Supplementary Materials for:

Viscoelastic phase patterning in artificial protein hydrogels

Peter B. Rapp, Bradley R. Silverman

correspondence to: peter.rapp@yale.edu

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Materials and Methods

Protein expression and purification. Plasmids encoding the artificial proteins were transformed into BL21 or BL21 (DE3) chemically competent *E. coli*. After overnight culture, cells were inoculated (inoculation ratios of 1:50 – 1:100) into 1 L flasks containing Terrific Broth (TB) supplemented with 100 – 200 mg ml^{-1} ampicillin. Cells were grown to an OD_{600} of 0.8 – 1.0 and then induced with 1 mM final concentration of isopropyl β-D-1 thiogalactopyranoside (IPTG). After 4-5 h, bacterial cultures were harvested by centrifugation for 5-10 min at 10,000*g*, followed by lysis with 8 M urea. Cell lysates were freeze-thawed at least once before being subject to high-power tip sonication for homogenization (50 mL of lysate from a 1 L culture was typically treated with 30 – 50 W for 10 min in 0.5 s pulses). Homogenized lysate was clarified by high-speed centrifugation (>30,000*g* for 1 h) and then subject to standard His-tag purification over Ni-NTA agarose beads (Qiagen) under denaturing conditions (8 M urea).

Protein dialysis and refolding. Denatured, purified protein (ca. 1 mg/mL starting in 50 – 100 mL 8 M urea plus phosphate buffer) was dialyzed against 4 L of distilled water at 4 °C. The water was changed repeatedly (5 – 6X) over the course of several days. Typically, protein precipitation in the dialysate was used as the dialysis endpoint, after which point the aqueous suspensions were lyophilized. This procedure, which was used for all of the experiments reported here, routinely gave gels that displayed cloud points near 40 °C (cf. Fig. 1C). Notably, subtle changes to the refolding procedure (e.g., changes in dialysis temperature, starting buffer identity or dialysate exchange frequency) could both raise and lower the observed cloud-point temperature of PEP networks above or below 40 °C. For example, frequent dialysate exchange of purified protein starting in urea plus Tris buffer gave protein batches that formed networks with undetectable cloud-points over the examined range (25 – 95 °C). We infer that the observed network LCST is not a simple function of the E midblock alone, but depends also on the folded state of the P endblocks, as well as on noncovalent interactions among E midblocks.

Hydrogel preparation. 100 mM phosphate buffer (pH 6.5 – 7.4) was added to lyophilized PEP protein and the suspension was placed on ice for 2 – 4 h to promote gelation. Fluorescent hydrogels were prepared by adding low concentrations of labeled PE_CP to normal PEP networks (typically, PE_CP:PEP mass ratios of 1:50 and 1:100 were used). Dye conjugation to cysteine-containing probes was performed as described previously (*1*).

Rheological analysis of gels. Oscillatory shear rheology was performed on 10% (*w*/*v*) PEP hydrogels using an ARES-RFS strain-controlled rheometer (TA Instruments) equipped with a cone-and-plate geometry. The outer edge of the plate was coated with mineral oil in order to minimize evaporation from the exposed gel. Strain sweeps identified a linear regime between 0.1 – 10% strain at 10 rad s^{-1}. Frequency sweeps were performed at a fixed strain amplitude of 1% between 0.01 and 100 rad s^{-1}. Temperature data was collected at 1% strain and 10 rad s^{-1}, at 5 °C intervals between 25 °C and 60 °C.

Cloud-point measurements. Protein solutions were prepared at concentrations ranging from 1 to 10% (*w*/*v*) in 100 mM phosphate buffer, pH 6.5 – 7.4. Solutions of PEP above 2 – 3% formed viscoelastic gels, whereas below 2% the solutions flowed easily. Solutions and gels

were loaded between the two halves of a disassembled quartz cuvette. The cuvette was assembled by pressing the two halves together, which sandwiched the gel within a 0.1 cm thick cavity. Roughly 400 μL of gel was required to fill the cuvette. The absorbance at 650 nm in response to temperature was monitored continuously on a Cary 100 Bio UV-Vis spectrometer equipped with temperature control. Gels were typically heated from 25 °C to 95 °C, held at 95 °C for 5 min, then cooled to 5 °C. The heating and cooling rates were held constant at 1 – 3 °C min^{-1}, with minimal differences observed between the faster and slower rates. Prior to cloud-point measurements on the "E_C" protein, a 5% solution was placed at 4 °C on a rotator plate for 3 – 4 days to promote oxidative crosslinking of the thiol groups.

