Supporting Information

Temperature-Regulated Activity of Responsive Polymer-Protein Conjugates Prepared by Grafting-from via RAFT Polymerization

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Materials. 2-Dodecylsulfanylthiocarbonylsulfanyl-2-methylpropionyl chloride (DMP-Cl) was prepared as previously reported.[1] 2,2′-Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako Pure Chemical Industries, Ltd. and was recrystallized three times from methanol. *N*-Isopropylacrylamide (NIPAM, TCI America) was recrystallized using hexanes. *N*,*N*-Dimethylformamide (DMF, Aldrich 99.9%), triethylamine (EMD Chemicals, 99.5+%), N,N,N′,N′-ethylenediaminetetraacetic acid (EDTA, Acros Organics, 99%), bovine serum albumin (BSA, Aldrich, Approx. 99%), tris(2-carboxyethyl)phosphine hydrochloride (Calbiochem, TCEP, 99.7 %), 5,5′-dithio-bis-(2-nitrobenzenzoic acid) (Ellman's reagent, Pierce), 4-nitrophenyl acetate (Alfa Aesar, 98+%), $CDCl_3$ (Cambridge Isotope, 99% D), and deuterium oxide (D_2O, Acros, 99.8% D) were used as received. Dialysis membranes (Spectra/Por® 7, molecular weight cut-off (MWCO): 50,000 Da) were obtained from Spectrum Laboratories, Inc. All other chemicals were purchased from VWR and used without further purification, unless otherwise noted.

Synthesis of *N*-(methoxycarbonyl)maleimide: This procedure is modified from an earlier reported procedure.[2] Maleimide (2.0 g, 20 mmol, 1.0 equiv.) was dissolved in ethyl acetate (80 mL) in a 150 mL round-bottom flask, and the solution was cooled to approximately 0 °C. A solution of *N*-methyl morpholine, (2.00 mL, 1.85 g, 21.0 mmol, 1.10 equiv.) in ethyl acetate (10 mL) was added dropwise over 10 min. A solution of methyl chloroformate, (2.00 mL, 2.46 g, 20.0 mmol, 1.0 equiv.) in ethyl acetate (5.0 mL) was added dropwise, and the solution was allowed to reach room temperature while stirring for 1 h. The solution was diluted with ethyl acetate (100 mL) and washed with saturated aqueous sodium bicarbonate solution, water, and saturated sodium chloride solution, successively. The organic layer was separated, dried over $MgSO_4$, and filtered. The supernatant was concentrated under reduced pressure to yield the product as a solid (2.6 g, 81 % yield). m_p = 60-61^oC. ^{1}H-NMR (400 MHz, ppm, $CDCl_3$): 6.88 (d, 2H, J = 0.6 Hz, -C***H***=C***H***-CO), 3.97 (s, 3H, COOC***H***$_3$). ^{13}C-NMR (100.6 MHz, ppm, $CDCl_3$): 165.5 (-CH=CH-***C***=O), 147.9 (-N-***C***$OOCH_3$), 145.1 (-***C***H=***C***H-C=O), 54.1 (-NCOO***C***H_3). IR (KBr, cm^{-1}): 3103, 3013 (C-Cs), 1773 (C=O), 1441 (C=Cs), 1261, 1137 and 848 (C-Cb). Elemental Analysis: Calcd for $C_6H_5NO_4$: C = 46.46%, H = 3.25%, N = 9.03%. Found: C = 46.30%, H = 3.87%, N = 8.25%.

Synthesis of *N*-[2-(2-hydroxyethoxy)ethyl]-maleimide: [3] 2-(2-Aminoethoxy)-ethanol (680 mg, 6.45 mmol, 1.0 equiv.) was dissolved in a saturated aqueous solution of sodium bicarbonate (20 mL). The solution was cooled to 0 ^oC and stirred for 10 min before adding *N*-(methoxycarbonyl)maleimide (1.0 equiv.). The resulting solution was stirred at

0 ^{o}C for 20 min and at room temperature for an additional 30 min before being extracted with chloroform (50 mL × 3), dried over $MgSO_4$, filtered, and concentrated under reduced pressure to yield a residual oil (1.08 g, 90%). ^{1}H-NMR (400 MHz, ppm, $CDCl_3$): 6.73 (s, 2H, -C***H***=C***H***-CO), 4.90 (br-s, 1H, OH), 3.76-3.73 (t, 2H, J = 5.42 Hz, -$OCH_2C\boldsymbol{H}_2OH$), 3.67-3.64 (m, 4H, -$C\boldsymbol{H}_2OC\boldsymbol{H}_2$-), 3.58-3.56 (t, 2H, J = 4.6 Hz, -$CONC\boldsymbol{H}_2CH_2O$-). ^{13}C-NMR (100.6 MHz, ppm, $CDCl_3$): 170.7 (-CH=CH-***C***=O), 134.1 (-***C***H=***C***H-C=O), 72.1 (-O***C***H_2CH_2OH), 68.2 (-$CONCH_2\boldsymbol{C}H_2O$-), 61.6 (-$OCH_2\boldsymbol{C}H_2OH$), 37.4 (N***C***$H_2CH_2O$-). IR (KBr, cm^{-1}): 3100, 2933 (C-Cs), 1773 (C=O), 1445 (C=Cs), 1265, 1140 and 848 (C-Cb).

