Complex coacervation of supercharged proteins with polyelectrolytes

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Experimental Section

General Methods. Unless otherwise noted, the chemicals and solvents used were of analytical grade and were used as received from commercial sources. All organic solvents were removed under reduced pressure using a rotary evaporator or vacuum oven. Purification of small molecules was achieved using a Biotage Isolera One system. Water (dd-H_2O) used as a buffer medium was deionized using a Millipore Milli-Q Academic purification system (Millipore). Centrifugations were performed with a Sorvall Legend Micro 21 (Thermo Scientific). Methyl quaternized poly(4-vinylpyridine) was purchased from Polymer Source (M_w/M_n = 1.20, M_n = 12,000). Myoglobin, α-chymotrypsinogen, and lysozyme were purchased from Sigma-Aldrich. RNase A was purchased from Akron Biotechnology.

NMR ^{1}H spectra were measured with a Bruker AVB-400 (400 MHz, 100 MHz) spectrometer. ^{1}H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CHCl_3$ (δ 7.26, singlet). The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. Multiplicities are reported as follows: s (singlet), t (triplet), q (quartet) or br (broad). Coupling constants are reported as a J value in Hertz (Hz).

Mass Spectrometry Protein bioconjugates were analyzed using an Agilent 1260 series liquid chromatograph (Agilent Technologies, USA) that was connected in-line with an Agilent 6130 single quadrupole LC/MS system equipped with a Turbospray ion source.

Gel Analyses For protein charge state analysis, native polyacrylamide gel electrophoresis (Native-PAGE) was carried out on a Mini-Protean apparatus (Bio-Rad, Hercules, CA), using a 9% precast polyacrylamide gel prepared as follows: The separating gel was made by combining 3 mL of 30% acrylamide/bisacrylamide solution with 2.5 mL 1.5 M tris buffer, pH 8.8, 4.5 mL dd-H_2O, and 100 μL 10% (w/v) ammonium persulfate. Tetramethylethylenediamine (TEMED, 8 uL) was added last to initiate the gelation. The stacking gel was prepared by combining 1 mL of 30% acrylamide/bisacrylamide solution with 2.5 mL 0.5 M tris buffer, pH 6.8, 6.5 mL dd-H_2O and 100 μL 10% (w/v) ammonium persulfate. Tetramethylethylenediamine (TEMED, 8 uL) was added last to initiate the gelation. The pH of the native running buffer was adjusted depending on the pI of the unmodified protein. Gels were run for 75-90 minutes at 120 V to separate the bands. Commercially available markers (NativeMark, Novex Life Technologies) were applied to at least one lane of each gel. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Fisher).

Gel permeation chromatography (GPC) Polymers were characterized using Waters 1515 isocratic pump with a Wyatt Optilab T-rEX refractometer and a Wyatt miniDAWN TREOS light

scattering detector employing *N*,*N*-Dimethylformamide with 0.02 M LiBr as the mobile phase with two ResiPore (Agilent) in-line columns.

UV/vis spectroscopy Turbidity measurements were performed using a Cary 50 Bio UV/Vis spectrophotometer or a Tecan 200 Infinite Pro plate reader.

Dynamic Light Scattering (DLS) DLS measurements were obtained using either a Wyatt Möbiuζ with a 532 nm laser or Wyatt Dyna Pro Plate Reader with an 830 nm laser. Data plots are shown as size distribution by intensity. Samples were measured three times.

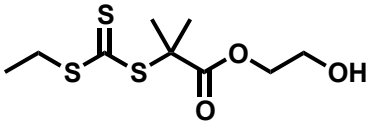
Circular Dichroism (CD) Solutions of the model proteins (0.5 mg/mL in 10 mM tris buffer, pH 8.0) were loaded into a quartz cuvette with a 1 mm path length. The samples were scanned using an Aviv Model 202 Circular Dichroism Spectrometer with a Peltier temperature controller. CD spectra were collected with 1 nm resolution and an averaging time of 5 s.

Transmission Electron Microscopy (TEM) TEM images were obtained at the MIT Center for Materials Science and Engineering facility using a FEI Tecnai G2 Spirit TWIN transmission electron microscope with 120 keV accelerating voltage. Samples (0.04 mg/mL in 10 mM tris buffer, pH 8.0) were prepared for TEM analysis by pipetting 10 μL of the samples onto Formvar-coated copper mesh grids (200 mesh, Electron Microscopy Sciences, Hatfield, PA) and after equilibration the samples were then wicked with filter paper. After excess sample was removed, the grids were allowed to dry in air.

Small Angle Neutron Scattering (SANS) Samples were prepared in 10 mM tris buffer, pH 8.0 in 100% D_2O at a final concentration of 20 mg/mL. The radius of gyration was determined by Guinier analysis (Supporting Information Figure S8) and the reduced data were fit to a smeared fuzzy spheres form factor.[1,2] The fit parameters are displayed in the table below.

Parameter	RNase A -14.2	standard deviation	Chymo. -18.0	standard deviation
mean radius (Å)	160.00	1.29	135.46	0.34
polydisp (sig/avg)	0.35	0.0059	0.21	0.0018
interface thickness (Å)	30.44	1.08	25.48	0.48
SLD sphere ($Å^{-2}$)	1.26E-06	0.020	8.66E-07	0.017
SLD solvent ($Å^{-2}$)	2.74E-06	0.036	3.13E-06	0.0090
Lorentz Scale	0.35	0.0085	0.66	0.012
Lorentz length (Å)	23.76	0.39	21.29	0.26
Bkg (cm^{-1} sr^{-1})	0.040	0.00012	0.037	0.00019

2-Ethylsulfanylthiocarbonylsulfanyl-2-methylpropionic Acid (EMP). The procedure for EMP synthesis was followed directly according to previous work.[3]



