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Los Angeles

**Synthesis of Homodimeric Protein-Polymer Conjugates via the Tetrazine-*trans*-Cyclooctene
Ligation**

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science
in Chemistry

by

Maltish Missael Lorenzo

2015

ABSTRACT OF THE THESIS

Synthesis of Homodimeric Protein-Polymer Conjugates via the Tetrazine-*trans*-Cyclooctene

Ligation

by

Maltish Missael Lorenzo

Master of Science in Chemistry

University of California, Los Angeles, 2015

Professor Heather D. Maynard, Chair

Described in this thesis is the use of controlled radical polymerization techniques to synthesize tetrazine end-functionalized telechelic polymers, which were employed to generate T4 Lysozyme homodimers. A mutant of T4 Lysozyme (V131C), containing a single surface-exposed cysteine residue, was modified via Michael addition with a protein-reactive *trans*-cyclooctene. Reversible addition-fragmentation chain transfer (RAFT) polymerization was used to obtain telechelic poly(N-isopropylacrylamide) (pNIPAAm) polymers with M_n ($^1\text{H-NMR}$) of 2.0 kDa and molecular weight dispersity, \mathcal{D} , (GPC) of 1.05. pNIPAAm was modified at both ends with (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol. As a control, a 2.0 kDa bis-carboxylic acid poly(ethylene glycol) was modified at both ends with (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol and a 2.0 kDa bis-maleimide pNIPAAm was also synthesized. Tetrazine-functionalized polymers were then ligated to a *trans*-cyclooctene modified Lysozyme (T4L-

TCO). Ligation of T4L-TCO to bis-tetrazine pNIPAAm resulted in 27.27% yield of the homodimeric conjugate, whereas bis-tetrazine PEG resulted in 16.75% and bis-maleimide pNIPAAm resulted in <1% yield after 1 hour with no significant increase in yield during extended incubation times up to 24 hours. This work shows that tetrazine chemistry results in a higher yield of dimeric protein conjugation for both PEG and CRP polymers.

The thesis of Maltish Missael Lorenzo is approved.

Jorge Torres

Yves F. Rubin

Heather D. Maynard, Committee Chair

University of California, Los Angeles

2015

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Acknowledgements and Dedication

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Introduction

Protein dimerization and formation of higher-order multimers is a ubiquitous and essential phenomenon in biological systems. Proteins in biological systems require interactions with other biomolecules, such as nucleotides, carbohydrates, and steroids, to induce cellular responses.¹ A high degree of protein-protein interaction exists in protein interaction networks, cascades, as well as in isolation.² Dimerization and oligomerization induces structural and functional advantages to proteins, such as improved stability and mediation of activity. Therefore, natural and synthetic multimerization of proteins has been extensively studied. For example, epidermal growth factor (EGF) is a cytokine involved in cell adhesion, proliferation, differentiation, and invasion. Current proposed models for induction of this cell signaling cascade involves dimerization of both EGF and EGF receptors (EGFRs).^{3,4,5} Another signaling mechanism that mammalian cells rely upon to maintain homeostasis is through guanosine triphosphate (GTP) binding proteins, or G proteins.⁶ G protein coupled-receptors (GPCRs) encompass the largest class of membrane proteins in the human genome and are involved in modulating cellular responses to extracellular signals including small molecules, peptides and proteins.^{7,8} There is growing evidence showing that GPCRs exist as dimers (or oligomers) and that these dimers may be important for G protein activation in some GPCR families.⁹ Dimerization can also be a mechanism for enzyme activation. Caspases, single-chain cysteine proteases, play essential roles in apoptosis (programmed cell death), necrosis, and inflammation.¹⁰ Studies have shown that under physiological conditions, caspase-9 exists as an inactive monomer. During apoptosis, caspase-9 dimerization is induced, resulting in the activation of the enzyme.¹¹

Many groups have studied synthetic protein dimerization. Imperiali and co-workers developed biomimetic dimeric ligands for ligand-receptor interaction studies. These ligands enhanced receptor affinity for the oligomeric system compared to monovalent forms.¹² Other methods for protein and peptide dimerization include protein or peptide stapling. Smith and co-workers designed a reversible stapling and unstapling mechanism of proteins and peptides capable of further modification through the use of a tetrazine handle.¹³ Synthetic oligomeric EGF has also been modified with an average of two EGFs units and shown to exhibit significantly higher cell affinity compared to free EGF in cells that over expressed EGFRs.¹⁴ There are many more examples where linkers such as oligo(ethylene glycol units) and protein specific ligands have been used to construct protein dimers.^{5,15,16,17,18} The significance of dimerization and oligomerization in nature has amplified the search for novel materials and methodology for the preparation of synthetic protein-polymer hybrids, including dimers and oligomers. These materials could display the advantageous properties of protein dimers coupled with the ability to enhance protein circulation that polymers provide.

The FDA has approved many PEGylated materials for use as a component in foods, cosmetics, and pharmaceuticals.¹⁹ Poly(ethylene glycol) (PEG) exhibits low pharmacological toxicity, enhanced blood circulation time and enhanced pharmacokinetic properties. PEG-protein conjugates were the first examples of polymers attached to proteins and remain popular for protein-polymer conjugations^{20,21} Controlled radical polymerizations (CRP) techniques offer an exciting way to synthesize PEG-alternative polymers with finely tuned physical properties. This is of interest because protein-polymer conjugates exhibit properties derived from both the biological and synthetic polymer materials.^{22,23,24} For example, a protein-polymer conjugate was

recently synthesized which contains a mimic of naturally occurring sulfonated heparin polymer. The protein benefits from the polymer's stabilization property while still retaining its bioactivity.

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Despite the aforementioned significance of protein dimerization, many protein–polymer conjugates used as pharmaceuticals and research consist of one protein bound to one or more polymers. The limited synthetic strategies for protein reactive polymers that yield desired physical properties may be the cause of the minimal examples of such conjugates. Exploration of new preparation methods to maximize applicability including increasing conjugation yield while minimizing the inherent heterogeneity of protein-polymer conjugates is important in the field. Reversible addition-fragmentation chain transfer (RAFT) polymerization is a commonly utilized CRP method due to its known versatility, ability to predetermine molecular weight, and dispersity.^{26,27} Polymer molecular weight and polymeric linker length have been shown to influence bioactivity of dimers.^{5,28} Therefore, the ability to control the degree of polymerization as well as polymer dispersity is important to consider.

Telechelic CRP polymers, polymeric molecules with reactive end groups, allow for the synthesis of dimeric species. These polymers can be prepared by a pre-polymerization approach where a bifunctional RAFT chain transfer agent (CTA) is modified with protein reactive end-groups. If the CTA approach is not viable, telechelic polymer chain end reactivity provides a convenient and facile way to install protein-reactive groups post-polymerization.²⁹ Lai et al. and Lui et al. used a trithiocarbonate CTA and typical free radical initiators to prepare polymers capped with carboxylic acid and alcohol functional groups.^{30,31} CTAs can act as handles for modification and have allowed for polymer end-groups with more elaborate functionalities. For

example, a protein reactive bifunctional pyridyl disulfide CTAs eliminates the need for post-polymerization modification.^{32,33,34,35} Other protein reactive functional groups available include thiazolidine-2-thione,³⁶ N-hydroxysuccinimidyl (NHS) esters,³⁷ biotin,^{32,38,39} aldehydes,^{40,41} and maleimide.^{42,43} CTA's and RAFT polymers containing alkynes functionalities, capable of 1,3-dipolar cycloadditions with an azide, have received a great deal of attention due to the high specificity.^{44,45} Typically, site-specific conjugation of polymers onto proteins is achieved through the utilization of amino acids. Surface exposed nucleophilic amino acids such as lysine, glutamate and cysteine are generally exploited for conjugation with activated polymers. If a protein does not contain any accessible amino acids, genetically engineered proteins, which display a single reactive amino acid on its outer surface, can be used for conjugations. Thiol groups offer an exceptional method for site-specific conjugation. At neutral or slightly acidic pH, maleimides are known to react with thiols approximately 1000 times faster than with amines.⁴⁶ In this thesis, we utilized T4 lysozyme. T4 Lysozyme was engineered to express a single surface cysteine mutation^{47,48} and subsequently used for bioconjugations.^{49,43,42}

Straightforward preparations of dimeric protein assemblies can be achieved with the use of such homo and heterotelechelic polymers synthesized by CRP. One of the first examples of CRP employed to synthesize homodimeric protein conjugates was in 2009.⁴³ Tao et. al employed RAFT polymerization from a bistrithiocarbonate CTA followed by radical exchange of the trithiocarbonates with a masked protein-reactive maleimide azo-initiator, and subsequent conjugation to the protein to yield homodimers.⁴³ Later, the same maleimide-thiol chemistry was employed to synthesize multimeric protein-polymer conjugates.⁴² Heterotelechelic biotin-maleimide poly(N-isopropylacrylamide) (pNIPAAm) was synthesized for the formation of

streptavidin (SAv)–bovine serum albumin (BSA) heterodimeric protein-polymer conjugates.³⁹ Similarly, homotelechelic polymers have also been synthesized by RAFT for protein conjugation.³⁴ In addition, bifunctional CTAs have been coupled to N-Boc-aminoxy ethanol or pyridyl disulfide ethanol, resulting in a bis-N-Boc-aminoxy CTA and a bis-pyridyl disulfide CTA, respectively. Conjugations of small molecules and peptides resulted in homotelechelic polymer conjugates and have the potential for protein dimerization.³³

Despite the availability of several preparation methods for homo and heterodimeric protein-polymer conjugates, the conjugation yields of such conjugates are not high. Liu et. al. intended to synthesize homodimers via a macro-RAFT agent approach, BSA–RAFT–BSA, but were unsuccessful.³⁴ Tao et. al. reported a 21% homodimer yield with the use of homotelechelic maleimide functionalized pNIPAAm as most of the protein remained in the unreacted monomeric state after a prolonged 16 hour conjugation time.⁴³ Low conjugation efficiency is likely due to the inherent steric hindrance exhibited during conjugation between polymer end-group and protein surface. Maleimide-thiol chemistry has also been used with varying spacer length to obtain a 30% yield for dimerization of oppositely charged proteins and less than 1% yield for similarly charged proteins, suggesting that charge can facilitate or limit dimer formation.⁵⁰ In this thesis, we describe research to address whether more a rapid ligation, specifically the tetrazine ligation, would increase dimer yield.

Inverse-electron-demand Diels-Alder reactions between tetrazine and strained double bonds are rapid bio-orthogonal reactions, and have been commonly employed in labeling cells^{51,52,53} and biomacromolecules.^{54,55,56} Tetrazines react with various dienes in inverse-electron-demand Diels-Alder reactions, and N₂ is the only byproduct released upon the

subsequent retro-[4+2] cycloaddition to complete the reaction. One of the most reactive dienophiles in this reaction is *trans*-cyclooctene, which is five orders of magnitude more reactive than the *cis*-cyclooctene counterpart.⁵⁷ The tetrazine-*trans*-cyclooctene ligation proceeds very rapidly (k_2 up to $10^6 \text{ M}^{-1}\text{s}^{-1}$) without a catalyst and at almost 100% conversion at micromolar concentrations and room temperature.⁵⁸ This ligation has been employed to prepare dimeric PEG-based conjugate species for studying receptor interactions through the use of a tetrazine-norbornene ligation.⁵ Due to the discriminating nature of this ligation and rate of the tetrazine-*trans*-cyclooctene ligation, protein dimerization yield can theoretically be increased and conjugation time significantly decreased compared to existing dimerization methods. This thesis details the comparison in yield of homodimeric protein-polymer conjugates synthesized via the tetrazine-*trans*-cyclooctene ligation versus maleimide-thiol chemistry with pNIPAAm prepared by CRP and PEG.

Experimental

Methods

4-(hydroxymethyl)benzonitrile was purchased from Alfa Aesar. Bis-carboxylic acid PEG was purchased from Jenkem Technology. All other chemicals were purchased from Sigma Aldrich. AIBN was recrystallized from acetone. NIPAAm monomer was recrystallized in hexanes prior to polymerization.

Analytical techniques

Polymerization and end group modification reactions were carried out using standard Schlenk techniques under an inert atmosphere of argon. NMR spectra were obtained on a 500 MHz DRX spectrometer with a delay time of 10 seconds for polymers and AV 400 MHz with a delay time of 2 seconds for small molecules. Mass spectra for small molecules were obtained with a direct analysis in real time (DART) mass spectrometer. 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 mixed D columns. LiBr (0.1 M) in dimethylformamide (DMF) at 40 °C as the eluent (flow rate: 0.60 mL/min). Calibration was performed using near monodisperse poly(methyl methacrylate) (PMMA) standards from Polymer Laboratories. SDS–PAGE was performed using Bio-Rad Any kD Mini-PROTEAN-TGX gels. SDS–PAGE protein standards were obtained from Bio-Rad (Precision Plus Protein Pre-stained Standards). For SDS–PAGE analysis, approximately 4 µg of protein was loaded into each lane. Fast protein liquid chromatography (FPLC) was performed on a Bio-Rad BioLogic DuoFlow chromatography system equipped with a GE Healthcare Life Sciences Superdex 75 10/300 column. Protein concentrations were determined using the Thermo Scientific Nanodrop 2000. Infrared absorption spectra were recorded on a PerkinElmer FT-IR instrument equipped with an ATR accessory.

