Synthetic Approach to Homodimeric Protein-Polymer Conjugates

Lei Tao,[†,‡] Catherine S. Kaddis,[†] Rachel R. Ogorzalek Loo,[§] Gregory N. Grover,[†,‡] Joseph A. Loo, [†,§] Heather D. Maynard[†,‡,*]

Department of Chemistry and Biochemistry, California NanoSystems Institute, and Department of Biological Chemistry, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

**maynard@chem.ucla.edu*

Supporting Information

[†] Department of Chemistry and Biochemistry

[‡] California NanoSystems Institute

[§] Department of Biological Chemistry, David Geffen School of Medicine

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Materials

Ethanethiol (Aldrich, 97%), 2-bromopropionic bromide (Aldrich, 99%), carbon disulfide (Reagent A. C. S., Fisher), pentaerythritol (Aldrich, 98%), *exo*-3, 6-epoxy-1, 2, 3, 6-tetra-hydrophthalic anhydride (Aldrich), and hen egg white lysozyme (Aldrich) were used as received. 2,2′-Azobisisobutyronitrile (AIBN) (Aldrich, 98%) was recrystallized twice from acetone. 4,4'-Azobis(4-cyano-valeric acid) (V501, Aldrich, 75+%) was dried under high vacuum for 12 h before use. *N*-Isopropylacrylamide (NIPAAm, Acros, 99%) was recrystallized from hexane, twice. Triethylamine (TEA) (Fisher, 99%) was stored over KOH pellets prior to use. Dichloromethane (DCM) (Fisher, 99%) was stirred over calcium hydride, distilled, and stored under nitrogen. Bromopropionate-functionalized pentaerythritol,[1] 2-[4-(2-hydroxy-ethoxy)-phenoxy]-ethanol,[2] 2-ethylsulfanylthiocarbonyl sulfanyl-propionic acid,[3] 2-[methyl-(7-nitro-benzo[1,2,5]oxadiazol-4-yl)-amino]-ethanol,[4] and 4-(3-hydroxy-propyl)-10-oxa-4-aza-tricyclo[5,2,1,$0^{2,6}$] dec-8-ene-3,5-dione V501 dimer (**4**)[3] were synthesized according to literature procedures. *Abbreviations:* Acetonitrile: ACN; chain transfer agent: CTA; dichloromethane: DCM; *N,N*-dimethylformamide: DMF; *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene] malononitrile: DCTB; deionized water: dH_2O; 4-(dimethylamino)pyridine: DMAP; gel permeation chromatography: GPC; Laser light scatter: LLS; molecular weight cut-off: MWCO; *N*-isopropylacrylamide: NIPAAm; phosphate buffer: PB; phosphate buffered

saline: PBS; sodium dodecyl sulfate polyacrylamide gel electrophoresis: SDS-PAGE; V131C T4 lysozyme: T4L; tris(2-carboxyethyl)phosphine hydrochloride: TCEP; Matrix-assisted laser desorption/ionization time of flight: MALDI-TOF.

Equipment

Polymerization and end group modification reactions were carried out using standard Schlenk techniques under an inert atmosphere of argon. R_f values refer to analytical thin layer chromatography (TLC) performed using pre-coated silica gel 60 F254 and developed in the solvent system indicated. Compounds were visualized with UV light (254 nm) or a basic solution (10% K_2CO_3 aqueous solution) of $KMnO_4$. Merck 60 (230-400 Mesh) silica gel was used for column chromatography. Gel permeation chromatography (GPC) analysis of the polymers was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, a UV-Vis detector SPD-10A VP and two Polymer Laboratories PLgel 5 μm mixed D columns (with guard column). LiBr (0.1 M) in DMF at 40 °C was used as the eluent (flow rate: 0.80 mL/min). Near-monodisperse poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories) were employed for calibration. Chromatograms were processed using the EZStart 7.2 chromatography software. The M_n by LLS was collected from a Viscotek GPCmax VE2001 GPC solvent/sample module with 270 Dual detector and VE 3580 RI detector. MALDI-TOF mass spectrometry was performed on an Applied Biosystems Voyager-DE STR and operated in linear mode with an external calibration. Polymer samples were prepared by mixing 5:1:5 v/v/v ratios of DCTB matrix (40 mg/mL in THF) to potassium trifluoroacetate (5 mg/mL in THF) to polymer (1 mg/mL in THF). NMR spectra were obtained on Bruker ARX500 and Bruker DRX400 spectrometers. Monomer conversions were calculated by ^{1}H NMR by monitoring the disappearance of the peaks corresponding to the vinylic protons with the CH of the

poly(NIPAAm) as the internal standard. SDS PAGE was carried out with 4-20% Tris-Glycine gels (Invitrogen, 1.0 mm x 12 well). Infrared absorption spectra were recorded on a PerkinElmer FT-IR instrument equipped with an ATR accessory. UV-VIS analysis was conducted on a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments).

