaissance, with recent discoveries revealing the important role of liquid-
36 liquid phase separation (LLPS) in biology ¹⁻³. LLPS of proteins and nucleic acids underlies the formation of
37 membraneless organelles, alternatively called biomolecular condensates, which are distinct intracellular
38 compartments that lack a delimiting membrane ^{2,3}. Biomolecular condensates contribute to numerous cell
39 functions, including stress response, gene regulation, and signaling ⁴. Conversely, aberrant phase
40 separation due to mutations and age-related processes is implicated in diseases such as
41 neurodegeneration and cancer ⁵. Deciphering the rules of self-assembly of biomolecular condensates has
42 therefore emerged as a promising avenue for elucidating fundamental principles of biological structure,
43 function, and dysfunction.

44 Despite significant recent progress in understanding the biophysics of biomolecular condensates,
45 many open questions remain ⁶. One key question is what molecular phenomena govern the spontaneous
46 assembly of condensates with core-shell or multiphasic structures. Another important question is how cells
47 tune the size of biomolecular condensates. Here, we sought to gain insight into both questions by examining
48 how amphiphilic, surfactant-like proteins contribute to the self-assembly and regulation of biomolecular
49 condensates. Amphiphiles are typically defined as molecules comprising separate hydrophilic and
50 hydrophobic parts. Here, we note the etymology (in Greek, “amphi” means both and “philia” means
51 friendship or love) and use the term amphiphile to describe proteins comprising one domain that has affinity
52 for biomolecular condensates and one domain that has affinity for the dilute phase.

53 Surfactants are substances, generally amphiphiles, that adsorb to interfaces and decrease
54 interfacial tension. Extracellularly, pulmonary surfactant lining the alveoli plays a vital role in lung physiology
55 by reducing the work of breathing ^{7,8}. However, the role of surfactants in the emulsion-like intracellular milieu
56 is just beginning to be explored ⁹. In biological systems, the surfactant-like protein Ki-67 prevents individual
57 chromosomes from coalescing during early stages of mitosis by forming a repulsive molecular brush layer
58 ¹⁰. Some biomolecular condensates are reminiscent of surfactant-laden emulsions, although their physical
59 chemistry remains to be elucidated. For instance, Atg19 forms a thin surface layer surrounding Ape1
60 condensates that is necessary for selective autophagy of Ape1 in yeast ¹¹. Inspired by such examples, we

61 hypothesized that a minimal system comprising surfactant-like proteins interacting with phase-separating
62 proteins could recapitulate enveloped condensate structures observed in nature.

63 Moreover, condensates exhibit a variety of multiphase and multilayer structures underpinning their
64 biological functions. Bre1 assembles as a shell surrounding Lge1 condensates, generating a catalytic
65 condensate that functions to accelerate ubiquitination of histone H2B in yeast ¹². The nucleolus is comprised
66 of coexisting liquid phases of differing interfacial tensions ¹³, while P granules contain coexisting liquid and
67 gel phases ¹⁴. Stress granules ¹⁵, nuclear speckles ¹⁶, paraspeckles ¹⁷, and reconstituted polypeptide/RNA
68 complex coacervates also exhibit core-shell structures sensitive to stoichiometry and competitive binding
69 ¹⁸⁻²¹. Functionally related condensates can remain in contact without coalescing, as in the case of stress
70 granules and P-bodies ²², or P granules and Z granules ²³. We asked whether amphiphilic proteins could
71 contribute to the complex morphologies of biomolecular condensates that have been observed within cells,
72 just as synthetic amphiphiles and surfactant systems exhibit rich structures and phase behaviors ²⁴.

73 Surfactant-like proteins could have additional important functional consequences, including but not
74 limited to modulating biomolecular condensate size, which in turn influences biochemical processes
75 through condensate size-dependent effects on molecular concentrations and diffusion ^{25,26}. Biomolecular
76 condensates are often observed in cells as multiple smaller droplets, rather than as a single larger droplet,
77 even though the latter is expected to be thermodynamically favored. Recent studies have attributed the
78 apparent metastability of biomolecular condensates in various contexts to surface charge ²⁷, cytoskeletal
79 caging ^{28,29}, membrane association ³⁰, and exhaustion of available binding sites ³¹; active processes can
80 also maintain the emulsified, multi-droplet state in vivo ³². An additional possibility, which we examine here,
81 is that surfactant proteins may help stabilize biomolecular condensates.

82 To address these questions, we adopted a bottom-up, bioinspired approach, seeking to leverage
83 a simplified system to shed light on the role of amphiphilic proteins in the self-assembly of biomolecular
84 condensates. We designed amphiphilic proteins containing an intrinsically disordered region (IDR) fused to
85 folded domains. The IDR is a phase-separating domain, whereas the folded domains are not. When one of
86 these amphiphilic fusion proteins is mixed at low concentrations with the IDR alone, the two proteins
87 assemble such that the amphiphilic protein forms a film that coats the IDR core. We demonstrate several
88 extensions of this observation, including the important finding that condensate size can be controlled by

89 varying surfactant protein concentration. Furthermore, when amphiphilic proteins with different folded
90 domains are mixed together with the core IDR, we observe competition between different surfactant
91 proteins for binding to the condensate interface. Interestingly, one family of amphiphilic proteins exhibits
92 varied morphologies, including multiphasic condensates, and we map the rich concentration-dependent
93 phase behavior. To gain mechanistic insight into these experimental observations, we present a
94 minimalistic computational model that recapitulates the range of behaviors observed experimentally by
95 varying the strength of interaction between domains. Our experiments and simulations suggest that
96 amphiphile-condensate assembly is determined by the strength of interaction between the amphiphile and
97 the IDR core, as well as interactions between the folded domain of the amphiphile. Taken together, this
98 work illustrates the diverse interfacial phenomena that can arise from interactions between condensates
99 and amphiphilic proteins, notably raising the possibility that surfactant proteins may play a significant role
100 in regulating the structure and function of biomolecular condensates.

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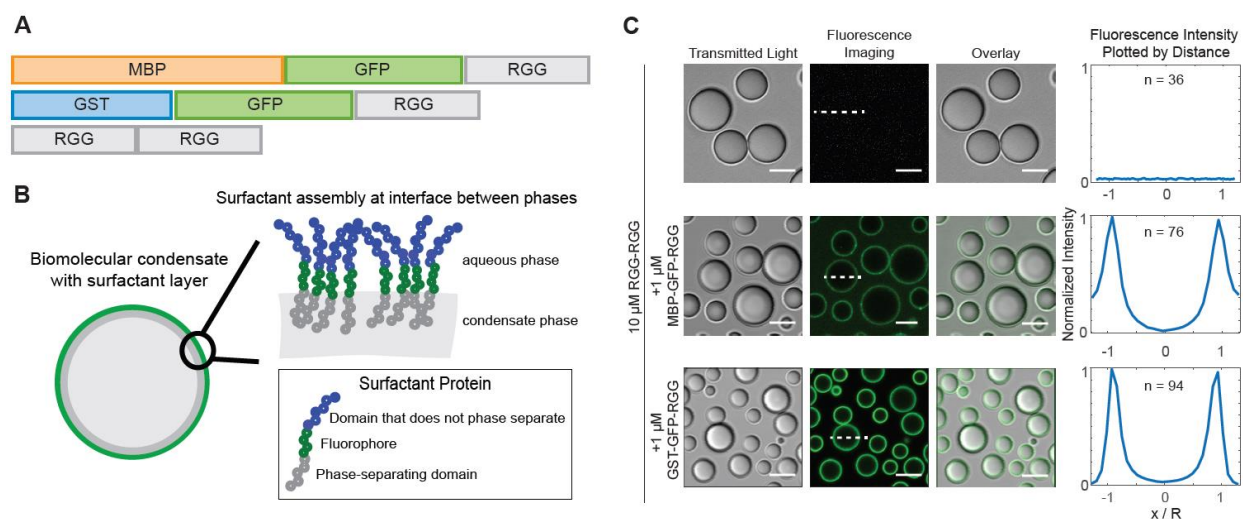
102 **Results**

103 **Designing Amphiphilic Proteins to Act as Biological Surfactants**

104 In prior work, we examined the partitioning of client proteins into biomolecular condensates³³. We
105 focused on the RGG domain from LAF-1, a prototypical arginine/glycine-rich intrinsically disordered protein
106 (IDP) involved in P granule assembly in *C. elegans*^{34–36}. Tandem repeats of the RGG domain (RGG-RGG)
107 phase separate in vitro at concentrations >1 μ M under physiological conditions. We found that RFP and
108 GST-RFP are excluded from RGG-RGG condensates; RFP denotes red fluorescent protein and GST
109 denotes glutathione S-transferase, which is widely used as a solubility-enhancing affinity tag in recombinant
110 protein production³⁷. In contrast, we observed that RFP fused to one or two RGG domains partitioned into
111 RGG-RGG condensates. These results demonstrated that client partitioning into or exclusion from RGG-
112 RGG condensates depends on the balance between the “RGG-philic” and “RGG-phobic” content of the
113 client protein. Here, we hypothesized that a third, intermediate outcome is possible: that with the right
114 balance, a client protein may localize to the interface between the condensate and dilute phases, coating
115 the condensate and displaying surfactant-like behavior.

