Heterotelechelic Polymers for Capture and Release of Protein-Polymer Conjugates

Karina L. Heredia,[a] Lei Tao,[a] Gregory N. Grover,[a] and Heather D. Maynard[a]*

[a]*Department of Chemistry and Biochemistry and California NanoSystems Institute, 607 Charles E. Young Drive East, University of California, Los Angeles, CA 90095*

**maynard@chem.ucla.edu*

Supporting Information

Experimental

Materials

Reacti-Bind neutravidin-coated 96-well plates, SuperBlock blocking buffer (BB), *o*-phenylenediamine hydrochloride (OPD), and high capacity streptavidin agarose resin were purchased from Pierce. Mouse anti-bovine serum albumin-horse radish peroxidase (anti-BSA-HRP) was purchased from US Biological. *N*-Isopropylacrylamide (NIPAAm, Sigma) was recrystallized twice from hexane; tetrahydrofuran (THF, Fisher) was distilled over sodium/benzophenone and stored under argon; and 2,2'azobisisobutyronitrile (AIBN) was recrystalized from acetone. 2-(Ethyl trithiocarbonate)propionic acid,[1] biotin-maleimide poly(*N*-isopropylacrylamide) (pNIPAAm) (**P3**) (M_n 18,400 Da, PDI 1.10 by gel permeation chromatography (GPC)) and the protected maleimide azo-initiator **3** were prepared as previously described.[2] V131C T4-lysozyme plasmid was obtained from Prof. Wayne Hubbell (UCLA) and was expressed and isolated from *E. coli* as reported in literature.[3, 4] All other chemicals were purchased from Sigma or Fisher.

Analytical Techniques

^{1}H and ^{13}C NMR spectra were acquired on a Bruker Avance 500 MHz or 600 MHz DRX and spectra were processed using Topspin 1.2 NMR software. UV-Vis spectra were obtained on a Biomate 5 Thermo Spectronic UV-Vis spectrometer using quartz cells. High resolution electrospray ionization mass spectrometry (ESI-MS) was performed on an IonSpec Ultima 7T ICR, (Varian Inc.). Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. TLC plates were pre-coated with silica gel 60 F254 and were developed in the indicated solvent systems. Merck 60 (230-400 Mesh) silica gel was used for column chromatography. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μm mixed D columns. LiBr (0.1 M) in DMF at 40 °C was used as the eluent (flow rate: 0.80 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) standards from Polymer Laboratories. Chromatograms were processed using the EZStart 7.2 chromatography software. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 4-15% TRIS-glycine precast gradient gels (Invitrogen), and samples were dissolved in TRIS buffer containing SDS, bromophenol blue and glycerol. ELISA was performed using a Bio-Tek Instruments, Inc. EL_X800 universal plate reader and analyzed using KCjunior Microplate Data Analysis Software.

Methods

Synthesis of biotinylated disulfide trithiocarbonate CTA 2. 2,2'-disulfane-diyldiethanol (6.16 g, 40 mmol), 2-(ethyl trithiocarbonate)propionic acid (2.10 g, 10 mmol), and 4-(dimethylamino)pyridine (DMAP) (0.122 g, 1 mmol) were dissolved in dry THF (50 mL). *N*,*N*′-