Synthesis of the RAFT agent, 2-dodecylsulfanylthiocarbonylsulfanyl-2-methylpropionic acid maleimido ethoxyethyl ester (Maleimide-CTA).

Maleimidoethoxy-ethanol (483 mg, 2.60 mmol, 1.00 equiv.) was dissolved in methylene chloride (5.0 mL) in a 50 mL round-bottom flask, and the solution was cooled to 0 °C. A solution of triethylamine (0.73 mL) in methylene chloride (5.0 mL) was added dropwise over 10 min. A solution of DMP-Cl (1.0 g, 2.6 mmol) in methylene chloride (5.0 mL) was added dropwise to the round-bottom flask. The solution was allowed to reach room temperature and stirred for 3 h before being concentrated under reduced pressure, diluted with diethyl ether (100 mL), and washed with saturated aqueous sodium bicarbonate solution, water, and saturated NaCl solution, successively. The organic layer was separated, dried over $MgSO_4$, and filtered. The supernatant was concentrated under reduced pressure to yield the product (0.80 g, 58 % yield) as a residual yellow solid. ^{1}H-

NMR (400 MHz, ppm, $CDCl_3$): 6.73 (s, 2H, -C***H***=C***H***-CO), 4.23-4.20 (t, 2H, *J* = 4.3 Hz, -OCH_2-C***H*****$_2$**-O-C=O), 3.74-3.71 (t, 2H, *J* = 5.4 Hz, -N-C***H*****$_2$**-CH_2-O-), 3.65-3.63 (t, 4H, *J* = 5.0 Hz, -N-CH_2-C***H*****$_2$**-O-C***H*****$_2$**-CH_2-OCO-), 3.30-3.26 (t, 2H, *J* = 7.3 Hz, -CH_2-C***H*****$_2$**-S-C=S), 1.70 (s, 6H), 1.67-1.64 (s, 2H, *J* = 7.5 Hz, -C***H*****$_2$**-CH_2-S-C=S) 1.39-1.27 (m, 18H, CH_3-C_9***H*****$_{18}$**-CH_2-CH_2S-C=S), 0.91-0.88 (t, 3H, *J* = 5.8 Hz, C***H*****$_3$**-C_9H_{18}-CH_2-CH_2S-C=S). ^{13}C-NMR (100.6 MHz, ppm, $CDCl_3$): 172.7 (-CH_2-O-***C***=O), 170.5 (-CH=CH-***C***O), 134.1 (-***C***H=***C***H-CO), 68.2 (-OCH_2-***C***H_2-O-C=O), 67.7 (-N-CH_2-CH_2-O-***C***H_2-CH_2-OCO-), 64.9 (-N-CH_2-***C***H_2-O-CH_2-CH_2-OCO-), 55.8 (-S-***C***$(CH_3)_2$-CO), 37.1 (-N-***C***H_2-CH_2-O-), 36.9 (-CH_2-***C(1)***H_2-S-C=S), 31.8 (-***C(2)***H_2-***C***H_2-S-C=S), 29.6 (-***C(3 & 4)***H_2-CH_2-S), 29.5 (-***C(5)***H_2-CH_2-S), 29.4 (-***C(6)***H_2-CH_2-S), 29.3 (-***C(7)***H_2-CH_2-S), 29.0 (-***C(8)***H_2-CH_2-S), 28.9 (-***C(9)***H_2-CH_2-S), 27.8 (-***C(10)***H_2-CH_2-S), 25.2 (-S-C(***C***$H_3)_2$-CO), 22.6 (-***C(11)***H_2-CH_2-S), 14.0 (***C(12)***H_3-C_9H_{18}-CH_2-CH_2S-C=S). FTIR (KBr, cm^{-1}): 2924 and 2853 (C-C*s*), 1711 (C=O), 1066 (C=S), 1123 and 825 (C-C*b*). Elemental Analysis. Calcd for $C_{25}H_{41}NO_5S_3$: C = 56.46%, H = 7.77%, N = 2.63%. Found: C = 56.40%, H = 7.94%, N = 1.99%.

Conjugation of BSA with Maleimide CTA to Obtain BSA macroRAFT Agent. BSA (0.2 g, 3.0 μmol, 1 equiv.) was dissolved in phosphate buffer (PB) solution (15 mL, pH 7.2) in a 20 mL glass vial equipped with a magnetic stir bar. The solution was purged with nitrogen for 40 min. A solution of maleimide-CTA (32 mg, 6.0×10^{-5} mol, 20 equiv.) in nitrogen-purged DMF (1.0 mL) was added dropwise, and the solution was stirred at 25 °C for 20 h. The reaction mixture was centrifuged twice (at 4,100 rcf for 20 min at 5

°C) to remove excess maleimide CTA. Subsequently, the supernatant was dialyzed against deionized (DI) water (6 × 18 L) for 36 h using a MWCO of 50,000 Da, and then lyophilized to isolate the BSA macroRAFT agent (BSA-macroCTA). Ellman's analysis (see below) was carried out to determine the number of sulfhydryl groups present in BSA before and after conjugation of the RAFT agent.

