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

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Abstract

Tetrazine end-functionalized telechelic polymers were synthesized by controlled radical polymerization (CRP) and employed to generate T4 Lysozyme homodimers. Mutant T4 Lysozyme (V131C), containing a single surface-exposed cysteine, was modified with a protein-reactive *trans*-cyclooctene (T4L-TCO). Reversible addition-fragmentation chain transfer (RAFT) polymerization yielded poly(N-isopropylacrylamide) (pNIPAAm) with a number average molecular weight (M_n by ¹H-NMR) of 2.0 kDa and a dispersity (by GPC) of 1.05. pNIPAAm was then modified at both ends by post-polymerization with 6-methyl tetrazine. For comparison, 2.0 kDa bis-tetrazine poly(ethylene glycol) (PEG) and 2.0 kDa bis-maleimide pNIPAAm were synthesized. Ligation of T4L-TCO to bis-tetrazine pNIPAAm or bis-tetrazine PEG resulted in protein homodimer in 38% yield and 37% yield, respectively, after only 1 hour, whereas bis-maleimide pNIPAAm resulted in only 5% yield of dimer after 24 h. This work illustrates the advantage of employing tetrazine ligation over maleimide thiol-ene chemistry for the synthesis of protein homodimer conjugates.

Introduction

Protein dimerization and formation of multimers is a ubiquitous and essential phenomenon in biological systems, and is often required for signal transduction and native protein activity.^{1,2} Therefore, the development of new divalent ligands and methodologies towards protein multimerization continues to be an expanding area of research in biology, biomedicine and biotechnology.^{3–10} By tethering proteins, the entropic cost of association can be paid upfront and/or multimerization results in a higher local concentration, often resulting in higher biological activities than monomeric protein.

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Supporting Information

Synthetic procedures for the synthesis of **1**, **3**, and **7**, expression and purification of mutant T4L(V131C), ¹H-NMR for all small molecules and polymers, ¹³C-NMR spectra for all small molecules, ESI-MS of T4L(V131C) and T4L-TCO, and activity studies.

Polymer conjugation to proteins leads to improved pharmacokinetics and stabilization. Many linear poly(ethylene glycol) (PEG)-protein monoconjugates have been approved by the FDA.^{11,12} Second generation protein-polymer conjugates, with additional benefits as compared to PEG including degradability, enhanced stabilization or activity, have been developed utilizing CRP techniques, which allows for the use of a variety of monomers and more complex polymeric structures.¹³ As a result of these advantages, polymer-based tethers, either PEG or those prepared by CRP, have been used for protein homodimerization, oligomerization, and multimerization.

The majority of the protein-polymer conjugates prepared via CRP are monoconjugates. Although there are fewer reported studies, protein-reactive telechelic polymers by CRP have been mainly synthesized by RAFT polymerization.^{14,15} This has been accomplished by employing a protein reactive chain transfer agent (CTA) to form the semi-telechelic polymer and then post polymerization modification to form the telechelic polymer^{16–20} or by polymerization involving a bis-functionalized protein reactive CTA.^{21–23} The former technique can readily provide polymer with the same or different protein-reactive group allowing for the synthesis of homo or hetero protein dimers, respectively. For example, Theato and coworkers synthesized poly(diethylene glycol monomethyl ether) utilizing an activated ester CTA and then transformed the omega end of the polymer to a biotin group via aminolysis.²⁰ The latter technique is straightforward and in one step produces polymers for homo dimer formation. Additionally, post polymerization modification of polymers synthesized by bis-functionalized CTAs yields protein-reactive telechelic polymers.^{24–26} For example, we developed homotelechelic polymers with maleimide end-groups by radical reaction of furan-protected maleimide azo initiators with thiocarbonylthiol groups.²⁴ After retro Diels Alder to form the maleimides, homodimerization of proteins was possible by Michael addition.

A major drawback to polymer-based protein dimerization methods is low yield. For example, Liu et. al. intended to synthesize homodimers via a macro-RAFT agent approach, but were unsuccessful.²³ We reported a 21% homodimer yield with the use of homotelechelic maleimide functionalized pNIPAAm after a 16 hour conjugation time.²⁴ Maleimide-thiol chemistry has also been used with small molecules of 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.²⁷ Recent developments in conjugation techniques and linkers may have the potential to produce higher yields of protein dimers.^{28–30} Here, we report the use of an extremely rapid bioorthogonal chemistry, specifically the tetrazine ligation, as a method to increase dimerization yield.