Synthesis of Polymers and Protein Conjugations

Synthesis of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (1).

(1) was synthesized using a slight modification of a previously reported protocol.⁵⁹ Zn(OTf)₂ (0.570 g, 1.56 mmol), and 4-(hydroxymethyl)benzonitrile (0.420 g, 3.12 mmol) were added in a

flame dried 15 mL round bottom flask under inert atmosphere, and dissolved in acetonitrile (1.28 g, 31.2 mmol, 1.3 mL). Anhydrous hydrazine (4.99 g, 0.155 mol, 4.89 mL) was added slowly to this mixture under argon flush. The flask was sealed and the mixture was stirred in an oil bath at 60 °C for 24 hours. The reaction solution was then allowed to cool to room temperature and sodium nitrite (2.15 g, 31.2 mmol) in 15 mL of water was slowly added to the slurry, followed by the slow addition of 2M hydrochloric acid during which the solution turned purple in color and the toxic nitrogen oxide gas evolved vigorously. Addition of 2M hydrochloric acid continued until gas evolution stopped at pH 2. The crude product was extracted with ethyl acetate until no purple color remained in the aqueous layer. The organic layers were combined, dried over magnesium sulfate and solvent was removed in vacuo. The purple crude product was purified using silica column chromatography in 3:2 ethyl acetate:hexanes to give the product as purple solid at 26% yield. ¹H-NMR (400 MHz, CDCl₃) δ: 1.75-1.86 (1H, m), 3.09 (3H, m), 4.84 (2H, s), 7.59 (2H, m), 8.58 (2H, m). ¹³C-NMR (400 MHz, CDCl₃) δ: 33.40, 68.13, 118.39, 118.89, 121.09, 132.91, 147.50, 150.14. IR: 3251, 2924, 2952, 2355, 2253, 2224, 2213, 2162, 2066, 2021, 1996, 1609, 1397, 1360, 1087, 1036, 1012, 888, 796 cm⁻¹. DART-MS [M+H]⁺ m/z calcd. for [C₁₀H₁₁N₄O]⁺ 203.0927, found 203.0915.

Synthesis of 2,2'-(Thiocarbonylbis(sulfanediyl))-dipropanoic Acid (2).

(2) was synthesized following a literature procedure with slight modifications.³³ KOH (6.60 g, 0.12 mol) was placed in a 200 mL round bottom flask equipped with a magnetic stir bar and then dissolved in 75 mL of water. Carbon disulfide (6.35 mL, 0.11 mol) was added, and the color turned light brown. 2-bromopropionic acid (4.73 mL, 0.05 mol) was then added dropwise. After

48 h, the solution was washed with dichloromethane (25 mL) five times to remove unreacted carbon disulfide. The aqueous layer was acidified to pH 5 with concentrated hydrochloric acid and then extracted with dichloromethane until the aqueous layer was no longer yellow. The organic layers were combined, dried with magnesium sulfate, and the solvent removed in vacuo to afford a yellow solid. The crude product was then dissolved in minimal dichloromethane and recrystallized in hexanes to give the product as a yellow powder in 52% yield. ¹H-NMR (400 MHz, CDCl₃) δ: 1.55–1.62 (6H, d), 4.75–4.83 (2H, q). ¹³C-NMR (400 MHz in CDCl₃) δ: 15.80, 48.51, 176.26, 219.33. IR: 2982, 1702, 1450, 1411, 1285, 1176, 1094, 1065, 980, 805. DART-MS [M+H]⁺ m/z calcd. for [C₇H₁₁O₄S₃]⁺ 254.9814, found 254.9796.

Synthesis of bis(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl) 2,2' (thiocarbonylbis(sulfanediyl)) dipropionate (3).

(2) (0.015 g, 0.0590 mmol) was placed in a round bottom flask equipped with a magnetic stirrer at 0 °C. (1) (0.025 g, 0.124 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 0.024 g, 0.124 mmol), and 4-dimethylaminopyridine (DMAP, 0.003 g, 0.0177 mmol) were added to the flask. The contents were dissolved with 2 mL of anhydrous dichloromethane. After 2 h, the reaction was warmed to 23 °C and stirred for 12 h. The solvent was then removed in vacuo and the crude product was purified by column chromatography in 2:3 ethyl acetate:hexanes obtaining a 65% yield. ¹H-NMR (400 MHz, CDCl₃) δ: 1.55–1.69 (6H, d), 2.96–3.19 (6H, m), 4.80–4.91 (2H, q), 5.17–5.36 (4H, m), 7.48–7.58 (4H, m), 8.52–8.62 (4H, m). IR: 2923, 1735, 1674, 1450, 1402, 1362, 1304, 1216, 1152, 1088, 1016, 940, 889, 839, 796 cm⁻¹. DART-MS [M+H]⁺ m/z calcd. for [C₂₇H₂₇N₈O₄S₃]⁺ 623.1311, found 623.1275.

4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)butanoic acid (4).

(4) was synthesized using a modification of a previously reported protocol.⁶⁰ γ -Aminobutyric acid (2.00 g, 19.40 mmol) and maleic anhydride (2.282 g, 23.3 mmol) were dissolved in 60 mL of glacial acetic acid and stirred at 120 °C for 6 h. The reaction mixture was poured into water after cooling to room temperature and extracted with ethyl acetate (50 mL) four times. The organic layers were combined, washed with brine, dried with magnesium sulfate, and solvent removed in vacuo to give the crude product. Purification was performed by recrystallization three times in 1:1 hexanes:ethyl acetate obtaining a 71% yield. ¹H-NMR (CDCl₃, 400 MHz) δ : 1.86-2.01 (2H, m), 2.33-2.44 (2H, t), 3.55-3.64 (2H, t), 6.65-6.82 (2H, s). ¹³C-NMR (CDCl₃, 400 MHz) δ : 24.0, 35.6, 41.08, 135.8, 170.5, 182.7. IR: 3086, 1695, 1447, 1412, 1371, 1227, 1146, 1096, 913, 836, 696 cm⁻¹.

Synthesis of rel-(1R, 4E, pR)-cyclooct-4-enol [5a (major)]

rel-(1R, 4E, pR)-cyclooct-4-enol (**5a**) was synthesized from (Z)-cyclooct-4-enol using a modification of a previously reported procedure.⁶¹ (Z)-cyclooct-4-enol (500 mg, 3.96 mmol) and methyl benzoate (550 mg, 4.04 mmol) sensitizer was dissolved in diethyl ether:hexanes 9:1 (50 mL) in a quartz flask. The reaction solution was exposed to light in a photoreactor equipped with a 450W medium pressure mercury lamp (Hanovia 451050) under constant stirring. At 60 minute intervals, the irradiation was stopped and the entire solution was passed through a column packed with silver nitrate (10%, w/w) impregnated silica (10 g), flushing with diethyl ether to trap the *trans* isomer. The solution that passed through was then transferred back into the quartz

flask and irradiation continued. After 12 cycles (6 hours) the irradiation was stopped and the silica was dried by air and added to a aqueous solution of ammonium hydroxide (30%, w/w) and stirred for 10 minutes after which diethyl ether (150 mL) was added and continued stirring for 5 more minutes. The crude product was then washed with H₂O and dried with magnesium sulfate. Evaporation of diethyl ether gave 152 mg mixture of trans/cis isomers. The isomers were separated by silica column chromatography in 1:1 ethyl acetate:hexanes in 30% yield. rel-(1R, 4E, pR)-cyclooct-4-enol (**5a**): ¹H-NMR (CDCl₃, 400 MHz) δ: 1.39 (1H, s), 1.48-1.75 (3H, m), 1.82-2.02 (4H, m), 2.18-2.43 (3H, m), 3.35-3.53 (1H, m), 5.26-5.43 (1H, m), 5.44-5.62 (1H, m); ¹³C-NMR (CDCl₃, 400 MHz) δ: 31.23, 32.66, 34.33, 41.08, 44.60, 77.75, 132.79, 135.08. IR: 3339, 3013, 2926, 2856, 2324, 1648, 1466, 1443, 1351, 1287, 1262, 1241, 1198, 1050, 1014, 987, 966, 893, 796, 726 cm⁻¹.

Synthesis of (E)-cyclooct-4-en-1-yl 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)butanoate (6).

(**4**) (325 mg, 1.77 mmol) was added to a 15 mL round bottom flask equipped with a magnetic stirrer and dissolved in dichloromethane (7 mL). Thionyl chloride (3.38 g, 28.4 mmol, 2.06 mL) was added to the reaction flask drop wise via syringe under inert argon. The solution was stirred and heated to reflux for 10h. The solvent and thionyl chloride were removed in vacuo and the crude solid was immediately used for the next step without purification. (**5a**) (200 mg, 1.59 mmol) and triethylamine (0.192 g, 1.90 mmol, 0.265 mL) were dissolved in anhydrous DCM (10 mL) and added drop wise to the crude reaction mixture at 0 °C. The reaction was stirred for 24 hours at 23 °C. The reaction was concentrated in vacuo and then loaded onto a silica gel column and run using 1:1 hexanes:ethyl acetate to give the product with impurities. The product was then

recrystallized three times in 1:1 diethyl ether:ethyl acetate in 26% yield. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 1.48-1.79 (4H, m), 1.83-2.00 (6H, m), 2.19-2.43 (5H, m), 3.50-3.63 (2H, t), 4.33-4.53 (1H, m), 5.38-5.76 (2H, m), 6.68 (2H, s). IR: 3088, 2934, 1726, 1439, 1406, 1379, 1353, 1327, 1202, 1176, 1095, 1065, 996, 947, 911, 883, 842, 809, 722, 693 cm^{-1} . DART-MS $[\text{M}+\text{H}]^+$ m/z calcd. for $[\text{C}_{16}\text{H}_{22}\text{NO}_4]^+$ 292.1543, found 292.1524.

Synthesis of bis-carboxylic acid PNIPAAm (7).

N-Isopropylacrylamide (NIPAAm, 0.410 g, 3.62 mmol), AIBN (0.0026 mg, 0.0157 mmol), and CTA (**2**) (0.040 g, 0.157 mmol) were charged into a Schlenk tube, dissolved in anhydrous THF (1 mL), and subjected to four freeze-pump-thaw cycles to degas the solution of any oxygen. The flask was refilled with argon and immediately transferred to a 70 °C oil bath to initiate the polymerization. Monomer conversions were calculated by $^1\text{H-NMR}$ by monitoring the disappearance of the peaks corresponding to the vinylic protons with the main chain CH (3.95-4.15 ppm) of the pNIPAAm as the internal standard. The polymerization was stopped after 2.5 h by rapidly cooling in liquid nitrogen and then exposing to atmosphere. The mixture was precipitated five times from cold hexanes, and then dialyzed (MWCO 1000) against MeOH for 12 hours and then H_2O for another 12 hours to remove unreacted monomer. The polymer was recovered as a yellow solid after freeze-drying, with a M_n ($^1\text{H-NMR}$) of 2.0 kDa and a \bar{D} (GPC) of 1.05. $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ : 0.90-1.30 (main chain, $\text{CH}(\text{CH}_3)_2$), 1.33-2.60 (main chain, CH_2 and main chain, CH), 3.83-4.20 (main chain, CH), 4.39-4.75 (first carbon adjacent to trithiocarbonate, SCH), 5.86-7.24 (main chain, NH). IR: 3302, 3083, 2973, 2930, 2877, 2365, 1709, 1640, 1542, 1458, 1386, 1367, 1259, 1171, 1130, 1082, 926, 885, 800 cm^{-1} .

Synthesis of bis-tetrazine pNIPAAm (8).