Ellman's assay. 5,5′-Dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent[4], 4 mg) was dissolved in 0.1 M PB (1 mL, pH 7.2, containing 2 mM EDTA) to prepare the Ellman's reagent solution. A portion of the sample to be analyzed (5 mg) was added to buffer (2.50 mL) mixed with the Ellman's reagent solution (50 μL) and incubated for 45 min at room temperature. The absorbance (λ_{max} = 412 nm) of the 5-thio-2-nitrobenzoic acid (TNB) product was measured by UV-vis spectroscopy. The thiol concentration was calculated from the Beer-Lambert law. (The molar extinction coefficient of TNB in our experimental conditions was determined to be 16,560 M^{-1} cm^{-1} at 412 nm.)

General Procedure for the RAFT Polymerization of NIPAM Using BSA-macroRAFT Agent. A typical RAFT polymerization procedure was as follows. NIPAM (67.6 mg, 0.597 mmol), BSA-macroCTA (40.0 mg, 0.597 μmol), PB pH 6.0 (1.8 mL) were sealed in a 8 mL glass vial equipped with a magnetic stir bar and purged with nitrogen for 30 min in an ice bath. A concentrated and nitrogen-purged solution of VA-044 (0.0030 mmol in 0.2 mL) in PB (pH 6.0) was added by syringe, and the reaction vial

was placed in a reaction block preheated to 26 °C. VA-044 was employed at a ratio of $[VA\text{-}044]_o$: $[CTA]_o$ = 5:1 because of its slow decomposition at room temperature ($k_d \approx 1.5\times10^{-6}$ s^{-1} at 25 °C). Samples were removed periodically by syringe for size exclusion chromatography (SEC) analysis and monomer conversion by ^1H NMR spectroscopy. The polymerization was quenched after a predetermined time, and the reaction mixture was diluted with approximately DI water (12 mL) and dialyzed against DI water (6×18 L) for 36 h using a membrane with MWCO 50,000 Da. The resulting solution was lyophilized to isolate the protein-polymer conjugates.

Thermally-Induced Precipitation. After the RAFT polymerization of NIPAM with the BSA-macroCTA, unconjugated BSA was removed from the BSA-PNIPAM conjugates by thermally induced precipitation. A solution of conjugate (30 mg/mL) in DI water was heated at 40 °C for 10 min, followed by centrifugation at 13,600 rcf at 25 °C for 1 min. The supernatant was decanted, and the precipitate was dissolved in DI water. The above procedure was repeated to remove unconjugated BSA from the BSA-PNIPAM bioconjugates. Finally, the precipitate was dissolved in DI water and lyophilized to isolate BSA-PNIPAM bioconjugates. It is important to note that efficient thermal precipitation of the conjugate required centrifugation and a relatively high solution concentration of 30 mg/mL.

Size Exclusion Chromatography (SEC). SEC of the cleaved PNIPAM was conducted in DMF with 50 mM LiBr at 55 °C at a flow rate of 1.0 mL/min (Viscotek GPC pump; columns: ViscoGel I-series G3000 and G4000 mixed bed columns: molecular weight

range 0-60×10^3 and 0-400×10^3 g/mol, respectively). Detection consisted of a Viscotek refractive index detector operating at λ = 660 nm, and a Viscotek model 270 series platform consisting of a laser light scattering detector (operating at 3 mW, λ = 670 nm with detection angles of 7° and 90°) and a four-capillary viscometer. Molecular weights were determined by conventional calibration based on polystyrene standards. For characterization of BSA, BSA-macroCTA, and BSA-polymer conjugates, SEC was conducted in water (containing 0.5% w/v NaN_3) at 23 °C with a flow rate of 0.7 mL/min (Viscotek GPC pump; column: Biosep-SEC-S 3000). Detection consisted of a Viscotek refractive index detector operating at λ = 660 nm, and a Viscotek UV-vis detector operating at λ = 360nm. Calibration was accomplished with poly(ethylene oxide) standards.

UV-vis Spectroscopy. UV-vis spectroscopy was conducted with an Ocean Optics UV-vis spectrophotometer (USB2000, Ocean Optics Inc.).

FTIR Spectroscopy. FTIR spectra were obtained with a Nicolet Magna-IR 560 E.S.P. spectrometer.

