Supporting information for

Oxidatively Responsive Chain Extension to Entangle Engineered Protein Hydrogels

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Calculation Details

1) The protein volume fraction in the hydrogel (φ) is calculated based on the following assumptions:

- The density of protein ρ is 1.3 g cm^{-3}.[1]
- The volume of the hydrogel is the sum of the solvent volume and the protein volume.

2) Averaged molecular weight (MW) and molecular weight distribution (MWD)

The weight fraction of each species is estimated using densitometry analysis. Due to the resolution limitation of SDS-PAGE, the high molecular weight species cannot be well separated. Therefore, only weight fractions (from three different SDS-PAGEs) of unimer to heptamer are used in the calculation. By using the MATLAB built-in lsqcurvefit algorithm, the MWD is fit to the theoretical Jacobson-Stockmayer distribution.[2] Briefly, assuming that the molecular size distribution for the chain fraction of the same form as the distribution in the ring-free case, namely, the Flory-Schulz distribution, the number of *n*-mer linear chains in the mixture is

$$C_n = Ax^n$$

where A is a normalization constant and x is the fraction of reacted endgroups in the chain fraction.

The number of *n*-mer rings in the mixture is assumed to be

$$R_n = Bx^n n^{-5/2}$$

where B is a constant.

Therefore, the weight fraction of n-mer, including chains and rings, is

$$w_n = \frac{n\left(C_n + R_n\right)}{\sum n\left(C_n + R_n\right)} = \frac{\frac{A}{B}nx^n + x^n n^{-3/2}}{\frac{A}{B}\frac{x}{\left(1-x\right)^2} + \sum x^n n^{-3/2}} = \frac{anx^n + x^n n^{-3/2}}{\frac{ax}{\left(1-x\right)^2} + \sum x^n n^{-3/2}}$$

The fitting parameters are a (the ratio of A to B) and x. Their values from nonlinear regression are 0.574 ± 0.378, and 0.610 ± 0.089, respectively. By using the values of a and x, other quantities can be calculated as follows.

The number and weight fractions of rings in the system is

$$n_r = \frac{\sum R_n}{\sum \left(C_n + R_n \right)} = \frac{\sum x^n n^{-5/2}}{\frac{ax}{\left(1-x\right)} + \sum x^n n^{-5/2}}$$

$$w_r = \frac{\sum n R_n}{\sum n \left(C_n + R_n \right)} = \frac{\sum x^n n^{-3/2}}{\frac{ax}{\left(1-x\right)^2} + \sum x^n n^{-3/2}}$$

The fraction of reacted functional groups in the system is

$$p = x + \left(1-x\right) w_r$$

The number and weight average degrees of polymerization of the chain fraction are

$$\bar{X}_{cn} = \frac{1}{1-x}, \quad \bar{X}_{cw} = \frac{1+x}{1-x}$$

The number and weight average degrees of polymerization of the ring fraction are

$$\bar{X}_{rn} = \frac{\sum x^n n^{-3/2}}{\sum x^n n^{-5/2}}, \qquad \bar{X}_{rw} = \frac{\sum x^n n^{-1/2}}{\sum x^n n^{-3/2}}$$

The number and weight average degrees of polymerization of the system are

$$\bar{X}_n = n_r \bar{X}_{rn} + \left(1-n_r\right) \bar{X}_{cn}, \qquad \bar{X}_w = w_r \bar{X}_{rw} + \left(1-w_r\right) \bar{X}_{cw}$$

The values above are listed in Table S1. The error bars in the calculation represent the 95 % confidence interval of the fitting parameter, calculated based on built-in nlparci algorithm.

Table S1. Summary of the average degrees of polymerization and molecular weights calculated from the Jacobson-Stockmayer theory.