(7) (0.050 g, 0.0262 mmol) was added to a flame-dried round bottom flask equipped with a magnetic stirrer and dissolved in anhydrous dichloromethane (800 μ L) at 0 $^{\circ}$ C. (1) (0.0133 g, 0.0656 mmol) EDC-HCl (0.0210 g, 0.0577 mmol) and DMAP (0.34 mg, 0.0105 mmol) were added to the solution. After 2 h, the reaction was warmed to 23 $^{\circ}$ C and stirred for 24 h. Dichloromethane was removed in vacuo and the crude product was redissolved in THF. The mixture was precipitated five times from cold hexanes, and then dialyzed (MWCO 1000) against MeOH for 12 hours and then H₂O for another 12 hours. The polymer was recovered as a purple solid after freeze-drying. *M_n* was calculated by ¹H-NMR by comparison of the peak integrations of the polymer main chain at 3.95-4.15 ppm and signal at 4.35-4.75 (2H). End-Group conversions were calculated from the ¹H-NMR spectra by comparison of the peak integrations at 4.35-4.75 ppm (2H), and using the sum of the integral values of the peaks at 3.10, 5.13-5.30, 7.58, 8.59 ppm divided by their respective theoretical values and then averaged. ¹H-NMR (CDCl₃, 400 MHz) δ : 0.9-1.24 (main chain, CH(CH₃)₂), 1.27-2.59 (main chain, CH₂ and main chain, CH), 3.10 (6H, m), 3.90-4.17 (main chain, CH), 4.40-4.73 (first carbon adjacent to trithiocarbonate, SCH), 5.13-5.30 (4H, m), 5.56-7.20 (main chain, NH), 7.58 (4H, m), 8.59 (4H, m). IR: 3344, 2971, 2114, 1739, 1647, 1542, 1365, 1217 cm⁻¹.

Synthesis of 2 kDa bis-tetrazine poly(ethylene glycol) (9).

2 kDa bis-carboxylic acid poly(ethylene glycol) (PEG) (100 mg, 0.0500 mmol) was placed in a 2-neck round bottom flask equipped with a magnetic stirrer. (1) (22 mg, 0.110 mmol), EDC-HCl

(21 mg, 0.110 mmol), and DMAP (1.22 mg, 0.0100 mmol) were added to the flask at 0 °C. The contents were dissolved with 2 mL of anhydrous DCM. The reaction mixture was stirred under argon for 12 h while letting the reaction warm to 23 °C. The mixture was then precipitated directly into cold diethyl ether and then dialyzed against methanol for 24 h and then water for 12 h. The product was then freeze dried and afforded a pink solid. End-Group conversions were calculated from the ¹H-NMR spectra setting the signal at 4.21-4.29 ppm equal to its theoretical value of 4H, and using the sum of the integral values of the signals at 3.10, 4.84, 7.58, 8.59 ppm divided by their respective theoretical values and then averaged. ¹H-NMR (CDCl₃, 400 MHz) δ: 3.10 (6H, m), 3.40-3.89 (main chain CH₂), 4.21-4.29 (4H, s), 5.29 (4H, s), 7.58 (4H, m), 8.59 (4H, m). IR: 2884, 2344, 1751, 1466, 1404, 1360, 1342, 1279, 1240, 1198, 1146, 1103, 1060, 961, 841 cm⁻¹.

Synthesis of furan protected bis-maleimide pNIPAAm (10).

(7) (0.050 g, 0.0262 mmol) was added to a flame-dried round bottom flask equipped with a magnetic stirrer and dissolved in anhydrous dichloromethane (800 μL) at 0 °C. A furan protected maleimide alcohol (0.0156 g, 0.0698 mmol), EDC-HCl (0.0254 g, 0.0698 mmol) and DMAP (0.22 mg, 0.00698 mmol) were added to the solution. After 2 h, the reaction was warmed to 23 °C and stirred for 24 h. Dichloromethane was removed in vacuo and the crude product was redissolved in THF. The mixture was precipitated five times from cold hexanes, and then dialyzed (MWCO 1000) against MeOH for 24 hours and then dried in vacuo. ¹H-NMR (CDCl₃, 400 MHz) δ: 0.9-1.25 (main chain, CH(CH₃)₂), 1.26-2.65 (main chain, CH₂ and main chain, CH), 2.85 (chain end, CHCHOCH), 3.57 (chain end, NCH₂), 3.81-4.22 (main chain, CH),

4.35-4.70 (first carbon adjacent to trithiocarbonate, SCH), 5.25 (chain end, CHOCH), 6.51 (chain end, CH=CH), 5.49–7.26 (main chain, NH).

Retro Diels-Alder reaction to form (11).

The difunctionalized pNIPAAm with furan protected maleimide end-groups (**10**) was maintained at 120 °C under vacuum for 3 h. The target polymer was obtained in 100 % yield by ¹H-NMR. Deprotection was monitored by the disappearance of the peaks at 6.51, 5.25, and 2.85 ppm and appearance of a new peak near 6.72 ppm. ¹H-NMR (CDCl₃, 400 MHz) δ: 0.9-1.25 (main chain, CH(CH₃)₂), 1.3-2.7 (main chain, CH₂ and main chain, CH), 3.57 (chain end, NCH₂), 3.95-4.15 (main chain, CH), 4.35-4.75 (first carbon adjacent to trithiocarbonate, SCH), 6.72 (chain end CH=CH), 5.39-7.39 (main chain, NH).

Expression, purification and characterization of mutant V131C T4 Lysozyme (12).

Expression and purification was performed as described in current literature with slight modifications.⁴⁹ E. coli host BL21(DE3) (Invitrogen) was used to express V131C T4 Lysozyme using an expression vector obtained from Prof. Wayne Hubbell (UCLA).^{47,48} Following the expression, the crude mixture was prepared for cation exchange chromatography. Extensive washes with lysis buffer, two column volumes of lysis buffer each with increasing NaCl content (in a gradient from 0 M to 0.2 M in 0.05 M steps), were used to elute Lysozyme. The eluted fractions were analyzed by SDS-PAGE under reducing conditions to determine which fractions contained Lysozyme. The 0.1 M and 0.15 M fractions were combined and further purified using centrifugal filtration (Amicon Ultra, MWCO 30,000) to separate from higher molecular weight

impurities. The isolated Lysozyme V131C was then concentrated by centrifugal filtration (Amicon Ultra, MWCO 10,000) and resuspended in degassed and filtered 100 mM phosphate buffer at pH 6.5 prior to mass determination. Masses were obtained by ESI-MS as described by Whitelegge et al.⁶² Briefly, the protein samples (10 μ L) were diluted in formic acid (90 μ L of 90% formic acid, ACS grade Fisher) before to size-exclusion chromatography (SEC) with ESI-MS. Zerocharged mass spectra were generated with BioMultiview (Applied Biosystems). M expected: 18607. M observed (ESI-MS): 18611

Synthesis of *trans*-cyclooctene labeled Lysozyme (T4L-TCO) (13).

DTT (2.33 μ L of 1 M stock solution) was added to a solution of Lysozyme (2.404 mg/mL in 260 μ L of 100 mM PBS at pH 6.5) with average of 0.74 thiols/protein (verified by Ellman's assay) and incubated at 4 $^{\circ}$ C for 1 h to ensure that the protein was completely reduced prior to conjugation. The reduced Lysozyme was then added dropwise to a solution of **(4)** (0.00169 g, 0.00581 mmol) in 20 μ L of DMSO. The solution was stirred at 23 $^{\circ}$ C for 30 minutes and then centrifuged to remove any precipitated **(6)**. Excess **(6)** was also removed by centrifugal filtration (Amicon Ultra, MWCO 10,000). This protocol was repeated twice until the average thiols/protein dropped to zero (verified by Ellman's assay) after modification. M expected: 18898. M observed (ESI-MS): 18898.

Typical Dimerization of T4L-TCO with (8).

bis-tetrazine pNIPAAm **(8)** (0.555 μ L of 1 mg/mL) was mixed with an aliquot of T4L-TCO (1.617 μ L of 4.948 mg/mL) in 100 mM PB pH 6.5. The reaction mixture was brought to a final

volume of 5 μ L with buffer and final protein concentration of 1.60 mg/mL and mixed at 4 °C for 24 hours. A time-point was taken at 1 hour to monitor yield. The sample was analyzed by SDS-PAGE under reducing conditions.

Typical Dimerization of T4L-TCO with (9).

bis-tetrazine PEG (9) (0.509 μ L of 1 mg/mL) was mixed with an aliquot of T4L-TCO (1.617 μ L of 4.948 mg/mL) in 100 mM PB pH 6.5. The reaction mixture was brought to a final volume of 5 μ L with buffer and final protein concentration of 1.60 mg/mL and mixed at 4 °C for 24 hours. A time-point was taken at 1 hour to monitor yield. The sample was analyzed by SDS-PAGE under reducing conditions.

Typical Dimerization of T4L-TCO with (11).

bis-maleimide pNIPAAm (11) (0.508 μ L of 1 mg/mL) was mixed with an aliquot of T4L (1.58 μ L of 5.048 mg/mL) in phosphate buffered saline (PBS) (pH 7.5 with 10 mM of TCEP and 10 mM of EDTA). The reaction mixture was brought to a final volume of 5 μ L with buffer and final protein concentration of 1.60 mg/mL and mixed at 4 °C for 24 hours. A time-point was taken at 1 hour to monitor yield. The sample was analyzed by SDS-PAGE under reducing conditions.

Determination of Conjugation Yield

Conjugation yields were assessed as described in literature.⁶³ Briefly, SDS-PAGE gels were scanned with an Epson Perfection 2480 scanner, saved as tiff files and analyzed using ImageJ software. Bands in each lane were selected by the rectangular selection tool, and intensities

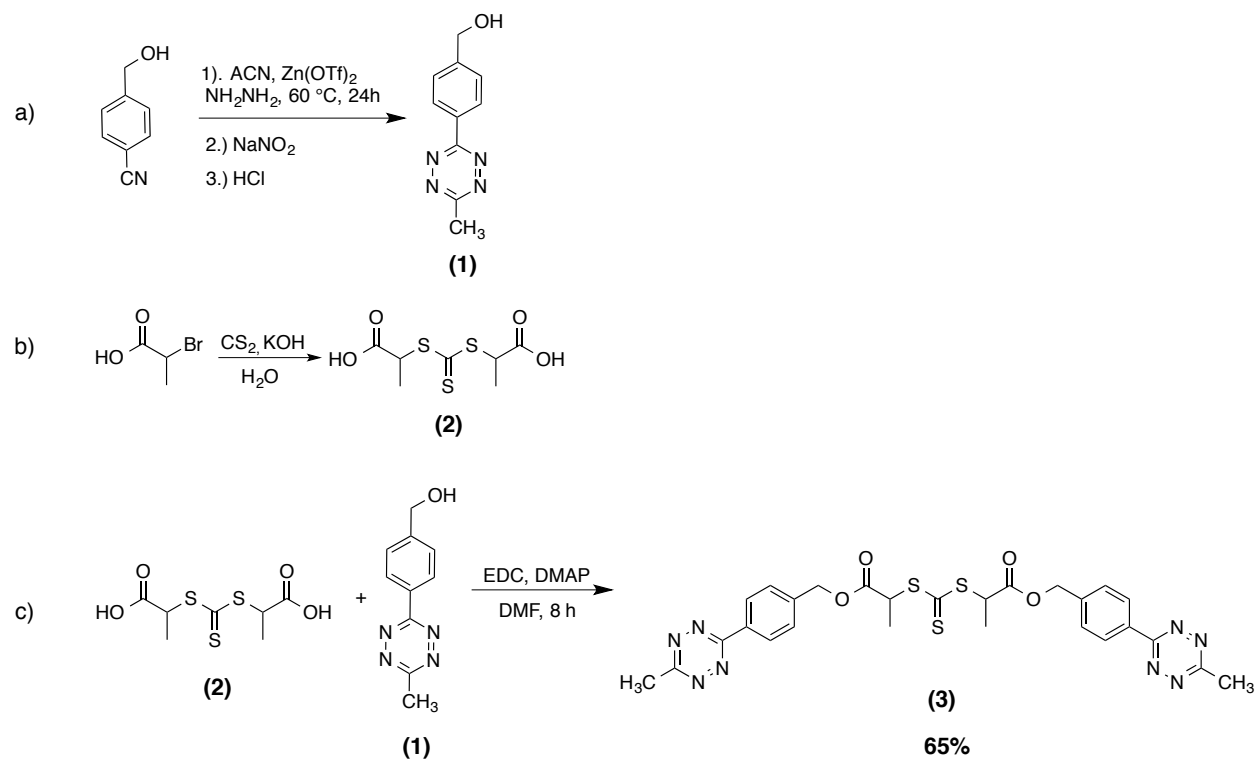
plotted with the gel analysis function. Percent yield was defined as the peak area of each protein band divided by the sum of the conjugates and unmodified protein areas.

Results and Discussion

Synthesis of Bis-Tetrazine CTA.