Synthesis of 2-hydroxyethyl 2-(((ethylthio)carbonothioyl)thio)-2-methylpropanoate (EMP-OH). To a stirred solution of EMP (817 mg, 3.6 mmol) in

tetrahydrofuran (THF) was added a solution of N,N-dicyclohexylcarbodiimide (1.65 g, 8.0 mmol) and 4-(dimethylamino)pyridine (135 mg, 1.1 mmol) in THF for a final reaction volume of 25 mL. The reaction mixture was stirred for 15 min and then 2 mL of ethylene glycol (35.8 mmol) was added and the mixture was stirred for an additional 21 h. The solution was then cooled to facilitate precipitation of the urea by-product. After cooling the mixture was filtered through a 0.45 micron PTFE syringe filter and the filtrate was concentrated. The crude product was purified via silica gel chromatography using a Biotage Isolera One system to yield 0.82 g of a bright yellow oil (84 % yield). ^{1}H NMR ($CDCl_3$, δ): 4.17 (t, 2H, J = 4.7), 3.73 (t, 2H, J = 4.7), 3.22 (q, 2H, J = 7.4), 2.31 (br s, 1H), 1.66 (s, 6H), and 1.26 (t, 3H, J = 7.4). ^{13}C NMR ($CDCl_3$, δ): 221.95, 173.20, 67.63, 60.80, 55.95, 31.28, 25.35, and 12.88. LRMS (ESI) calculated for $C_9H_{16}S_3O_3$ ($[M+Na]^+$) 291.02, found 291.0.

Myoglobin activity assay. Initial enzyme activity was measured *in situ* in 96 well-plates with a Tecan Infinite 200 Pro plate reader. To a solution of myoglobin (0.2 μM) in 10 mM tris buffer, pH 8.0 was added 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 10-500 μM) as a solution in 10 mM tris buffer, pH 8.0. The background absorbance was measured at 414 nm and then hydrogen peroxide (100 μM) was added to the solution to initiate the reaction. The absorbance at 414 nm was monitored every 15 s for 10 min. Comparative tests were taken at an ABTS concentration of 175 μM and a H_2O_2 concentration of 100 μM.

References:

(1) Kline, S. R. *Journal of Applied Crystallography* **2006**, *39* (6), 895.
(2) Stieger, M.; Pedersen, J. S.; Lindner, P.; Richtering, W. *Langmuir* **2004**, *20* (17), 7283.
(3) Thomas, C. S.; Glassman, M. J.; Olsen, B. D. *ACS Nano* **2011**, *5* (7), 5697.

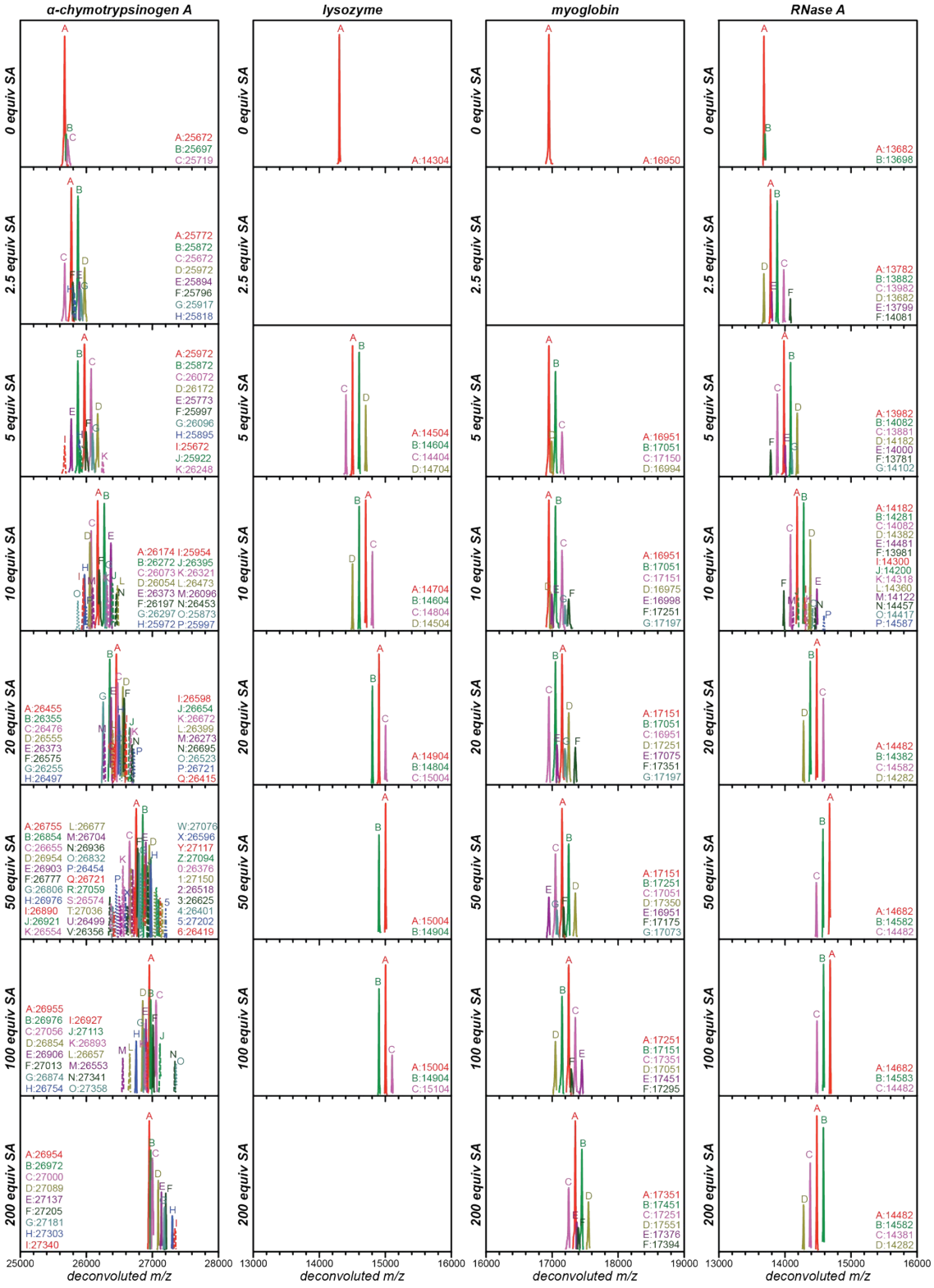


Figure S1. Deconvolued LC-MS data for each of the supercharged proteins.