Confocal microscopy, photobleaching and patterning. Imaging of spontaneous pattern formation and phase separation in labeled PEP hydrogels was performed on a Zeiss LSM 880 confocal microscope (488 nm with 10 – 20X objectives) equipped with a "Delta T" heated stage programmed to cycle between 25 °C and 50 °C within 60 seconds (Bioptechs, Butler PA). Labeled gels were placed on an ITO-coated, thermally conductive Delta T culture dish and sealed beneath a coverslip using 120 µm Secure-Seal spacers (Life Technologies). Image analysis was performed in MATLAB. FRAP experiments were performed on a Zeiss LSM 880 equipped with a 25 mW Argon laser. Recovery curves were fit to an effective Fickian diffusion model using the MATLAB function *nlinfit (1, 2).*

Patterning experiments were performed on a Zeiss LSM 5 Exciter equipped with a 25 mW Argon laser (458, 488 and 514 nm) and a 25 mW Diode (405 nm) laser. All laser lines, at maximum power, were typically activated during the photobleach. Bleach spot sizes ranged from 100 – 2000 μm^2. Bleaching of a 500 μm^2 (a = 12.5 µm radius) circle at a scan rate of 4 µm s^{-1} required roughly 2000 scans to ensure efficient bleaching of fluorescein. Bleaching of larger spot sizes (2000 μm^2, a = 25 µm) was usually performed at a scan rate of 1 µm s^{-1}. Typical bleaching times varied from 2 – 10 min, depending on the size of the bleach spot and the total number of scans (2000 – 5000 scans). The total incident power emitted from the Argon laser during a typical fluorescein photobleach was measured to be ~1 mW using a power meter. At 1 mW incident power, the average irradiance (power density) was estimated to be 50 W cm^{-2}. After bleaching of the desired pattern, the sample was allowed to recover fluorescence for 15 – 30 min. Gels were then slowly heated to 50 °C at rate of 2 – 3 °C min^{-1} using a standard heated stage and an aluminum coverslip mount.

Oxidative crosslinking studies. Gels were prepared at 5% in 100 mM phosphate buffer pH 7, supplemented with 25 μM free fluorescein or Rose Bengal, with or without 100 mM NaN_3. Gels were sealed between two coverslips, separated by a 0.030 in sheet of PDMS. Sample irradiation was performed using a Coherent Innova 70 CW Ar-Ion laser. The total incident beam power at 488 nm was fixed at 250 – 300 mW using a circular beam spot with a diameter of roughly 0.8 cm. The irradiance (power density) was estimated to be 0.5 W cm^{-2}. Gels were irradiated for 2 h, then solubilized in 8 M urea and crosslinking was assessed by SDS-PAGE under non-reducing conditions.