Dynamic Light Scattering. Dynamic light scattering (DLS) was conducted with a Malvern Zetasizer Nano-S equipped with a 4 mW, 633 nm He-Ne laser, and an Avalanche photodiode detector at an angle of 173°. The temperature of the polymer solutions (0.2 wt %, filtered through 0.45μ syringe filter) was regulated within an error of ± 0.1 °C.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF). MALDI-TOF MS was employed to confirm functionalization of BSA with the RAFT agent moiety. Analysis of the high molecular weight BSA-PNIPAM conjugates (M_n > 300,000 g/mol) proved more difficult, and no conclusive results were obtained. Spectra of BSA and BSA macroCTA were collected using a Bruker Daltonics Autoflex II mass spectrometer equipped with a N_2 laser (λ=337 nm) with a pulse width of 3 ns, a 2 GHz acquisition digitizer, and a pulsed ion extraction source operated with flexControl 2.4 software. Data analysis was performed using flexAnalysis 2.4 software. Protein solutions (1 mg/mL) were prepared by dissolving in acetonitrile/water (1:1 v/v) with 0.1% trifluoroacetic acid. The matrix, sinapinic acid, was dissolved in the same solvent system at a concentration of 25 mg/mL. Equal volumes of matrix and analyte were mixed, vortexed, and spotted onto a stainless steel target. Each spectrum consists of at least 200 average scans. The molar mass of the native BSA and BSA-macroCTA were calculated to be 66,450 and 66,867 g/mol. The MALDI-TOF spectra are provided in Figure S1.

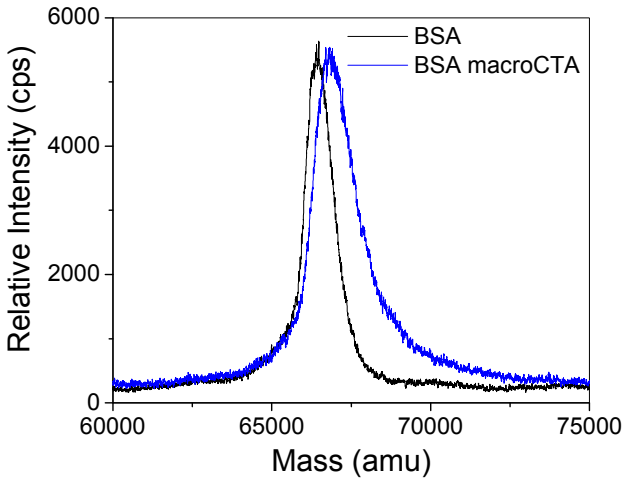

Figure S1. MALDI-TOF spectra for BSA and BSA-macroCTA.

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectroscopy was conducted with a Bruker Avance 400 spectrometer operating at 400 and 100 MHz, respectively. Conversion of NIPAM was determined from the integration ratio of the vinyl protons at 6.02-6.15 ppm (2H from NIPAM monomers) to the isopropyl -C***H***-$(CH_3)_2$ area of both monomer and PNIPAM at 3.7-3.9 ppm. As is commonly reported, no appreciable resonance signals from BSA could be observed in the ^{1}H NMR spectrum under the measurement conditions (Figure S2).

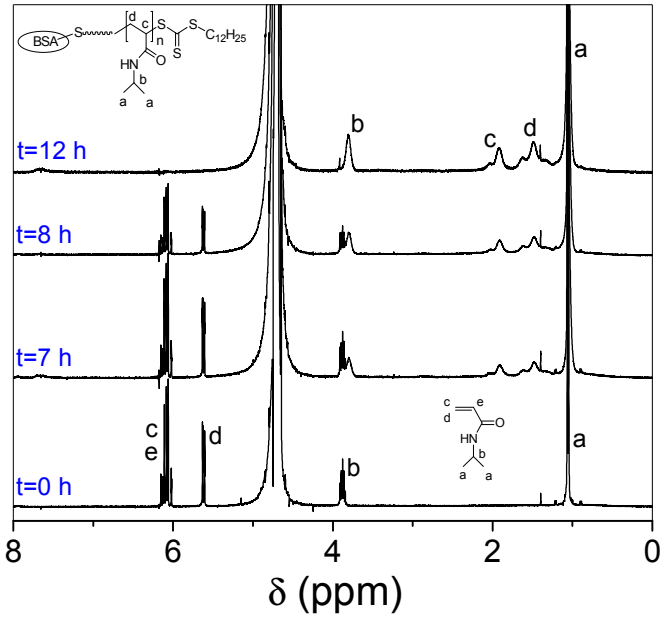

Figure S2. ^{1}H NMR spectra obtained after the polymerization of NIPAM in the presence of BSA-macroCTA as a function of time (PB pH 6.0 at 26 °C, [NIPAM] = 0.3 M).

Lower Critical Solution Temperature (LCST) Measurements. The BSA-PNIPAM conjugates and PNIPAM were dissolved in DI water (0.2 mg/mL) and cooled at 4 °C for 75 h to ensure complete dissolution. The samples were transferred to a quartz cuvette and warmed from 24 °C to 48 °C over 30 min in a preheated temperature block equipped with a digital thermometer. The cuvette was quickly removed from the preheated temperature block and the absorbance at 500 nm was measured by a UV-vis spectrometer. The LCST was defined as the temperature where 10% of the maximum absorbance was observed.[5] The results are shown in Figure S3.