The inverse-electron-demand Diels-Alder reaction between tetrazine and strained double bonds is a rapid bio-orthogonal reaction, and has been commonly employed in labeling cells^{31–33} and biomacromolecules.^{34–37} One of the most reactive partners in this reaction is *trans*-cyclooctene, which is five orders of magnitude more reactive than the *cis*-cyclooctene counterpart.³⁸ 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 at room temperature.³⁹ This ligation has been employed to prepare dimeric PEG-based

conjugate species for studying receptor interactions through the use of a tetrazine-norbornene ligation,⁴ but has not been explored with CRP-based polymers. Due to the discriminating nature of this ligation and rate of the tetrazine-*trans*-cyclooctene ligation, we hypothesized that homotelechelic tetrazine-polymers will enhance protein dimerization yields at significantly decreased times compared to existing dimerization methods. Herein we describe the synthesis of homodimers of T4 lysozyme through functionalization of the protein with a *trans*-cyclooctene followed by ligation to both ends of a bis-tetrazine pNIPAAm or PEG. Homodimerization yield is compared to the Michael addition of bis-maleimide pNIPAAm to mutant V131C T4 lysozyme.

Experimental Section

Materials and 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. Azobisisobutyronitrile (AIBN) was recrystallized from acetone. NIPAAm monomer was recrystallized in hexanes prior to polymerization. A furan protected maleimide alcohol (shown in Scheme 1a), was synthesized as previously described.^{18,40} Mutant V131C T4 Lys was prepared as previously described.^{41,42} Polymerization and end group modification reactions were carried out using standard Schlenk techniques under an inert atmosphere of argon. The synthesis of **1**, **3**, and **7** are described in the Supporting Information.

Analytical techniques

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, with 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. Conjugation yields were assessed by SDS-PAGE gel electrophoresis followed by ImageJ software assessment of band intensities.⁴³ Lower critical solution temperatures (LCST) were obtained in quartz cells on a Hewlett-Packard HP8453 diode array UV-Vis spectrophotometer fitted with a Peltier temperature control.

Synthesis of Telechelic Polymers

Synthesis of bis-carboxylic acid pNIPAAm (2)—*N*-Isopropylacrylamide (NIPAAm, 0.410 g, 3.62 mmol), AIBN (2.6 mg, 16 μ mol), and bis-carboxylic acid CTA (**1**) (0.040 g, 0.157 mmol) were charged into a Schlenk tube, dissolved in anhydrous tetrahydrofuran (THF) (1 mL), and subjected to four freeze-pump-thaw cycles to remove 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 (5.53–5.45 ppm) compared to the growing polymer chain N-CH (4.15–3.95 ppm). The polymerization was stopped at 77% conversion after 2.5 hours by rapidly cooling in liquid nitrogen and exposing to atmosphere. The mixture was precipitated five times from cold hexanes, and then dialyzed (MWCO 1000) against MeOH for 12 hours followed by H₂O for an additional 12 hours to remove unreacted monomer. The polymer was recovered as a yellow solid after freeze-drying. M_n was calculated by comparing the peak integrations ($^1\text{H-NMR}$) of the polymer (N-CH, 4.15–3.95 ppm) with the first monomer unit next to the trithiocarbonate (-S₂C-S-CH, 4.35–4.75 ppm). M_n ($^1\text{H-NMR}$) = 2.0 kDa and (GPC) = 1.05. $^1\text{H-NMR}$ (CDCl₃, 500 MHz) δ : 7.24–5.86 (17H, side chain NH), 4.75–4.39 (2H, -S₂C-S-CH-), 4.20–3.83 (18H, side chain N-CH), 2.60–1.33 (46H, backbone), 1.30–0.90 (106H, side chain NCH(CH₃)₂) ppm. 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 (4)—Bis-carboxylic acid pNIPAAm **2** (50 mg, 25 μ mol) was added to a flame-dried round bottom flask equipped with a magnetic stirrer and dissolved in anhydrous dichloromethane (DCM) (800 μ L) at 0 °C. (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol **3** (13 mg, 64 μ mol) 1-ethyl-N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)-HCl (21 mg, 110 μ mol) and dimethylaminopyridine (DMAP) (0.34 mg, 2.8 μ mol) were added to the solution. After 2 hours, the reaction was warmed to 23 °C and allowed to stir for an additional 24 hours. DCM 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 followed by H₂O for an additional 12 hours. The polymer was recovered as a purple solid after freeze-drying. End-group conversion was calculated by comparing the integration corresponding to the first monomer unit next to the trithiocarbonate (-S₂C-S-CH, 4.73–4.40 ppm) to the new end-group integrations including the aromatic =CH at 8.59 and 7.58 ppm, -COOCH₂ from 5.30–5.13 ppm, and -N₂-C-CH₃ at 3.10 ppm. End group conversion was 94%. $^1\text{H-NMR}$ (CDCl₃, 500 MHz) δ : 8.59 (4H, aromatic =CH), 7.58 (4H, aromatic =CH) 7.20–5.56 (19H, side chain, NH), 5.30–5.13 (4H, end group methylene, -COOCH₂) 4.73–4.40 (2H, -S₂C-S-CH), 4.17–3.90 (20H, N-CH), 2.59–1.27 (46H, backbone), 1.24–0.9 (118H, side chain NCH(CH₃)₂) ppm. IR: 3344, 2971, 2114, 1739, 1647, 1542, 1365, 1217 cm⁻¹.