Dicyclohexylcarbodiimide (DCC) (2.27 g, 11 mmol) was then added and the contents was stirred at 23 °C for 16 h, and then filtered to remove the formed solids. The solvent was removed *in vacuo* and the crude product was purified by silica gel chromatography (4:1 hexane: ethyl acetate (EtOAc) yielding 2-((2-hydroxyethyl) disulfanyl)ethyl 2-(ethylthio-carbonothioylthio) propanoate as a yellow oil in 72 % yield (2.50 g). δ ^{1}H NMR (600 MHz, $CDCl_3$): 4.81 (q, J = 7.4 Hz, 1H, $CH_3C\mathbf{H}$), 4.43-4.39 (m, 2H, O=COC$\mathbf{H}_2$), 3.88 (t, J = 5.8 Hz,2H, COC$\mathbf{H_2}CH_2S$), 3.38-3.34 (m, 2H, $CH_3C\mathbf{H_2}S$), 2.93 (t, J = 6.6 Hz, 2H, SC$\mathbf{H_2}CH_2O$), 2.88 (t, J = 5.7 Hz, 2H, $HOCH_2C\mathbf{H_2}S$), 1.60 (d, J = 7.4 Hz, 3H, C$\mathbf{H_3}$CH), 1.35 (t, J = 7.4 Hz, 3H, C$\mathbf{H_3}CH_2S$); δ ^{13}C NMR (600 MHz, $CDCl_3$): 221.91, 171.22, 63.62, 60.38, 47.89, 41.74, 36.83, 31.72, 16.85, 13.09; IR (cm^{-1}): 3365, 2927, 2870, 1730, 1646, 1448, 1400, 1377, 1297, 1247, 1151, 1066, 1042, 1028, 873, 810, 775; ESI-MS observed (predicted): [M+Na] 368.97 (368.98), [M+K] 384.94 (384.95); UV-Vis (MeOH): λ_{max}(SC=SS) = 306 nm.

Biotin (244 mg, 1.0 mmol), 2-((2-hydroxyethyl) disulfanyl) ethyl 2-(ethylthio-carbonothioylthio) propanoate (380 mg, 1.1 mmol) and DMAP (12.2 mg, 0.1 mmol) were dissolved in dry DMF (15 mL). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (460 mg, 2.4 mmol) was then added and the solution was stirred at 23 °C for 48 h. The DMF was removed *in vacuo*, and the crude product was purified by silica gel chromatography (9:1 dichloromethane (DCM): methanol (MeOH) yielding CTA **2** as a yellow solid in 82 % yield (470 mg). δ ^{1}H NMR (600 MHz, $CDCl_3$): 4.77 (q, J = 7.4 Hz, 1H, SC$\mathbf{H}CH_3$), 4.48 (dd, J = 5.0 Hz, 7.6 Hz, 1H, NHC$\mathbf{H}$CHS), 4.39-4.35 (m, 2H, CH_2C=OOC$\mathbf{H}_2$), 4.30-4.27 (m, 3H, C$\mathbf{H}_2$OC=OCH & NHC$\mathbf{H}CH_2$), 3.36-3.32 (m, 2H, SC$\mathbf{H_2}CH_3$), 3.14-3.11 (m, 1H, NHCHC$\mathbf{H}$S), 2.91-2.86 (m, 5H, C$\mathbf{H}_2$SSC$\mathbf{H}_2$ & NHCHC$\mathbf{H}$HS), 2.71 (d, J = 12.7 Hz, 1H, NHCHCH$\mathbf{H}$S), 2.33 (t, J = 7.5 Hz 2H, C$\mathbf{H_2}C$=OOCH_2), 1.73-1.60 (m, 4H, SCHC$\mathbf{H}_2$ & $SCHCH_2CH_2C\mathbf{H}_2$), 1.58 (d, J

= 7.4 Hz, 3H, C**H**$_3$CHS), 1.45-1.39 (m, 2H, CHCH$_2$C**H**$_2$), 1.33 (t, *J* = 7.4 Hz, 3H, C**H**$_3$CH$_2$S); δ ^{13}C NMR (600 MHz, $CDCl_3$): 221.76, 173.47, 171.01, 163.93, 63.46, 62.11, 62.04, 60.21, 55.58, 47.83, 40.64, 37.39, 36.90, 33.85, 31.63, 28.42, 28.28, 24.79, 16.79, 13.03; IR (cm^{-1}): 3194, 2919, 2860, 1731, 1697, 1449, 1378, 1302, 1247, 1149, 1069, 1027, 861, 809, 724; ESI-MS observed (predicted): [M+Na] 595.05 (595.05), [M+K] 611.03 (611.02); UV-Vis (MeOH): λ_{max} (SC=SS) = 306 nm.