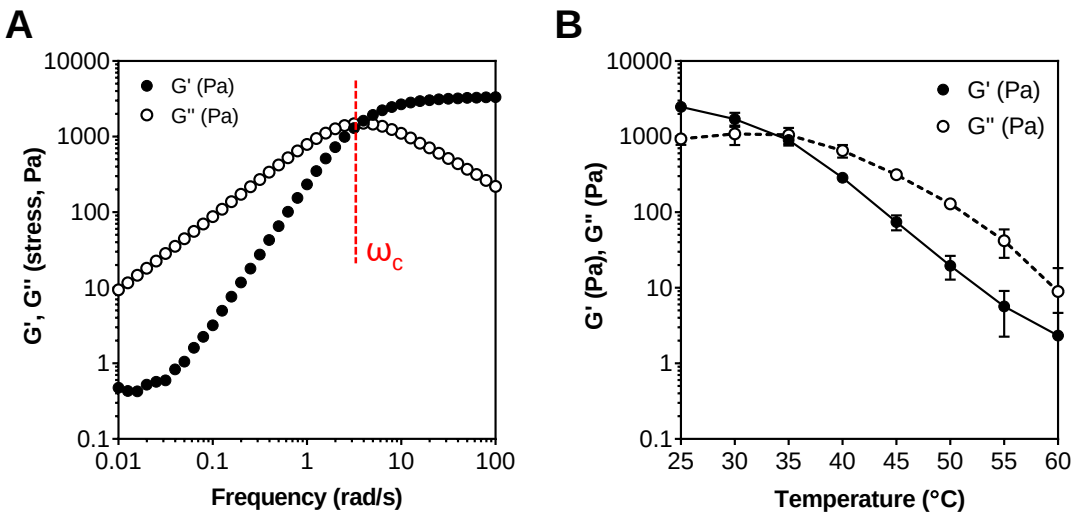

Fig. S1

Oscillatory shear rheometry of PEP hydrogels. (**A**) Frequency sweep of 10% (*w*/*v*) gels prepared in 100 mM phosphate buffer at a fixed strain amplitude of 1% (25 °C). The network behaves as a viscous liquid at low frequencies ($G' < G''$) but transitions to elastic-dominated behavior at high frequencies ($G' > G''$). This transition occurs at a critical frequency ω_c, corresponding to the dominant stress relaxation mode of the physical network. (**B**) Storage (G') and loss (G'') moduli were measured for a 10% gel at 1% strain, 10 rad s^{-1} at various temperatures (n = 2, mean ± SD). Although the viscous loss modulus dominates at high temperatures, weak crosslinking is still evident.

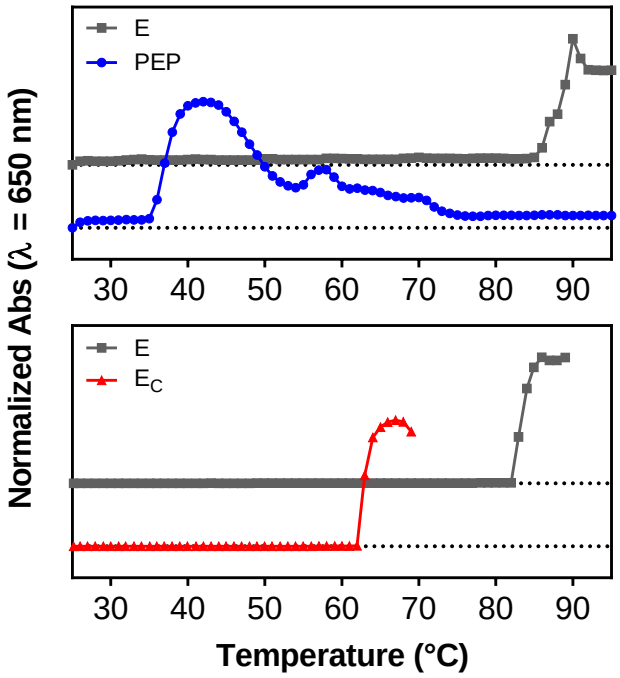

Fig. S2

Crosslinking affects the LCST of elastin-like proteins. Proteins were dissolved at a concentration of 5% in 100 mM phosphate buffer (pH 6.5 – 7.4) and heated to 95 °C at a rate 1 °C min^{-1}. The onset of turbidity was monitored at 650 nm. (*Top*) The predicted LCST of E based on its repeat sequence (70 °C) is close to its observed transition temperature (80 - 90 °C), whereas the transition temperature of gelled PEP is much lower (38 °C). (*Bottom*) The presence of an oxidized thiol (dimerization) in E_C depresses its LCST by ~20 °C relative to E.