Synthesis of furan protected bis-maleimide pNIPAAm—Bis-carboxylic acid pNIPAAm **1** (35 mg, 17.5 μ mol) was added to a flame-dried round bottom flask equipped with a magnetic stirrer and dissolved in anhydrous DCM (800 μ L) at 0 °C. The furan protected maleimide alcohol (15.6 mg, 70.0 μ mol), EDC-HCl (25.4 mg, 132 μ mol) and DMAP (0.22 mg, 1.8 μ mol) were added to the solution. After 2 hours, the reaction was

warmed to 23 °C and allowed to stir for an additional 24 hours. DCM 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. The product was resubjected to the same conditions a second time to increase percent end-group functionalization. End-group functionalization was determined by comparing the integrations corresponding to the first monomer unit next to the trithiocarbonate ($-S_2C-S-CH$, 4.70-4.35 ppm) to the new end-group integrations at 5.25 (OCH) and 2.85 (CONCOCH). After two subjections end-group modification was 93%. 1H -NMR ($CDCl_3$, 500 MHz) δ : 7.26-5.49 (side chain NH and vinylic CH), 5.25 (4H, -OCH), 4.70-4.35 (2H, $-S_2C-S-CH$), 4.22-3.81 (24H, -NCH), 3.81-3.40 (4H, N-CH₂), 2.85 (4H, CONCOCH), 2.65-1.26 (66H, backbone), 1.25-0.9 (130H, side chain NCH(CH₃)₂) ppm.

Retro Diels-Alder deprotection to form bis-maleimide pNIPAAm (5)

The furan protected bis-maleimide pNIPAAm was heated to 120 °C under vacuum for 3 hours. Complete deprotection was observed by 1H -NMR. Deprotection was monitored by the disappearance of the vinylic CH peak at 6.51, the -OCH peak at 5.25, and the CONCOCH peak at 2.85 ppm. The appearance of a new peak near 6.72 ppm for the deprotected maleimide (NCOCH=CH) was observed. 1H -NMR ($CDCl_3$, 400 MHz) δ : 7.39-5.39 (side chain NH and vinylic CH), 4.75-4.35 ($-S_2C-S-CH$), 4.15-3.95 (-NCH), 3.57 (N-CH₂), 2.70-1.30 (backbone), 1.25-0.9 (side chain NCH(CH₃)₂) ppm.