Typical RAFT polymerization of NIPAAm with CTA 2. In a typical experiment, 1:250:0.1 molar equivalents of **2**, NIPAAm, and AIBN were weighed into a Schlenk tube. DMF was added, and the contents was subjected to a minimum of 3 freeze-pump-thaw cycles prior to immersion into a 70 °C oil bath. The polymerization was stopped by cooling and opening the flask to the atmosphere, followed by dialysis against methanol to afford biotin-SS-pNIPAAm-trithiocarbonate. δ ^{1}H NMR (500 MHz, CD_3OD): 7.98-7.62 (N**H**, pNIPAAm), 4.52-4.49 (m), 4.36-4.30 (m), 3.96 (bs, C**H**$(CH_3)_2$, pNIPAAm), 2.97-2.92 (m), 2.72 (d, *J* = 12.6 Hz), 2.40-2.36 (m), 2.08 (bs, CH$_2$C**H**C=O, pNIPAAm), 1.59 (bs, C**H**$_2$CHC=O, pNIPAAm), 1.42, 1.33, 1.17 (bs, CH(C**H**$_3$)$_2$, pNIPAAm). M_n g/mol: (theory) 21,200 Da; (GPC): 34,200 Da; PDI: 1.15; (^{1}H NMR) 23,540 Da. UV-Vis (MeOH): λ_{max} = 306 nm.

Typical radical cross-coupling of trithiocarbonate end-functional polymers with protected maleimide azo-initiator 3. In a typical radical cross-coupling, **P2** (34 mg, 3.3 μmol) and **3** (49.0 mg, 70.9 μmol) were loaded into a Schlenk tube, and then evacuated and argon-refilled three times. A 1/1 v/v solution of DMF/dioxane (660 μL) was added and the reaction flask was subjected to three freeze-pump-thaw cycles. The reaction flask was immersed into a 70 °C oil bath. After four hours the reaction flask was removed from the oil bath, cooled and opened to the atmosphere. The solvent was removed *in vacuo* and the polymer was purified by dialysis

against 1:1 EtOAc:MeOH (MWCO 6-8,000 Da) to afford **P2**. δ ^{1}H (500 MHz, CD_3OD) 7.96-7.60 (NH, pNIPAAm), 6.55 (s), 5.16 (s), 4.51-4.48(m), 4.35 (m), 3.97 (bs, C**H**$(CH_3)_2$), 3.57 (m), 2.98-2.92 (m), 2.72 (d, *J* = 12.0 Hz), 2.08 (bs, CH_2C**H**C=O), 1.59 (bs, C**H**$_2$CHC=O), 1.16 (bs, CH(C**H**$_3)_2$). M_n g/mol: (GPC): 35,800 Da; PDI: 1.13.

Typical regeneration of maleimide functional group to form biotin-maleimide pNIPAAm. P2 (1.0 mg, 0.085 μmol) was loaded into a Schlenk tube and then dissolved in MeOH (100 μL). The solvent was removed *in vacuo*, and while under vacuum the flask was placed in a 120 °C oil bath for 2 h to afford **P4**. ^{1}H NMR (500 MHz, CD_3OD) δ ^{1}H NMR (500 MHz, CD_3OD): 7.97-7.60 (N**H**, pNIPAAm), 6.83 (d, *J*=4.8 Hz), 4.52-4.48 (m), 4.35-4.30 (m), 3.96 (bs, C**H**$(CH_3)_2$, pNIPAAm), 3.62-3.60 (m), 2.99-2.92 (m), 2.72 (d, *J* = 14.6 Hz), 2.42-2.36 (m), 2.09 (bs, CH_2C**H**C=O, pNIPAAm), 1.58 (bs, C**H**$_2$CHC=O, pNIPAAm), 1.16 (bs, CH(C**H**$_3)_2$, pNIPAAm); M_n g/mol: (GPC) 35,700 Da; PDI: 1.17.