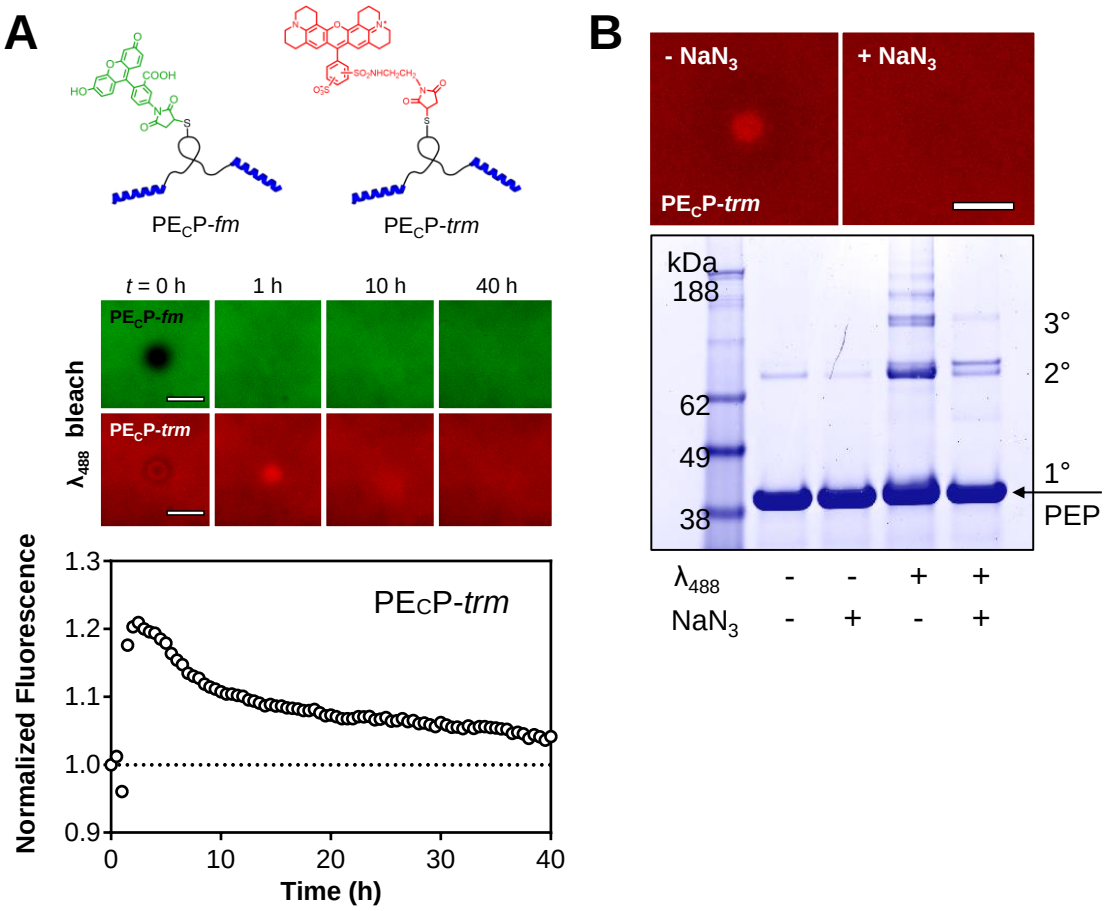

Fig. S3

Photobleaching promotes covalent interchain crosslinking and subsequent probe enrichment in bleach spots. (**A**) 5% gels were labeled with green (PE$_C$P-*fm*, 490Ex/525Em) and red (PE$_C$P-*trm*, 596Ex/615Em) probes and photobleached (spot radius a = 25 µm, λ_{488} bleach). Fluorescence recovery was monitored at 25 °C. Red probes diffused into photobleached volumes and remained enriched for several hours. Scale bar = 100 µm. (**B**) (*Top*) Photobleaching promotes covalent interchain crosslinking by singlet oxygen generation. The presence of 100 mM NaN_3, a strong singlet oxygen quencher, prevented red probe enrichment in 5% gels labeled with red and green probes (λ_{488} bleach). (*Bottom*) Bulk irradiation of 5% gels (488 nm, 500 mW cm^{-2}) containing 25 µM free fluorescein promoted covalent multimer formation of PEP chains. Multimerization was suppressed by the presence of 100 mM NaN_3. Similar results were obtained replacing fluorescein with Rose Bengal, a highly efficient singlet oxygen generator. All scale bars = 100 µM.