Methods

Synthesis of 1:

2-Ethyl sufanylthiocarbonyl sulfanyl-propionic acid (4.60 g, 22 mmol), 2-[4-(2-hydroxy-ethoxy)-phenoxy]-ethanol (1.98 g, 10 mmol) and DMAP (0.12 g, 1.0 mmol) were dissolved in 60 mL of dry THF and DCC (5.15 g, 25 mmol) was added. The system was stirred at 23 °C under argon for 6 h, the solid was filtered and the solvent was removed under vacuum. The crude product was purified by column chromatography on silica gel (ethyl acetate:hexanes 1:8) to yield the product as a yellow oil. (3.08 g, 53.0%). ^{1}H NMR (400.13 MHZ, $CDCl_3$)/ppm: 6.84 (s, 4H, C_6H_4), 4.83 (q, J = 7.6 Hz, 1H, $CH_3C\underline{H}$), 4.45-4.47 (m, 4H), 4.11-4.13 (m, 4H), 3.33 (t, *J* = 7.6 Hz, 4H, $CH_3C\underline{H_2}S$), 1.60 (d, *J* = 6.9 Hz, 6H, $C\underline{H_3}CH$), 1.33 (t, *J* = 7.6 Hz, 6H, $C\underline{H_3}CH_2S$); ^{13}C NMR (100.61 MHZ, $CDCl_3$)/ppm: 221.90 (C=S), 171.30 (C=O), 153.27 (C on the benzene ring), 116.05 (CH on the benzene ring), 66.69 ($\underline{C}H_2OC=O$), 64.29 ($CO\underline{C}H_2$), 48.05 ($CH_3\underline{C}H$), 31.72 (CH_2S), 16.94 ($\underline{C}H_3CH$), 13.15 ($\underline{C}H_3CH_2$). IR (cm^{-1}): 2927, 2867, 1731, 1505, 1448, 1374, 1303, 1210, 1153, 1065; MALDI-TOF: $M+K^+$ expected (observed): 621.00 (620.99).

CTA retention by UV-vis:

A series of standard solutions containing **1** ranging from 2.81 x 10^{-5} M to 3.51 x 10^{-6} M were prepared in volumetric glassware in methanol. The absorbance of each sample was measured at 306 nm (λ_{max} SC=SS). A line was fit to these data points to give a molar absorptivity (ε) of 30,528 M^{-1} cm^{-1} (Figure S1).

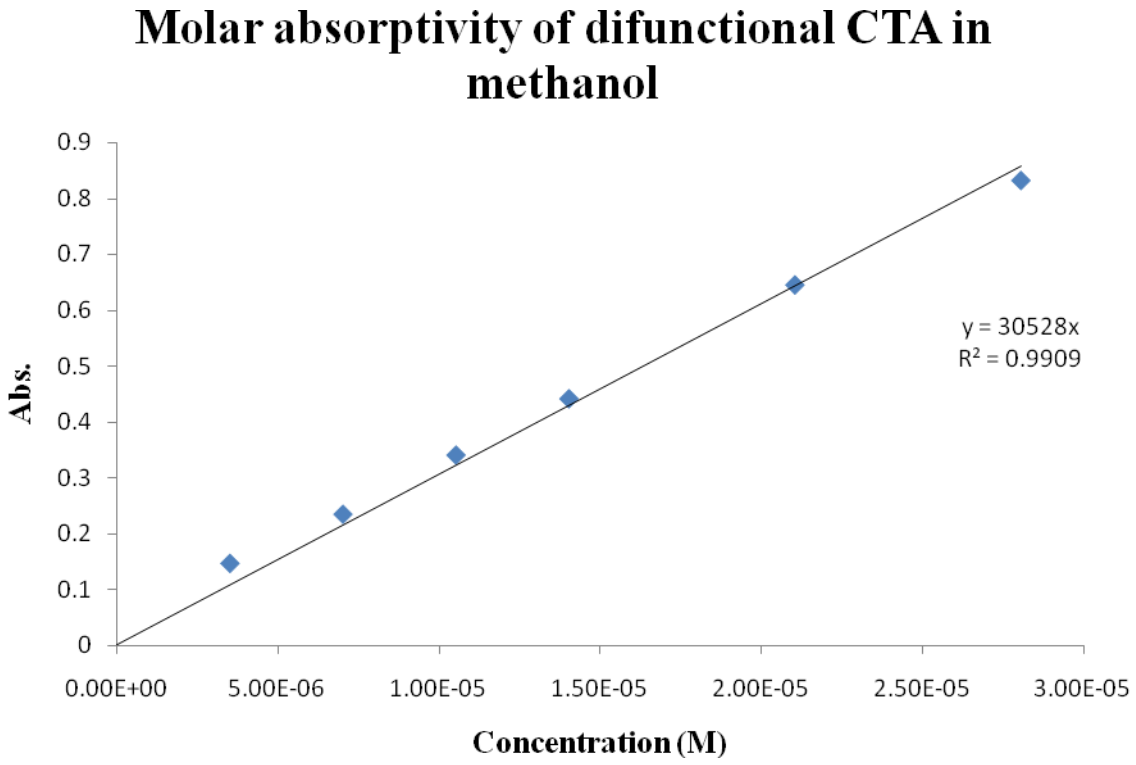
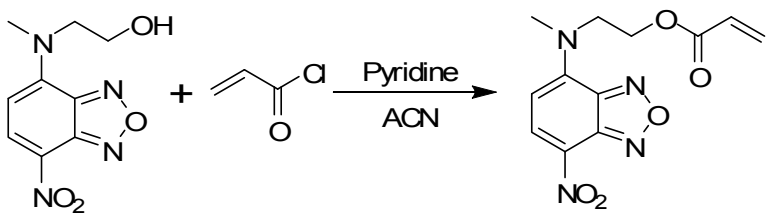