116 We therefore asked whether we could engineer amphiphilic proteins that assemble as a film on the

117 surface of RGG-RGG condensates. We designed a family of amphiphilic fusion proteins containing a
118 phase-separating domain (RGG-philic) fused to a non-phase separating domain (RGG-phobic) (SI
119 Appendix Fig. 1). Two representative protein constructs from this family are MBP-GFP-RGG and GST-
120 GFP-RGG (Fig. 1A). MBP denotes maltose binding protein, which like GST is a well-known affinity tag that
121 enhances protein solubility^{37–40}. GFP denotes enhanced green fluorescent protein with the monomerizing
122 A206K mutation. By combining RGG, GST, MBP, and fluorescent protein domains, our aim was to generate
123 amphiphilic fusion proteins with RGG-philic and RGG-phobic parts. We hypothesized that upon mixing the
124 amphiphilic proteins with RGG-RGG, the poorly soluble RGG domain in the amphiphiles would interact with
125 and orient towards the inner RGG-RGG condensate phase, while the RGG-phobic MBP and GST domains
126 would interact with and orient towards the outer dilute phase (Fig. 1B).



127

128 **Figure 1: Amphiphilic proteins MBP-GFP-RGG and GST-GFP-RGG form a film around RGG-RGG.**
129 **A**, Schematic domain diagram for MBP-GFP-RGG, GST-GFP-RGG, and RGG-RGG. Length is
130 proportional to number of base pairs. **B**, Schematic model of the interaction between amphiphilic proteins
131 and RGG-RGG. The amphiphilic protein is depicted at the interface between the two phases, surrounding
132 the RGG-RGG core, with the phase-separating RGG domain facing the RGG-RGG core and the non-
133 phase-separating GST or MBP domain facing the aqueous phase. **C**, Transmitted light and fluorescence
134 imaging of the film formed by mixing RGG-RGG and MBP-GFP-RGG or GST-GFP-RGG in a 10:1
135 concentration ratio (10 μ M RGG-RGG, 1 μ M MBP-GFP-RGG or GST-GFP-RGG). For this and
136 subsequent figures, buffer was 150 mM NaCl, 20 mM Tris, pH 7.5. Right: Fluorescence intensities were
137 quantified as line profiles across individual condensates, which were normalized and averaged. n, number
138 of condensates. Scale bars, 5 μ m.
139

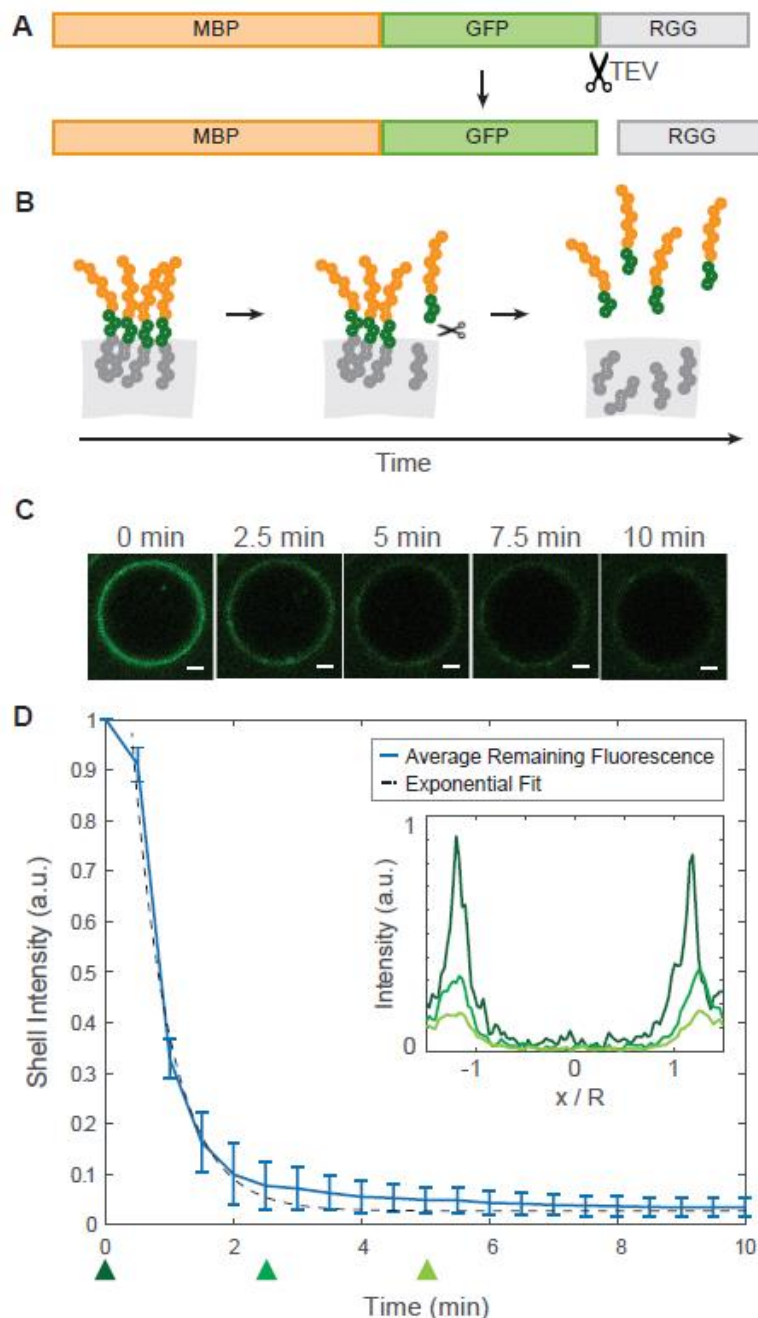
140 Amphiphilic Proteins Coat Biomolecular Condensates, Resembling Surfactants

141 To test our hypothesis, we mixed RGG-RGG with the amphiphilic proteins GST-GFP-RGG or MBP-
142 GFP-RGG in physiological buffer and used microscopy to observe the resulting assemblies. In these binary

143 mixtures, RGG-RGG phase separated into spherical protein droplets, as expected. Remarkably, MBP-
144 GFP-RGG formed green rings around the RGG-RGG condensates, as observed by confocal fluorescence
145 microscopy (Fig. 1C). Similarly, GST-GFP-RGG mixed with RGG-RGG formed green rings around the
146 RGG-RGG condensates. We analyzed fluorescence intensity line profiles drawn radially across
147 condensates in the confocal images, showing localization of GFP mainly at the condensate surface, as
148 opposed to within or outside the condensates (Fig. 1C). Quantification of the partitioning of both surfactant-
149 like proteins indicates a subtle difference. While GST-GFP-RGG localized almost entirely to the interface,
150 MBP-GFP-RGG exhibits an elevated concentration in the dilute phase (ratio of concentrations at interface
151 : condensate : buffer for GST-GFP-RGG is 1 : 0.05 : 0.01 and for MBP-GFP-RGG is 1 : 0.05 : 0.3). This
152 suggests a potentially higher affinity between the GST-GFP-RGG protein and RGG-RGG condensate when
153 compared to MBP-GFP-RGG. In both cases, these data demonstrate that the amphiphilic proteins adsorb
154 to and form a layer at the condensate surface, reminiscent of surfactant-stabilized emulsions. This
155 surfactant-like assembly was observed even upon shuffling the sequence of the RGG domains used to
156 form the droplet core, suggesting that the adsorption does not depend on the precise sequence of the
157 phase-separating IDP (SI Appendix Fig. 2).

158 To confirm the mechanism by which the amphiphilic proteins adsorb to the interface, we examined
159 how the fluorescence localization changes when the RGG-philic and RGG-phobic regions of the amphiphilic
160 protein are uncoupled. We therefore included a tobacco etch virus (TEV) protease recognition site
161 immediately N-terminal to the RGG domain of MBP-GFP-RGG, so that upon treatment with TEV protease,
162 MBP-GFP would be liberated from RGG (Fig. 2A). We hypothesized that when TEV protease is added to
163 RGG-RGG condensates enveloped by an MBP-GFP-RGG film, the cleaved RGG domain from the
164 surfactant protein would partition into the RGG-RGG condensates, whereas the MBP-GFP would partition
165 into the surrounding dilute phase, so the fluorescence intensity of the ring would decrease (Fig. 2B). Indeed,
166 using time-lapse confocal microscopy, we observed the intensity of the fluorescent ring rapidly diminish
167 following TEV protease addition (Fig. 2C-D). The fluorescence intensity of the rings, $f(t)$, decays
168 approximately exponentially with time. The data is consistent with first-order kinetics, $f(t) = a * e^{-k*t}$,
169 which is expected for an enzyme-catalyzed reaction in which the enzyme is not saturated by the substrate
170 ([GST-GFP-RGG] of 1 μ M \ll Michaelis constant K_m of TEV \sim 61 μ M⁴¹). As a control, we performed the

171 same experiment without TEV protease to account for photobleaching and observed only slow, linear
172 decrease in fluorescence intensity (SI Appendix Fig. 3). Collectively, these results support the model that
173 MBP-GFP-RGG and related proteins localize to the condensate interface due to their amphiphilic nature.