protein, equiv. succinic anhydride	expected charge	# lysines	# mod.	positive residues	negative residues	negative to positive ratio	charge density (expected)	molecular weight	# of charged residues	max charge
trypsin,0	7.9	14.00	0.00	17.00	9.00	0.53	2961.98	23305.00	26.00	-19.00
lysozyme, 0	6.9	6.00	0.00	18.00	11.00	0.61	2063.73	14303.00	29.00	-14.00
RNaseA, 0	3.9	10.00	0.00	15.00	11.00	0.73	3488.88	13690.00	26.00	-17.00
α-chymo., 0	3.9	14.00	0.00	19.00	15.00	0.79	6565.23	25666.00	34.00	-19.00
lysozyme, 5	2.5	3.29	2.71	15.29	12.71	0.83	5803.62	14574.39	28.00	-15.71
RNaseA, 2.5	1.0	8.53	1.47	13.53	12.47	0.92	13953.30	13836.84	26.00	-18.47
lysozyme, 10	0.5	2.28	3.72	14.28	13.72	0.96	29287.97	14675.06	28.00	-16.72
α-chymo., 2.5	0.7	12.38	1.62	17.38	16.62	0.96	37742.30	25827.51	34.00	-20.62
myoglobin, 0	0.0	19.00	0.00	22.00	22.00	1.00	8066730.35	16951.00	44.00	-24.00
myoglobin, 5	-1.6	18.20	0.80	21.20	22.80	1.08	-10586.92	17031.17	44.00	-24.80
myoglobin, 10	-2.2	17.89	1.11	20.89	23.11	1.11	-7656.75	17062.20	44.00	-25.11
α-chymo., 5	-2.0	11.04	2.96	16.04	17.96	1.12	-12941.09	25962.24	34.00	-21.96
myoglobin, 20	-3.5	17.24	1.76	20.24	23.76	1.17	-4863.18	17126.97	44.00	-25.76
mCherry, 0	-6.0	8.00	0.00	33.00	39.00	1.18	-4658.55	28137.00	72.00	-51.00
BSA, 0	-17.2	59.00	0.00	83.00	100.00	1.20	-3864.31	66463.00	183.00	-120.00
myoglobin, 50	-4.1	16.98	2.02	19.98	24.02	1.20	-4236.92	17153.35	44.00	-26.02
RNaseA, 5	-2.6	6.71	3.29	11.71	14.29	1.22	-5308.05	14018.76	26.00	-20.29
myoglobin, 100	-5.7	16.16	2.84	19.16	24.84	1.30	-3030.81	17235.39	44.00	-26.84
lysozyme, 20	-4.1	-0.03	6.03	11.97	16.03	1.34	-3631.83	14905.68	28.00	-19.03
amylase, 0	-13.1	17.00	0.00	35.00	48.00	1.37	-3582.40	47022.00	83.00	-66.00
α-chymo., 10	-6.3	8.90	5.10	13.90	20.10	1.45	-4173.01	26175.90	34.00	-24.10
lysozyme, 50	-5.3	-0.64	6.64	11.36	16.64	1.46	-2812.11	14966.68	28.00	-19.64
β-lactoglobulin, 0	-9.1	15.00	0.00	19.00	28.00	1.47	-2020.24	18367.00	47.00	-32.00
lysozyme, 100	-5.9	-0.93	6.93	11.07	16.93	1.53	-2540.93	14995.69	28.00	-19.93
myoglobin, 200	-10.2	13.92	5.08	16.92	27.08	1.60	-1719.77	17459.00	44.00	-29.08
α-chymo., 20	-11.0	6.54	7.46	11.54	22.46	1.95	-2402.86	26412.23	34.00	-26.46
RNaseA, 10	-11.5	2.28	7.72	7.28	18.72	2.57	-1258.21	14462.11	26.00	-24.72
RNaseA, 200	-12.9	1.58	8.42	6.58	19.42	2.95	-1127.04	14532.22	26.00	-25.42
α-chymo., 50	-18.0	3.01	10.99	8.01	25.99	3.25	-1483.40	26765.35	34.00	-29.99
RNaseA, 20	-13.9	1.08	8.92	6.08	19.92	3.27	-1050.37	14581.71	26.00	-25.92
RNaseA, 100	-14.2	0.91	9.09	5.91	20.09	3.40	-1026.51	14598.70	26.00	-26.09
RNaseA, 50	-14.8	0.65	9.35	5.65	20.35	3.60	-991.38	14625.28	26.00	-26.35
α-chymo., 100	-20.4	1.83	12.17	6.83	27.17	3.98	-1318.15	26883.10	34.00	-31.17
α-chymo., 200	-22.5	0.77	13.23	5.77	28.23	4.89	-1198.93	26989.08	34.00	-32.23

does not coacervate | forms bulk coacervate but not coacervate micelles | forms bulk coacervate did not test coacervate micelles | forms bulk coacervate and coacervate micelles

Figure S2. Table of different charge parameters for the model proteins used in this study. Proteins that formed bulk coacervate and complex coacervate core micelles are highlighted.