Figure S1: UV-vis analysis of difunctional CTA.

Using the molecular weight calculated from MALDI-TOF MS for **3** (MW = 8,990), three polymer concentrations were prepared in volumetric flasks in methanol. The absorbance at 306 nm was measured for each sample. The percentage of trithiocarbonate end-group was determined using the calculated molar extinction coefficient of 30,528 M^{-1} cm^{-1}; the average end group percentage was 91%.

Synthesis of NBD Monomer 2:



2-[methyl-(7-nitro-benzo[1,2,5]oxadiazol-4-yl)-amino]-ethanol (0.238 g, 0.10 mmol) and pyridine (0.158 g, 0.20 mmol) were dissolved in 10 mL of ACN. The solution was kept in ice-water bath under argon and acryloyl chloride (0.109 g, 0.12 mmol) was added by degassed syringe. The solution was stirred at 23 °C for 4 h, and the volatile molecules were removed under vacuum. The remaining crude product was purified by column chromatography on silica gel (dichloromethane) to yield the product as a red waxy solid. (0.230 g, 78.8%). ^{1}H NMR (400.13 MHZ, $CDCl_3$)/ppm: 8.44 (d, 1H, J = 8.9 Hz, $CHCNO_2$), 6.30 (d, 1H, J = 17.2 Hz, CHCN), 6.18 (d, 1H, J = 8.9 Hz, C=CH), 6.04-5.97 (m, 1H, C<u>H</u>=CH_2), 5.82 (d, 1H, J = 9.1 Hz, C=CH), 4.53 (t, 2H, J = 4.7 Hz, CH_2OC=O), 4.47 (br s, 2H, NCH_2), 3.48 (s, 3H, CH_3N); ^{13}C NMR (100.61 MHZ, $CDCl_3$)/ppm: 165.81, 145.67, 144.85, 135.46, 134.09, 132.19, 129.19, 127.70, 101.97, 61.94, 54.45, 47.55. IR (cm^{-1}): 2335, 1714, 1606, 1551, 1408, 1325, 1233, 1183, 1060, 978, 820; MALDI-TOF: M+Na^+ expected (observed): 315.07 (315.09).

Synthesis of difunctional poly(NIPAAm) (3). NIPAAm (2.26 g, 20.0 mmol), **1** (116 mg, 0.20 mmol), **2** (12 mg, 0.041 mmol) and AIBN (3.3 mg, 0.02 mmol) were dissolved in 5 mL of *N,N*-dimethylformamide (DMF). After three freeze-pump-thaw cycles, the Schlenk tube was placed into an 80 °C oil bath. Samples were taken out with a degassed syringe for conversion analysis by ^{1}H NMR. The polymerization was quenched after the conversion reached 82 %. Most of the DMF was removed under high vacuum at 40 °C and the polymer was then purified by dialysis against methanol (molecular weight cut off, MWCO ~ 3,500). ^{1}H NMR (400.13 MHZ,

$CDCl_3$)/ppm: 7.10-5.63 (main chain, NH), 6.84 (chain end, aromatic protons), 4.01 (main chain, CH), 3.34 (chain end, SCH_2), 2.09 (main chain, CH), 1.83-1.63 (main chain, CH_2), 1.14 (main chain, $CH(CH_3)_2$).

Aminolysis of the difunctional poly(NIPAAm) (3): Difunctionalized poly(NIPAAm) (10 mg, 1.1 x 10^{-3} mmol) was dissolved in 10 mL of methanol and 0.10 mL of butylamine was added. The solution was kept at 60 °C for 15 h. After removing the volatile molecules under vacuum, the remaining polymer was used directly for analysis. The expected M_n was calculated assuming complete removal of the core and amide formation with butyl amine.