174

175 **Figure 2: Desorption of surface-associated amphiphilic protein triggered by proteolytically**
176 **cleaving the dissimilar domains. A**, Schematic depicting the location of the TEV protease cleavage site
177 inserted into MBP-GFP-RGG, between the GFP and RGG domains. **B**, Schematic model of the result of
178 adding TEV protease to the RGG-RGG and MBP-GFP-RGG system. Cleavage of the amphiphilic protein
179 results in the release of the fluorescently tagged MBP domain into the aqueous solution and retention of

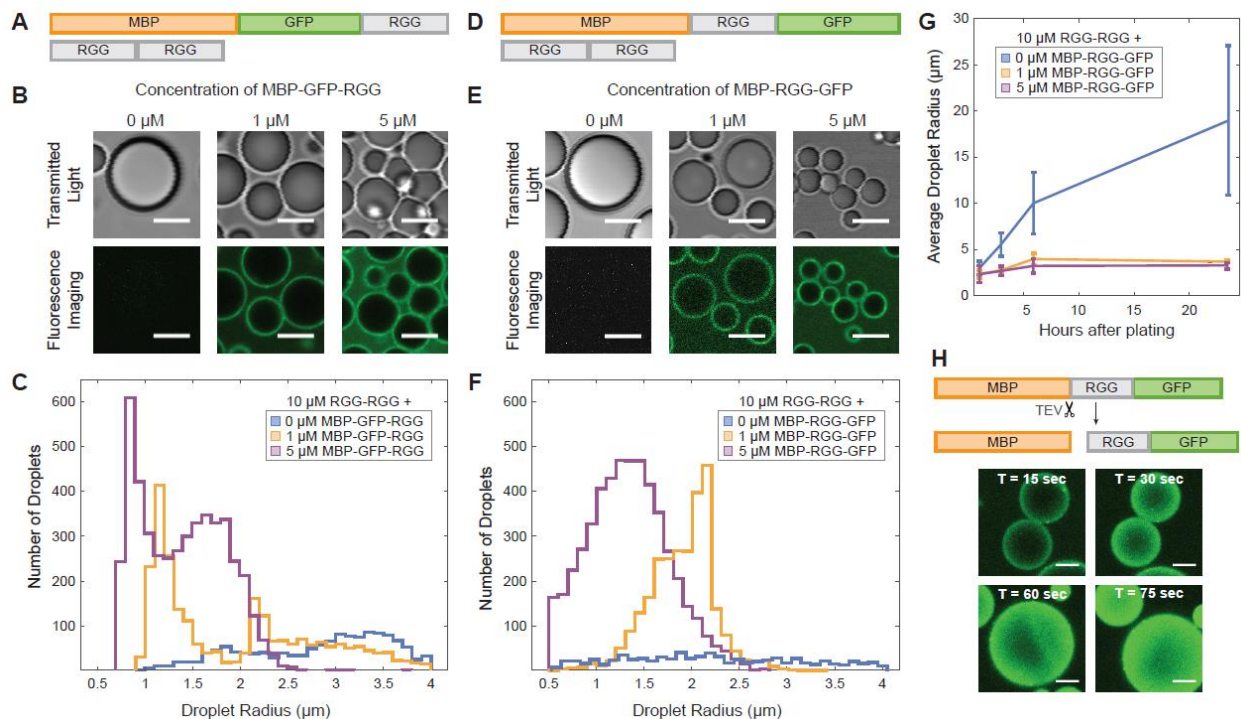
180 the RGG domain by the condensed phase. **C**, Representative droplet images at 0, 2.5, 5, 7.5 and 10
181 minutes after TEV protease addition. Scale bars, 1 μm . **D**, Total fluorescence intensity of the shell region
182 decreases over time with the release of MBP-GFP following cleavage of MBP-GFP-RGG by TEV
183 protease, which was added at $t = 0$ (plot shows average of $n = 87$ droplets). Inset, normalized average
184 fluorescence intensity line profiles at time 0 (dark green), 2.5 minutes (medium green) and 5 minutes (light
185 green). 5 mM DTT was added to the buffer in accordance with recommended TEV protease protocols.
186

187 **Surfactant-like Proteins Control Condensate Size**

188 In conducting the aforementioned TEV protease experiments, we observed that removing the
189 protein film from around the RGG-RGG condensates triggered droplet fusion events. We sought to further
190 understand this result, asking whether the amphiphilic proteins can control condensate size by acting as
191 biological surfactants and stabilizing condensates against fusion. We therefore measured droplet sizes
192 upon mixing RGG-RGG with various concentrations of the amphiphilic protein MBP-GFP-RGG (Fig. 3A).
193 As the concentration of MBP-GFP-RGG increased, the droplet sizes decreased significantly, as observed
194 by brightfield and confocal microscopy (Fig. 3B). This would be expected for surfactants in a surfactant-
195 poor regime, where there is no excess of emulsifier ⁴². Since the condensates displayed a wide range of
196 sizes for any given MBP-GFP-RGG surfactant concentration, we plotted histograms of droplet size, further
197 revealing a shift from larger condensates in the absence of surfactant to numerous smaller condensates as
198 surfactant concentration increases (Fig. 3C). Interestingly, size distribution appears bimodal in several
199 conditions, which is reminiscent of observations in nanocrystal growth where the smaller-size mode
200 corresponds to primary nucleation, and the larger-size mode to the nanoparticles growing by aggregation
201 ⁴³. Total condensate volume is approximately conserved across samples with differing surfactant
202 concentrations ($\pm 19\%$), supporting the theory that surfactant proteins do not inhibit phase separation but
203 rather stabilize droplet size. Motivated by studies on synthetic triblock polymer surfactants, comprised of a
204 hydrophobic block sandwiched between hydrophilic blocks, we wondered how our results may differ if the
205 RGG domain were placed as the middle block of a surfactant protein. We therefore switched the order of
206 the fluorescent and phase-separating domains, forming MBP-RGG-GFP. We confirmed that MBP-RGG-
207 GFP also coats RGG-RGG condensates, appearing as fluorescent rings under confocal microscopy.
208 Similar to MBP-GFP-RGG, increasing concentrations of MBP-RGG-GFP resulted in decreased RGG-RGG
209 droplet size (Fig. 3D-F).

210 Using this system, we also measured how RGG-RGG droplet size changes over time with or

211 without MBP-RGG-GFP surfactant. Without a stabilizing mechanism, and provided that droplets do not age,
212 condensates would be expected to grow over time due to coalescence and Ostwald ripening ⁴⁴. We
213 examined droplet size at timepoints of 1, 3, 6, and 24 hours, finding that without surfactant, average droplet
214 size indeed grew significantly over time (from average radius of 3.0 μm at hour 1 to 19.0 μm at hour 24). In
215 stark contrast, we observed only a slight size increase for the surfactant-coated condensates (from average
216 radius of 2.3 μm at hour 1, to 3.7 and 3.3 μm at hour 24, for 1 μM and 5 μM MBP-RGG-GFP, respectively).
217 These data illustrate the lasting stabilizing effect of the surfactant layer (Fig. 3G). To explore the implications
218 of these results, we further examined how droplet size changes upon triggered removal of the surfactant
219 layer. We added TEV protease to RGG-RGG condensates coated with MBP-x-RGG-GFP, where x denotes
220 a TEV protease recognition sequence, and we measured the change in cross-sectional area of the
221 condensates over time. When the TEV protease cut site is placed such that MBP is liberated alone and
222 GFP remains attached to RGG, TEV protease treatment causes the green fluorescence to relocalize from
223 the interface to the interior of the condensates (Fig. 3H). Strikingly, removal of the surfactant layer triggered
224 rapid fusion events, leading to a significant increase in droplet size. A majority of fusion events and size
225 increase occurred within the first five minutes after TEV addition (SI Appendix Fig. 4), after which droplet
226 size stabilized. As a control, we added TEV to a sample containing only RGG-RGG. Though scattered
227 fusion events occurred due to the creation of flow, the average droplet size did not change significantly.
228 Overall, our results suggest that condensate size can be controlled by surfactant proteins, dependent on
229 surfactant concentration and tunable via stimuli that manipulate the interfacial properties.