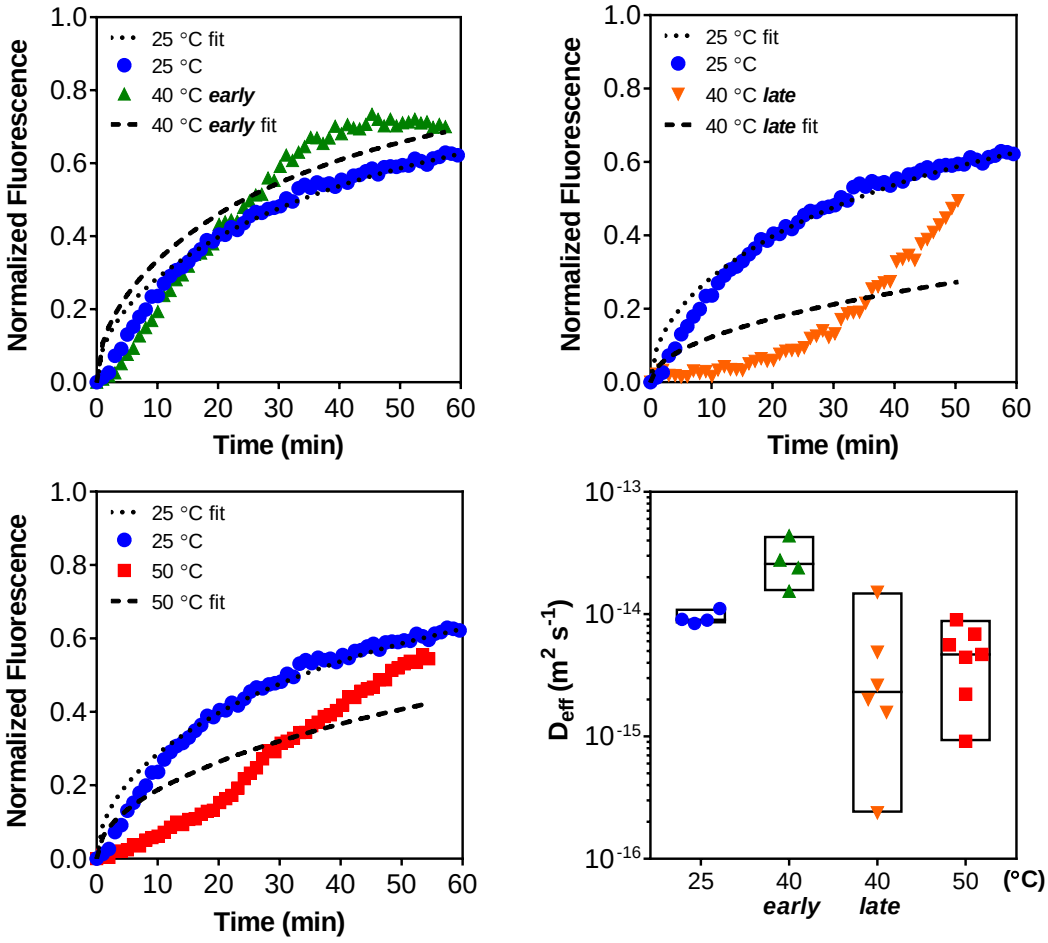

Fig. S4

Anomalous fluorescence recovery behavior above the LCST is poorly fit by standard FRAP models. Shown are representative FRAP traces acquired in a 10% PEP gel at 40 °C and 50 °C, along with their corresponding fits assuming Fickian diffusion. Imprecision in estimating D_{eff} at or above the LCST is attributable both to poor curve fitting as well as to the heterogenous character of the phase separated samples ($n \geq 4$ per temperature; floating bars depict median as well as min/max estimates).