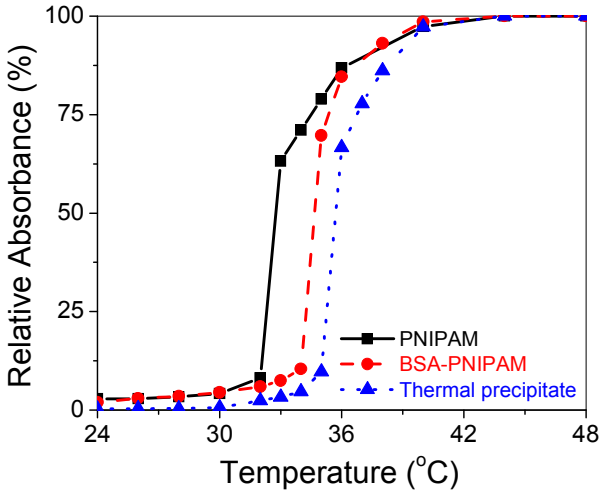

Figure S3. Plot of relative absorbance at 500 nm versus temperature for 0.2 wt % solution in DI water of PNIPAM (■, solid line, M_n = 48,000, PDI = 1.35), BSA-PNIPAM (●, dashed line), and thermally precipitated BSA-PNIPAM (▲, dotted line) bioconjugates.

Dynamic Light Scattering (DLS): BSA-PNIPAM was dissolved in DI water (1 mg/mL) and incubated at 4 °C for 24 h. The resulting solution was filtered through a 0.45 micron syringe filter, and DLS measurements were recorded over a temperature range of 20 to 45 °C (1 °C increments, 30 min equilibration at each temperature). A dramatic increase in size, indicative of the LCST, was observed at 34 °C, in good agreement with the value of 35 °C determined by turbidimetry.

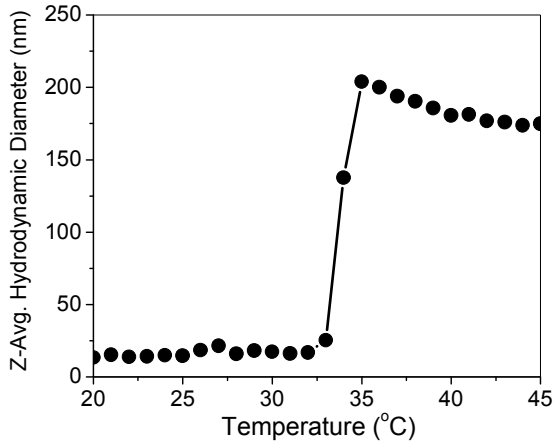

Figure S4. Hydrodynamic diameter versus temperature for BSA-PNIPAM in DI water.

Figure S5 shows representative size distributions obtained by DLS at temperatures below and above the LCST of BSA-PNIPAM conjugates. As expected, the BSA-PNIPAM conjugate (15 nm at 25 °C) was larger than BSA (4.4 nm, 25 °C). Upon heating an aqueous solution of the conjugates, a dramatic increase in hydrodynamic diameter was observed, consistent with intermolecular aggregation upon dehydration of the tethered PNIPAM chains. BSA-PNIPAM formed uniform aggregates at 45 °C with size of approximately 175 nm. There was no appreciable change in size of BSA at 45 °C (3.9 nm).

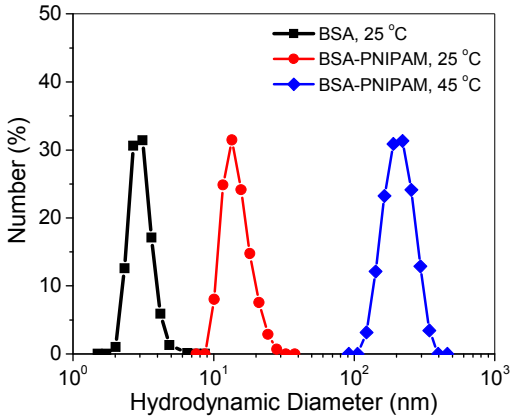

Figure S5. Hydrodynamic **s**ize distributions as determined by DLS for BSA at 25 °C, BSA-PNIPAM at 25 °C, and BSA-PNIPAM at 45 °C.

SEC Measurements at Different Wavelength: SEC measurements for BSA, BSA-macroCTA, BSA-PNIPAM conjugates, and PNIPAM were carried out with a $\lambda = 360$ nm UV-Vis detector wavelength. There were no peaks observed at this wavelength for PNIPAM (M_n = 48,000, PDI = 1.35). A peak was observed for BSA and the BSA-macroCTA. For BSA-PNIPAM conjugates (with unconjugated BSA present), there were two small peaks due to the presence of unconjugated BSA and BSA-PNIPAM conjugates. The thermally precipitated BSA-PNIPAM conjugates with no unconjugated BSA showed only one peak. The results are shown in Figure S4.