	Chain fraction	Ring fraction	Total
$\bar{X}_n$	2.57 ± 0.58	1.17 ± 0.04	1.96 ± 0.40
$\bar{M}_n$ (kDa)	162.4 ± 36.6	73.9 ± 2.5	123.9 ± 25.3
$\bar{X}_w$	4.13 ± 1.17	1.42 ± 0.13	3.43 ± 0.95
$\bar{M}_w$ (kDa)	261.0 ± 73.9	89.7 ± 8.2	216.8 ± 60.0

3) Stress-strain curve and toughness in tensile experiments

The stress-strain curve is obtained from the force-displacement relation. The engineering stress is calculated using the initial cross sectional area 2 mm × 4 mm in the dog bone. The engineering strain is calculated using the 8 mm as the initial length for approximation. We choose this value instead of the 10 mm gauge length accounting for non-uniform deformation within the specimen. Use of a video extensometer at early deformation enabled accurate calculation of the strain field in the specimen. It was verified that the calculated strain based on 8 mm initial length was within the range of the strain values calculated from video extensometer. True stress and strain are calculated via the following relations:

$$\varepsilon_T = \ln\left(1+\varepsilon_E\right)$$

$$\sigma_T = \sigma_E\left(1+\varepsilon_E\right)$$

where ε_E is the engineering strain, ε_T is the true strain, σ_E is the engineering stress and σ_T is the true stress.

The toughness of topologically entangled gels is calculated as the area under the engineering stress-strain curve. Three independent experiments were performed and the error bars reported in the text are the standard deviation of the measurement.

4) Entanglement molecular weight in gels

The entanglement molecular weight is calculated as[3]

$$M_e = \frac{4}{5}\frac{\rho\varphi RT}{G_e}$$

where R is the universal constant 8.314 J K^{-1} mol^{-1}, T is the absolute temperature, G_e is the entanglement plateau modulus. Three independent experiments for hydrogel *o*-Cys-P_4-Cys at 15 (w/v)% were performed and the error bars reported in the text are standard deviation of the measurement. This relative standard deviation is used for estimating the error bars for the entanglement plateau modulus at 17.5 (w/v)% and 20 (w/v)%.

5) The number of elastically effective chains after chain extension

For protein Cys-P_4-Cys (monomer) in the reduced state, there are 3 elastically effective chains per molecule. The theoretical maximum number of elastically effective chains per protein monomer for a linear n-mer extended protein is given by 4 - 1/n, whereas it is a constant of 4 for

all the looping species. The theoretical molecular weight distributions of the linear and ring molecules are calculated from the Jacobson-Stockmayer theory mentioned previously. Therefore, the total number of elastically effective chains per monomer is

$$\upsilon = \frac{(4-1/n)C_n}{\sum(C_n+R_n)} + \frac{4R_n}{\sum(C_n+R_n)} = 4 - \frac{\sum C_n/n}{\sum(C_n+R_n)} = 4 - \frac{\sum \frac{ax^n}{n}}{\frac{ax}{(1-x)} + \sum x^n n^{-5/2}}$$

$$= 4 + \frac{a\ln(1-x)}{\frac{ax}{(1-x)} + \sum x^n n^{-5/2}}$$

Substituting the numerical values from the fit gives υ = 3.68.

6) The fraction of elastically effective chains in the protein hydrogel

$$v_{\text{eff}} = \frac{G'_{\infty}(\text{expt})}{G'_{\infty}(\text{theo})} = \frac{G'_{\infty}(\text{expt})}{\upsilon RT}$$

where υ is the molar density of elastically effective chains. For P_4, υ = 3c, c being the protein molar concentration in the unit of mol m^{-3}; for *o*-Cys-P_4-Cys, υ = 3.68c. As a result, the theoretical prediction of the increase in the fraction of elastically effective chains should be 1.23.

7) The entanglement volume fraction (φ_e)

The sticky reptation theory[4] predicts that the entanglement volume fraction follows the equation

$$\varphi_e = \left(\frac{N_{e0}}{N}\right)^{3\nu-1}$$

where N_{e0} is the number of monomers between entanglements in a melt, N is the degree of polymerization of the polymer, and υ is the Flory exponent.