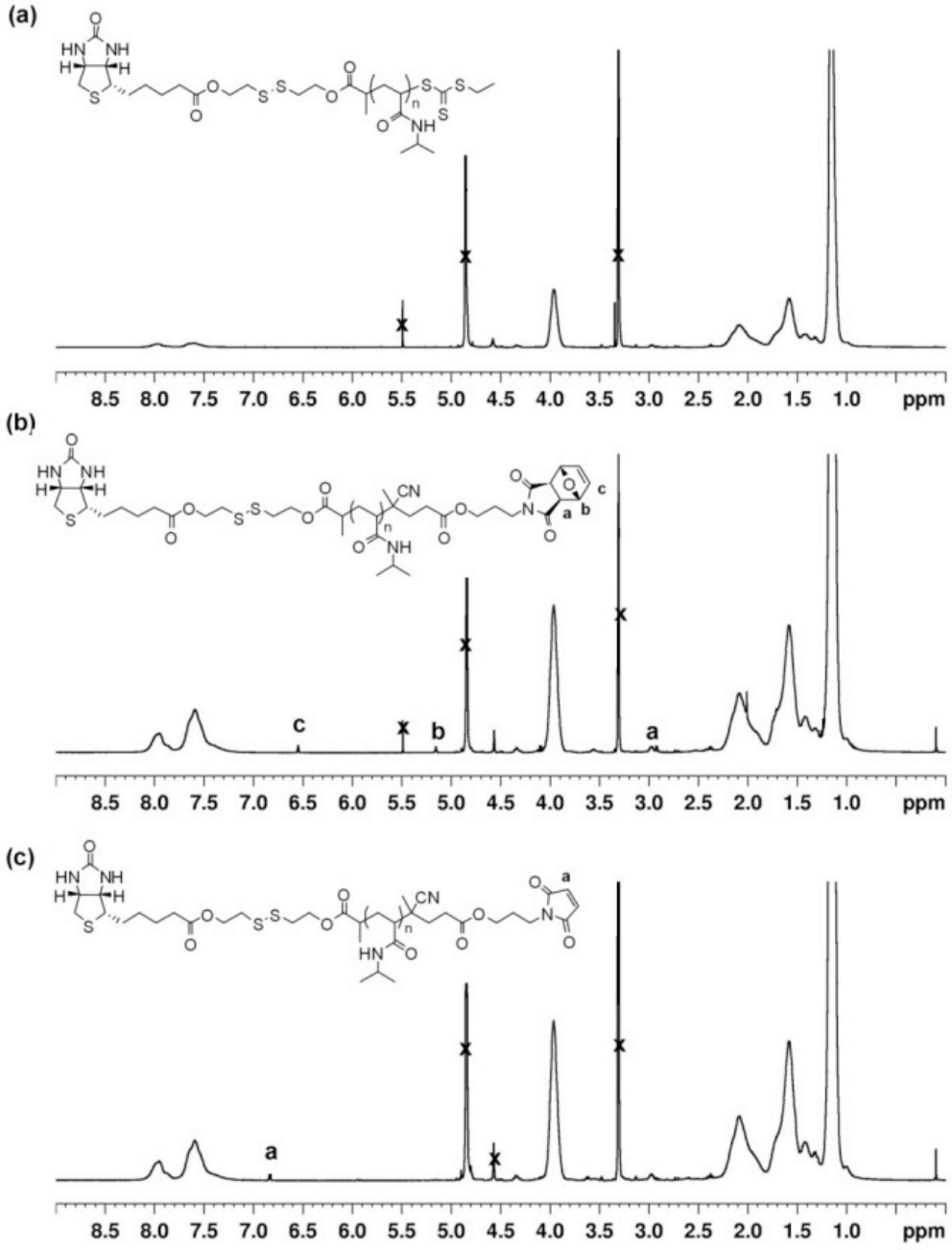

Figure S1. ^{1}H NMR spectra of pNIPAAm prepared from CTA **1** (CD_3OD). (a) biotin-SS-pNIPAAm-trithiocarbonate; (b) biotin-SS-pNIPAAm-protected maleimide (**P2**); (c) biotin-SS-pNIPAAm-maleimide (**P4**). X = solvent.