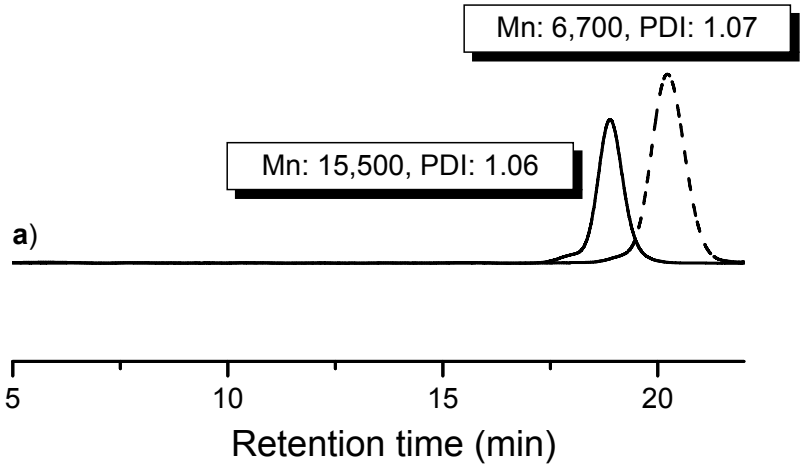


Figure S2. GPC traces of polymer before and after aminolysis of core.

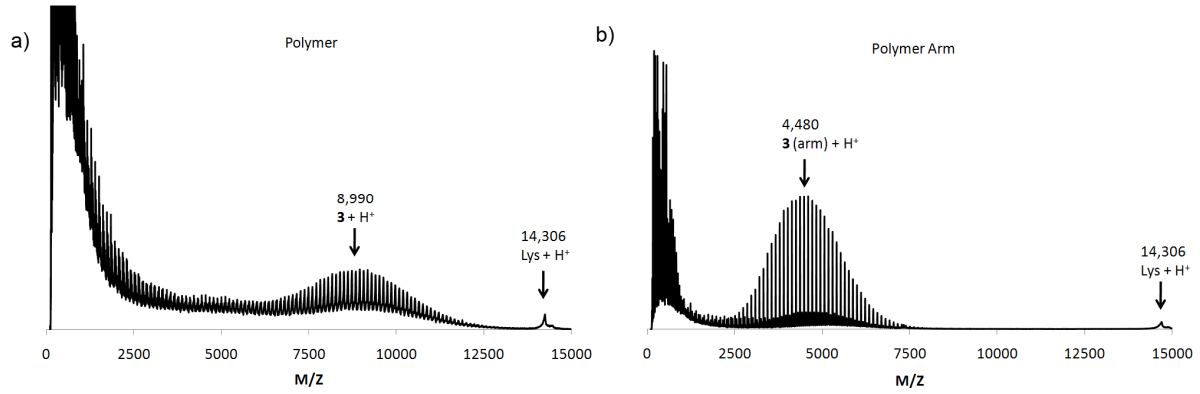


Figure S3. MALDI-TOF MS spectra of a) **3** before and b) after aminolysis of the core calibrated to hen egg white lysozyme.

End group modification with 4. Poly(NIPAAm) **3** (90 mg, M_n ~ 9,230, 9.8 x 10^{-3} mmol) and **4** (276 mg, 0.40 mmol) were dissolved in 4 mL of dioxane:DMF (1:1). The Schlenk tube was subjected to three freeze-pump-thaw cycles, and placed into a 70 °C bath for 4 h. The dioxane and DMF were removed under vacuum at 40 °C. The crude material was taken up in 10 mL of methanol and any precipitates removed by centrifugation. The methanol solution was then dialyzed against ethyl acetate/methanol (1:1, MWCO: 3,500) for 72 h to give the protected maleimide difunctionalized polyNIPAAm. ^{1}H NMR (400.13 MHZ, $CDCl_3$)/ppm: 7.10-5.66 (main chain, NH), 6.85 (chain end, aromatic protons), 6.51 (chain end, CH=CH), 5.25 (chain end, CHOCH), 4.01 (main chain, CH), 3.57 (chain end, NCH_2), 2.85 (chain end, C<u>H</u>CHOCH), 2.08 (main chain, CH), 1.84-1.63 (main chain, CH_2), 1.15 (main chain, $CH(CH_3)_2$). UV-Vis analysis at 306 nm gave an absorbance of 0, which suggested no retention of the trithiocarbonate chain-end after the reaction.

Retro Diels-Alder reaction to form 5. The difunctionalized poly(NIPAAm) with furan protected maleimide end-groups was maintained at 120 °C under vacuum for 1 h. The target polymer **5** was obtained in 100 % yield. ^{1}H NMR (400.13 MHZ, $CDCl_3$)/ppm: 7.10-5.66 (main

chain, NH), 6.84 (aromatic protons), 6.72 (maleimide chain end, CH=CH), 4.01 (main chain, CH), 2.08 (main chain, CH), 1.83-1.63 (main chain, CH_2), 1.14 (main chain, $CH(CH_3)_2$).