After chain extension, the total number of monomers in the polymer is

$N = 2.57 \times 714 = 1835$

A good solvent quality is assumed, giving the Flory exponent υ as 0.588.

Due to the difficulty of obtaining the entanglement segment length in a melt for a protein, N_{e0} is estimated to be 100, bounded between 37~40 (for flexible PEO) to 128~130 (for rigid PS). [5]

Thus the entanglement volume fraction is estimated as

$$\varphi_e = \left(\frac{N_{e0}}{N}\right)^{3\nu-1} = \left(\frac{100}{1835}\right)^{3\times 0.588-1} = 0.1083$$

From linear rheology, the entanglement effect is observed in gels at concentration 15 (w/v)% and above. Therefore the entanglement volume fraction should be somewhere between 0.0877 and 0.1034. The prediction from sticky reptation is close to the experimental result.

8) Estimation of overlap concentration of strands between stickers

The overlap concentration between stickers, namely the C_{10} domain, can be estimated using the radius of gyration (measured by quasi-elastic light scattering from Shen *et al.*)[6]

$$c^* = \frac{3M}{4\pi N_A R_g^3} = \frac{3\times 7870.17\times 10^{-3}}{4\pi\times 6.023\times 10^{23}\times\left(5\times 10^{-9}\right)^3} = 25.0\left(\frac{\text{kg}}{\text{m}^3}\right) = 25\left(\frac{\text{mg}}{\text{mL}}\right)$$

which gives the overlap volume fraction

$$\varphi_s = \frac{c^*}{\rho} = \frac{25}{1300} = 0.019$$

An alternative is to use the result given by the sticky Rouse and sticky reptation to estimate the overlap volume fraction

$$\varphi_s = N_s^{1-3\nu} = 90^{1-3\times 0.588} = 0.032$$

where N_s is the number of monomers in the C_{10} domain. The two results calculated above are close to each other.

Considering the fact that the C_{10} domain only takes up ca. 62% of the total molecular weight of Cys-P_4-Cys, the corrected overlap volume fraction of C_{10} becomes 0.031 ~ 0.052. All the hydrogels investigated here are above the overlap concentration of the C_{10} domain. At low concentrations, with $\varphi_s < \varphi < \varphi_e$, the protein dynamics can be modeled by the sticky Rouse theory. Once the protein volume fraction exceeds φ_e, the dynamics should be explained by sticky reptation. At all concentrations, the strands between stickers are below the entanglement molecular weight. In experiments, no entanglement signature was observed when $PC_{10}P$ gels are

prepared at high concentration, even up to 30 (w/w)%, corresponding to a protein volume fraction of 0.248.[7] Therefore, in the entangled state, the chain extended hydrogels are in the regime $\varphi_e < \varphi < \varphi_{ren}$ and $\varphi_{ren} < \varphi < \varphi_{le}$ according to the sticky reptation theory.

Supplemental Figures

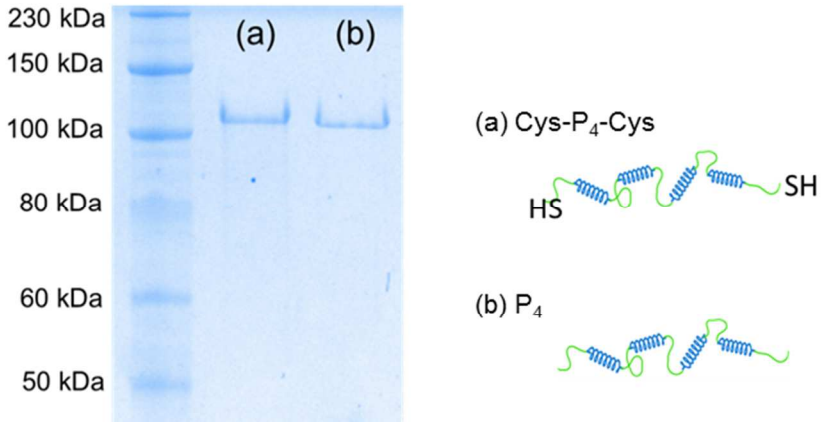

Figure S1. SDS-PAGE showing the purity of proteins (a) Cys-P_4-Cys and (b) P_4. The proteins run on the gel with an apparent molecular weight equal to approximately twice the true molecular weight. The weak binding affinity to the C_{10} domains results in reduced effective charge and electrophoretic mobility, an effect which has been previously observed for structurally similar proteins. [8]