In order to synthesize homodimeric protein-polymer assemblies, several protein-reactive polymers were synthesized. To prepare bis-reactive pNIPAAms, a bis-carboxylic acid CTA was first synthesized (Scheme 1b), which can be functionalized pre or post-polymerization with tetrazine. During our first attempt, (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine, or 6-methyltetrazine amine, was coupled onto the bis-carboxylic acid CTA in extremely low yield (18%, data now shown). The amine most likely reacted by cleaving the trithiocarbonate faster than coupling to the carboxylic acid groups. Aminolysis of trithiocarbonates is widely used for RAFT end-group conversion, resulting in the formation of a thiol end-group.^{64,65} A tetrazine-containing alcohol was then synthesized (Scheme 1a) and coupled to the CTA. Specifically, (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (**1**) was chosen due to its known stability in aqueous environments and the hydroxyl functionality should not react with the CTA.⁶⁶ The stability of the tetrazine suggests compatibility with CRP and bioconjugation conditions. Tetrazine (**1**) was synthesized through the reaction of a benzonitrile and acetonitrile in the presence of hydrazine and a lewis acid catalyst, $\text{Zn}(\text{OTf})_2$.⁵⁹ We were able to obtain (**1**) in 26% yield; a higher yield has been reported with the use of $\text{Ni}(\text{OTf})_2$ which could be used in the future. Sodium nitrite and HCl were used to oxidize the dihydrotetrazine intermediate to yield

the tetrazine. **(1)** was coupled to both ends of **(2)** to afford a bis-tetrazine CTA as a purple solid at 65% yield.



Scheme 1. Synthesis of RAFT CTAs **(2)** and **(3)**

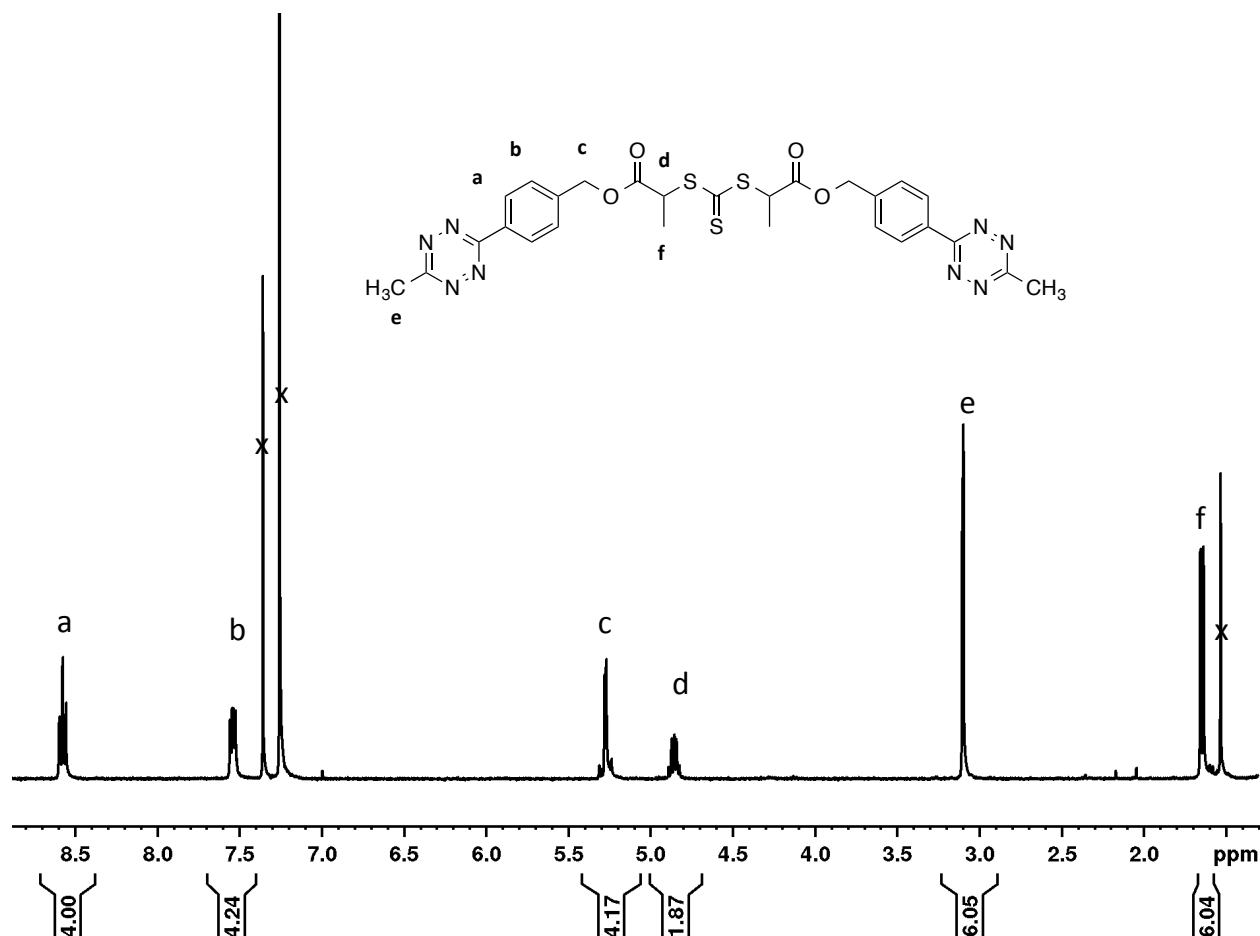
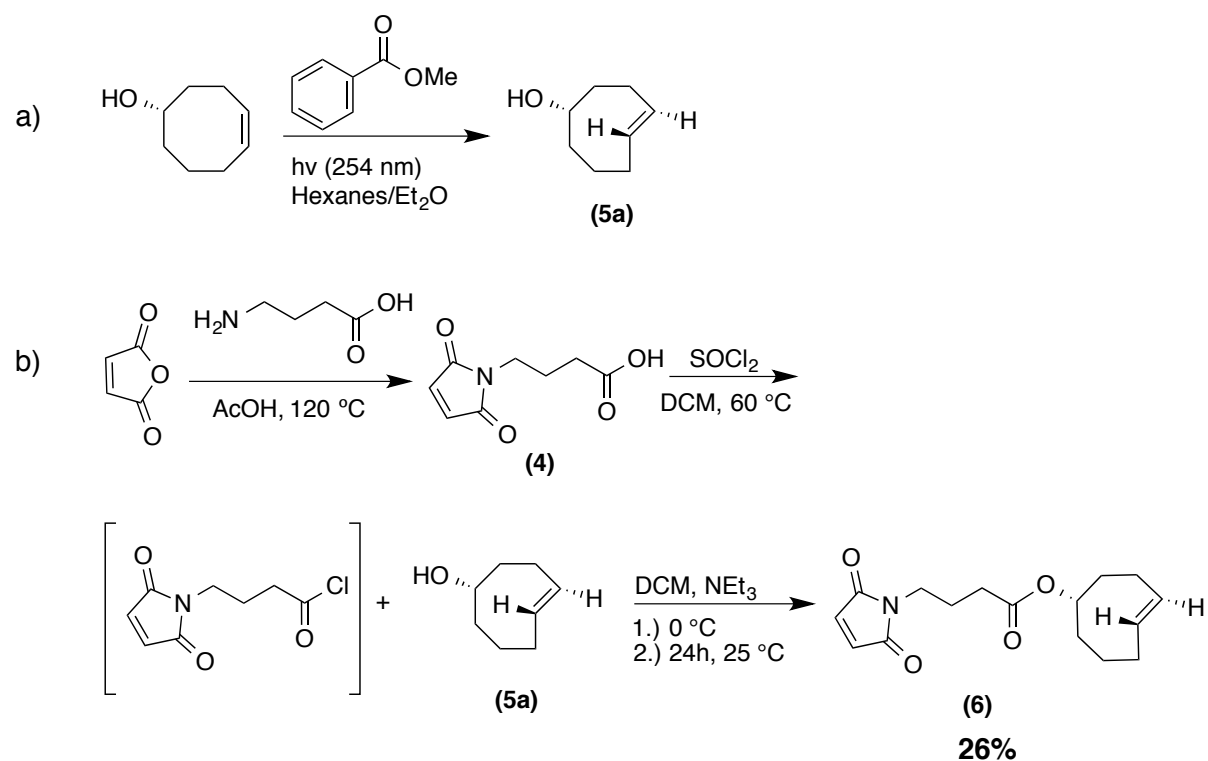


Figure 1. ¹H-NMR spectrum (CDCl₃) of bis-tetrazine CTA (**3**)

Synthesis of protein reactive *trans*-Cyclooctene. The other component of the tetrazine ligation, *trans*-cyclooctene, was synthesized using a slightly modified procedure from literature (Scheme 2a).⁶¹ *Trans*-cyclooctene was selected for this ligation since it has been reported that it is one of the most reactive dienophiles, owing this to the highly strained 8-membered ring. *Trans*-cyclooctene is also five orders of magnitude more reactive than *cis*-cyclooctene towards tetrazines; therefore only the *trans* isomer was utilized for this ligation.⁵⁷ (**5a**) was synthesized from (Z)-cyclooct-4-enol by photoisomerization in the presence of a sensitizer, methyl benzoate, to yield both *trans* and *cis* alkene products. After irradiation, the reaction mixture was passed

through a column packed with silver nitrate-impregnated silica which forms a stable complex with the *trans* isomer, whereas the *cis* isomer binds weakly and is eluted out.⁶¹ The *trans* isomer then was isolated from silver nitrate by breaking coordination with addition of ammonium hydroxide followed by extraction with ether. Any minor *cis* isomer impurity was removed by column chromatography. *Trans*-cyclooctene was functionalized to bear a protein reactive maleimide since the use of maleimide-thiol chemistry has been shown to be very effective in modifying proteins with one surface-exposed thiol.^{49, 54} Attempts for esterification to make **(6)** began by activating the carboxylic acid of **(4)** through the use of coupling agents including carbonyldiimidazole (CDI), and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC). However, thionyl chloride proved to be the easiest method and **(6)** was synthesized in 26% yield (Scheme 2b). Techniques, including extractions and column chromatography were attempted to purify this product but we did not obtain a high level of purity until multiple re-crystallizations were preformed (Figure 2). The compound was utilized in the later steps to modified T4L.



Scheme 2. Synthesis of protein reactive *trans*-cyclooctene (**6**)

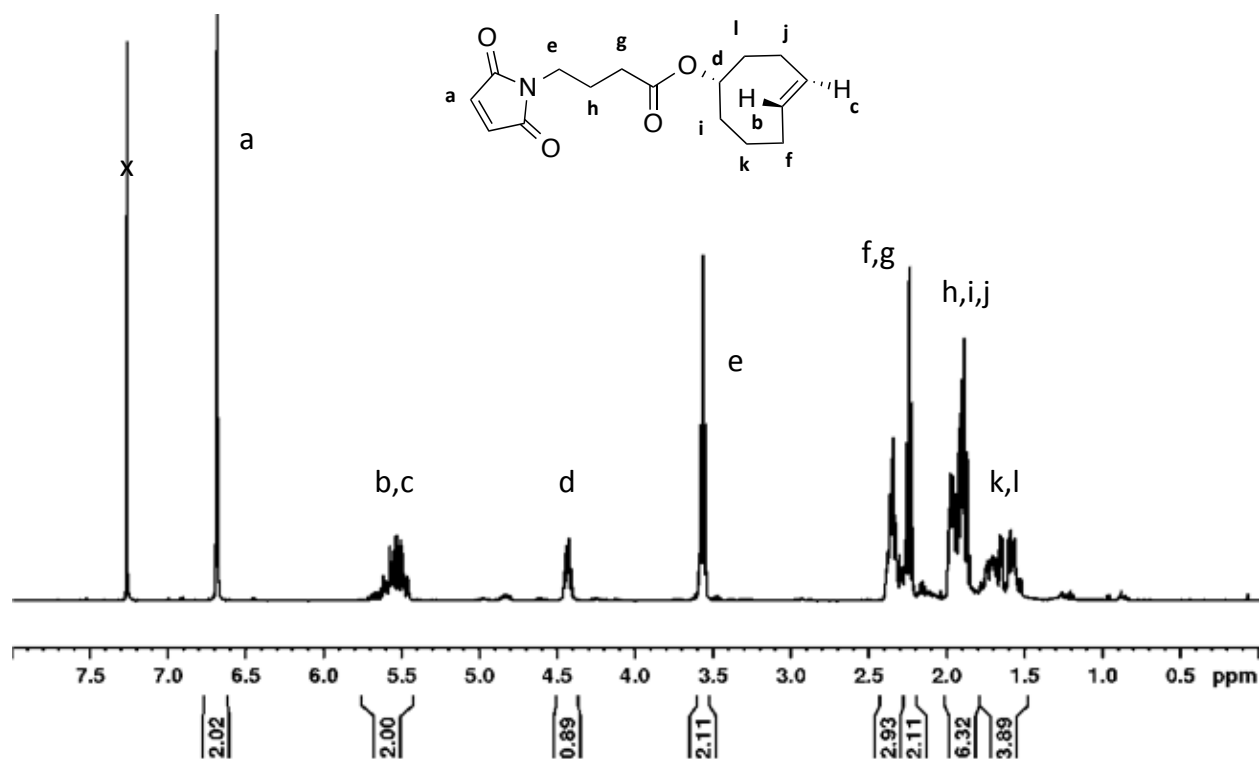
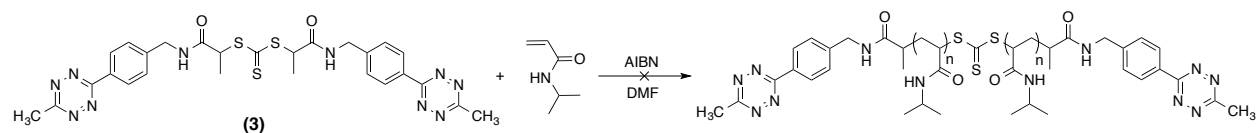