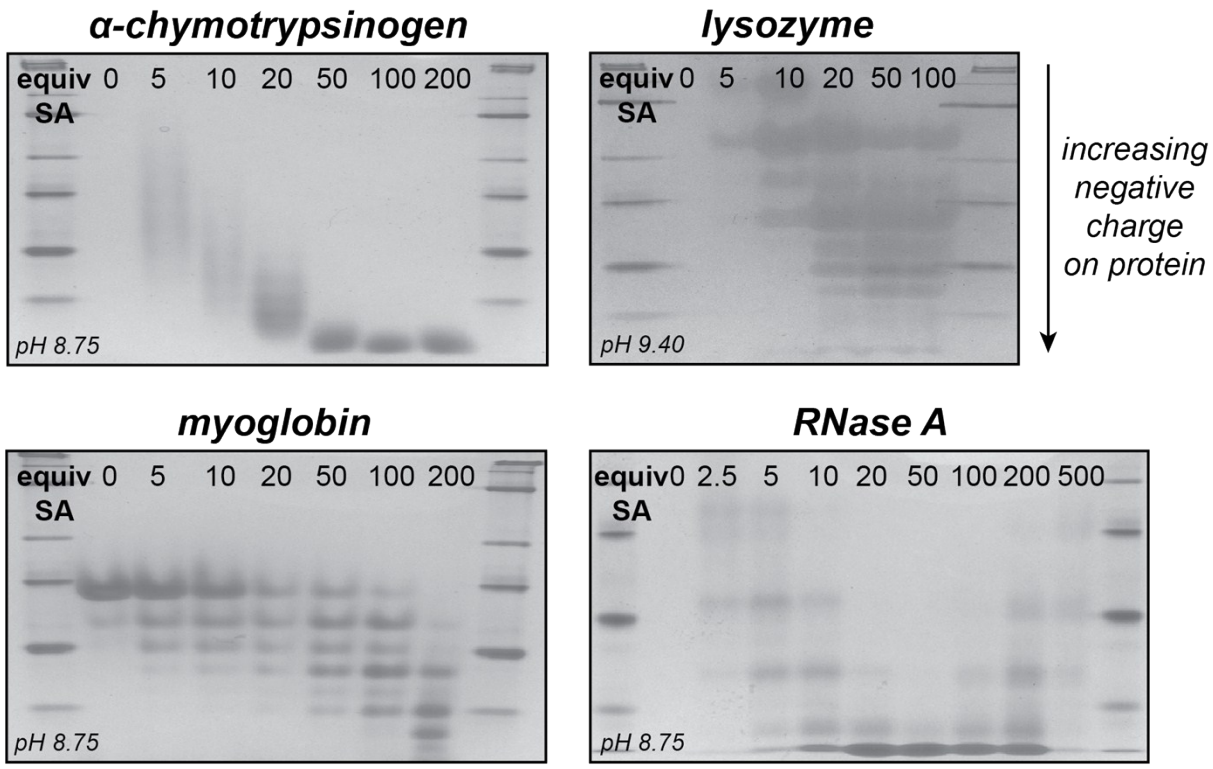
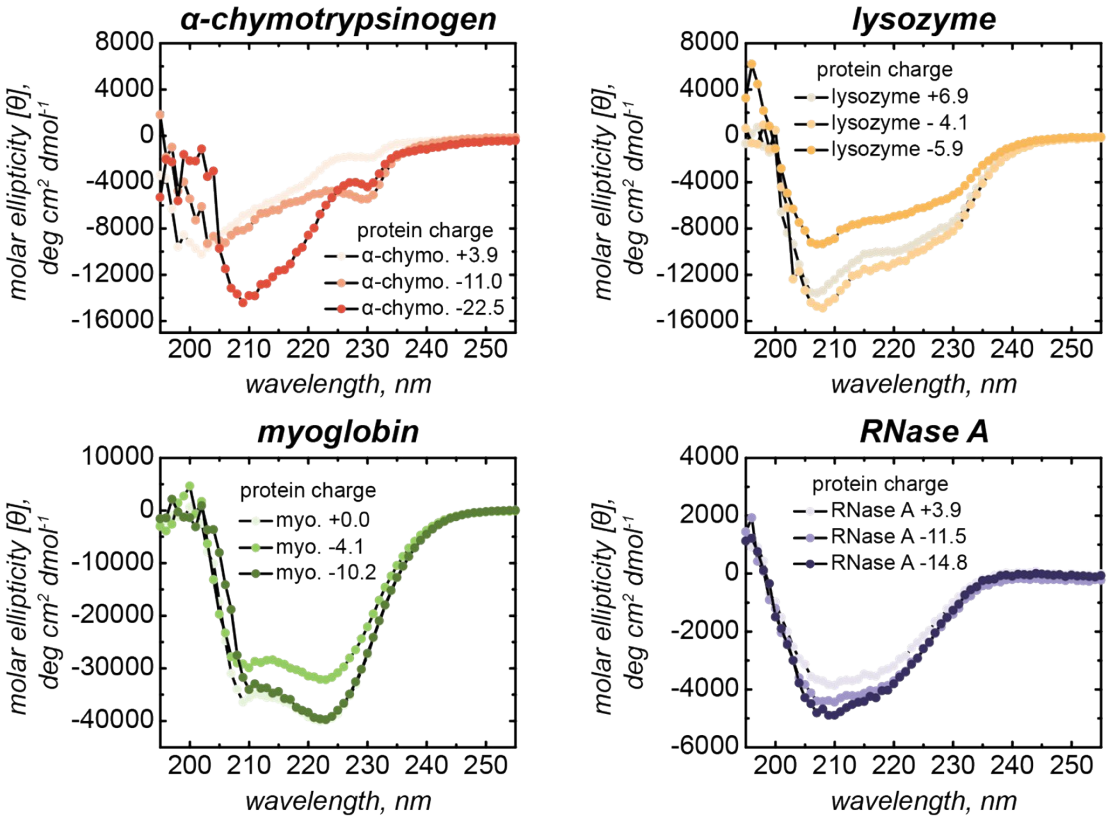


Figure S3. Native PAGE analysis of the supercharged proteins shows an increase in the negative charge on the protein after treatment with increasing amounts of succinic anhydride (SA). The Native PAGE was performed at the indicated pH on each gel to maximize the separation of the supercharged species while permitting the analysis of proteins that were positively charged at pH 8.0.



	α-helix (%)	β-sheet (%)	other (%)
chymo. expected	14	32	54
chymo. +3.9	9.7	31.6	58.6
chymo. -11.0	13.4	29.2	57.3
chymo. -22.5	9.2	22.6	68.2
lyso. expected	41	10	49
lyso. +6.9	32.4	15.8	51.8
lyso. -4.1	37.1	14.7	48.1
lyso. -5.9	14.7	30.2	55.0
myo. expected	73	0	27
myo. +0.0	75.7	0	24.2
myo. -4.1	68.0	0	32.0
myo. -10.2	52.6	4	43.3
RNase A expected	20	35	45
RNase A +3.9	7.4	37.1	55.5
RNase A -11.5	8.9	36.1	55.0
RNase A -14.8	10.6	34.9	54.6

Figure S4. Circular dichroism of unmodified and supercharged model proteins (0.5 mg/mL) was performed in 2.5 mM tris buffer, pH 8.0 The expected secondary structure was determined from the crystal structure. Only the most supercharged variants of lysozyme and myoglobin demonstrated substantial changes to the secondary structure.