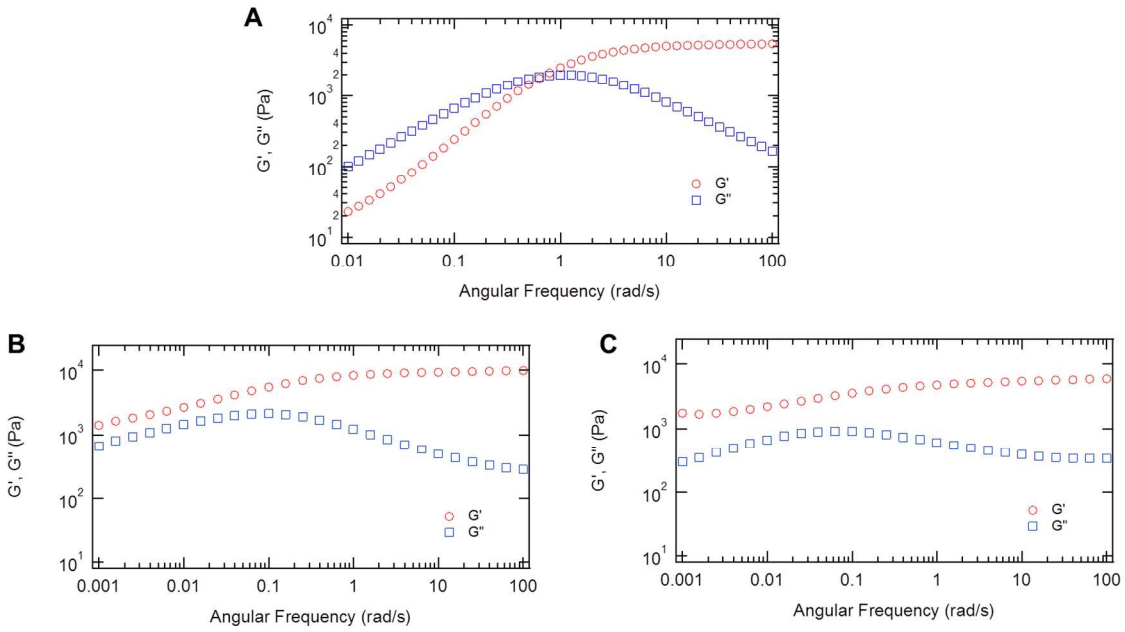

Figure S2. The entanglement plateau modulus is oxidatively-responsive. (A) Adding TCEP to a chain extended protein hydrogel can eliminate the chain entanglement effect. A five-fold excess TCEP was added to reduce the as-formed disulfide. The drop in the high frequency plateau is believed to come from the pH change.[9] (B) Upon mixing with 0.5 μL 30 wt% hydrogen peroxide, the entanglement feature is recovered. (C) Hydrogel *o*-Cys-P4-Cys processed by another oxidation method: DTT (5× excess) was used to reduce the disulfide, and the hydrogel was then mechanically mixed periodically before loading on the rheometer. Again the drop in high frequency plateau is possibly due to the pH environment change, while the entanglement plateau remains unaffected.