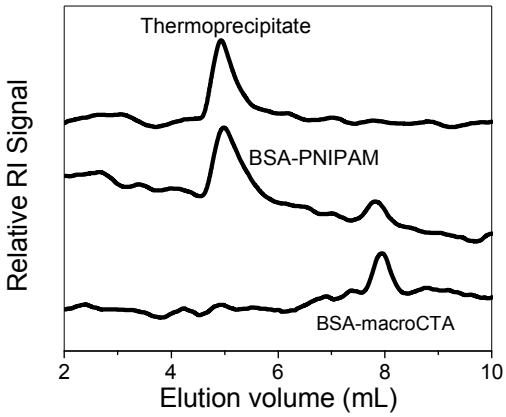

Figure S6. SEC UV-vis traces at λ = 360 nm of BSA-macroCTA, BSA-PNIPAM conjugates and thermally precipitated BSA-PNIPAM with no unconjugated BSA.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (PAGE). PAGE was performed with a Bio-Rad Laboratories Mini-Protean 3 Cell System using 12% Precise™ Protein Gels (Pierce) at 100 V voltage and 120 mA current for 45 min. Samples were dissolved in DI water ([BSA] = 0.12 mg/mL), mixed with 5 μL lane marker sample buffers (Pierce), and heated at 95 ºC for 5 min (samples were heated only for reducing conditions) before loading. Staining was accomplished with coomassie blue. PAGE results are shown in Figure S5 for BSA and conjugates in reducing and non-reducing conditions. Because the PNIPAM was attached to the protein via an ester linkage, the resulting conjugates were thermally and reductively stable.

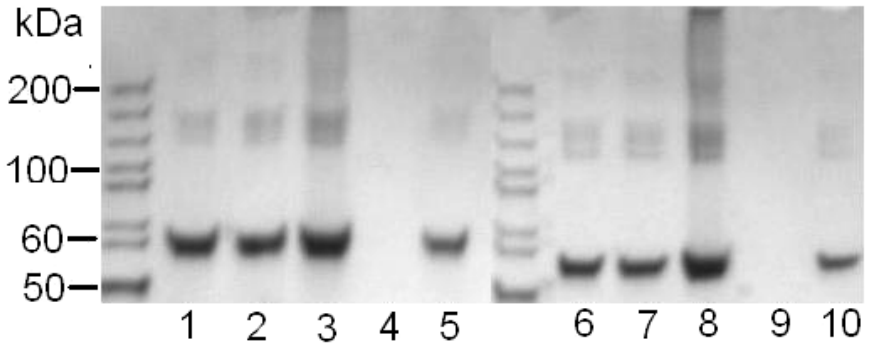

Figure S7. PAGE of BSA and conjugates; Lanes 1-5 : reducing conditions and Lanes 6-10: non-reducing conditions. Lanes 1, 6 : BSA, Lanes 2, 7: BSA-macroCTA, Lanes 3, 8: BSA-PNIPAM (obtained from the polymerization of [NIPAM] : [BSA-macroCTA] : [VA-044] = 1000 : 1 : 5.0 at 26 °C in PB pH 6.0, Conversion of NIPAM = 75 %), Lanes 4, 9: PNIPAM (M_n = 48,000, PDI = 1.35) (control), Lanes 5, 10: physical mixture of BSA and PNIPAM (control).

Bioactivity assay of BSA and BSA-PNIPAM conjugates: The bioactivity of BSA and the BSA conjugates was determined by observing the absorbance associated with the hydrolysis product of 4-nitrophenyl acetate in a manner similar to a previously published procedure.[6] A BSA or BSA conjugate solution (50 μL, BSA concentration: 0.27 mM) in PB (pH 8.0) was mixed with a solution of 4-nitrophenyl acetate (10 mM) dissolved in acetonitrile (10 μL) and PB (0.94 mL, pH 8.0) by centrifuging for 5 min at 6,000 rpm. After incubating at room temperature for 30 min, the absorbance at λ = 405 nm was measured for each sample to evaluate the activity. Measurements were performed with three different samples (activities reported as the average of 3 measurements ± standard deviation).

The bioactivity at of BSA and BSA-PNIPAM at 40 °C (above the LCST of the PNIPAM conjugate) was measured according to the following procedure. A BSA or BSA conjugate solution (50 μL, BSA concentration: 0.27 mM) in PB (pH 8.0) was mixed with a solution of 4-nitrophenyl acetate (10 mM) dissolved in acetonitrile (10 μL) and PB (0.94 mL, pH 8.0) by centrifuging for 5 min at 6,000 rpm. After incubating at 40 °C for 25 min, the solution was kept at room temperature for 5 minutes and the absorbance at λ = 405 nm was measured for each sample to evaluate the activity. The percentage bioactivity of the BSA-PNIPAM conjugate is reported with reference to BSA incubated at 40 °C.