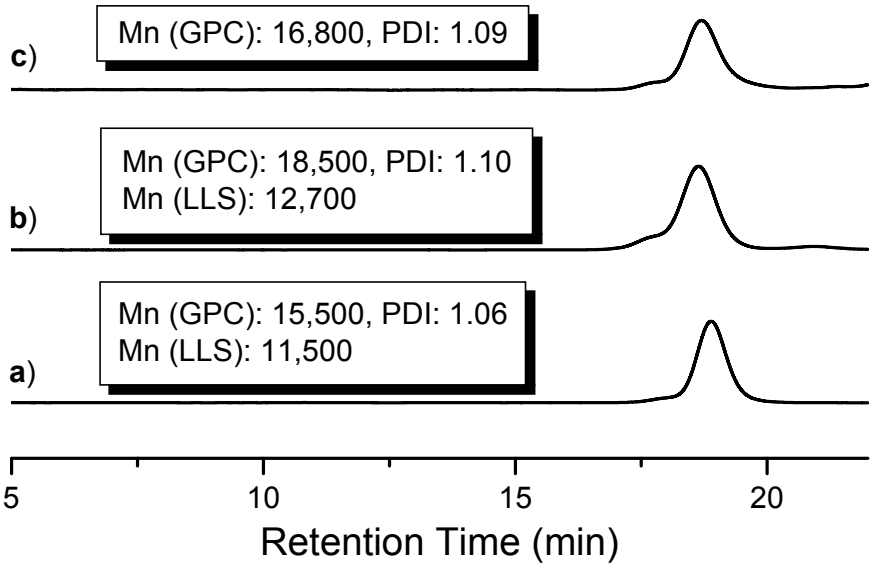


Figure S4. GPC traces of the polymers before and after radical exchange. a) difunctional poly(NIPAAm) **3**; b) poly(NIPAAm) with furan protected maleimide; c) poly(NIPAAm) with maleimide chain ends (**5**).