230

231 **Figure 3: Amphiphilic proteins MBP-GFP-RGG and MBP-RGG-GFP act as surfactants by stabilizing**
 232 **small condensates.** **A**, Domain schematics depicting surfactant protein MBP-GFP-RGG and core protein
 233 RGG-RGG. **B**, Transmitted light and confocal fluorescence imaging of the film formed by mixing RGG-
 234 RGG (10 μM) with different concentrations of MBP-GFP-RGG. Droplet size becomes smaller as MBP-
 235 GFP-RGG concentration increases. Scale bars, 5 μm . **C**, Histogram depicting significant shift from a small
 236 number of large condensates when no surfactant protein is added, to a large number of small condensates
 237 with increasing surfactant protein concentration ($p < 0.005$ based on one-way ANOVA followed by Tukey's
 238 post hoc test). **D**, Schematic depicting MBP-RGG-GFP and RGG-RGG. **E**, Micrographs depict
 239 progressively smaller droplet sizes with increasing concentrations of MBP-RGG-GFP, similar to the result
 240 for MBP-GFP-RGG. Scale bars, 5 μm . **F**, Histogram depicting shift in number and size of condensates
 241 with increasing concentrations of MBP-RGG-GFP ($p < 0.005$ based on one-way ANOVA followed by
 242 Tukey's post hoc test). **G**, Surfactant stabilization of droplet size is sustained after 3, 6, and 24 hours ($p <$
 243 0.05 based on one-way ANOVA followed by Tukey's post hoc test). **H**, TEV protease cleaves the MBP-
 244 RGG-GFP construct between MBP and RGG, releasing MBP and causing partitioning of RGG-GFP into
 245 the RGG-RGG condensates. Rapid fusion events can be observed upon disassembly of the MBP-RGG-
 246 GFP layer. Scale bars, 1 μm .
 247

248 Rich Phase Behavior with Increasing Concentrations of GST-GFP-RGG

249 Next, we investigated the impact of altering the stoichiometry of the system. The experiments in
 250 which we varied the concentrations of MBP-GFP-RGG and MBP-RGG-GFP suggested that the amphiphilic
 251 protein can accumulate in the continuous phase as the surface becomes saturated (Fig. 3B,E). An
 252 alternative scenario, however, is that at high concentrations, the amphiphilic protein could itself phase
 253 separate, resulting in multiphasic behavior. A growing body of literature supports the existence and
 254 important role of multiphasic behavior in biological ^{13,14,45} and polymer-based systems ⁴⁶. We therefore

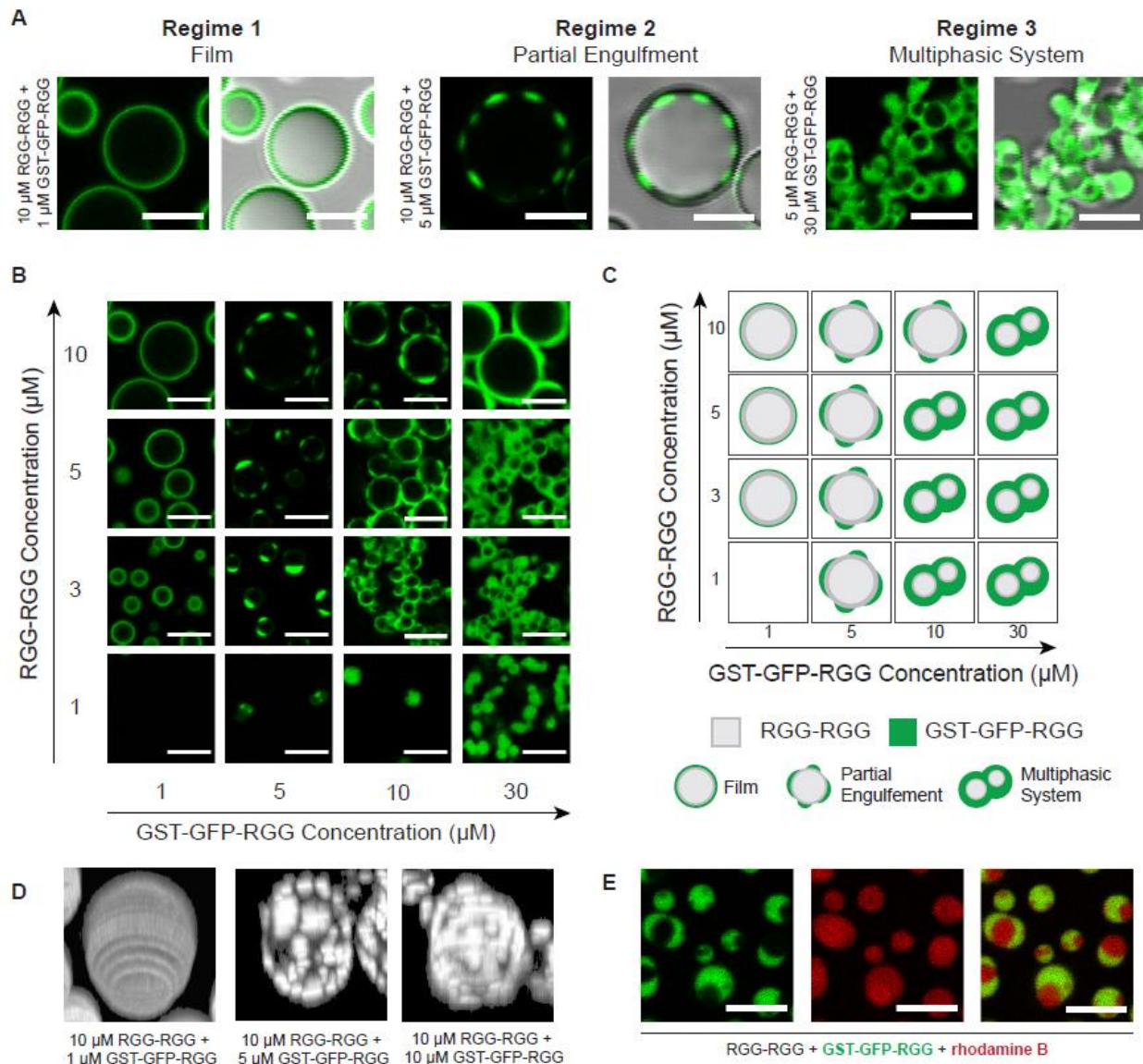
255 considered whether this occurs in our system, focusing on GST-GFP-RGG based on its lower partition
256 coefficient in the continuous phase as compared to the MBP-based surfactants (Fig. 1C). For these
257 experiments we used buffer consisting of 150 mM NaCl, 20 mM Tris, pH 7.5 with no reducing agent added.

258 Remarkably, we observed rich phase behavior in a system of varying concentrations of RGG-RGG
259 plus GST-GFP-RGG. The various assemblies can be categorized into three regimes. As described
260 previously, at low concentration of GST-GFP-RGG relative to RGG-RGG, we observed that RGG-RGG
261 condensates are coated with a film of GST-GFP-RGG (Fig. 4A, left). With increasing relative concentration
262 of GST-GFP-RGG to RGG-RGG, a transition occurs to a second regime where GST-GFP-RGG phase
263 separates and forms puncta that partially engulf the core droplet (Fig. 4A, middle). Upon still further
264 increasing the relative concentration of GST-GFP-RGG to RGG-RGG, the two proteins assemble into a
265 multiphasic system where a continuous GST-GFP-RGG phase envelops RGG-RGG droplets (Fig. 4A,
266 right). The formation of a GST-GFP-RGG phase at high concentrations, whereas MBP-based proteins did
267 not display such behavior, implies a higher strength of interactions between GST domains or between GST
268 and the RGG-RGG core.

269 To further explore this range of behaviors, we mapped the phase space by varying the
270 concentrations of both RGG-RGG and GST-GFP-RGG (Fig. 4B). When 1 μ M GST-GFP-RGG was added
271 to 3, 5, or 10 μ M RGG-RGG, we observed films of GST-GFP-RGG coating the RGG-RGG condensates,
272 agreeing with our prior observations. When the concentration of GST-GFP-RGG was increased to 5 μ M,
273 we observed the formation of partially engulfed RGG-RGG droplets. At a GST-GFP-RGG concentration of
274 10 μ M, we observed partial engulfment at high RGG-RGG concentration (10 μ M), shifting to multiphasic
275 behavior at lower RGG-RGG concentration. Finally, increasing the concentration of GST-GFP-RGG further
276 to 30 μ M resulted in a greater volume of the GST-GFP-RGG phase. We can schematically summarize the
277 phase diagram based on these qualitative categorizations for each concentration pair (Fig. 4C).

278 Next, we generated 3-dimensional (3D) reconstructions of condensates from confocal z-stacks to
279 show the degree of coverage of GST-GFP-RGG surrounding the RGG-RGG phase. In the first regime, with
280 low GST-GFP-RGG concentration, the 3D reconstruction showed a homogenous fluorescent film
281 surrounding the RGG-RGG condensate (Fig. 4D, left panel). At higher concentrations of GST-GFP-RGG,
282 the 3D reconstructions revealed small GST-GFP-RGG condensates decorating larger core RGG-RGG

283 condensates (Fig. 4D, middle panel). Still further increase of GST-GFP-RGG concentration resulted in
284 increased surface coverage by the GST-GFP-RGG phase (Fig. 4D, right panel). Next, to better visualize
285 the multiphasic regime at relatively low RGG-RGG and high GST-GFP-RGG concentrations, we added a
286 small amount of rhodamine B to the system (Fig. 4E). The rhodamine strongly partitioned into both the
287 RGG-RGG and GST-GFP-RGG phases (partition coefficients of 21 and 17, respectively, defined as the
288 ratio of rhodamine fluorescence in each condensate phase to that of the dilute phase). This confirms that
289 the voids observed in the GST-GFP-RGG phase are composed of condensed RGG-RGG, rather than
290 buffer. Finally, we tested the liquidity of the GST-GFP-RGG phase compared to the RGG-RGG phase using
291 fluorescence recovery after photobleaching (FRAP). We observed a striking difference: GST-GFP-RGG
292 exhibited slow and incomplete recovery (approximately 10% recovery after 1 minute), compared to the
293 faster and almost complete RGG-RGG recovery (approximately 70% after 1 minute; SI Appendix Fig. 5)³³.
294 This FRAP data suggests that GST-GFP-RGG has a higher viscosity compared to RGG-RGG.