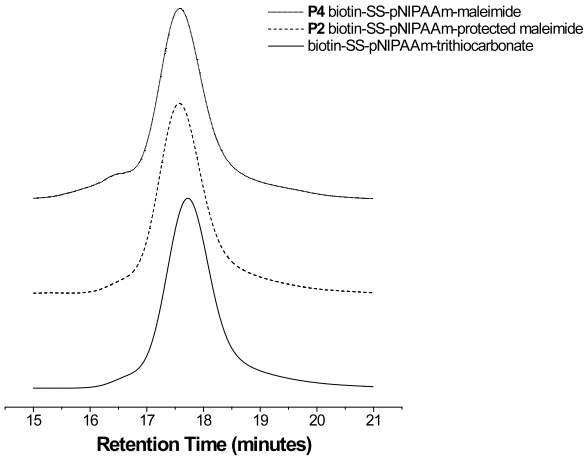

Figure S2. GPC chromatograms of pNIPAAm prepared from CTA **2**.

Typical Synthesis of polymer-T4L conjugates. 1 mL of TCEP disulfide reducing gel was placed into a plastic column and washed with phosphate buffered saline (PBS) (0.5 M PB and 0.15 M NaCl, pH 7.0). Then, 800 μL of T4L (1.9 mg/mL in dH_2O) was added and the slurry was bubbled with argon for 60 minutes. The protein was added drop wise to a degassed solution of **P4** (1.57 mg/mL in PBS, pH 7.0) and the conjugation proceeded at 4 °C for 24 hours. An alloquot was removed for SDS PAGE.

Immobilization and removal of P4-T4L on SAv-agarose. High capacity streptavidin resin (1 mL) was transferred to a plastic column and then washed with PBS (pH 7.0, 20 column volumes). 100 μL of crude **P4**-T4L was loaded onto the resin and incubated at 4 °C for 12 hrs. The resin was then washed extensively with PBS to remove unbound T4L until no absorbance at 280 nm was observed. 1 mL of PBS containing 10 mM TCEP was then added, and the slurry was incubated at 22 °C for 2 hours and then washed with PB (0.1 M PB, 0.20 M NaCl, pH 7.0) to elute the T4L-pNIPAAm conjugate. All fractions containing an absorbance at 280 were

combined, concentrated by centrifugal filtration (Amicon, MWCO 5,000) and analyzed by SDS PAGE.

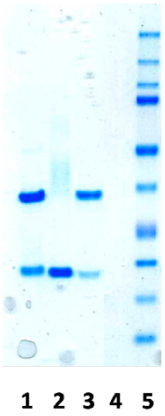

Figure S3. SDS-PAGE of V131C T4 lysozyme (T4L) conjugates. Lane 1: T4L, lane 2: crude T4L + **P4**, lane 3: T4L + biotin-SS-pNIPAAm-furan protected maleimide, lane 4: blank, lane 5: protein marker. Lanes 1-3 under non-reducing conditions

Formation of biotin-pNIPAAm-BSA (conjugate 1). In typical experiment, biotin-pNIPAAm-maleimide (**P3**, 1.94 mg) was dissolved in 200 µL of phosphate buffer (PB) (0.05 M, 25 mM ethylenediaminetetraacetic acid (EDTA), 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), pH 7.0). 200 µL of a BSA stock solution (1 mg/mL in PB; 0.05 M, 25 mM EDTA, 5 mM TCEP, pH 7.0) was added to the polymer solution. The sample was incubated at 4 °C for 12 hrs. The conjugate was purified by gel filtration (Sephadex G100) and eluted with dH_2O. The absorbance of each fraction was measured at 280 nm and fractions containing protein were lyophilized and then used for SDS-PAGE and enzyme-linked immunosorbent assays (ELISA).