Table S1

Plasmids and amino acid sequences for all artificial proteins described in the text. Protein coding sequences were confirmed by double-stranded DNA sequencing. Each "P" domain is highlighted in blue, and the "E" domain is highlighted in gray. Cysteine residues are in red.

Plasmid	Protein	Molecular Weight (Da)
pET15b-PEP	PEP	32,047
MKGSHHHHHHHVDGSGSGSGSGSGSGAPQMLRELQETNAALQDVRELLR QQVKEITFLKNTVMESDASGSGSGSGSGSGSGLDGHGVGVPGVGVPGVGV PGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPGVG VPGEGVPGVGVPGVGELYAVTGRGDSPASSAPIATSVPGVGVPGVGVPGE GVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPGVGVPG EGVPGVGVPGVGVPGGLLDGSGSGSGSGSGSGAPQMLRELQETNAALQDV RELLRQQVKEITFLKNTVMESDASGSGSGSGSGSGSGLEMHHHHHHK*		
pET15b-PE$_C$P	PE$_C$P	32,151
MKGSHHHHHHHVDGSGSGSGSGSGSGAPQMLRELQETNAALQDVRELLR QQVKEITFLKNTVMESDASGSGSGSGSGSGSGLDGHGVGVPGVGVPGVGV PGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPGVG VPGEGVPGVGVPGVGELCYAVTGRGDSPASSAPIATSVPGVGVPGVGVPGE GVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPGVGVPG EGVPGVGVPGVGVPGGLLDGSGSGSGSGSGSGAPQMLRELQETNAALQDV RELLRQQVKEITFLKNTVMESDASGSGSGSGSGSGSGLEMHHHHHHK*		
pQE80L-E	E	17,670
MKGSSHHHHHHVDGHGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVG VPGVGVPGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGELYA VTGRGDSPASSAPIATSVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPG VGVPGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGG LLE WKKM*		
pQE80L-E$_C$	E$_C$	17,706
MKGSSHHHHHHVDGHGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGVG VPGVGVPGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGELYA VTGRGDSPACSAPIATSVPGVGVPGVGVPGEGVPGVGVPGVGVPGVGVPG VGVPGEGVPGVGVPGVGVPGVGVPGVGVPGVGVPGEGVPGVGVPGVGVPGG LLE WKKM*		

Movie S1

Programmed emergence of a sponge-like morphology at 50 °C during early stage phase separation in a 10% PEP hydrogel (related to Fig. 2). Total elapsed time 10 min. Scale bar = 100 μm.

Movie S2

A global view of spontaneous pattern evolution at 50 °C during late stage coarsening of PEP suggests a breakdown of self-similarity and the absence of a characteristic length scale (related to Fig. 2). Aqueous phase coarsening by droplet ripening and elastically hindered droplet coalescence are also clearly observed. Total elapsed time 16 h. Scale bar = 1 mm.

Movie S3

Viscoelastic "snapping" of a late stage coacervate tendril illustrates the persistence of viscoelastic coarsening mode at 50 °C (related to Fig. 2). Total elapsed time 1 h. Scale bar = 50 μm.

Movie S4

Photobleaching enables transient patterning of non-equilibrium phase shapes during phase separation onset in a PEP hydrogel (related to Fig. 3). Shown is fluorescence recovery of a square photobleached pattern over 30 min, followed by heating of the gel to 50 °C for 1.5 h. Heating matures the photobleached region into a cylindrically patterned coacervate domain. Total elapsed time 2 h. Scale bar = 50 μm.

Supplementary References

1. P. B. Rapp *et al.*, Analysis and Control of Chain Mobility in Protein Hydrogels. *J. Am. Chem. Soc.* **139**, 3796-3804 (2017).
2. B. L. Sprague *et al.*, Analysis of binding reactions by fluorescence recovery after photobleaching. *Biophys. J.* **86**, 3473-3495 (2004).