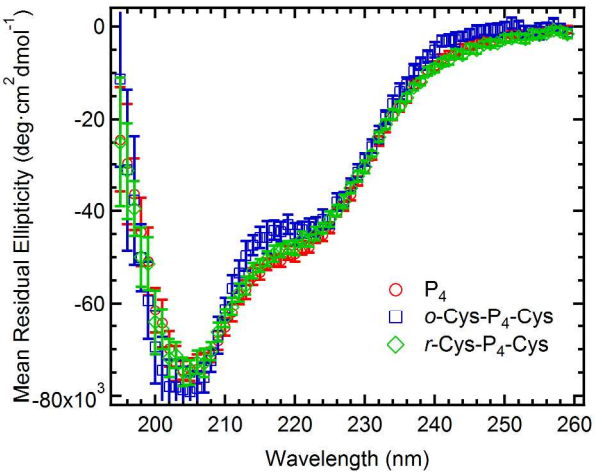

Figure S3. Circular dichroism (CD) experiment showing that the secondary structure of the coiled-coils remains unchanged among proteins. The CD data is analyzed using the CONTINLL algorithm (providing good estimates of the α-helices content in the protein) in the CDPro package with a basis set of 43 soluble and 13 membrane proteins.[10, 11] Estimated α-helices contents in P_4, *o*-Cys-P_4-Cys (oxidized, chain extended Cys-P_4-Cys) and *r*-Cys-P_4-Cys (reduced Cys-P_4-Cys) are 67.0 %, 74.0 % and 65.9%, respectively.

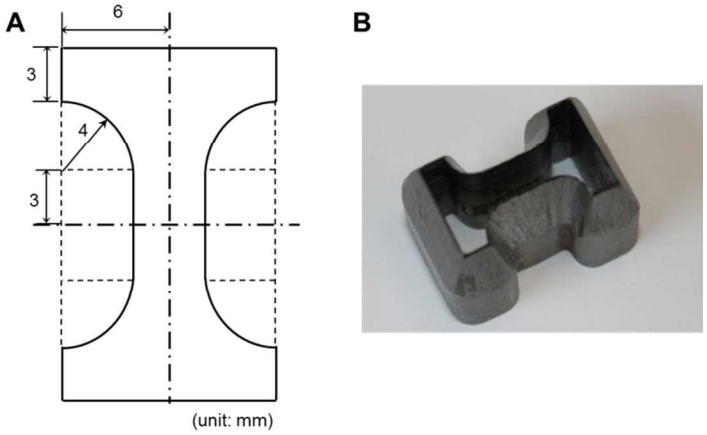

Figure S4. (A) An engineering drawing of the dog bone dimension; (B) A photograph of the dog bone cutter.