Figure 2. ^1H -NMR spectrum (CDCl_3) of protein reactive *trans*-cyclooctene (**6**)

RAFT Polymerization of NIPAAm with CTA (2). The first attempt to synthesize the bis-tetrazine pNIPAAm was through the use of a RAFT CTA bearing the tetrazine end-groups. Normally, this approach is very successful in ensuring polymer end-groups without the need of post-polymerization modification. Polymerization using the ester derivative of the bis-tetrazine CTA followed (Scheme 3). Three different reaction conditions were attempted in order to make the bis-tetrazine polymer (Table 1). The ¹H-NMR of the product after precipitation and dialysis for poly3 reveals the presence of obscure peaks in the aromatic and alkyl region, which could not be assigned (Figure 3). These signals were present in all three polymerizations performed using CTA (3). The emergence of peaks in the aromatic and aliphatic regions could be explained by decomposition of the tetrazine end-group or a possible Diels-Alder reaction between tetrazine and the alkene of N-isopropylacrylamide monomer. The [4+2] cycloaddition would cause a change in electronic environment, creating new aromatic peaks. The conversion of the alkene to a saturated product is suggested by the emergence of peaks in the alkyl region. The cycloaddition was considered as a potential problem prior to planning the polymerization and CTA synthesis. Due to the deactivated nature of the alkene, and the higher stability of (1) compared to other tetrazines, it was assumed that cycloaddition would not occur. The use of a radical azo-initiator, such as 2,2'-Azobis(4-methoxy-2,4-dimethyl valeronitrile) (V70), that decomposes at near room temperatures may allow for the polymerization of (3) since lower temperatures may slow or prevent the cycloaddition.



Scheme 3. Typical RAFT polymerization of NIPAAm poly3

Reaction	Ratio [CTA]:[M]:[I]	°C	Target Mw	Mw by ¹ H-NMR
Poly1	1:23:0.1	60 °C	2 kDa	x
Poly2	1:125:0.1	70 °C	9.9 kDa	12.1 kDa
Poly3	1:100:0.3	70 °C	8 kDa	9.6 kDa

Table 1. Conditions and results for RAFT polymerizations using the bis-tetrazine CTA (3) and NIPAAm monomer

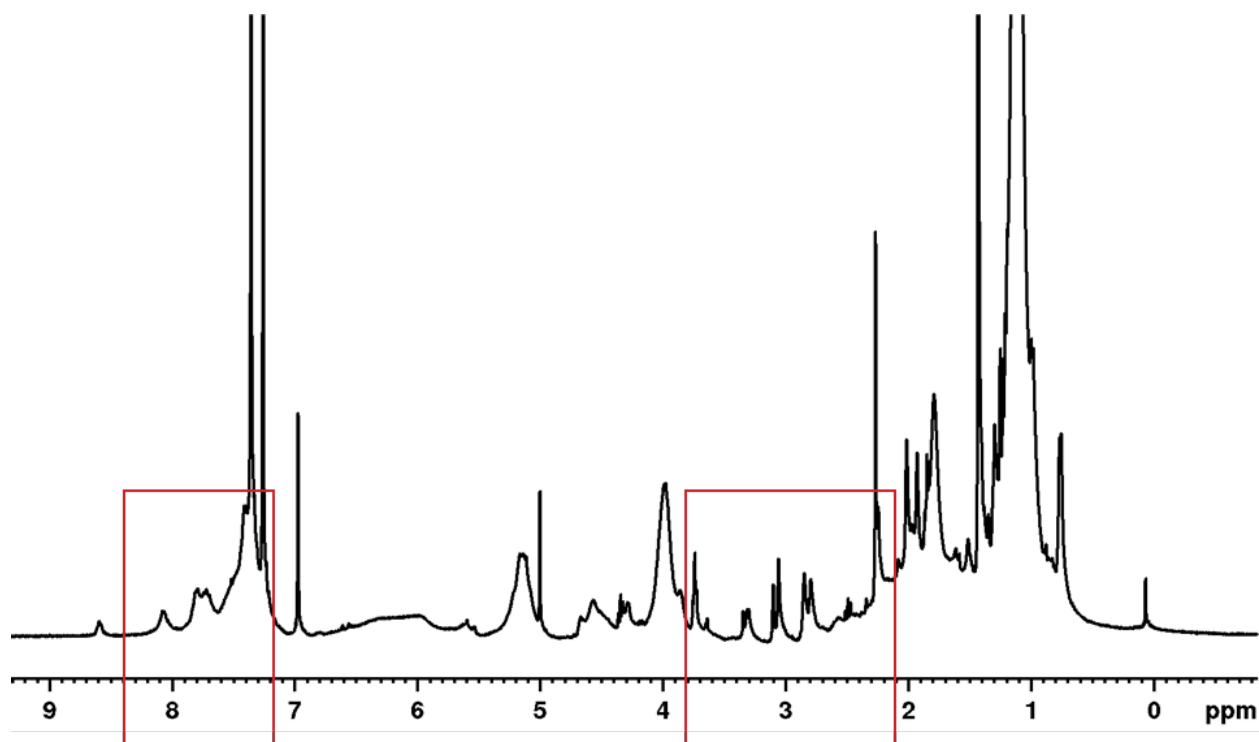
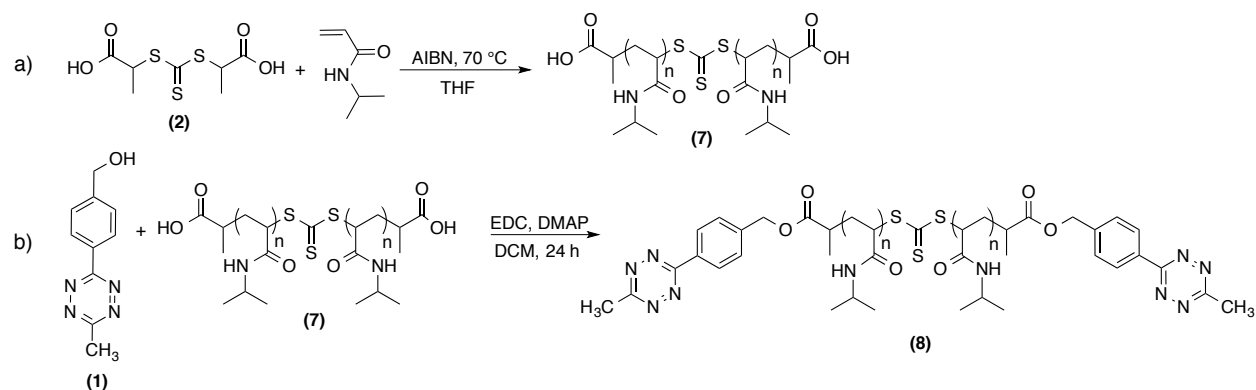


Figure 3. ¹H-NMR (CDCl₃) of **poly3** after purification.

Synthesis of bis-tetrazine pNIPAAm. Due to the side-reactivity of tetrazine under RAFT conditions, post-polymerization modification of bis-carboxylic acid pNIPAAm was next pursued. **(8)** was synthesized in two steps (Scheme 4) starting with polymerization of NIPAAm with **(2)** at a target molecular weight of 2.0 kDa. The polymerization reached 77% conversion after approximately 2.5 hours and was immediately stopped. The polymer was characterized by ¹H-NMR and GPC. M_n (¹H-NMR) of **(7)** was 2.0 kDa, \bar{D} (GPC) was 1.05 and M_n (GPC) was 1.2 kDa. The large difference in M_n could be attributed to the difference in hydrodynamic volume of **(7)** compared to the poly(methyl methacrylate) standards used for GPC calibration. By NMR, the carboxylic acid end group is not observed but was evident by IR. Precipitation, dialysis in H₂O, and freeze-drying afforded **(7)** (Figure 4). The near mono-disperse nature of the polymer will allow for well-defined protein-polymer conjugates. Next, bis-carboxylic acid pNIPAAm was functionalized with tetrazine. EDC coupling of **(1)** to the carboxylic acid end-group of **(7)** was carried out three times to achieve 94% modification by ¹H-NMR (Figure 5).



Scheme 4. Synthesis of bis-carboxylic acid pNIPAAm **(7)** and subsequent EDC coupling to yield bis-tetrazine pNIPAAm **(8)**.

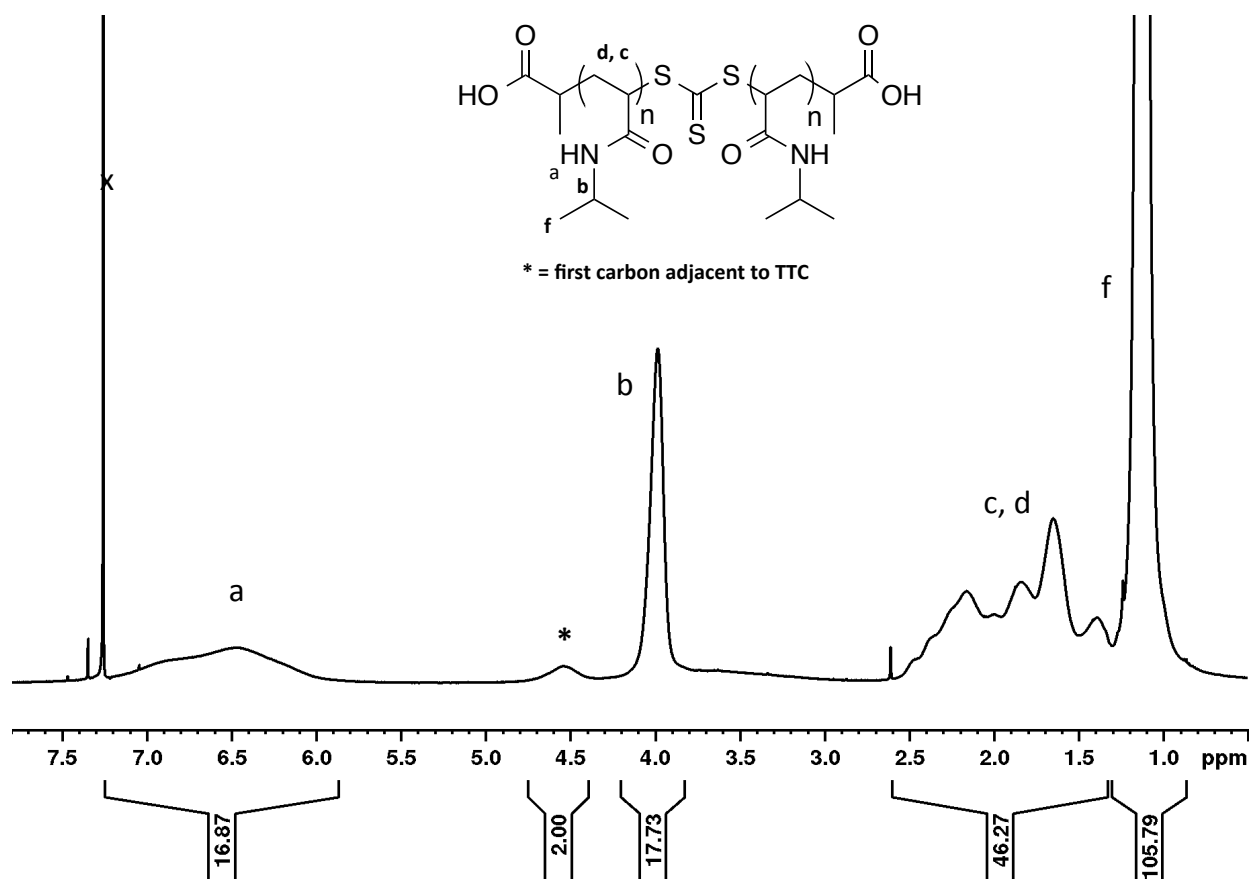


Figure 4. ^1H -NMR (CDCl_3) of bis-carboxylic acid pNIPAAm (7).