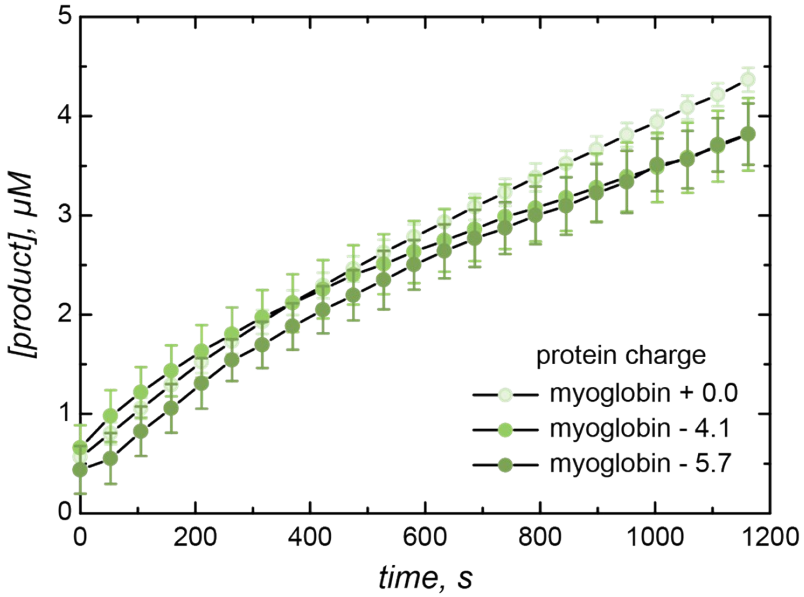


Figure S5. Initial activity of unmodified and supercharged myoglobin as measured by an ABTS/H_2O_2 activity assay shows little difference in activity after modification.

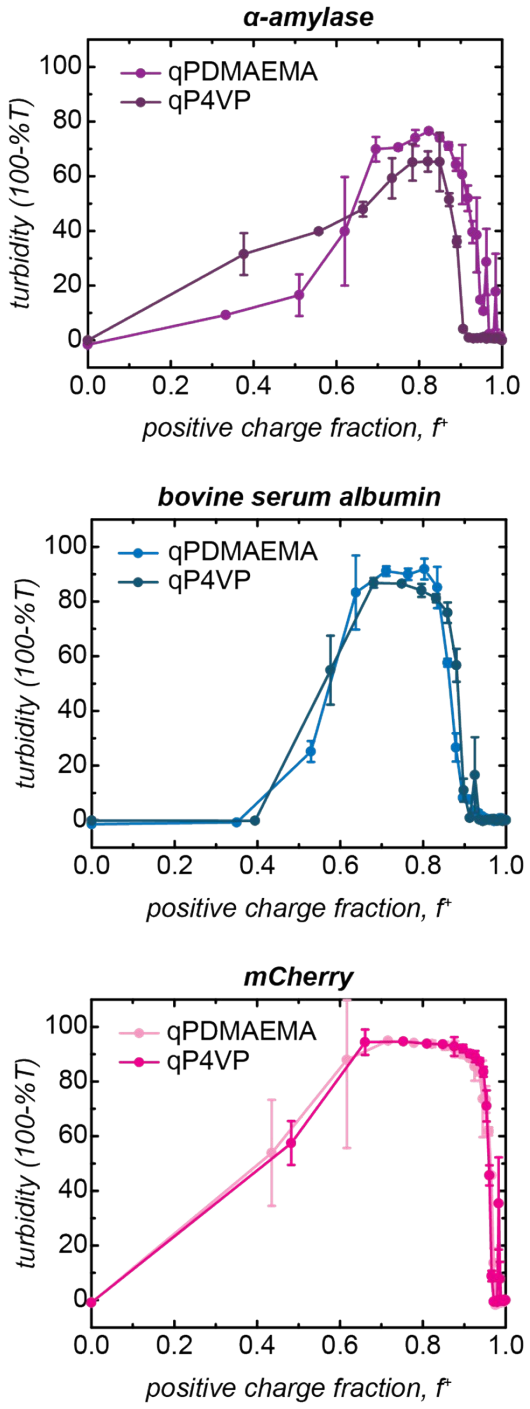


Figure S6. Bulk coacervation with other model proteins was monitored by changes in turbidity as a function of charge fraction in 10 mM tris buffer, pH 8.0 at a total polyelectrolyte concentration of 2 mg/mL.