Synthesis of bis-tetrazine PEG (6)

2 kDa bis-carboxylic acid poly(ethylene glycol) (PEG) (135 mg, 68 μ mol) was placed in a 2-neck round bottom flask equipped with a magnetic stirrer. **3** (30 mg, 0.15 mmol), EDC-HCl (54 mg, 0.28 mmol), and DMAP (0.87 mg, 7.1 μ mol) 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 hours and allowed to warm to 23 °C. The mixture was then precipitated directly into cold diethyl ether and then dialyzed against methanol for 24 hours followed by H₂O for an additional 12 hours. The product was then freeze-dried affording a pink solid. End group conversions were calculated from the 1H -NMR spectra by comparing the integrations from 4.29-4.21 ppm (PEG-O-CH₂COO-) to the new end-group integrations at 8.59 ppm (aromatic CH), 7.58 ppm (aromatic CH), and 3.10 ppm (-N₂-C-CH₃). The final end-group conversion was 92%. 1H -NMR ($CDCl_3$, 500 MHz) δ : 8.59 (4H, aromatic CH), 7.58 (4H, aromatic CH), 5.29 (4H, CO₂CH₂-aryl), 4.29-4.21 (4H, PEG-O-CH₂COO-), 3.89-3.40 (223H, backbone), 3.10 (6H, -N₂-C-CH₃) ppm. IR: 2884, 2344, 1751, 1466, 1404, 1360, 1342, 1279, 1240, 1198, 1146, 1103, 1060, 961, 841 cm⁻¹.

Synthesis of Homodimeric Protein-Polymer Conjugates

Synthesis of *trans*-cyclooctene labeled Lysozyme (T4L-TCO)—Mutant V131C T4L (260 μ L, 0.63 mg, 34 nmol) was incubated with 1 mM dithiothreitol (DTT) in 100 mM PB at pH 6.5 in a thermoshaker at 1500 rpm, 4 °C, for 1 hour. The reduced T4L was then added dropwise to a solution of maleimide *trans*-cyclooctene (**7**) (1.69 mg, 5.8 μ mol) in 20 μ L of dimethylsulfoxide (DMSO). The solution was stirred at 23 °C for 45 minutes and then purified 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. ESI-MS m/z expected (observed): 18898 (18898) (see Figure S10).

Synthesis of (T4L-Tz)₂-pNIPAAm—Bis-tetrazine pNIPAAm (**4**) (2.1 μ g, 0.89 nmol) was mixed with an aliquot of T4L-TCO (30 μ g, 1.6 nmol) in 100 mM PB pH 6.5. The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. A time-point was taken at 1 hour and 24 hours to monitor yield by SDS-PAGE. After 1 hour homodimer was formed in 38% yield with no significant increase after 24 hours.

Synthesis of (T4L-Mal)₂-pNIPAAm—Bis-maleimide pNIPAAm (**5**) (2.0 μ g, 0.88 nmol) was mixed with an aliquot of T4L (30 μ g, 1.6 nmol) in 100 mM PB, pH 7.5, 10 mM of tris(2-carboxyethyl)phosphine (TCEP), 10 mM of ethylenediaminetetraacetic acid (EDTA). The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. A time-point was taken at 1 hour and 24 hours to monitor yield by SDS-PAGE. After 1 hour homodimer was formed in <1% yield and after 24 hours the yield increased to 5%.

Synthesis of (T4L-Tz)₂PEG—Bis-tetrazine PEG (**6**) (1.9 μ g, 0.80 nmol) was mixed with an aliquot of T4L-TCO (30 μ g, 1.6 nmol) in 100 mM PB pH 6.5. The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. A time-point was taken at 1 hour and 24 hours to monitor yield by SDS-PAGE. After 1 hour homodimer was formed in 37% yield with no significant increase after 24 hours.

Control Conjugations with Excess bis-Reactive Polymer

Synthesis of T4L-Tz-pNIPAAm-Tz—Bis-tetrazine pNIPAAm (**4**) (208 μ g, 88 nmol) was mixed with an aliquot of T4L-TCO (50 μ g, 2.6 nmol) in 100 mM PB pH 6.5. The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. The reaction was analyzed by SDS-PAGE, and primarily monoconjugate was formed at 56% yield.

Synthesis of T4L-Mal-pNIPAAm-Mal—Bis-maleimide pNIPAAm (**5**) (106 μ g, 45 nmol) was mixed with an aliquot of T4L (15 μ g, 0.81 nmol) in 100 mM PB, pH 7.5, 10 mM TCEP, 10 mM EDTA. The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. The reaction was analyzed by SDS-PAGE, and primarily monoconjugate was formed at 81% yield.