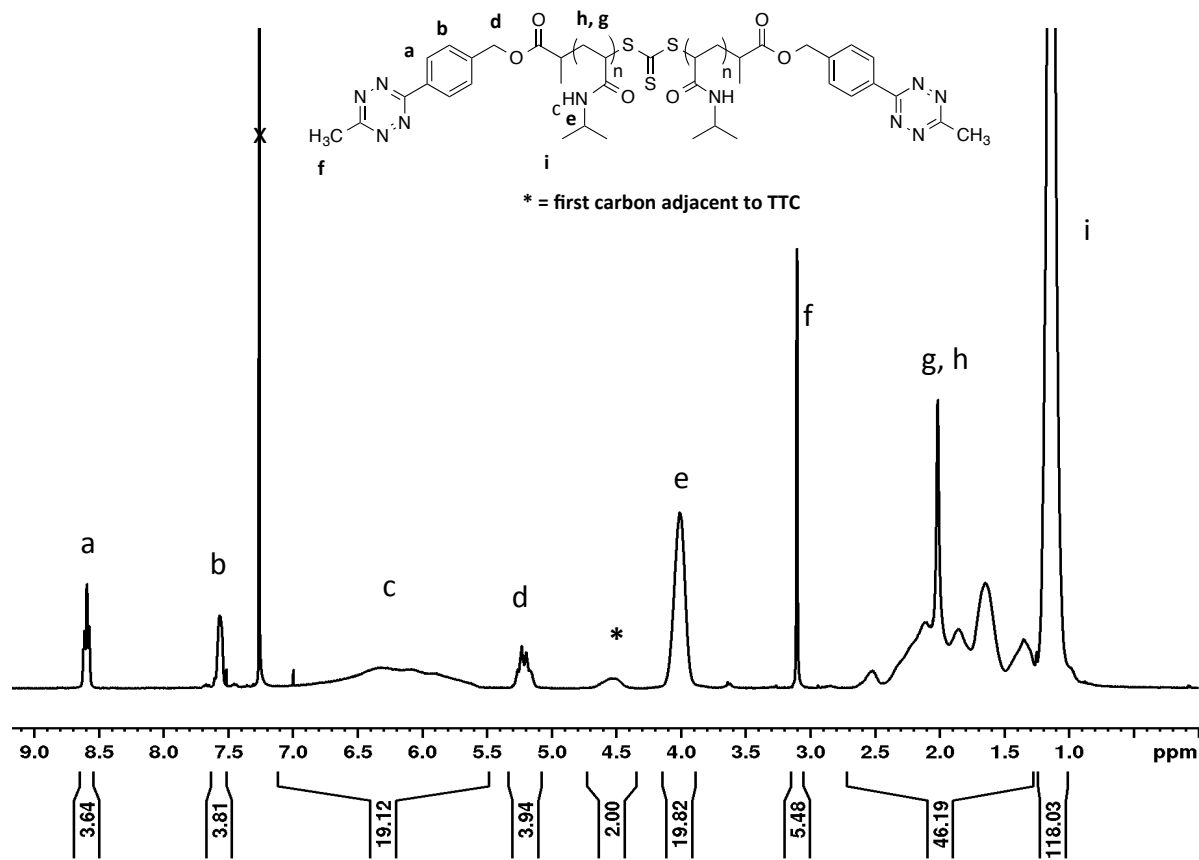
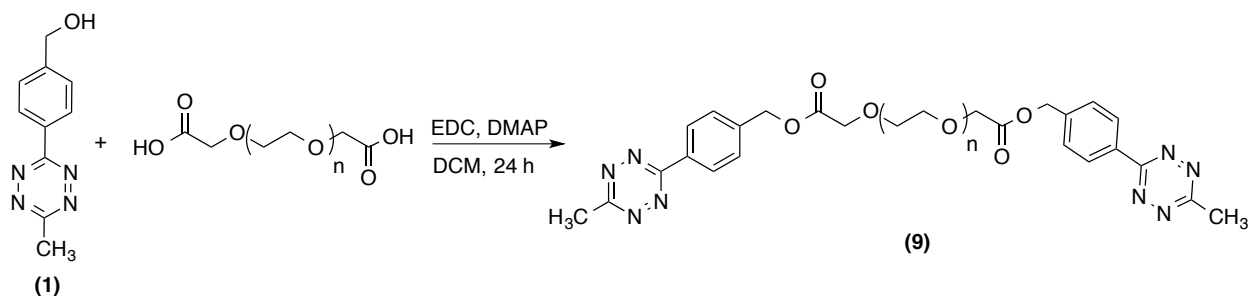


Figure 5. ^1H -NMR (CDCl_3) of bis-tetrazine pNIPAAm (**8**).

Synthesis of control polymers. Bis-tetrazine PEG (**9**) was synthesized using commercially available bis-carboxylic acid PEG (2 kDa) by EDC coupling of (**1**) (Scheme 5). The reaction was subjected three times until an average of 93% modification was achieved by ^1H -NMR (Figure 6). The polymer was prepared to serve as a comparison between polymers made by CRP and ones not made by CRP. Additionally, we synthesized a bis-maleimide pNIPAAm (**11**) polymer from (**7**) (Scheme 6). The maleimide-thiol chemistry between unmodified T4L and bis-maleimide pNIPAAm was made to compare the yield of maleimide cysteine Michael addition to the yield obtained by the tetrazine-*trans*-cyclooctene ligation. A protected maleimide alcohol was coupled by EDC to both ends of (**7**). Deprotection of (**10**) to give (**11**) was easily achieved by

heating the polymer to 120 °C under vacuum for 3 h with no further purification. The ^1H -NMR spectrum of **(11)** showed that deprotection occurred successfully, evident by the disappearance of the ^1H -NMR signals near 6.5, 5.25, and 2.85 ppm and appearance of a higher shifted signal at 6.72 ppm belonging to maleimide (Figure 7). These exact parameters are consistent with those described in literature.⁴³



Scheme 5. Synthesis of bis-tetrazine PEG **(9)**.

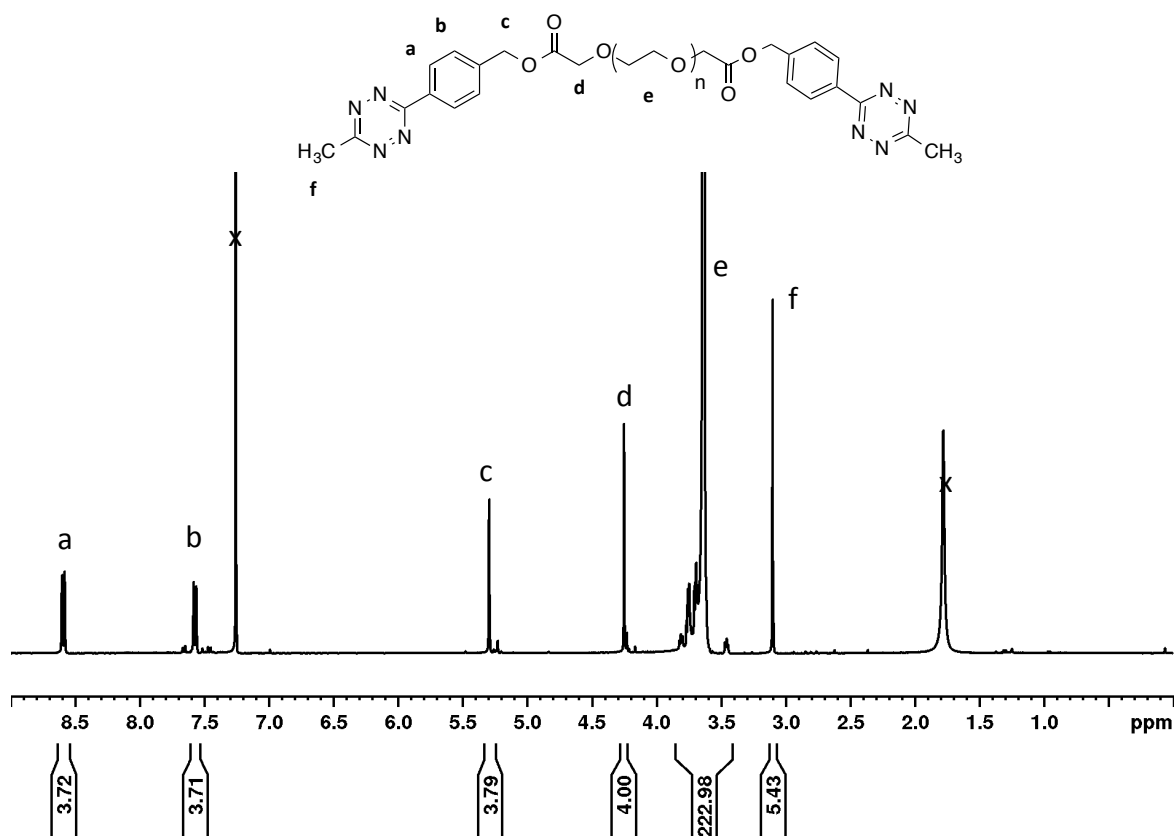
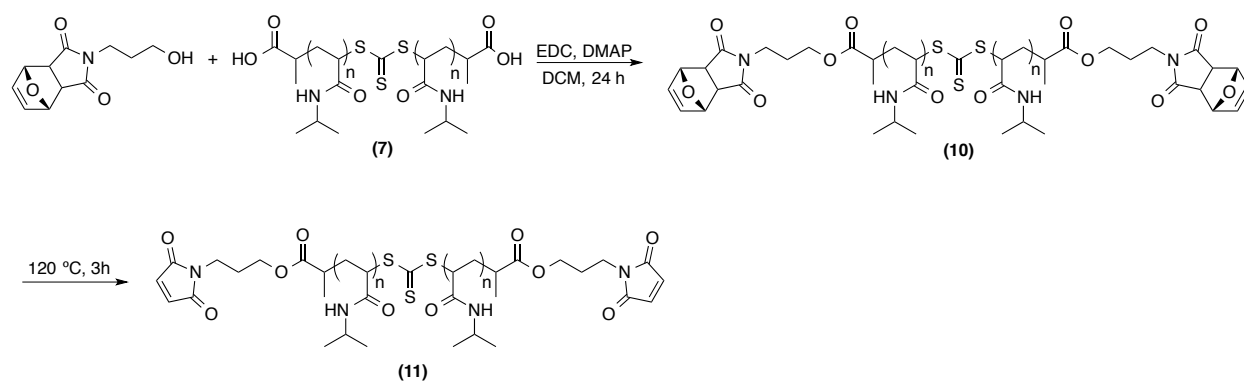


Figure 6. ^1H -NMR (CDCl_3) of bis-tetrazine PEG **(9)**.



Scheme 6. Synthesis of bis-maleimide pNIPAAm (**11**)