Synthesis of biotin-SS-pNIPAAm-BSA (conjugate 2). P4 (1.0 mg) was dissolved in 100 µL of PB (0.1 M Na_2HPO_4, 0.1M NaH_2PO_4, 50 mM EDTA, pH 7.2). 50 µL of a BSA stock solution (1mg/mL in same buffer) was added and incubated at 4 °C for 12 hrs. The crude

conjugate was purified by gel filtration (Sephadex G100) and fractions containing an absorbance at 280 nm were combined and lyophilized and used for ELISA.

Typical ELISA of BSA-pNIPAAm conjugates (conjugate 1 and conjugate 2). Neutravidin-coated 96-well plates were washed with BB (3 x 200 μL, 10 min in between each wash with shaking). Then, 80 μL of BB was added to each well followed by 20 μL of sample (0.005 mg/mL BSA determined by measuring absorbance at 280 nm) and the plate was incubated at 23 °C for 1 hr with shaking. The wells were then washed with BB (3 x 200 μL). Next, anti-BSA-HRP (100 μL of a 1:2000 dilution in BB) was added to each well and the plate was incubated at 23 °C for 1 hr with shaking. The wells were then washed with BB (6 x 200 μL). Next, 100 μL of OPD substrate (1 mg/mL OPD in 0.5 M citrate-PB with 0.01% H_2O_2, pH 5.0) was added to each well. After 30 min the absorbance at 450 nm was measured. The absorbance of the wells containing only anti-BSA-HRP was subtracted from readings as a background measurement. Binding of unmodified BSA was compared to the BSA-conjugate to evaluate binding efficiency of the BSA-pNIPAAm conjugates. Experiments were repeated a minimum of six times. Data sets were analyzed using 2-tailed student's t-test, and significance was reported at $p < 0.05$.

ELISA control 1: blocked biotin-binding sites. The plate was washed with BB, then 100 μL of a biotin solution (2.1 mM in BB) was added to each well. After 1 hr at 23 °C with shaking, the wells were washed with BB (3 x 200 μL). 80 μL of BB and 20 μL of each sample were added to each well and the assay proceeded as described above.

ELISA control 2: Unpurified conjugate 1 compared with BSA and polymer. The plate was washed with BB (3 x 200 μL). Then 90 μL of BB and 10 μL of crude **conjugate 1** (0.003 mg/mL BSA and 1.33 mg/mL **P3**), BSA (0.003 mg/mL) or **P1** (1.33 mg/mL) were added

to the well (repeated six times). The samples were incubated at 23 °C for 1 h and the wells were then washed with BB (3 x 200 µL) to remove unbound protein and polymer. The remainder of the assay was performed as described above.

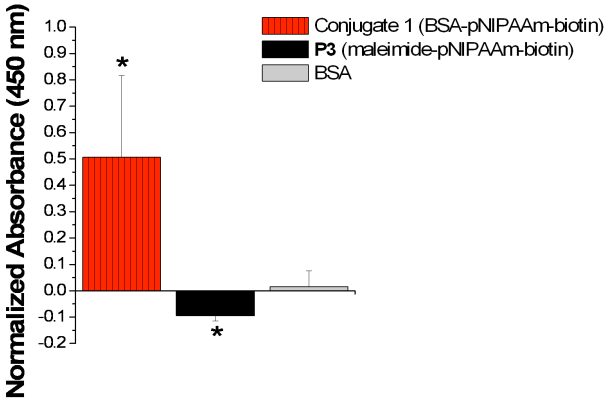

Figure S4. Control ELISA: comparison of crude conjugate with telechelic **P3** and BSA ($p < 0.05$ for conjugate vs. polymer and BSA control).

Glutathione surface reduction of conjugate 2 ELISA. After washing the plate with BB, 80 µL of BB containing 10 mM glutathione followed by 20 µL of the BSA-pNIPAAm-SS-biotin conjugate (**conjugate 2,** 0.005 mg/mL BSA) was added to each well. **Conjugate 2** (0.005 mg/mL BSA) and BSA (0.005 mg/mL) without glutathione were used as a comparison. The samples were left to incubate for 1 hr at 23 °C with shaking and then washed with BB (3 x 200 µL). The remainder of the assay was performed as described above.

References

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