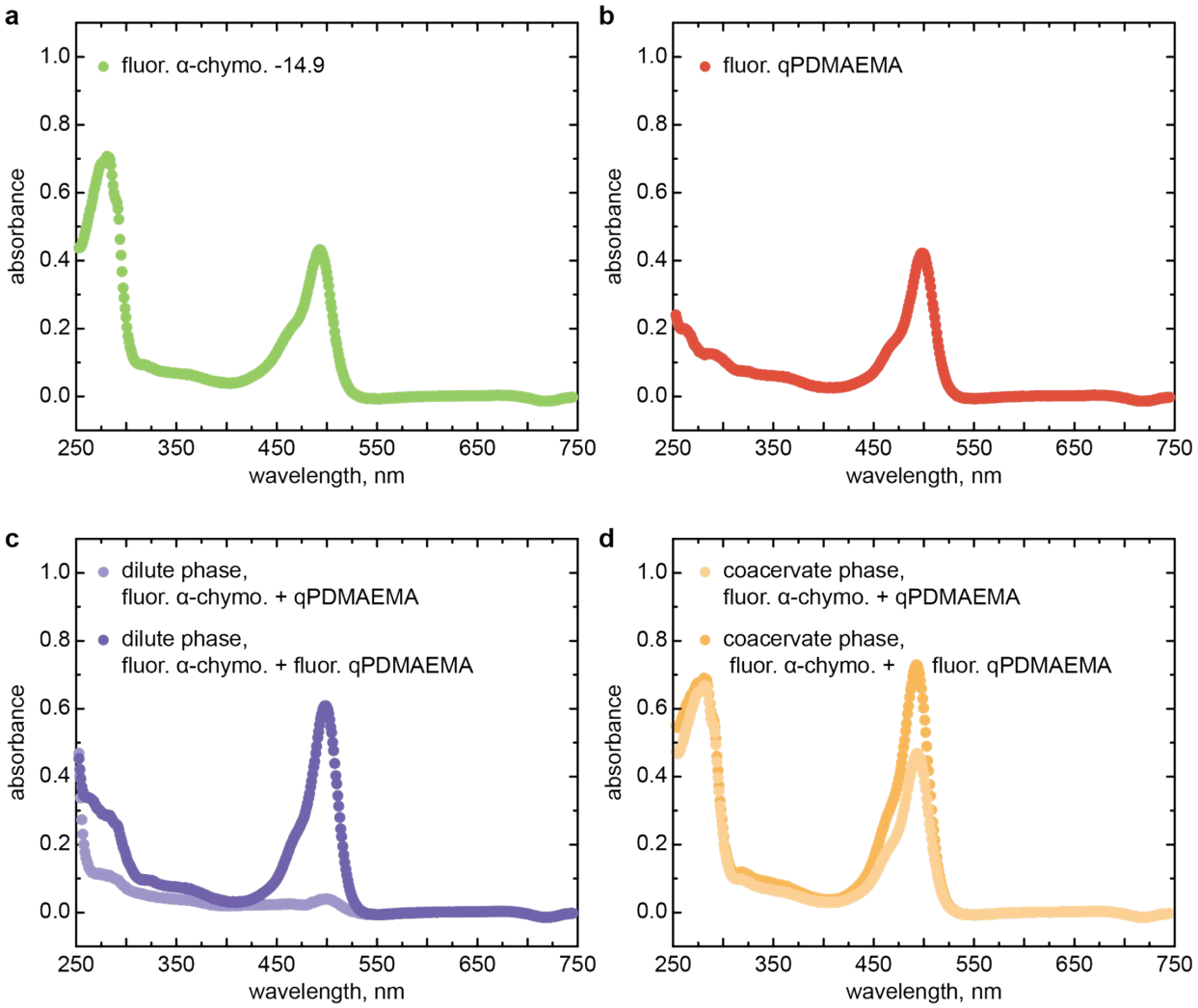


Figure S7. Representative UV-Vis spectra used to determine the composition of protein and polymer in the coacervate phase. Absorbance spectra of (a) fluorescein modified supercharged α-chymotrypsinogen, (b) fluorescein modified qPDMAEMA, (c) the isolated dilute phase and (d) the isolated coacervate phase. qPDMAEMA and α-chymotrypsinogen were mixed at a total polyelectrolyte concentration of 4 mg/mL in 10 mM tris buffer, pH 8.0 at the midpoint of coacervation (qPDMAEMA weight fraction 0.28, $f^{+} = 0.70$). Samples were diluted 2-fold (dilute phase) or 10-fold (initial and coacervate phase) in order to obtain absorbance maxima between 0.1-1.0 absorbance units.