295

296 **Figure 4: Rich phase behavior of the system depends on stoichiometry of RGG-RGG : GST-GFP-**
 297 **RGG.** **A**, Classification system for the morphologies formed by mixing different ratios of RGG-RGG to
 298 GST-GFP-RGG. **B**, Representative images of condensates formed across concentrations of RGG-RGG
 299 and GST-GFP-RGG tested, depicting a range of phase behaviors. **C**, Application of classification system
 300 to the assemblies observed in the phase diagram, including film, partial engulfment, and multiphasic
 301 complexes. **D**, Three-dimensional volume renderings of representative condensates depicting (left) film,
 302 (middle) low coverage partial engulfment, (right) high coverage partial engulfment. **E**, Dual-color imaging
 303 of multiphasic system (incorporating rhodamine B) to confirm presence of RGG-RGG in engulfed phase.
 304 Scale bars, 5 μ m.
 305

306 **GST-based surfactants outcompete MBP-based surfactants at binding to the condensate interface**

307 At first glance, GST-based and MBP-based amphiphiles exhibit similar behavior when either of the
 308 amphiphilic proteins is mixed at low concentration (1 μ M) with RGG-RGG (10 μ M), forming core

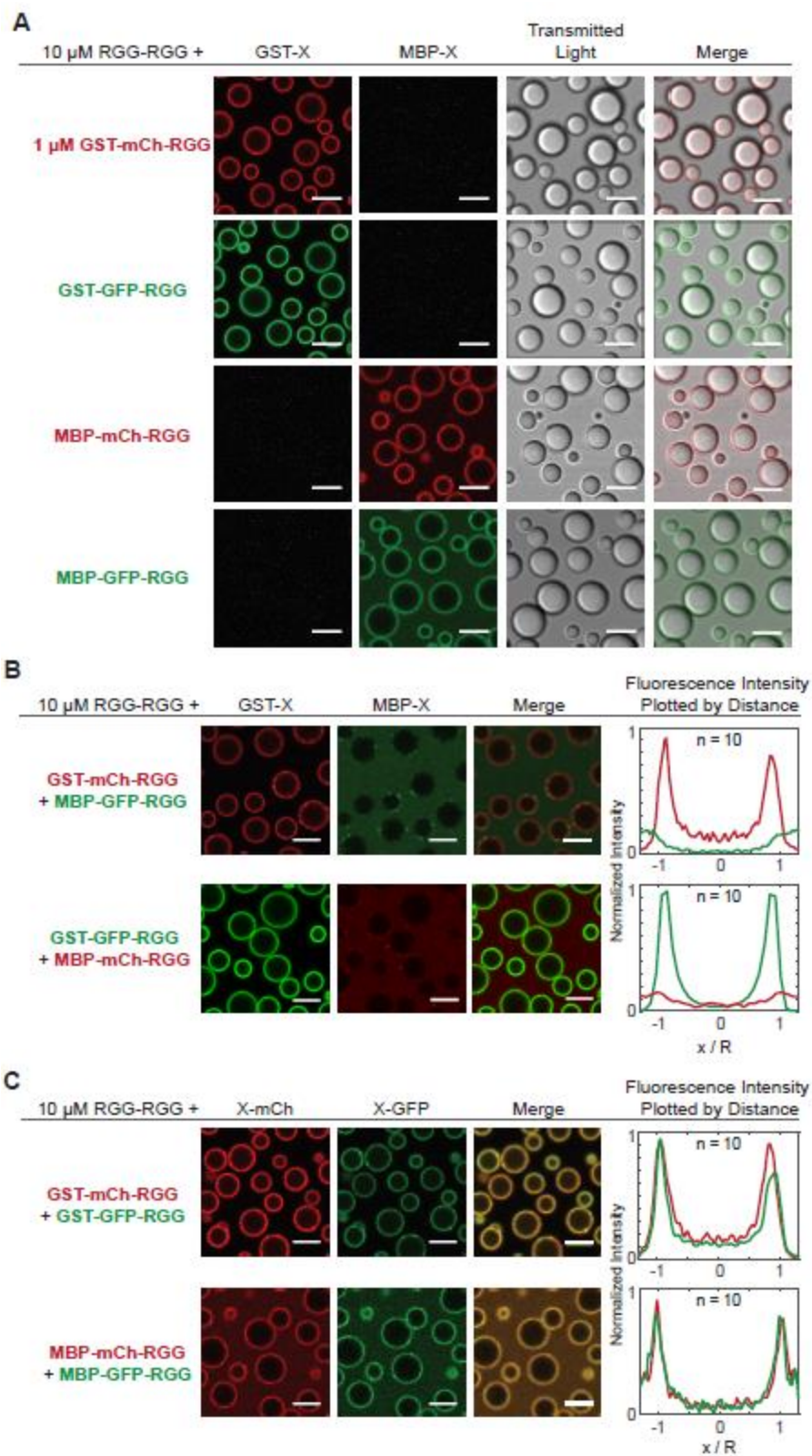
309 condensates enveloped by thin surfactant films. However, we previously noted subtle differences in
310 partitioning of GST-GFP-RGG vs. MBP-GFP-RGG in this regime. Whereas GST-GFP-RGG was highly
311 localized to the droplet surface, MBP-GFP-RGG was somewhat less so, with measurable MBP-GFP-RGG
312 fluorescence in the continuous phase (Figs. 1C and 3B) – suggesting these two amphiphilic proteins have
313 different affinities to the RGG-RGG condensate surface. This notion is further supported by the differing
314 behaviors of these proteins at increased concentrations, wherein GST-GFP-RGG phase separates
315 preferentially at the RGG-RGG droplet surface while excess MBP-GFP-RGG accumulates in the
316 continuous phase. This raises the question of how GST-based and MBP-based surfactants would behave
317 upon mixing RGG-RGG concurrently with both GST-based and MBP-based surfactants. Based on our prior
318 observations, we hypothesized that the two amphiphiles may compete for binding to the interface.

319 To begin, we needed to examine the effects on biomolecular assembly of varying the fluorescent
320 protein. Both the GST-based and MBP-based surfactants displayed consistent behavior when GFP was
321 replaced with the monomeric red fluorescent protein mCherry (Fig. 5A). Irrespective of fluorophore,
322 individually the amphiphilic proteins (1 μ M) colocalized to the surface of RGG-RGG (10 μ M) droplets. This
323 allowed us to assess interactions between the amphiphilic proteins, focusing on the role of GST vs. MBP,
324 regardless of the fluorophore.

325 In agreement with our hypothesis, when we mixed the two classes of surfactant proteins together
326 with RGG-RGG, GST-based proteins displaced MBP-based proteins from the condensate interface (Fig.
327 5B). When GST-mCherry-RGG and MBP-GFP-RGG together were mixed with RGG-RGG, we observed
328 red rings of GST-mCherry-RGG surrounding RGG-RGG droplets, while MBP-GFP-RGG appeared mostly
329 in the continuous phase. Likewise, when GST-GFP-RGG was mixed with MBP-mCherry-RGG, green rings
330 of GST-GFP-RGG formed while red fluorescence was distributed throughout the continuous phase,
331 indicating MBP-mCherry-RGG was displaced from the interface. Plotting intensity versus radial distance
332 confirmed that proteins with a GST tag remained adsorbed to the surface, whereas MBP-tagged proteins
333 were displaced, irrespective of the fluorescent tag used. As a control, we sought to further verify that the
334 choice of fluorophore has negligible impact on amphiphilic protein adsorption to the RGG-RGG surface (Fig
335 5C). We mixed RGG-RGG, GST-GFP-RGG, and GST-mCherry-RGG. Separately, we mixed RGG-RGG
336 plus the two MBP-based proteins with differing fluorophores. In both cases, we observed colocalized

337 fluorescent rings in the mCherry and GFP channels via confocal microscopy, resulting in yellow rings in the
338 merged channel view. This suggests that amphiphiles with the same RGG-phobic domain do not displace
339 each other, and are therefore likely to have similar binding affinities to the interface.

340 Finally, we asked whether this competition phenomenon is concentration dependent. To test this,
341 we prepared RGG-RGG (10 μ M) plus MBP-mCherry-RGG (1 μ M) with varying GST-GFP-RGG
342 concentrations (SI Appendix Fig. 6). Interestingly, with only 0.1 μ M GST-GFP-RGG, MBP-mCherry-RGG
343 remained enriched at the RGG-RGG interface. As we increased the concentration of GST-GFP-RGG, it
344 out-competed MBP-mCherry-RGG for binding, causing decreased red fluorescence at the droplet surface.
345 These results suggest that GST-tagged surfactant proteins displace MBP-tagged surfactant proteins
346 through competitive binding at the interface. This phenomenon may be due to higher solubility of the MBP
347 tag relative to the GST tag. The higher solubility of MBP-based proteins can also be observed in the sample
348 containing MBP-mCherry-RGG and MBP-GFP-RGG, where the continuous phase is more fluorescent as
349 compared to in the system containing GST-mCherry-RGG and GST-GFP-RGG (Fig. 5C). Concomitantly,
350 the strong interactions between the GST domains suggested by its multiphasic behavior (Fig. 4) may also
351 be contributing to localizing the GST-based constructs at the interface.