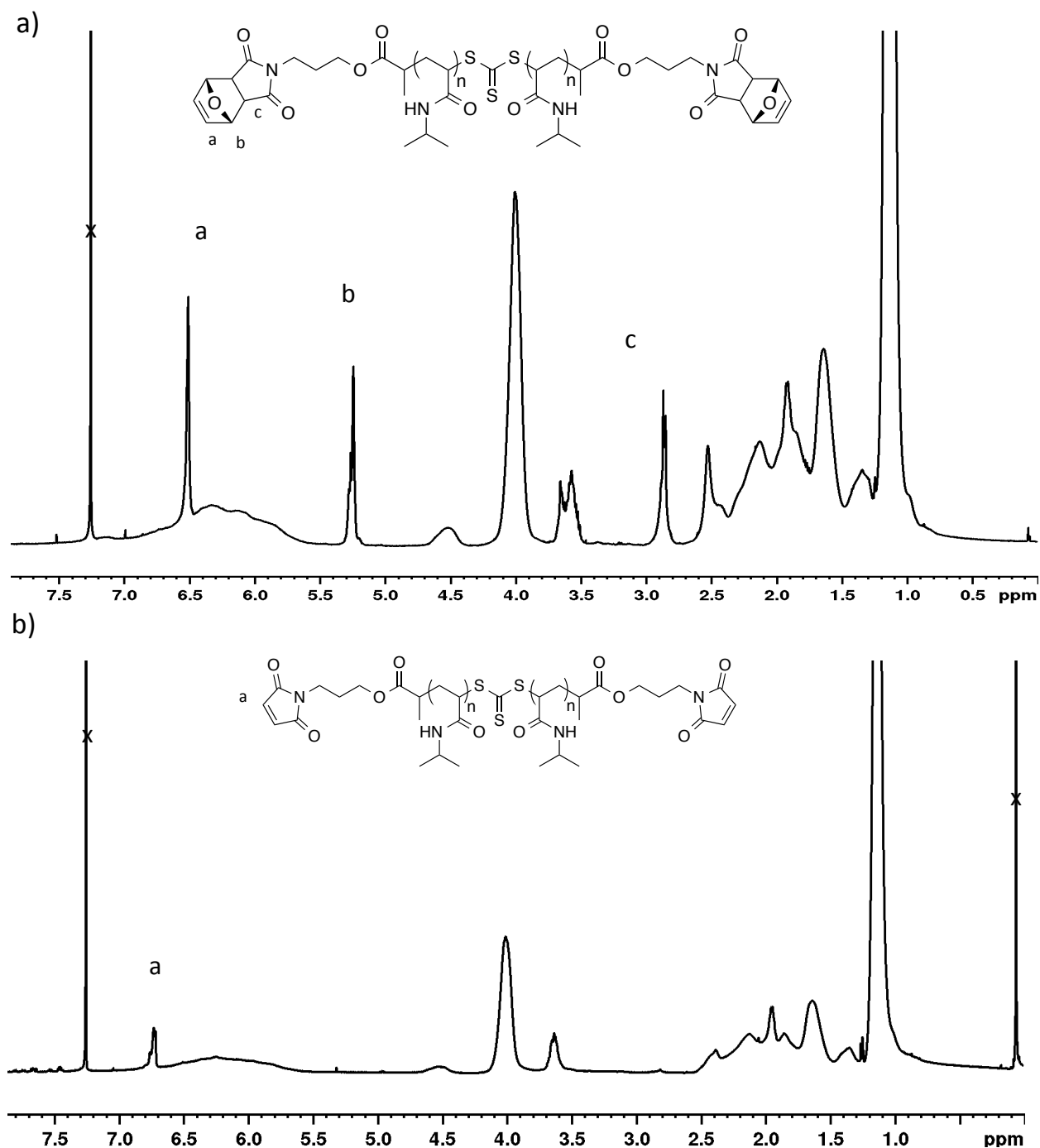
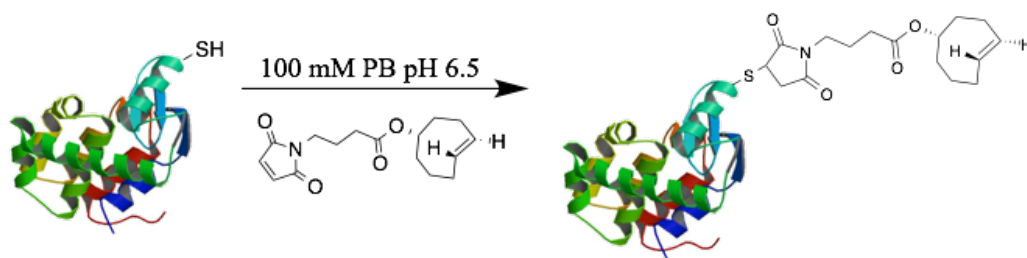


Figure 7. ^1H -NMR (CDCl₃) of bis-maleimide pNIPAAm (**11**) before and after deprotection.

Conjugation of protein reactive *trans*-cyclooctene (6**) to T4L.** After expression and purification, an average free thiol content of 0.74 thiols per T4L was determined by Ellman's

assay. The conjugation reaction was performed in a 100 mM phosphate buffer at pH 6.5. A low pH was used to ensure site-specific conjugation to the single surface exposed thiol group. It is known that in the presence of neutral or slightly acidic buffers, thiol groups are 1000 times more reactive and selective towards Michael acceptors compared to amines.⁴⁶ Using a high pH could be problematic since other nucleophilic groups are present on the protein surface. We tested various solvents including MeOH, DMF, ACN, and DMSO to introduce hydrophobic (**6**) into aqueous solution. Minimal organic solvent has been used before to introduce hydrophobic molecules into aqueous solution with retention of protein activity.⁴⁹ If the use of organic solvents is not desired, it is possible to introduce a hydrophilic group, such an oligo(ethylene glycol) group, to induce aqueous solubility of a protein reactive molecule. T4L was reduced using DTT prior to modification. A solution of (**6**) in DMSO was made in a three fold excess to DTT. The freshly reduced T4L was added dropwise into the DMSO solution containing (**6**) (Scheme 7). An overall 10% DMSO was present in the reaction mixture and found to allow efficient conjugation. Excess (**6**) precipitated quickly upon addition of T4L in buffer. After 30 minutes of incubation, any precipitate was removed by centrifugation and the supernatant was concentrated by centrifugal filtration (MWCO 10000). Centrifugal filtration also served to removed any unreacted DTT and (**6**). Modification of T4L to form T4L-TCO was monitored by electrospray-ionization mass spectroscopy (ESI-MS) (Figure 8) and no unreacted protein was detected. The change in mass corresponds to exactly one *trans*-cyclooctene moiety.



Scheme 7. Modification of reduced T4L with (6). (PDB #1LYD)

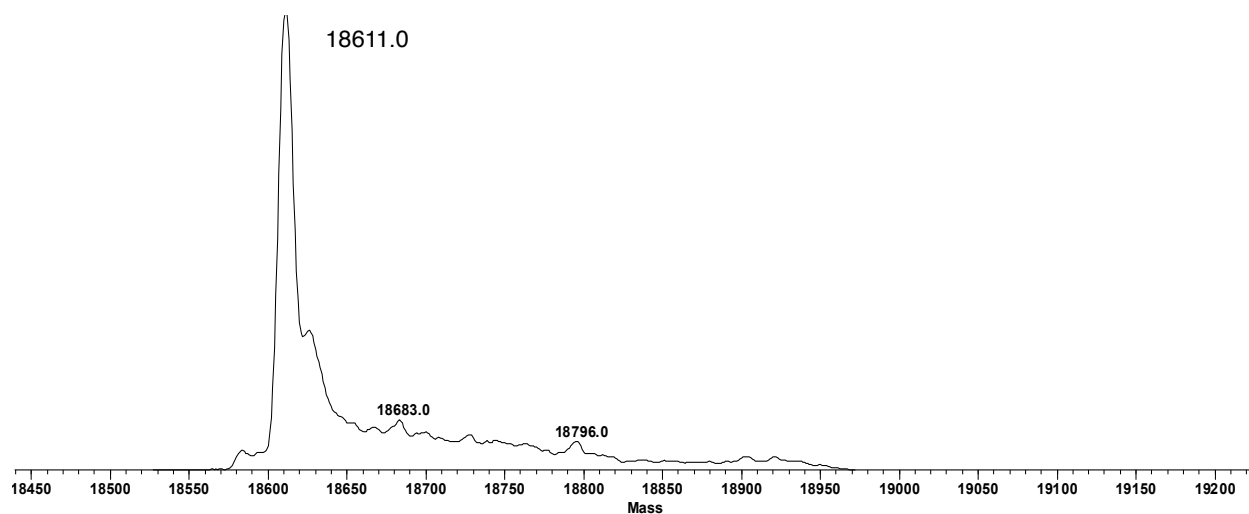


Figure 8a. ESI-MS of T4L before modification with (6)

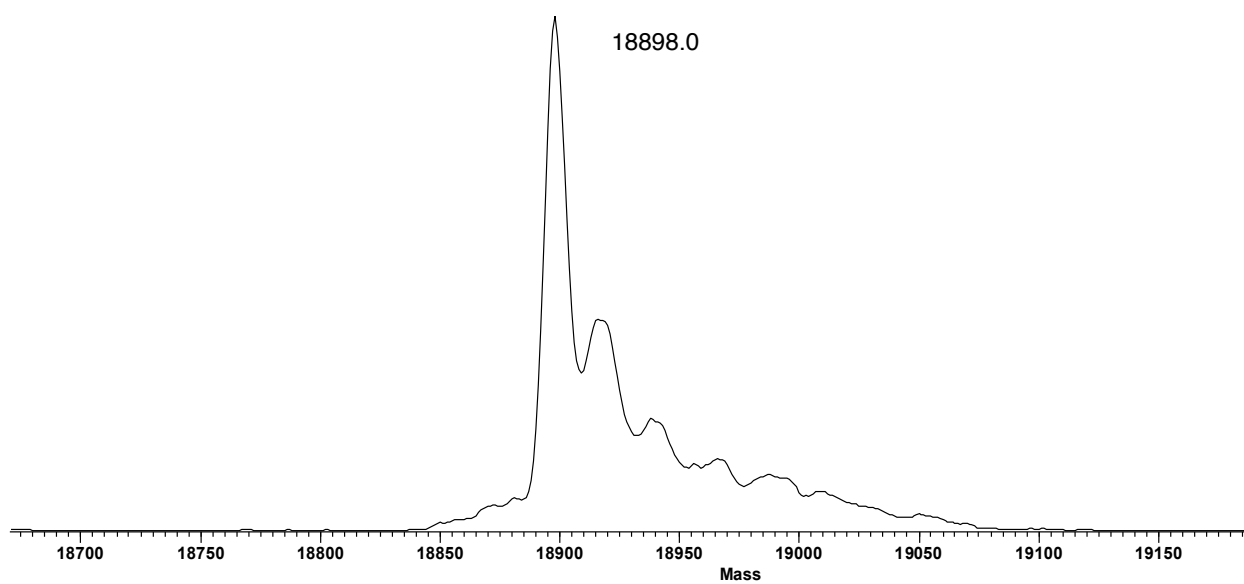
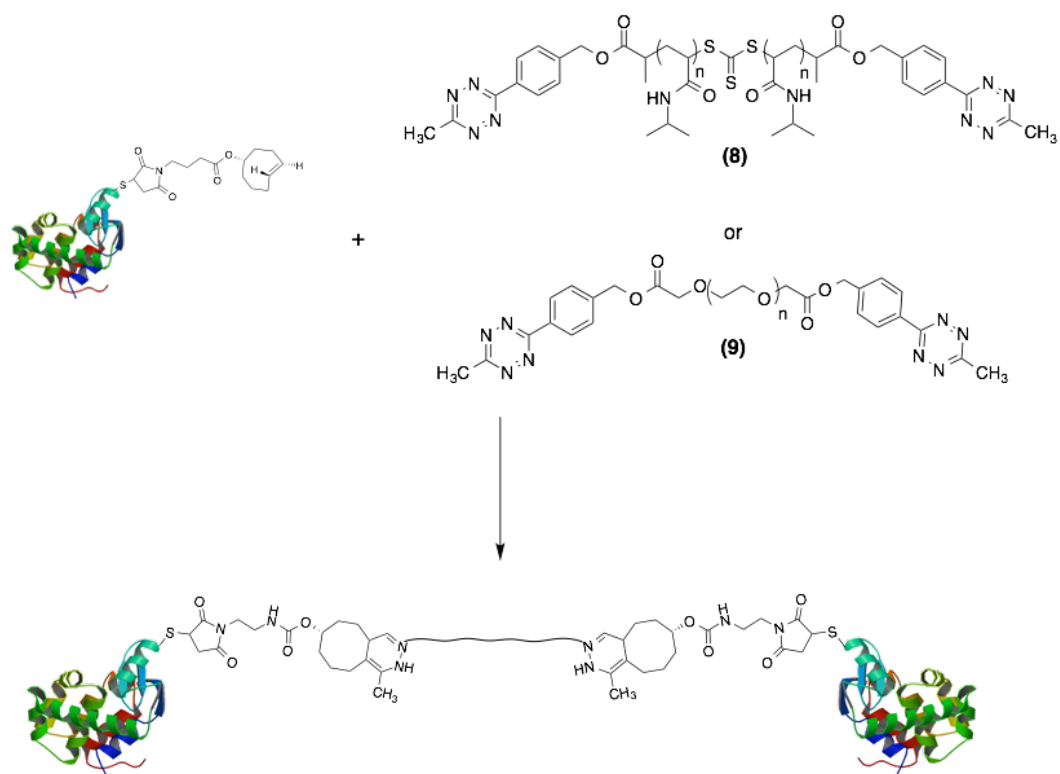


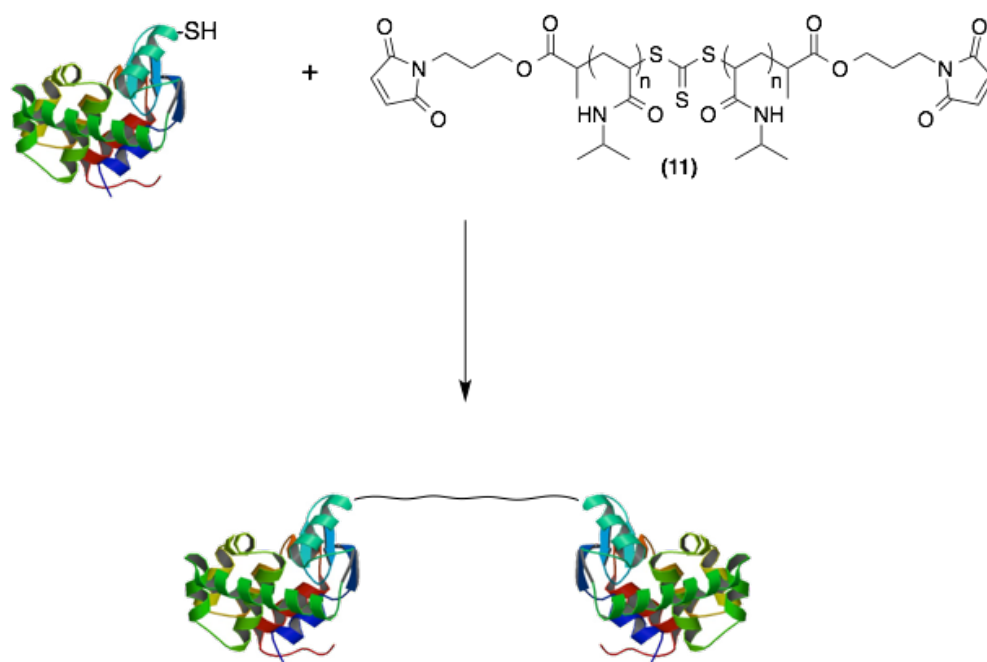
Figure 8b. ESI-MS after modification of T4L with (6).