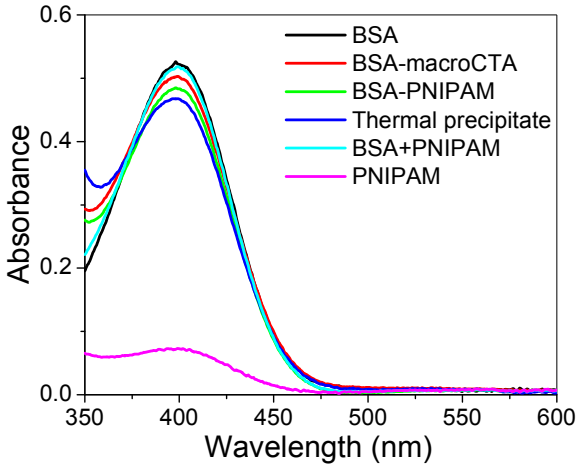

Figure S8. UV-vis spectra from the room temperature bioactivity assay of BSA, BSA-macroCTA, BSA-PNIPAM conjugates, and PNIPAM (control).

To investigate the effect of thermal cycling on the bioactivity of BSA-PNIPAM conjugates, assays were also conducted after multiple heating/cooling cycles. The procedure was similar to that described above with the following exceptions. Ten

samples of BSA-PNIPAM were prepared, and the activity of one sample was measured at 25 °C as described above. The remaining samples were heated and cooled multiple times with one sample being removed and having its bioactivity measured at 45 and 25 °C during each cycle. The process was repeated for a total of 5 heating/cooling cycles. The results are shown in the main text in Figure 2b.

While thermal cycling of some temperature-responsive protein conjugates has been reported to lead to reduced enzymatic activity, our studies demonstrate the activity of BSA is largely retained. This can potentially be attributed to the rather robust nature of BSA, as compared to more sensitive proteins.[78]

In addition to 25 and 40 °C, bioactivity assays for the BSA-PNIPAM conjugates were conducted at other elevated temperatures. In each case, the percentage activity was calculated with respect to pure BSA at the same temperature and protein concentration. As seen from Figure S9, activity was measured at 25, 35, 40, 50, and 75 °C. Essentially no difference was observed between the temperatures that were above the LCST. This is consistent with the sharp transitional nature of the LCST and offers further proof that the reduced activity was not a result of thermal denaturing, in which case the bioactivity would be expected to decrease with increased temperature.

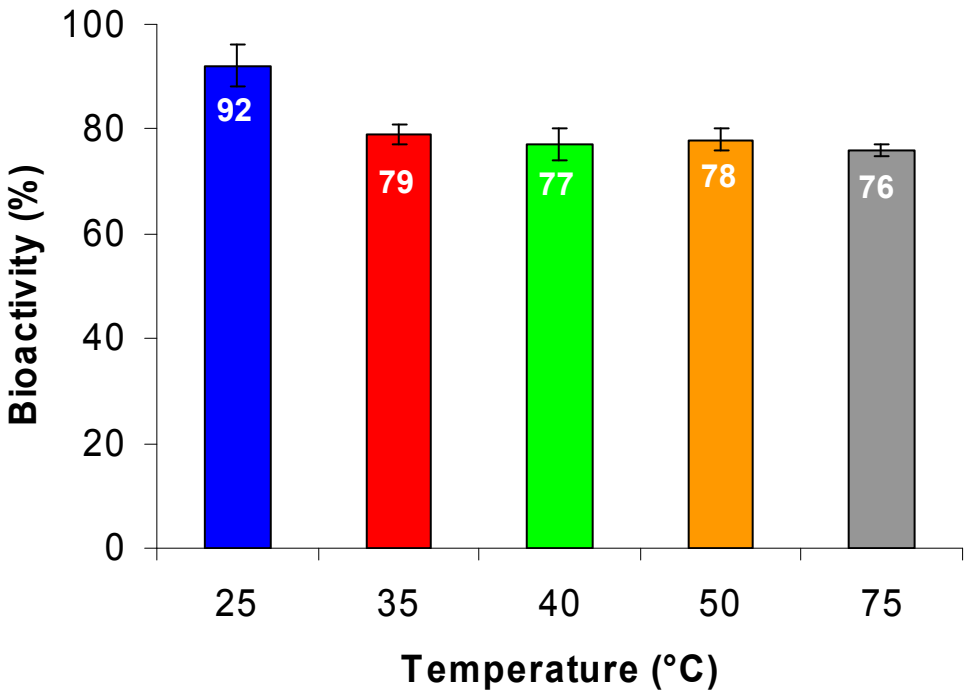

Figure S9. Bioactivity of BSA-PNIPAM conjugates as a function of temperature.

Circular dichroism: Circular dichroism (CD) spectra were measured at 20 ± 1 °C with a Jasco - 810 spectropolarimeter (Jasco, Tokyo, Japan) in sodium phosphate buffer (pH 7.14) and a cell with path length of 0.1 cm (bandwidth = 1 nm; step resolution = 0.1 nm; scan speed = 50 nm/min; response time = 0.25 s).