352

353 **Figure 5: Competitive adsorption to the surface of RGG-RGG condensates when two amphiphilic**
 354 **proteins are introduced. A, Films form when a single amphiphilic protein (1 μ M) is mixed with RGG-**

355 RGG (10 μ M), independent of soluble domain (GST or MBP) and fluorophore (mCherry or GFP)
356 combination used. **B**, Combining two dissimilar amphiphilic proteins with RGG-RGG revealed competitive
357 adsorption. Proteins with a GST domain adsorb to the RGG-RGG droplet surface, irrespective of
358 fluorophore included. Amphiphilic proteins with an MBP domain do not adsorb to the surface.
359 Fluorescence intensity line profiles quantified across single condensates confirm GST-containing proteins
360 preferentially adsorb to the surface of the RGG-RGG condensates. n, number of condensates. **C**,
361 Simultaneous adsorption, rather than competition, is observed when combining RGG-RGG with two
362 similar amphiphilic proteins with the same soluble domain (GST or MBP), but different fluorophores. Scale
363 bars, 5 μ m. Fluorescence intensity line profiles quantified across single condensates confirm simultaneous
364 adsorption. n, number of condensates.
365

366 **Domain Interactions Determine Behavior of Amphiphilic Proteins**

367 We aimed to synthesize a mechanistic explanation for the divergent behaviors we observed with
368 MBP- and GST-based amphiphilic proteins. To summarize these behaviors, GST-based amphiphiles
369 display low partitioning in solution and phase separate at concentrations above 1 μ M. MBP-based
370 amphiphiles are relatively more available in solution and do not phase separate at high concentrations,
371 rather controlling condensate size. When mixed together, GST-based amphiphiles outcompete MBP-based
372 amphiphiles for the surface of RGG-RGG condensates. Our analyses also rule out that the fluorophore has
373 a significant impact on amphiphile behavior, indicating that properties intrinsic to GST and MBP are critical.
374 To account for the behaviors observed, we hypothesized that the relative strength of interactions between
375 domains, i.e., homotypic interactions between MBP : MBP and GST : GST, and heterotypic interactions
376 between amphiphilic proteins and the RGG-RGG core, determine both the degree of partitioning of the
377 amphiphile to the surface as well as the formation of hierarchical structures. As mentioned earlier, strong
378 interactions between either GST : RGG or GST : GST could be driving the formation of the multiphasic
379 systems observed.

380 To explore the role of protein interactions further, we built a computational model that could
381 recapitulate our experimental observations and provide insight into the role of interactions in determining
382 amphiphilic protein behavior. This highly simplified model allowed us to simulate multicomponent phase
383 separation using molecular dynamics (MD) simulations. The computational efficiency of the model allows
384 us to scan a wide parameter space of inter-domain interactions and protein concentrations. Based on our
385 experimental observations, two assumptions were made: (1) the fluorescent tag does not play a major role
386 in dictating the observed behavior and hence can be excluded from this simplified model; (2) among all
387 interactions possible, homotypic interactions between RGG domains are the strongest, supported by its

388 high propensity to phase separate. All other heterotypic and homotypic interaction strengths in the system
389 are scaled with respect to the strength of homotypic RGG interaction.

390 We first examined a two-component system with RGG-RGG and either MBP-RGG or GST-RGG.
391 To decide on a suitable range of strengths for heterotypic interactions (RGG : GST and RGG : MBP) and
392 homotypic interactions (GST : GST and MBP : MBP), we spanned the parameter space searching for results
393 that approximated our experimental observations. To do so, we calculated the dilute, interfacial, and dense
394 phase concentrations of the molecules across a range of interaction strengths and found those values
395 where molecules are enriched in a similar pattern as observed in our experiments (SI Appendix Figs. 8, 9).
396 Our aim was to identify interaction criteria where GST-RGG and MBP-RGG form a layer surrounding RGG-
397 RGG and upon increasing the concentration of GST-RGG and MBP-RGG, we observe phase separation
398 and accumulation in the dilute phase for the two cases, respectively.

399 Results from the simulation allow us to make several conclusions regarding the strength of
400 homotypic and heterotypic interactions between the GST, MBP, and RGG domains. First, if RGG : GST or
401 RGG : MBP interaction strengths were high, we would observe partitioning of GST-RGG and MBP-RGG
402 within the RGG-RGG condensates, which is not observed experimentally (SI Appendix Figs. 7C-D, 9).
403 Interaction between the RGG domains in GST-RGG and MBP-RGG is sufficient to enrich those molecules
404 at the surface of RGG-RGG condensates; additional interaction between the folded and phase separating
405 domains shift the nature of the amphiphilic protein towards partitioning within the RGG-RGG core (SI
406 Appendix Figs. 7C-D, 9). Interestingly, this informs our search for the type of interaction that is responsible
407 for driving GST-GFP-RGG phase separation, suggesting that GST : RGG interactions may play less of a
408 role compared to GST : GST interactions in driving GST-GFP-RGG phase separation. Second, if MBP :
409 MBP or GST : GST interaction strength is too low, we lose the formation of a layer of GST-RGG or MBP-
410 RGG surrounding the RGG-RGG core, showing that homotypic interaction between either of the folded
411 domains is important for their enrichment at the RGG-RGG core surface (SI Appendix Figs. 7A-B, 9).

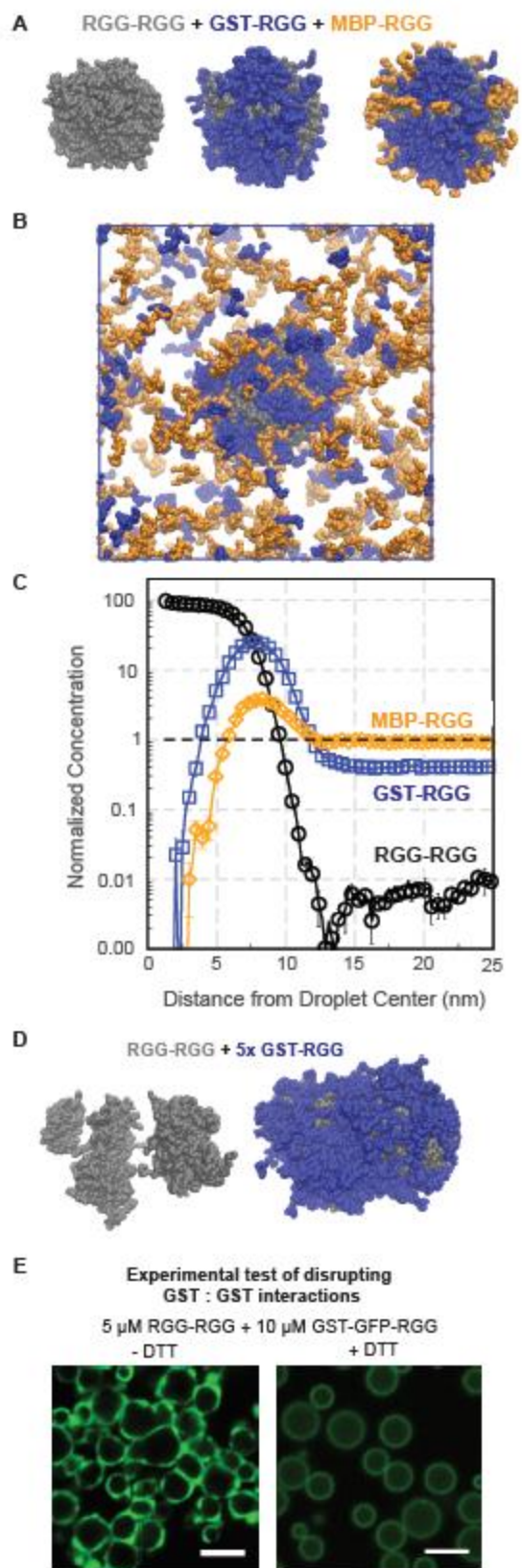
412 Next, we examined a three component system with RGG-RGG, GST-RGG, and MBP-RGG. Within
413 the parameter space explored, we were able to identify values for the strength of RGG : GST, RGG : MBP,
414 GST : GST and MBP : MBP interactions where the model agrees with our experimental data. We set the
415 GST : GST interaction strength to be greater than or equal to 0.8 times the RGG : RGG interaction strength

416 and the MBP : MBP interaction strength to be less than or equal to 0.2 times that of RGG : RGG interaction
417 strength. Results show RGG-RGG phase separating into droplets (Fig. 6A, left panel), GST-RGG becoming
418 highly enriched at the droplet surface (Fig. 6A, middle panel), while MBP-RGG displays a weaker
419 localization to the interface (Fig. 6A, right panel). Their simulated behaviors in the dilute phase also agrees
420 with that observed experimentally, with GST-RGG being relatively more enriched at the RGG-RGG
421 interface compared to MBP-RGG that is more present in the surrounding solution (Fig. 6B). We can also
422 quantify the distribution of each component relative to the component's overall concentration (Fig. 6C).
423 RGG-RGG becomes 100 times more concentrated as it phase separates into condensates centralized at r
424 $= 0$. GST-RGG is highly enriched (~50x) at the interface of RGG-RGG droplets at around $r = 7$ nm, and
425 thus reducing its relative concentration outside the droplet. In comparison, though we still observe some
426 localization of MBP-RGG at the interface, it mostly remains in the surrounding solution.