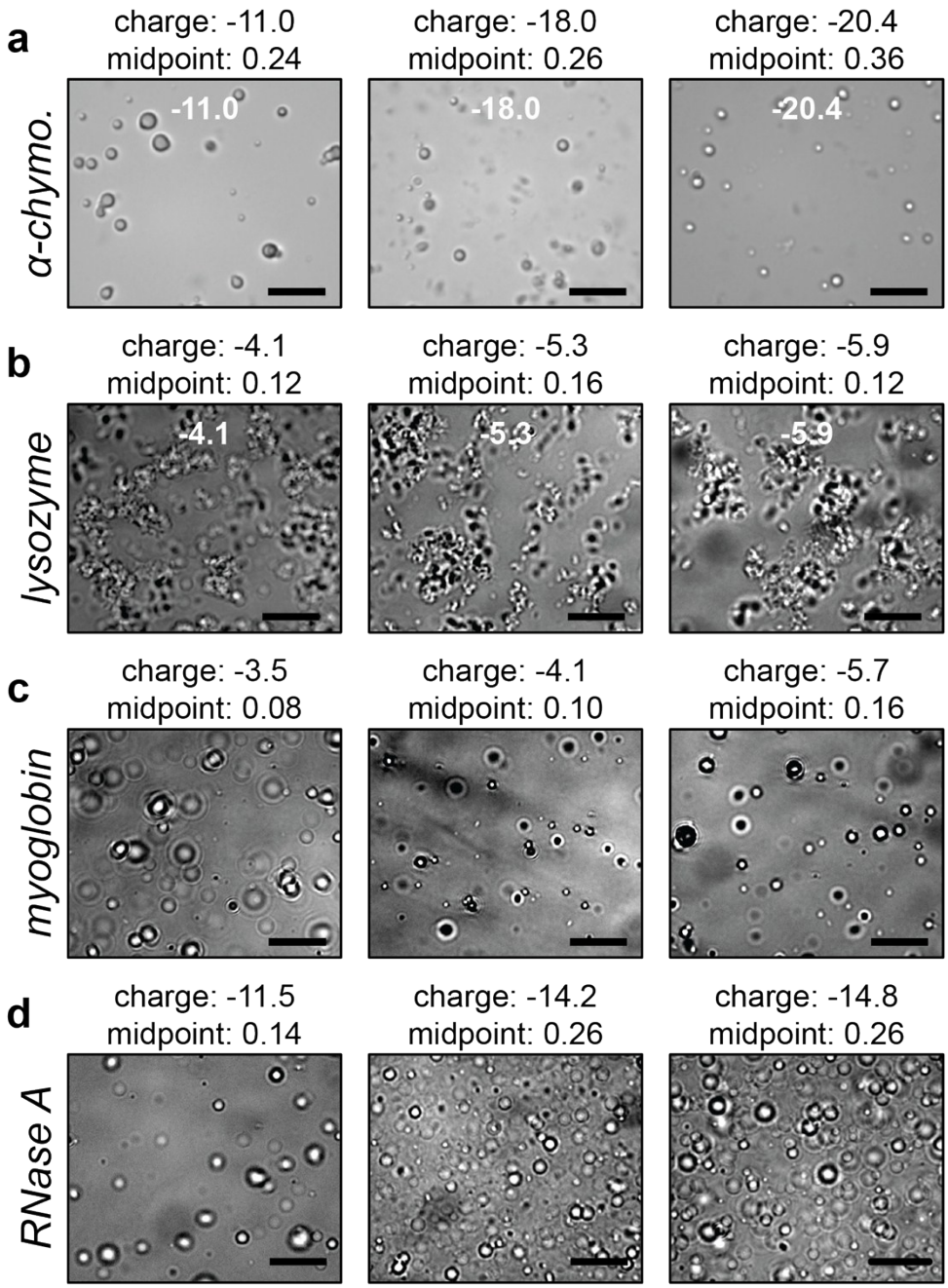


Figure S8. Bright field optical micrographs showing the formation of liquid coacervates or solid precipitates resulting from mixing (a) α-chymotrypsinogen, (b) lysozyme, (c) myoglobin, or (d) RNase A with qPDMAEMA at the midpoint of coacervation determined from bulk turbidity titrations. The expected charge and the midpoint of coacervation (expressed as the polymer weight fraction) are listed for the supercharged model proteins. The components were mixed at a total polyelectrolyte concentration of 2 mg/mL in 10 mM tris buffer, pH 8.0.

		qPDMAEMA			*qP4VP*		
protein, equiv. succinic anhydride	**expected charge**	**polymer weight fraction at midpoint**	**charge fraction, f⁺ at midpoint**	**protein-to-polymer ratio at midpoint**	**polymer weight fraction at midpoint**	**charge fraction, f⁺ at midpoint**	**protein-to-polymer ratio at midpoint**
α-chymo., 0	3.9	-	-	-	-	-	-
α-chymo., 2.5	0.7	-	-	-	-	-	-
α-chymo., 5	-2.0	0.10	0.82	9.00	0.08	0.82	11.50
α-chymo., 10	-6.3	0.14	0.69	6.14	0.16	0.76	5.25
α-chymo., 20	-11.0	0.24	0.72	3.17	0.30	0.81	2.33
α-chymo., 50	-18.0	0.26	0.63	2.85	0.32	0.74	2.13
α-chymo., 100	-20.4	0.36	0.71	1.78	0.34	0.73	1.94
α-chymo., 200	-22.5	0.38	0.71	1.63	0.38	0.75	1.63
lysozyme, 0	6.9	-	-	-	-	-	-
lysozyme, 5	2.5	-	-	-	-	-	-
lysozyme, 10	0.5	-	-	-	-	-	-
lysozyme, 20	-4.1	0.12	0.62	7.33	0.16	0.74	5.25
lysozyme, 50	-5.3	0.16	0.64	5.25	0.20	0.74	4.00
lysozyme, 100	-5.9	0.12	0.54	7.33	0.20	0.72	4.00
myoglobin, 0	0.0	-	-	-	-	-	-
myoglobin, 5	-1.6	-	-	-	0.06	0.71	15.67
myoglobin, 10	-2.2	-	-	-	0.04	0.56	24.00
myoglobin, 20	-3.5	0.08	0.59	11.50	0.06	0.54	15.67
myoglobin, 50	-4.1	0.10	0.61	9.00	0.08	0.60	11.50
myoglobin, 100	-5.7	0.16	0.66	5.25	0.16	0.70	5.25
myoglobin, 200	-10.2	0.32	0.73	2.13	0.28	0.73	2.57
RNase A, 0	3.9	-	-	-	-	-	-
RNase A, 2.5	1.0	-	-	-	-	-	-
RNase A, 5	-2.6	0.06	0.52	15.67	0.04	0.56	24.00
RNase A, 10	-11.5	0.14	0.40	6.14	0.14	0.45	6.14
RNase A, 20	-13.9	0.22	0.50	3.55	0.20	0.52	4.00
RNase A, 50	-14.8	0.26	0.54	2.85	0.24	0.56	3.17
RNase A, 100	-14.2	0.26	0.55	2.85	0.22	0.54	3.55
RNase A, 200	-12.9	0.22	0.51	3.55	0.20	0.53	4.00

Figure S9. Table of the midpoint coacervation values for the supercharged protein substrates. The midpoint of coacervation is listed as a function of polymer weight fraction, positive charge fraction, and protein-to-polymer ratio.