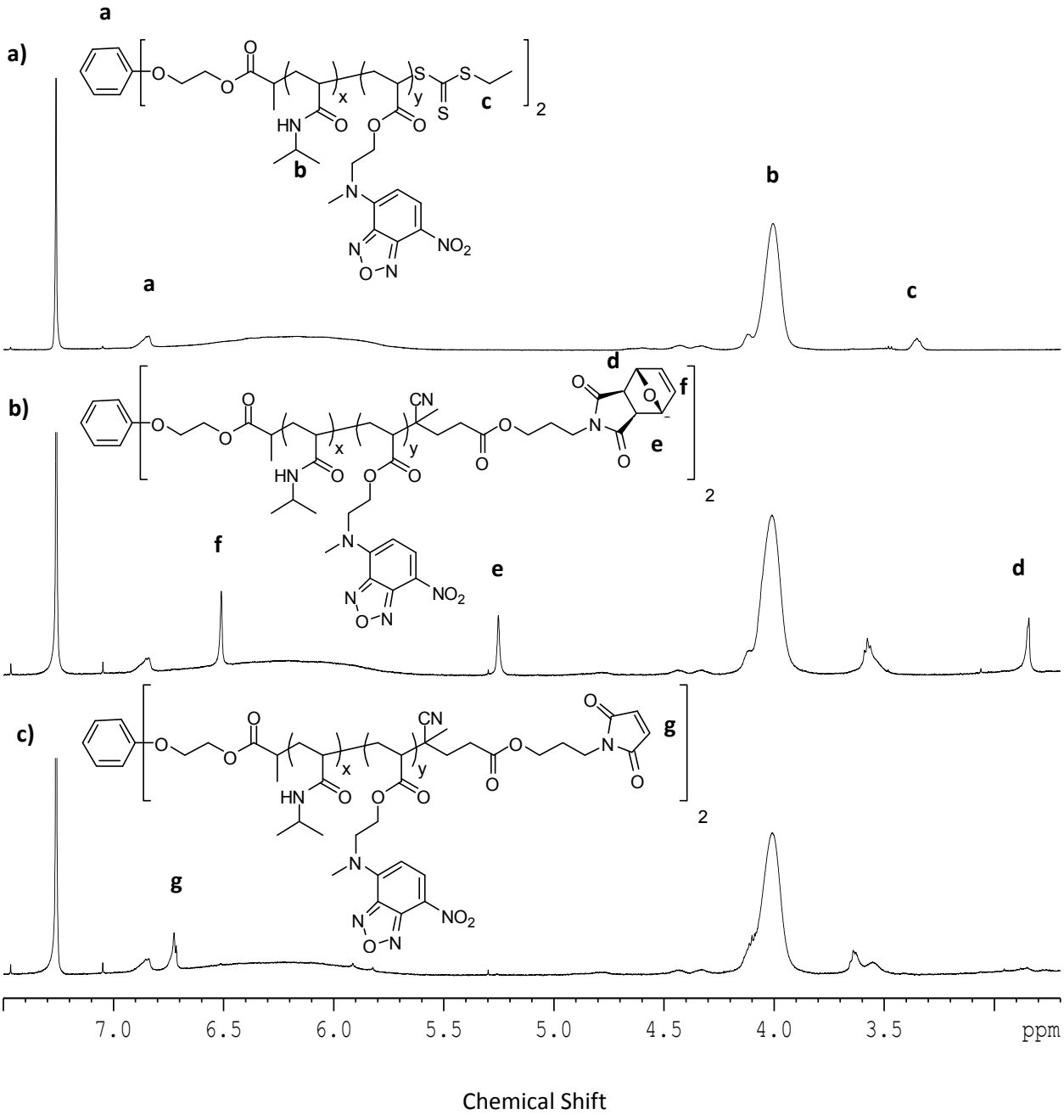


Figure S5. ^{1}H NMR spectra ($CDCl_3$) of the difunctional polymers before and after introduction of the protein-reactive end group. a) Poly(NIPAAm) **3**; b) poly(NIPAAm) with furan protected maleimide end groups; c) poly(NIPAAm) **5**.

Conjugation of T4L and 5. T4L (3.17 mg, 1.69 mg/mL) in tris (tris(hydroxymethyl)aminomethane) (Tris) buffer, pH 7.4 and **5** (0.5 mg, 1.0 mg/mL) in

phosphate buffered saline (PBS), pH 7.5 with 10 mM of TCEP and 10 mM of EDTA were mixed and maintained at 4 °C with gentle mixing for 16 h. The salts were removed by centrifugation filtration (MWCO: 10,000). The conjugate was then purified by gel filtration (Sephadex G100) and eluted with deionized water (dH_2O). The absorbance of each fraction was measured at 280 nm and fractions containing protein were combined and concentrated by centrifugal filtration (MWCO: 10,000). The sample was analyzed by SDS PAGE.

Purification of dimeric protein-polymer conjugate.

The T4L-poly(NIPAAm) dimer conjugate solution was loaded on a cation exchange chromatography (HiTrap SP HP, 5 mL, Amersham Biosciences). The column was washed using 10 mL of Tris buffer pH 7.4 with a gradient of increasing NaCl concentration. Fractions were collected, concentrated by centrifugal filtration (MWCO: 10,000) and used for SDS-PAGE analysis.

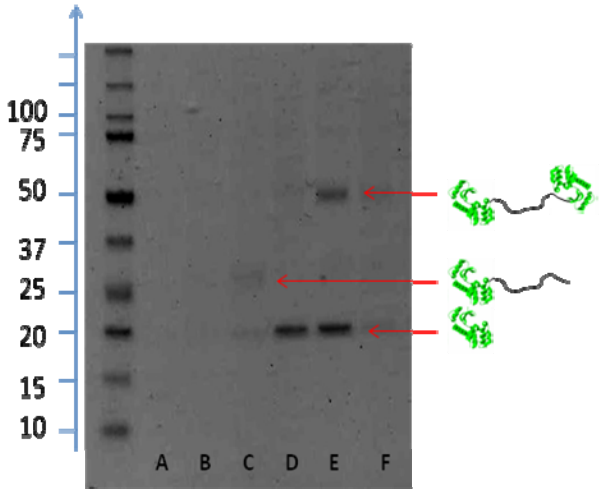


Figure S6. SDS-page of the fractions with gradient NaCl concentration. A: 0.10 M; B: 0.12 M; C: 0.14 M; D: 0.16 M; E: 0.18 M; F: 0.20 M.

Electrospray Ionization – Gas-Phase Electrophoretic-Mobility Macromolecule Analysis (ESI-GEMMA). Samples were diluted 10 to 200 times in 20 mM ammonium acetate, pH 8 or

20 mM acetic acid. Amicon Ultra-4 centrifugal filter devices (Millipore) or Micro Bio-Spin 6 chromatography columns (Bio-Rad) were used for samples subjected to buffer exchange. Details of the ESI-GEMMA instrument are described in literature.[5] ESI-GEMMA (TSI Inc., St. Paul, MN) consists of an Electrospray Aerosol Generator 3480, Electrostatic Classifier 3080 with Nano-DMA 3085, and Ultrafine Condensation Particle Counter 3025A. The ESI source ionizes the sample particles, the DMA separates the particles based on their electrophoretic mobility in air, and the CPC counts detected particles. Sample solutions were introduced in the ESI source through a fused silica capillary tube (polyimide coated, 25 cm long, 25 μm I.D. and 150 μm O.D.) at a flow rate of 70 nL/min and voltage of 2 to 3 kV. To promote particle charge reduction, the gases present inside the ESI neutralizing chamber were ionized by a ^{210}Po α-radiation source (5 mCi, model P-2042 Nucleospot local air ionizer, NRD, Grand Island, NY, USA). The DMA sheath flow was set to 20 L/min and the voltage was scanned -10 to -10,000 volts for 120 sec. to sample the EMD range of 2-56 nm. The DMA voltage was scanned and data recorded by Aerosol Instrument Manager Software (TSI Inc). PeakFit Version 4.12 (SeaSolve Software Inc., San Jose, CA) was used to centroid EMD peaks of interest. Data points were smoothed using Igor Pro 4.0.8.0 (Wavemetrics, Inc., Lake Oswego, OR). Molecular weights were calculated from the experimentally determined EMD using the formula for the volume of a sphere and density as described below.