CD bands in the amide region (170-250 nm) give information about peptide bonds and the secondary structure of the protein and are frequently analyzed to monitor changes in secondary structure. In particular, the α-helix content displays a characteristic CD spectrum in the far-UV region. Native BSA is reported to contain about 55% α-helical structure. The CD spectra of BSA exhibited two negative peaks at 208 and 222 nm,

which is typical for an α-helix structure (Figure S10). The incorporation of the CTA unit to BSA resulted in no noticeable decrease in band intensity or shift of peaks, indicating that the CTA induced no decrease in the helix structure and did not lead to unfolding of the constitutive polypeptides of the protein (despite the DMF cosolvent was used during BSA-macroCTA synthesis). No significance difference in the peak intensity was observed for BSA-PNIPAM, as compared to BSA, indicating the protein in the conjugate retained its secondary structure.

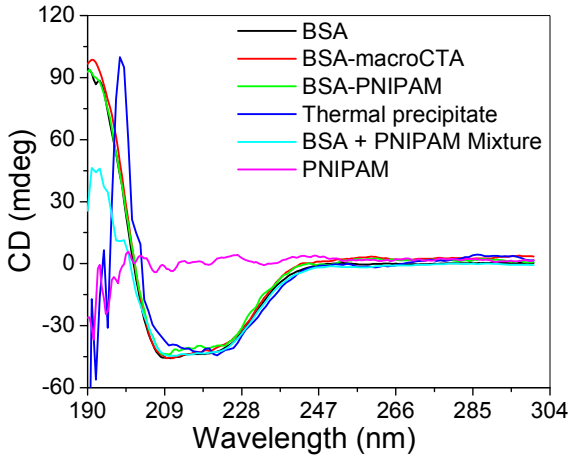

Figure S10. Circular dichroism spectra of BSA, BSA-macroCTA, BSA-PNIPAM conjugates (free BSA present), thermal precipitate (BSA-PNIPAM conjugates without free BSA), BSA + PNIPAM physical mixture, and PNIPAM (M_n = 48,000, PDI = 1.35).

Decomposition of BSA from BSA-PNIPAM Bioconjugates: Decomposition[9] of BSA from the BSA-PNIPAM conjugates with different PNIPAM chain lengths was conducted by reacting with TCEP at room temperature in DI water according to the

following general procedure. BSA-PNIPAM (0.09 μmol BSA, 1 equiv.) and TCEP (9 μmol, 100 equiv.) were dissolved in DI water (5 mL), purged with nitrogen for 30 minutes, and the solutions were stirred at room temperature 5 days. The remaining polymer was isolated by dialyzing against DI water and freeze-drying. Essentially quantitative decomposition of BSA was confirmed by gravimetric analysis. Aqueous SEC analysis and SDS-PAGE (Figure S11) of the lyophilized material showed complete disappearance of BSA. SEC of the filtered product in DMF allowed determination of the polymer molecular weight.

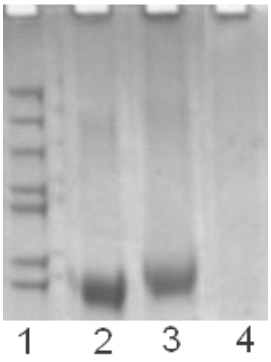

Figure S11. PAGE results; Lane 1: MW markers, Lane 2: BSA-macroCTA, Lane 3: BSA-PNIPAM (obtained from the polymerization of $[NIPAM]_o$: $[BSA\text{-}macroCTA]_o$: $[VA\text{-}044]_o$ = 1000 : 1 : 5.0 at 26 °C in PB pH 6.0, Conversion of NIPAM = 75 %), Lane 4: Product obtained after the decomposition of BSA from the BSA-PNIPAM bioconjugate.

References

(1) Gondi, S. R.; Vogt, A. P.; Sumerlin, B. S. *Macromolecules* **2007,** *40*, 474.

(2) Keller, O.; Rudinger, J. *Helvetica Chimica. Acta.* **1975**, *58*, 531.

(3) (a) Heredia, K. L.; Bontempo, D.; Ly, T.; Byers, J. T.; Halstenberg, S.; Maynard, H. D. *J. Am. Chem. Soc.* **2005**, *127*, 16955. (b) Weber, R. W.; Boutin, R, H.; Nedelman M. A.; Lister-James, J.; Dean, R. T. *Bioconjugate Chem*. **1990**, *1*, 431.

(4) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70.

(5) Shimoboji, T.; Ding, Z. L.; Stayton, P. S.; Hoffman, A. S. *Bioconjugate Chem.* **2002**, *13*, 915.

(6) (a) Tildon, J. T.; Ogilvie, J. W. *J. Biol. Chem*. **1972**, *247*, 1265. (b) Means, G. E.; Bender, M. L. *Biochem*. **1975**, *14*, 4989.

(7) Chen, G.; Hoffman, A. S. Bioconjugate Chem. 1993, 4, 509.

(8) Shimoboji, T.; Larenas, E.; Fowler, T.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2003,** *14,* 517

(9) Liu, J.; Bulmus, V.; Barner-Kowollik, C.; Stenzel, M. H.; Davis, T. P. *Angew. Chem. Int. Ed.* **2007**, *46*, 3099.