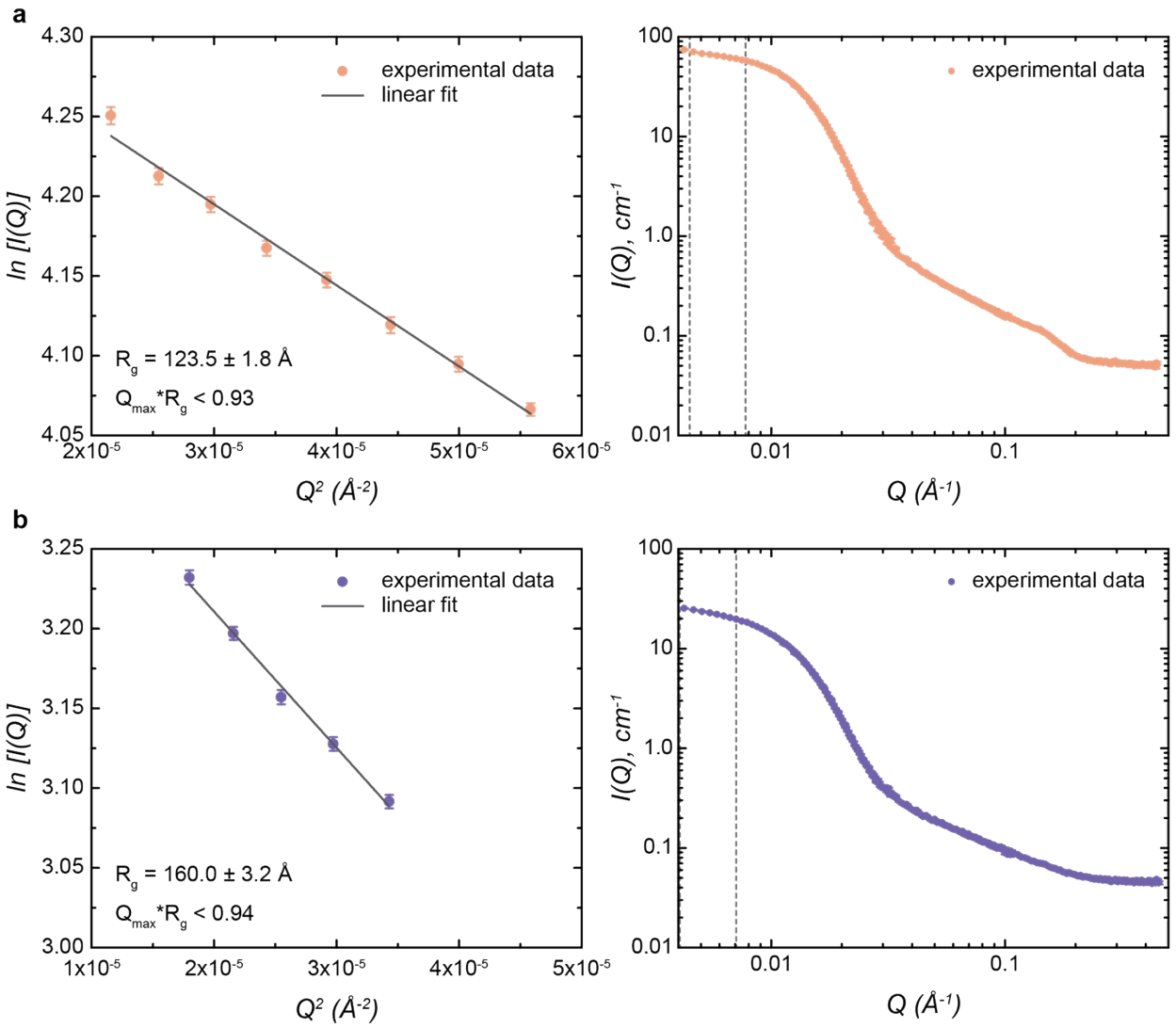


Figure S10. Guinier analysis was performed by fitting the slope of the following equation to determine the particle radius of gyration, R_g.

$$\log I(Q) = \log I(0) - \frac{R_g^2}{3}Q^2, \textit{ in the limit } QR_g \ll 1$$

The left plot shows the Guinier analysis fits and the right plot shows the bracketed data range used for Guinier analysis (dashed vertical lines) for (a) micelles with α-chymotrypsinogen (-18.0) and (b) micelles with RNase A (-14.2).

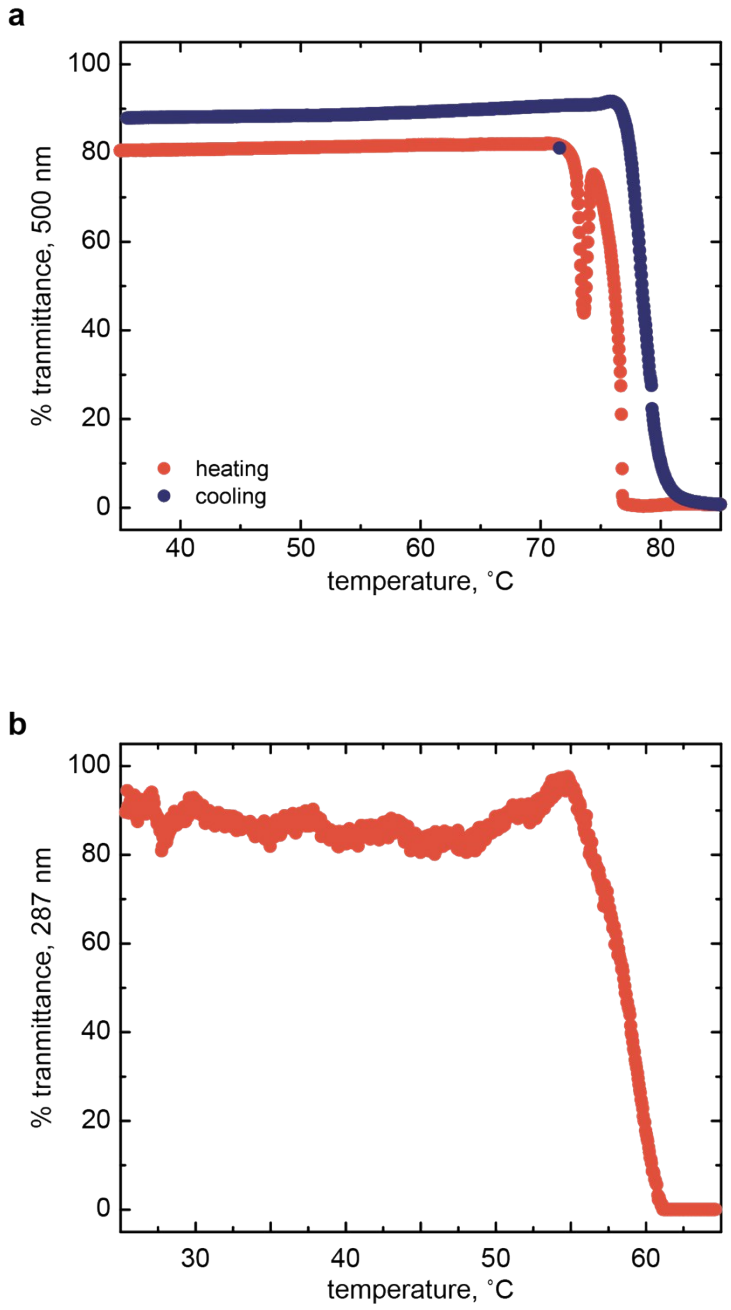


Figure S11. Thermal transitions of components of the complex coacervate core micelles. (a) The lower critical solution temperature (LCST) of the POEGMA neutral block was determined. The % transmittance of a 10 mg/mL solution of POEGMA in 10 mM tris buffer, pH 8.0 was monitored as the temperature was increased 0.1 °C/min from 35 °C to 85 °C and then cooled 0.1 °C/min to 35 °C. (b) The thermal denaturation of unmodified RNase A (10 mg/mL in 10 mM tris buffer, pH 8.0) was evaluated by monitoring tyrosine absorbance at 287 nm as the solution was warmed 0.1 °C/min from 25 °C to 65 °C.