Table S1 lists the EMD of the peaks identified in the ESI-GEMMA spectra of the protein-polymer conjugate samples. Molecular weights were calculated using the formula for density and the volume of a sphere: MW=V*d*Na where d is the effective density, Na is Avogadro's number and the volume, $V = (EMD)^3\pi/6$. A density of 0.58 g/cm^3 was used[5] for the molecular weight calculations of the protein-polymer conjugates synthesized from **5**.

Table S1. Calculated molecular weights of protein-polymer conjugate determined from ESI-GEMMA data.

Protein-Polymer Conjugate		
EMD (nm)	**Apparent MW (Da, d=0.58 g/cc)[1-3]**	**Assignment[4]**
4.6	1.8×10^4	L
5.3	2.7×10^4	P_2 + L
6.5	5.0×10^4	P_2 + 2L

[1]Based on the standard deviation of the effective density, a mass accuracy of $\pm$10% is estimated.
[2]Molecular weight was calculated using the formula: $MW=V*d*N_a$ where d is the effective density, N_a is Avogadro's number and the volume, $V = (EMD)^3\pi/6$.
[3]Density of protein and protein complexes calculated from reference 5.
[4]Abbreviations are defined as follows: L is T4 lysozyme; P_2 is the two armed polymer **5**; P_2 + L is the monomer linked two armed polymer; P_2 + 2L is the dimer linked two armed polymer.

Liquid Chromatography-Mass Spectrometry and Protein Identification.

In-gel tryptic digestion and LC-MS/MS were used to identify peptides associated with T4 lysozyme in the marked gel bands.

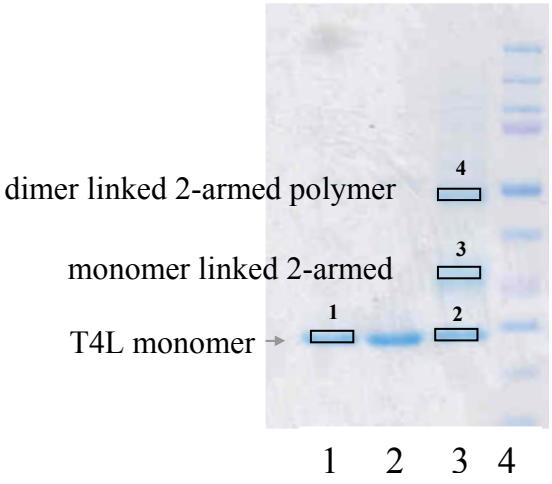


Figure S7. SDS-page analysis. Lanes 1) T4L; 2) T4L + protected polymer; 3) T4L: maleimide-difunctional polymer; 4) Protein Marker.

Coomassie-blue stained gel bands of interest were manually excised, then digested with sequencing-grade trypsin (Promega, Madison, WI). Resulting tryptic peptides were extracted using standard protocol.[6]

LC-MS/MS of peptide mixtures were performed on Applied Biosystems (Foster City, CA) QSTAR Pulsar XL (QqTOF) mass spectrometer equipped with nanoelectrospray interface (Protana, Odense, Denmark) and LC Packings (Sunnyvale, CA) nano-LC system. The nano-LC was equipped with a homemade precolumn (150 μm x 5 mm) and analytical column (75 μm x 150 mm) packed with Jupiter Proteo C12 resin (particle size 4 μm, Phenomenex, Torrance, CA). The dried peptides were resuspended in 1% formic acid solution. Six μL of sample solution were loaded onto the precolumn for each LC-MS/MS run. The precolumn was washed with the loading solvent (0.1%FA) for 4 min before the sample was injected onto the LC column. The eluents used for the LC were 0.1%FA (solvent A) and 95%ACN containing 0.1%FA (solvent B). The flow rate was 200 nL/min, and the following gradient was used: 3% B to 6% B in 6 sec, 6% B to 24% B in 18 min, 24% B to 36% B in 6 min, 36% B to 80% B in 2 min, and maintained at 80% B for 7.9 min. The column was finally equilibrated with 3% B for 15 min prior to the next run. Electrospray ionization was performed using a 30 μm (i.d.) nano-bore stainless steel online emitter (Proxeon, Odense, Denmark) and a voltage set at 1900 V.