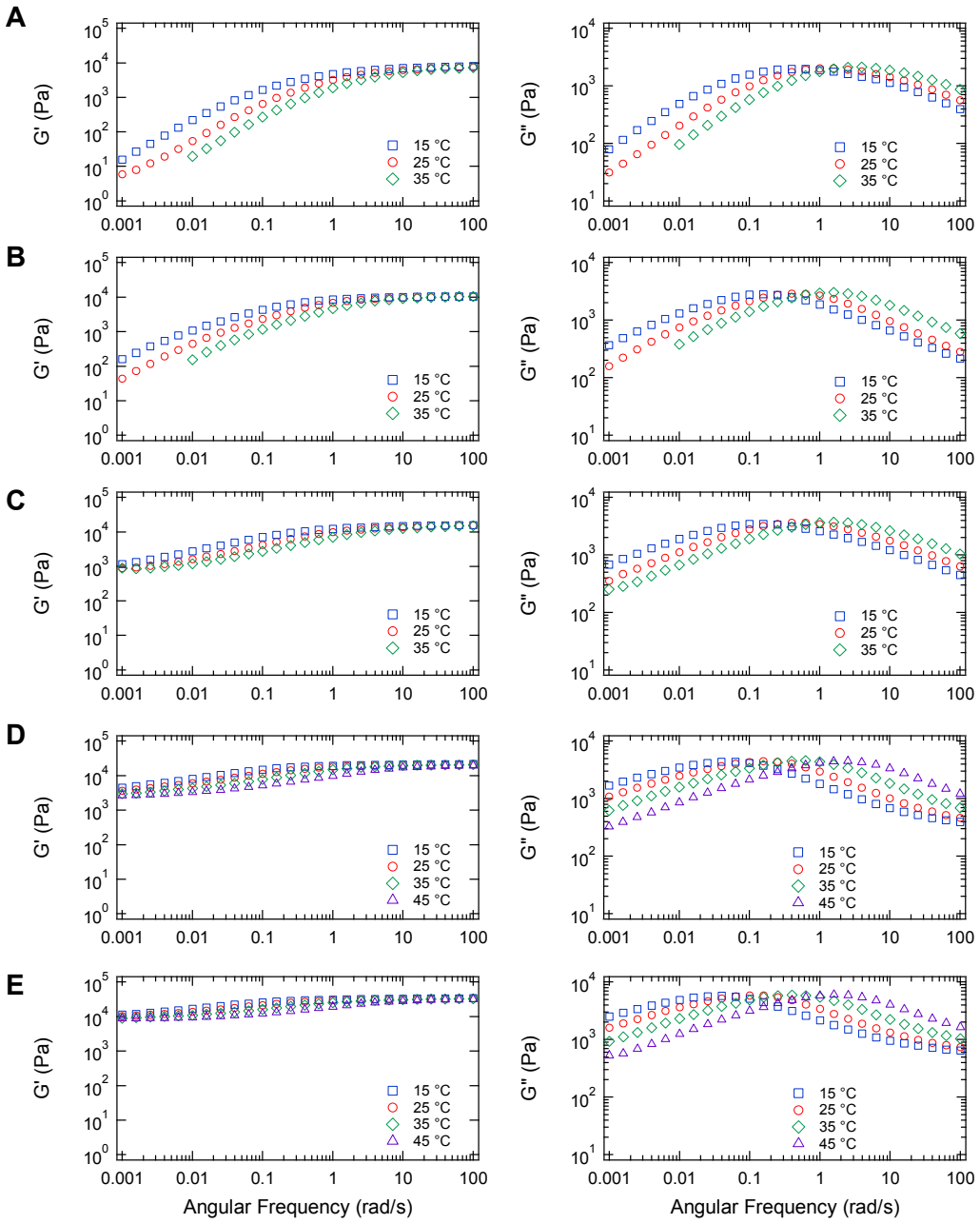

Figure S5. Frequency sweeps of hydrogel *o*-Cys-P4-Cys as a function of temperature (at 15, 25 and 35 ºC, some at 45 ºC) at different concentrations (A) 10.0, (B) 12.5, (C) 15.0, (D) 17.5 and (E) 20.0 (w/v)%).