427 To build on these observations, we explored whether the interaction values examined in the prior
428 simulation could also produce the multiphasic behavior observed with GST-GFP-RGG. We performed a
429 simulation of a binary mixture of RGG-RGG and GST-RGG, with GST-RGG concentration 5 times higher
430 than used previously. Remarkably, the model predicts GST-RGG phase separation, represented by a
431 dense layer of GST-RGG surrounding the RGG-RGG core. In some scenarios, multiple RGG-RGG cores
432 can be observed within a GST-RGG phase, similar to the experimental observation of a multi-phasic system
433 (Fig. 6D). A reasonable explanation for this behavior is that by splitting the RGG-RGG droplet into smaller
434 clusters, there is an increase in surface area for GST-RGG to interact with RGG-RGG molecules.
435 Furthermore, these results support that strong homotypic interactions between GST domains drive the
436 propensity of GST-based amphiphiles to be highly enriched at the condensate surface and to phase
437 separate at higher concentrations.

438 Based on this information, we explored potential drivers for the strong GST : GST interaction. It is
439 known that GST has a tendency to dimerize, as well as oligomerize due to 4 exposed cysteine residues at
440 the protein's surface. The dissociation constant K_d of GST is approximately 1 μ M, above which dimerization
441 is preferred.⁴⁷ We hypothesized these homotypic interactions could contribute to the tendency of GST-GFP-
442 RGG to phase separate, as seen in our experimental observations. To test this hypothesis, we assessed
443 the impact of adding a reducing agent that would disrupt the disulfide bonds between GST domains (Fig.

444 6E). Addition of dithiothreitol (DTT) to a solution of GST-GFP-RGG and RGG-RGG results in the formation
445 of a homogenous film of amphiphilic protein surrounding the RGG-RGG core, as well as increased
446 partitioning in the RGG-RGG phase, irrespective of the concentration of GST-GFP-RGG used. This is in
447 contrast to the partial engulfment and multiphasic behavior observed at high GST-GFP-RGG
448 concentrations in the absence of DTT (Fig. 4, Fig. 6E). This data therefore confirms that GST : GST
449 interactions are necessary for phase separation of the GST-based amphiphiles. Collectively, these
450 molecular simulations and experiments provide mechanistic insight into the differences between the two
451 classes of surfactant proteins tested.



452

453 **Figure 6: Simulations of RGG-RGG, GST-RGG, and MBP-RGG interactions. A, Simulation snapshots**

454 of a condensate formed from a ternary mixture with equal concentrations of RGG-RGG (grey), GST-RGG
455 (blue), and MBP-RGG (orange). All images are from the same snapshot, highlighting surface
456 concentrations of different sets of components. GST-RGG displays a higher degree of coverage of the
457 RGG-RGG surface compared to MBP-RGG. **B**, Simulation snapshot displaying the condensed and dilute
458 phases from part A. Higher concentration of MBP-RGG relative to GST-RGG is observed in the dilute
459 phase. **C**, Quantification of protein concentration distribution in space. Protein concentration is normalized
460 to the total concentration of each species in the solution. Mixing MBP-RGG and GST-RGG with RGG-
461 RGG results in greater GST-RGG localization to the surface while MBP-RGG remains in solution. **D**, MD
462 simulation of RGG-RGG with 5x GST-RGG concentration. RGG-RGG forms multiple cores (left)
463 surrounded by a GST-RGG phase (right). **E**, Confocal fluorescence imaging of RGG-RGG (5 μ M) and
464 GST-GFP-RGG (10 μ M) without DTT (left) and with DTT (5 mM, right). Addition of DTT to RGG-RGG and
465 GST-GFP-RGG mixture results in loss of multiphasic behavior by disruption of GST : GST disulfide bonds.
466 Scale bars, 5 μ m.
467

468 **Discussion**

469 In this paper, we demonstrate that surfactant-like proteins can contribute to the structure and
470 regulation of biomolecular condensates. We report that a two-component in vitro system, consisting of a
471 phase-separating protein and an amphiphilic protein, can reconstitute a range of condensate structures
472 observed in nature. The amphiphilic protein is composed of a domain that is prone to phase separate and
473 a domain that does not phase separate, so at suitable concentrations it adsorbs to the surface of a phase-
474 separated droplet, forming a film. Analogous to surfactant films that stabilize emulsified oil droplets, we
475 discovered that surfactant proteins can stabilize the size of protein condensates. We also found that we
476 can enzymatically disassemble the surfactant film and thereby trigger droplet fusion, demonstrating tunable
477 control of condensate properties by enzymatic manipulation of surfactant proteins. Furthermore, by varying
478 the concentrations of RGG-RGG and the GST-based amphiphiles, a rich phase behavior can be observed,
479 reminiscent of the multiphase and multilayer structures exhibited by naturally occurring condensates. Our
480 results highlight that different amphiphilic proteins can vary in their behavior, one consequence of which is
481 competitive adsorption. Using a simplified model, we show that the strength of interaction between protein
482 domains determine the type of behavior the amphiphilic protein displays. Future studies should further
483 examine the effect on surfactant behavior of mutagenesis, choice of domains, orthogonality of the system
484 and domain architecture for both the core and surfactant proteins.

485 One outstanding question that our work helps address is how the size of biomolecular condensates
486 is controlled^{48,49}. Thermodynamics suggests that smaller droplets should fuse into a single large
487 condensate to reduce the interfacial area between the condensed and dilute phases, thereby minimizing

488 the free energy of the system. However, frequently, cellular condensates coexist as small droplets that do
489 not fuse. Several explanations have been proposed and are likely at play ^{39,40, 57,32}. Our work provides
490 insight into an important alternative mechanism through which droplet size may be regulated. For certain
491 amphiphilic proteins that we tested, presence of the adsorbed surface layer stabilized droplets against
492 fusion. Relatedly, one recent computational study demonstrated that low-valency client molecules that
493 partition with scaffold proteins could theoretically modulate condensate size by acting as surfactants and
494 affecting droplet surface tension ⁵². Our findings raises the possibility that cells could be leveraging
495 surfactant-like, amphiphilic proteins to control organelle surface tension and size. To elucidate the
496 mechanism through which size stabilization is occurring in our system, further research quantifying the
497 surface tension of condensates is necessary. There are also future directions in classifying the type of
498 surfactant and emulsion observed in our system, given the important distinction between emulsions and
499 microemulsions in their behavior and applications ⁵³.

500 Our results also contribute to the growing body of literature on naturally-occurring, multi-layered
501 condensates. The coexistence of multiple phases or other hierarchical structures has been demonstrated
502 to play important roles in cellular function, such as in the organization of protein-RNA vesicles⁵⁴, TDP-43
503 droplets ⁵⁵, the nucleolus ⁵⁶, FMRP/CAPRIN1 droplets ⁴⁵, stress granules ⁵⁷ and P granules ¹⁴. These
504 hierarchical structures have been described using various terminologies, including core-shell structures ¹²,
505 liquid spherical shells ⁵⁵, and Pickering emulsions ⁵⁸. These structures have been associated with several
506 roles, including catalytic shells ¹² and selective barriers ⁵⁵. Beyond the field of liquid-liquid phase separation
507 of IDPs, the film morphologies we observe also resemble naturally occurring plasma membrane vesicles ⁵⁹
508 as well as apolipoprotein micelles ⁶⁰. Hence, multiphasic or multilayer morphologies can display a wide
509 range of behaviors and play a variety of roles in cells. Our engineered system of two proteins is reminiscent
510 of several of these behaviors and assemblies and can therefore be useful for understanding the principles
511 of formation of multi-layered condensates. A potential future direction is to conduct a bioinformatic search
512 for naturally-occurring intracellular proteins with amphiphilic properties and systematically test whether
513 these proteins, if any, partition to the surface of known membraneless organelles.

514 Our system is also similar to other synthetic systems and may also serve as a base platform for
515 designing new bio-inspired materials. Related to our work, formation of spherical micelles have been

516 observed with elastin-like polypeptides (ELPs)⁶¹, amphiphilic polymers^{62,63,64}, as well as plasma proteins
517 forming core-shell structures at the air-water interface⁶⁵. Furthermore, there is a growing field concerned
518 with developing condensates with controllable material properties with applications in several areas such
519 as therapeutic delivery, metabolic engineering, and chemical separations^{66–68}. We demonstrate that we
520 can develop a multi-phase system with controllable surface patterning depending on the construction of a
521 modular surfactant protein. Future investigations could aim at imparting function onto our film system such
522 as by engineering two-step reactions.

523 Membrane-forming lipids are the prototypical example of biological amphiphiles and are essential
524 for the function of membrane-bound compartments⁶⁹. But what role do amphiphilic proteins play in the
525 formation and function of membrane-less compartments? Our work advances the concept that amphiphilic
526 proteins may contribute to the assembly and size regulation of membrane-less organelles. Simple systems
527 comprising phase-separating and amphiphilic proteins, such as those described here, can be further
528 leveraged as models for elucidating fundamental principles governing self-assembly of film and multiphasic
529 hierarchical condensates. This work aims to serve as a basis for investigating the potentially widespread
530 role of amphiphilic proteins in biology.

531

532 **Methods**

533 **Cloning**

534 All genes of interest were cloned into pET vectors in frame with C-terminal 6x-His tags. RGG-RGG
535 was cloned as previously described³³. Other constructs were cloned by PCR and DNA assembly
536 (NEBuilder HiFi DNA Assembly Master Mix; New England Biolabs). The RGG domain used here is the N-
537 terminal IDR (residues 1-168) of *C. elegans* P granule protein LAF-1³⁴. Gene sequences were verified by
538 Sanger sequencing (Genewiz).