Protein identification was accomplished utilizing the Mascot database search engine (Matrix Science, London, UK). All searches were performed against Swiss-Prot protein sequence database. The following variable modification were allowed: acetylation of the N-terminus, carbamidomethylation of cysteines, oxidization of methionines, cyclization of N-terminal glutamine to Pyro-Glu, cyclization of N-terminal glutamic acid to Pyro-Glu, N-isopropylcarboxamidomethylation of cysteines, and phosphorylation of tyrosine, serine, and

threonine. A fragment mass tolerance of 0.3 Da, peptide mass tolerance of 0.5 Da, and maximum missed cleavage of 1 was set. To validate the accuracy of the identifications and find additional peptides associated with the T4-lysozyme mutant, MS/MS spectra were manually inspected.

Table S2. Identified peptides associated with T4-lysozyme.

Band	**Protein Score**[1]	**Peptide Sequence**	**Peptide**[3,4]
1	468	1 - 8	M[a]NIFEMLR (46)
		20 - 35	DTEGYYTIGIG HLLTK (64)
		36 - 43	SPSLNAAK (54)
		53-60	N(T)NGVITK*
		66 - 76	LFNQDVDAAVR (72)
		84 - 95	LKPVYDSLDAVR (58)
		138 - 145	WYNQTPNR (34)
		148-154	RVITTFR (31)
		155 - 162	TGTWDAYK (35)

Band	**Protein Score**[1]	**Peptide Sequence**	**Peptide**[2]
2	289	2 - 8	NIFEM[a]LR (36)
		20 - 35	DTEGYYTIGIG HLLTK (61)
		36 - 43	SPSLNAAK (51)

		36-48	SPSLNAAKSELDK (45)
		61-76	DEAEKLFNQDVDAAVR (40)
		66 - 76	LFNQDVDAAVR (82)
		84-95	LKPVYDSLDAVR (58)
		138 - 145	WYNQTPNR (37)
		155 - 162	TGTWDAYK (29)

Band	**Protein Score**[1]	**Peptide Sequence**	**Peptide**[2]
3	219	1 - 8	M[a]NIFEMLR (37)
		20 - 35	DTEGYYTIGIGHLLTK (66)
		36 - 43	SPSLNAAK (56)
		84-95	LKPVYDSLDAVR (60)
		138-145	WYNQTPNR (37)

Band	**Protein Score**[1]	**Peptide Sequence**	**Peptide**[2]
4	300	1 - 8	MNIFEM[a]LR (40)
		2 - 8	NIFEM[a]LR (29)
		20 - 35	DTEGYYTIGIGHLLTK (67)
		36 - 43	SPSLNAAK (56)
		61-76	DEAEKLFNQDVDAAVR (68)

66 - 76	LFNQDVDAAVR (78)
84-95	LKPVYDSLDAVR (46)
138 - 145	WYNQTPNR(34)
155 - 162	TGTWDAYK (55)

[a]Oxidation of methionine.
[1]Protein scores for T4-lysozyme (accession #P00720) assigned by the Mascot search engine using the Swiss-Prot protein database.
[2] Mascot peptide ion scores are listed in parentheses. A Mascot score of greater than 44 was considered a significant match by the search engine. The MS/MS spectra for all peptides included in the table were manually examined to validate the accuracy of the identifications.
[3] Mascot peptide ion scores are listed in parentheses. A Mascot score of greater than 45 was considered a significant match by the search engine. The MS/MS spectra for all peptides included in the table were manually examined to validate the accuracy of the identifications.
[4] Additional peptides associated with the V131C T4-lysozyme were manually assigned (*). V131C T4L differs from T4L (P00720) by 3 amino acids. Peptide 53-60 containing C54T was identified in bands 1 and 5 and peptide 126-135 containing V131C was identified in band 5. Peptide 97-119 containing C97A was not assigned.

References

1. J. Bernard, A. Favier, L. Zhang, A. Nilasaroya, T. P. Davis, C. Barner-Kowollik and M. H. Stenzel, *Macromolecules*, 2005, **38**, 5475-5484.
2. T. Ikeda, M. Asakawa, K. Miyake and T. Shimizu, *Chem. Lett.*, 2004, **33**, 1418-1419.
3. K. L. Heredia, G. N. Grover, L. Tao and H. D. Maynard, *Macromolecules*, DOI: 10.1021/ma8022712.
4. B. Moon, T. R. Hoye and C. W. Macosko, *Polymer*, 2002, **43**, 5501-5509.
5. C. S. L. Kaddis, S. H.; Yin, S.; Berhane, B.; Apostol, M. I.; Kickhoefer, V. A.; Rome, L. H.; Loo, J. A. , *J. Am. Soc. Mass Spec.*, 2007, **18**, 1206-12016.
6. A. W. Shevchenko, M.; Vorm, O.; Mann, M., *Anal. Chem.*, 1996, **68**, 850-858.