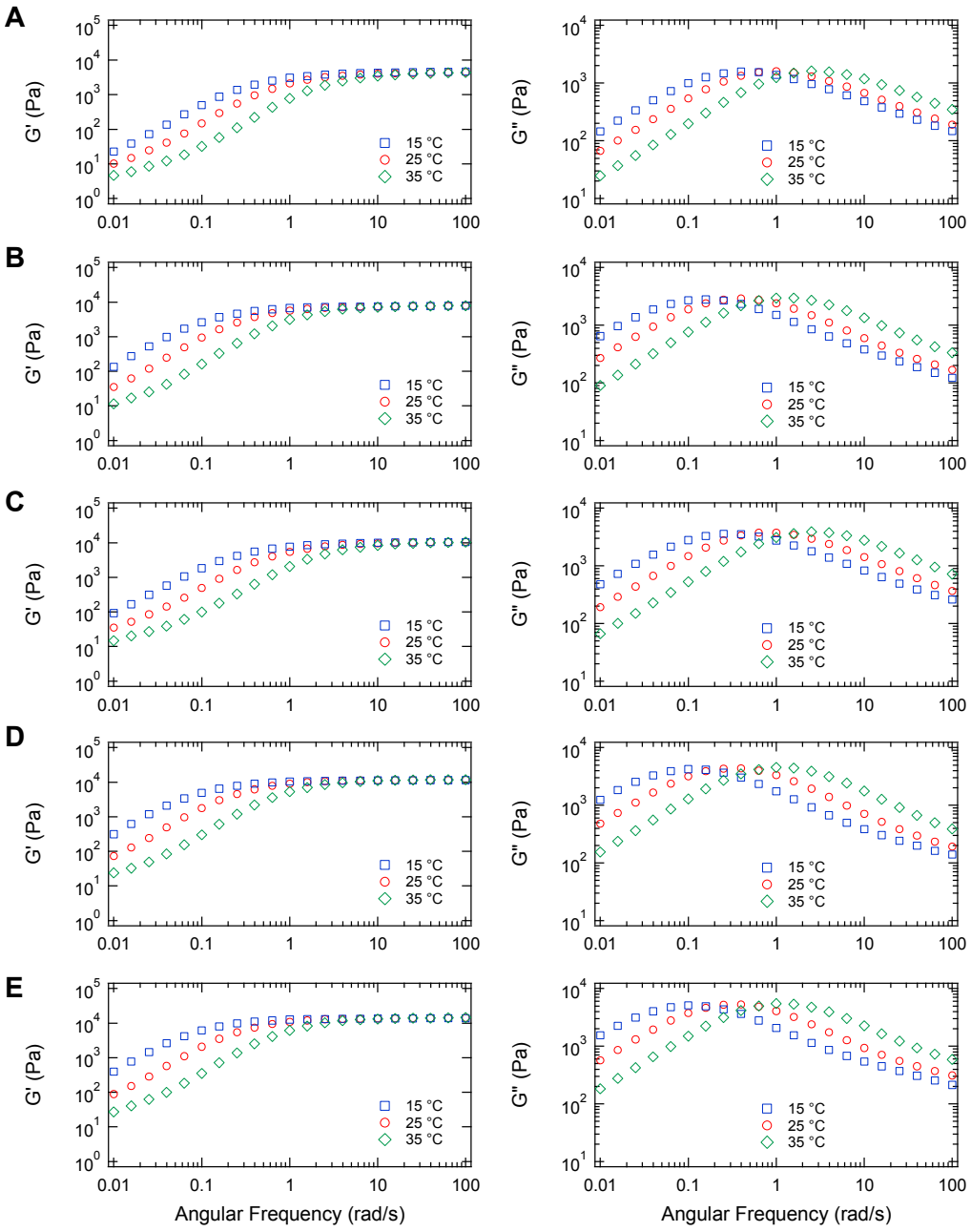

Figure S6. Frequency sweeps of hydrogel P_4 as a function of temperature (at 15, 25 and 35 ºC) at different concentrations (A) 10.0, (B) 12.5, (C) 15.0, (D) 17.5 and (E) 20.0 (w/v)%).

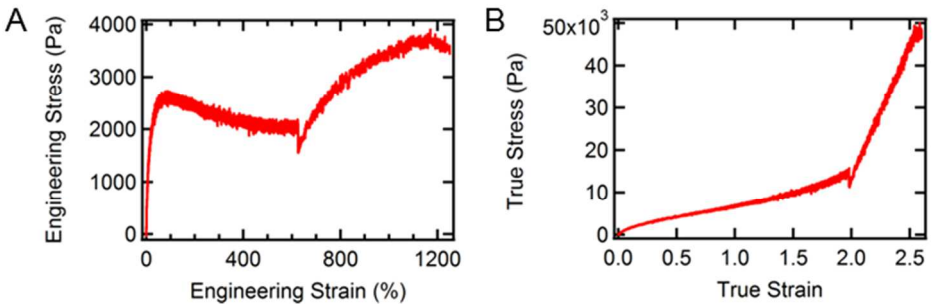

Figure S7. Stress-Strain curve of hydrogel o-Cys-P4-Cys under tensile loading with a shortened relaxation period of 1 min. (A) Engineering stress-strain; and (B) True stress-strain.

References:

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