Dimerization of T4L-TCO. The T4L-TCO conjugates were then prepared for dimerization reactions (Scheme 8). T4L-TCO (**13**) in 100 mM PB pH 6.5 was added to a solution containing tetrazine functionalized polymers (Figure 9). To determine the effects of excess protein on conjugate yield, we screened various conditions (Table 2). No significant increase in dimer yield was seen with higher equivalents of protein, therefore a 2:1 ratio of protein to polymer was used in subsequent reactions. During the preparation, all pNIPAAm polymers were difficult to fully solvate at ambient temperature. pNIPAAm's lower critical solution temperature (LCST) property, the temperature at which the polymer becomes insoluble, has been reported to decrease linearly with increasing salt concentration.⁶⁷ This could account for why no dimer was obtained after 1h (Figure 9a). To solve the solubility issue, all pNIPAAm samples were kept at 4 °C during the preparation and the conjugation temperatures. Additionally, we tested several solvent systems . pNIPAAm was prepared in a buffer/organic mixture to that gave a final 10% organic solution in the dimerization reaction. We found that the control, an additional 10% H₂O instead of organic solvent, performed just as well (Figure 9b). To determine whether a lower buffer ionic strength can lead to increased yields, the stock protein solution was exchanged to 5 mM PB pH 6.5, a 20 fold decrease in ionic strength and conjugated to all polymers. Buffer exchange, however, resulted in significant protein precipitation and loss so we continued with 100 mM PB pH 6.5. A gradual decrease in salt concentration may have prevented the protein loss. The later polymer solutions were prepared with an addition of H₂O to give an overall “10% additional” H₂O in the final mixture (Figure 9c). Bis-maleimide pNIPAAm (**11**) - T4L conjugation buffer was prepared as described in literature.⁴³ For all conjugation reactions, the yields after an extended conjugation time of 24 h did not improve significantly. This may reveal the inherent steric hinderance for

synthesis of homodimeric protein assemblies. Also, the conversion of *trans*-cyclooctene to *cis*-cyclooctene in solution over time is potentially problematic since *cis*-cyclooctene is five times less reactive than the *trans* isomer.^{68, 57} The yields for the maleimide-thiol conjugation reactions were significantly lower and almost undetectable compared to that of the tetrazine-*trans*-cyclooctene ligation under these reactions conditions. It has not escaped our attention that polymer concentrations can also be factor limiting conjugation yields and can be increased in the conjugation reactions to further optimize yields. Overall, the tetrazine-*trans*-cyclooctene ligation dimerizations gave higher yields compared to maleimide-thiol chemistry. The sensitivity of pNIPAAm's LCST was found to impede protein dimerization at temperatures above it's LCST. Bis-tetrazine pNIPAAm gave overall slightly higher yields than bis-tetrazine PEG under optimized conditions (27.27%), and both gave significantly higher yields than bis-maleimide pNIPAAm (Figure 9c). For future studies and optimization of dimerization yields, a more reactive tetrazine, such as (4-(1,2,4,5-tetrazin-3-yl)phenyl)methanol, could be incorporated onto polymer end-groups. Additionally, a higher degree of buffer conditions can be screened such as varying pH and ionic strength.



Scheme 8a. Dimerization T4L-TCO with (8) or (9)



Scheme 8b. Dimerization T4L-TCO with (11)

Polymer	[T4L-TCO]: [Polymer]	% Solvent in Polymer Solution	T (°C)	% Yield (1 h)	% Yield (24 h)
(8)	1:3:1	-	25 °C	0.81	1.78
	2:1	-	25 °C	1.4	0.23
	4:1	-	25 °C	0.76	0
(9)	1:3:1	-	25 °C	22.86	25.64
	2:1	-	25 °C	26.16	27.67
	4:1	-	25 °C	27.47	29.13
(8)	10:1	-	4 °C	16.96	16.52
	2:1	10 % H ₂ O	4 °C	26.98	26.48
	2:1	10 % DMSO	4 °C	21.23	22.60
	2:1	10 % ACN	4 °C	20.74	19.42
	2:1	10 % MeOH	4 °C	20.37	20.33
(8)	2:1	10 % H ₂ O	4 °C	27.27	29.41
(9)	2:1	10 % H ₂ O	4 °C	16.75	15.58
(11)	2:1	10 % H ₂ O	4 °C	0.88	1.92

Table 2. Dimerization results of T4L-TCO with (8), (9), or (11) under various conditions.

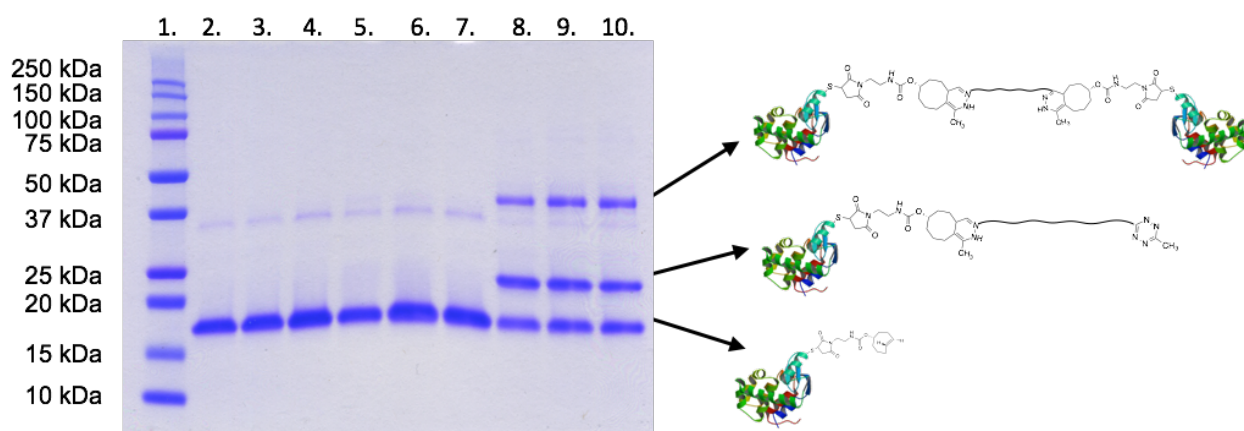


Figure 9a. Coomassie stained SDS-PAGE of dimerization reactions at 25 °C after 1 h with adjusted contrast. (1) protein ladder, (2) 2.0 µg T4L-TCO, (3) 3.0 µg T4L-TCO, (4) 4.0 µg T4L-TCO, (5) 1.3:1 T4L-TCO:Tz-pNIPAAm-Tz, (6) 2:1 T4L-TCO:Tz-pNIPAAm-Tz, (7) 4:1 T4L-TCO:Tz-pNIPAAm-Tz, (8) 1.3:1 T4L-TCO:Tz-PEG-Tz, (9) 2:1 T4L-TCO:Tz-PEG-Tz, (10) 4:1 T4L-TCO:Tz-PEG-Tz.

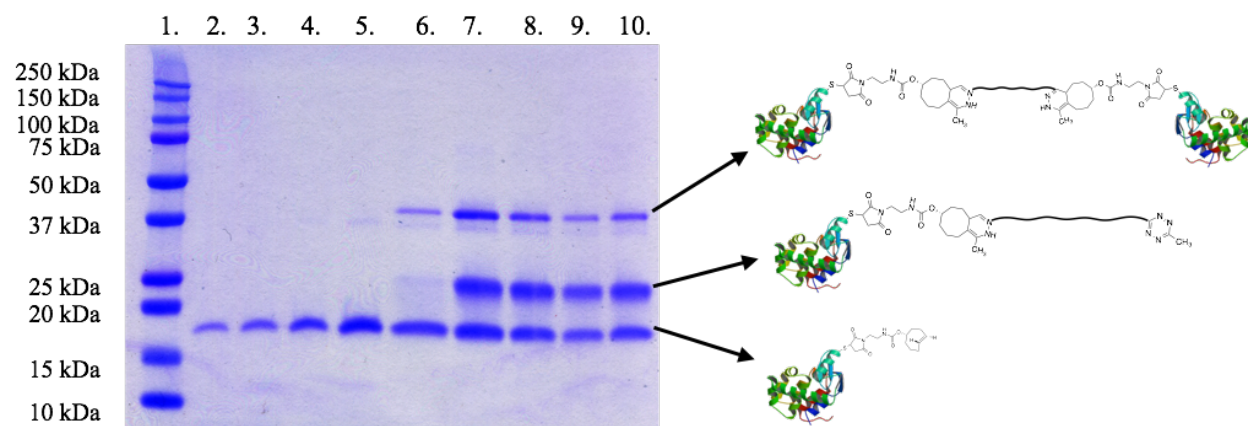


Figure 9b. Coomassie stained SDS-PAGE of dimerization reactions at 4 °C after 1 h with adjusted contrast. (1) protein ladder, (2) 0.5 μg T4L-TCO, (3) 1.0 μg T4L-TCO, (4) 1.5 μg T4L-TCO, (5) 2.5 μg T4L-TCO, (6) 10:1 T4L-TCO:Tz-pNIPAAm-Tz, (7) 2:1 T4L-TCO:Tz-pNIPAAm-Tz in 10% H_2O , (8) 2:1 T4L-TCO:Tz-pNIPAAm-Tz in 10% DMSO, (9) 2:1 T4L-TCO:Tz-pNIPAAm-Tz in 10% ACN, (10) 2:1 T4L-TCO:Tz-pNIPAAm-Tz in 10% MeOH

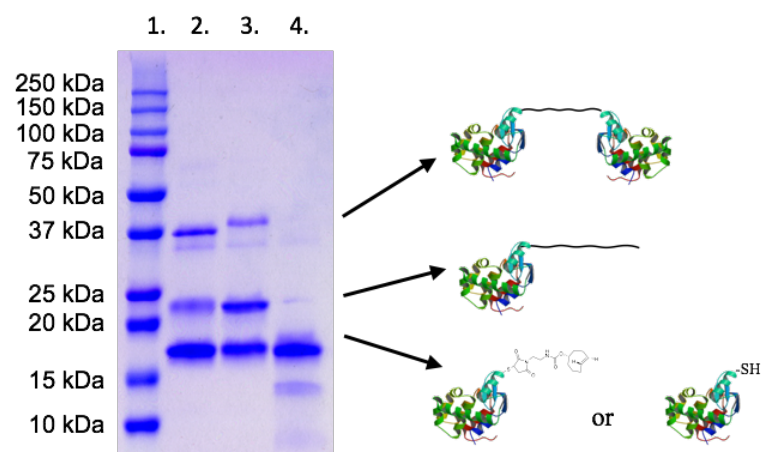


Figure 9c. Coomassie stained SDS-PAGE of dimerization reactions at 4 °C after 1 h with adjusted contrast. (1) protein ladder, (2) 2:1 T4L-TCO:Tz-pNIPAAm-Tz, (3) 2:1 T4L-TCO:Tz-PEG-Tz, (4) 2:1 T4L:mal-pNIPAAm-mal

Conclusions

Well-defined polymers were synthesized by RAFT and modified at both ends with tetrazine.

Mutant T4 Lysozyme containing one surface-exposed free cysteine was successfully modified

with a protein reactive *trans*-cyclooctene moiety via Michael addition to the cysteine. Dimerization of the polymers was conducted and yields were assed after 1 hour. Tetrazine functionalized polymer yielded dimers at significantly reduced incubation time (1 h). We obtained the higher yield with bis-tetrazine pNIPAAm (27.27%) compared to bis-tetrazine-PEG dimerization (16.75%) and bis-maleimide pNIPAAm controls (<1%). Therefore, this work demonstrated that the tetrazine-*trans*-cyclooctene ligation can be employed to synthesize homodimeric protein CRP-polymer conjugates with higher yields than commonly utilized maleimide-functionalized CRP polymers.

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