539 **Protein expression and purification**

540 For bacterial expression, plasmids were transformed into BL21(DE3) competent *E. coli* (New
541 England BioLabs). Colonies picked from fresh plates were grown for 8h at 37 °C in 1 mL LB + 1% glucose
542 while shaking at 250 rpm. This starter culture (0.5 mL) was then used to inoculate 0.5 L cultures. For

543 surfactant proteins (GST- and MBP- based constructs), cultures were grown overnight in 2 L baffled flasks
544 in Terrific Broth medium (Fisher Scientific) supplemented with 4 g/L glycerol at 18°C while shaking at 250
545 rpm. Once the OD₆₀₀ reached approximately 1, expression was induced with 500 μM isopropyl β-D-1-
546 thiogalactopyranoside (IPTG). For RGG- RGG, cultures were grown in 2 L baffled flasks in Autoinduction
547 medium (Formedium) supplemented with 4 g/L glycerol at 37 °C overnight while shaking at 250 rpm. The
548 pET vectors used contained a kanamycin resistance gene; kanamycin was used at concentrations of 50
549 μg/mL in cultures⁷⁰. After overnight expression at 18 °C or 37 °C, bacterial cells were pelleted by
550 centrifugation at 4100 x g at 4 °C. Pellets were resuspended in lysis buffer (1 M NaCl, 20 mM Tris, 20 mM
551 imidazole, Roche EDTA-free protease inhibitor, pH 7.5) and lysed by sonication. Lysate was clarified by
552 centrifugation at 25000 x g for 30 minutes at 25 °C. The clarified lysate was then filtered with a 0.22 μm
553 filter. Lysis was conducted on ice, but other steps were conducted at room temperature to prevent phase
554 separation.

555 Proteins were purified using an AKTA Pure FPLC with 1 mL nickel-charged HisTrap columns
556 (Cytiva) for affinity chromatography of the His-tagged proteins. After injecting proteins onto the column, the
557 column was washed with 500 mM NaCl, 20 mM Tris, 20 mM imidazole, pH 7.5. Proteins were eluted with
558 a linear gradient up to 500 mM NaCl, 20 mM Tris, 500 mM imidazole, pH 7.5. Proteins were dialyzed
559 overnight using 7 kDa MWCO membranes (Slide-A-Lyzer G2, Thermo Fisher) into physiological buffer (150
560 mM NaCl, 20 mM Tris, pH 7.5), with the exception that GST-GFP-RGG was dialyzed into high salt buffer
561 (1M NaCl, 20mM Tris, pH 7.5). RGG-RGG was dialyzed at 45°C to inhibit phase separation because phase-
562 separated protein bound irreversibly to the dialysis membrane; other proteins were dialyzed at room
563 temperature.

564 Proteins were snap frozen in liquid N₂ in single-use aliquots and stored at -80 °C. For microscopy
565 experiments, protein samples were prepared as follows: RGG-RGG protein aliquots were thawed above
566 the phase transition temperature. MBP- and GST- based proteins were thawed at room temperature. GST-
567 GFP-RGG protein aliquots were concentrated with a centrifugal concentrator (Amicon Ultra Centrifugal
568 Filters, Millipore). Proteins were then mixed with 20 mM Tris, 150 mM NaCl, pH 7.5 with the exception of
569 GST-GFP-RGG which was mixed with a combination of 20 mM Tris, 150 mM NaCl, pH 7.5 and 20 mM Tris,
570 0 mM NaCl, pH 7.5 buffers to obtain a final solution containing 150 mM NaCl. All experiments were

571 conducted with a buffer concentration of 20 mM Tris, 150 mM NaCl, pH 7.5. Protein concentrations were
572 measured based on their absorbance at 280 nm using a Nanodrop spectrophotometer (ThermoFisher);
573 RGG-RGG and GST-GFP-RGG were mixed in a 1:1 ratio with 8 M urea to prevent phase separation during
574 concentration measurements.

575 **SDS-PAGE**

576 For chromatographically purified proteins, SDS-PAGE was run using NuPAGE 4-12% Bis-Tris gels
577 (Invitrogen) and stained using a Coomassie stain (SimplyBlue SafeStain; Invitrogen).

578 **Microscopy: phase behavior, TEV, and FRAP**

579 RGG-RGG was mixed with MBP-based and/or GST-based amphiphiles at the desired protein
580 concentrations while maintaining the buffer conditions at 150 mM NaCl, 20 mM Tris, pH 7.5. Protein
581 samples were plated on 16-well glass-bottom dishes (#1.5 glass thickness; Grace Bio-Labs) that were
582 coated with 5% Pluronic F-127 (Sigma-Aldrich) for a minimum of 10 minutes. The chambers were washed
583 with buffer solution prior to plating the protein samples.

584 Imaging was performed on a Zeiss Axio Observer 7 inverted microscope equipped with an LSM900
585 laser scanning confocal module and employing a 63x/1.4 NA plan-apochromatic, oil-immersion objective.
586 GFP was excited to fluoresce with a 488 nm laser, and mCherry was excited with a 561 nm laser. Confocal
587 fluorescence images were captured using GaAsP detectors. Transmitted light images were collected with
588 either the ESID module or an AxioCam 702 sCMOS camera (Zeiss), in both cases using a 0.55 NA
589 condenser.

590 Time-resolved experiments with TEV protease were performed by adding 1 μ L TEV protease (New
591 England Biolabs) after the 3rd time point to an imaging well containing 100 μ L protein sample. Size
592 dependence experiments were performed by mixing RGG-RGG with 1 or 5 μ M MBP-RGG-GFP and imaged
593 after 1 hour of equilibration. Experiments with dithiothreitol (DTT) were done by mixing it with protein sample
594 at 5mM. All microscopy experiments were conducted at 14-17 °C.

595 **Droplet image analysis**

596 Image analysis and data processing were performed in MATLAB R2020b. The fluorescence
597 intensity profile of the condensates with fluorescent-tagged proteins was measured by using the Hough
598 Transform to identify droplet locations and drawing a line that spanned the droplet diameter plus 1/4th of a

599 radius length in each direction across the droplets. Line-scan graphs were generated in MATLAB. The
600 number of analyzed condensates (n) is indicated in each figure. The total fluorescence intensity of the shell
601 region of the condensates was measured by manually tracking fluorescence over a background threshold
602 across the z-stack over time. Total intensity graphs were generated in MATLAB. Condensate size was
603 calculated using MATLAB's inbuilt Hough Transform function. Box and whisker plot and scatter plots were
604 generated in Excel. Line graphs were generated in MATLAB.

605 **Molecular dynamics simulations**

606 We represent the individual protein sequences i.e., RGG (A), GST (B) and MBP (C) as 20 monomer
607 long disordered domains with identical harmonic bonds connecting all monomers in a domain and also
608 connecting domains with each other to form tandem A-A, A-B and A-C chains. The total interaction energy
609 in our simplistic model includes contributions from this harmonic bond potential and short-range van der
610 Waals (vdW) interactions between monomers,

$$611 \quad U_{tot} = \sum_{i,j} \phi_{ij}^{vdw} + \sum_{i=1}^{N-1} k_b (r_{i,i+1} - r_0)^2, \quad (1)$$

612 where k_b is the spring constant = 20 kJ/Å², and r_0 is the equilibrium bond length = 3.81 Å in the harmonic
613 bond potential, and $N = 40$ is the total number of beads in a tandem chain. The interactions between
614 monomers i and j are represented using the following Ashbaugh-Hatch functional form with $\sigma_{ij} = 5$ Å and
615 $\epsilon = 0.2$ kcal/mol for all heterotypic and homotypic pairs,

$$616 \quad \phi_{ij}(r) = \begin{cases} \phi_{ij}^{LJ}(r) + (1 - \lambda_{ij})\epsilon, & r \leq 2^{1/6}\sigma_{ij} \\ \lambda_{ij}\phi_{ij}^{LJ}(r), & r > 2^{1/6}\sigma_{ij}, \end{cases} \quad (2)$$

617 where ϕ_{ij}^{LJ} is the standard Lennard-Jones (LJ) potential shown below:

$$618 \quad \phi_{ij}^{LJ}(r) = 4\epsilon \left[\left(\frac{\sigma_{ij}}{r} \right)^{12} - \left(\frac{\sigma_{ij}}{r} \right)^6 \right] \quad (3)$$

619 Here λ_{ij} controls the interaction strength between monomers i and j .

620 All simulations were conducted at 298K with 250 chains of A-A while the number of X-A (X = B or
621 C) chains were varied, in a cubic periodic box of size 50 nm. The simulations were conducted using

622 HOOMD-Blue and coexistence densities were calculated using the protocol mentioned in our previous work
623 71.

624

625 **Data Availability Statement**

626 All the quantitative analyses discussed in this paper were generated based on computer codes that we will
627 make publicly available (links will be made available upon publication).

628

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638

639 **Competing Interest Statement**

640 The authors declare no competing interests.

641

642 **Author Contributions**

643 FMK, BF, and BSS conceived and designed research. FMK, BF, and BSS conducted wet lab experiments.
644 RMR and JM designed the computer experiments. RMR conducted the molecular simulations. BSS and
645 JM supervised research. FMK, BF, RMR, JM, and BSS wrote manuscript.

646

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