Synthesis of T4L-Tz-PEG-Tz—Bis-tetrazine PEG (**6**) (191 μ g, 81 nmol) was mixed with an aliquot of T4L-TCO (50 μ g, 2.6 nmol) in 100 mM PB pH 6.5. The reaction mixture was brought to a final protein concentration of 1.60 mg/mL and mixed on a thermoshaker at 1500 rpm at 4 °C for 24 hours. The reaction was analyzed by SDS-PAGE, and primarily monoconjugate was formed at 56% yield.

LCST Determination

A solution of pNIPAAm, T4L or (T4L-Tz)₂-pNIPAAm was diluted with DPBS (pH 7.4) to 0.2 mg/mL in a quartz cuvette. The cuvette was placed in a UV-Vis spectrophotometer with Peltier temperature control. The temperature of the system was elevated at a rate of 0.5 °C per minute. The temperature was held for 30 seconds prior to measuring absorbance. Absorbance at 600 nm was measured every 0.5 °C. LCST was determined at 10% of the maximum absorbance.

Results and Discussion

Synthesis of Telechelic Polymers

In order to prepare the required telechelic polymers by CRP we polymerized NIPAAm utilizing a bis-carboxylic acid CTA (**1**) followed by post-polymerization modification with a tetrazine alcohol (**3**, Scheme 1a). RAFT polymerization of NIPAAm is well-documented,^{44,45} resulting in well-defined polymers, as well as high yielding protein modifications and was therefore chosen as a model system for CRP-based homodimerization studies. Polymerization of NIPAAm with AIBN and CTA **1** at 70 °C over 2.5 hours gave bis-carboxylic acid pNIPAAm **2** with an M_n of 2.0 kDa and low dispersity (1.05). Post-polymerization modification via carbodiimide coupling with (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (**3**) led to bis-tetrazine pNIPAAm **4** with 94% modification of the chain ends. An alternative route to bis-tetrazine pNIPAAm **4** where CTA **1** was first functionalized with tetrazine was also attempted, but RAFT polymerization conditions were found to be incompatible with the reactive tetrazine groups. Reaction between the tetrazine and *N*-Isopropylacrylamide was observed by the emergence of new peaks in the aromatic and aliphatic regions of the ¹H-NMR at 70 °C, the temperature used to initiate AIBN in RAFT (data not shown). At 25 °C, no reaction was observed by ¹H-NMR, so it may be possible to polymerize directly from the tetrazine functionalized CTA using an initiator that decomposes at a lower temperature.

To compare the tetrazine ligation to typical Michael addition reactions with maleimide-functionalized CRP polymers, the same bis-carboxylic acid pNIPAAm **2** was again modified via carbodiimide coupling, this time employing a previously reported furan-protected maleimide propanol^{18,40} (Scheme 1a). Two subsections to the same coupling conditions yielded the furan-protected bis-maleimide pNIPAAm in 93% yield. Complete deprotection upon heating to 120 °C for 3 hours gave bis-maleimide pNIPAAm **5**.

Additionally, the tetrazine ligations of pNIPAAm polymers, which are more bulky, were compared to that of linear PEG polymers. Thus, 2 kDa PEG bis-carboxylic acid was modified with tetrazine alcohol **3** via carbodiimide coupling, giving bis-tetrazine PEG **6** in 92% yield (Scheme 1b). The ¹H-NMR spectra for all three polymers utilized in protein conjugations is shown in Figure 1. ¹H-NMR analysis of the purified homotelechelic polymers confirmed the bis-functionalization of the tetrazine polymers and deprotection of the furan-protected bis-maleimide pNIPAAm.

Synthesis of Homodimeric Protein-Polymer Conjugates

Site-specific modification is imperative to synthesize pure homodimer conjugates. Therefore a mutant T4 Lysozyme, containing a single surface-exposed free cysteine (cysteine 131) was expressed using a vector provided by Prof. Wayne Hubbell (UCLA)^{41,42} resulting in pure T4L V131C (Figure 2, lane 2). Complete modification of this cysteine through Michael addition to the small-molecule maleimide *trans*-cyclooctene **7** (Scheme 2, a) was observed after incubation for one hour at 4 °C by ESI-MS (Figure S10).

Homodimerizations of T4L-TCO with bis-tetrazine pNIPAAm **4** or PEG **6** (Scheme 2b) were carried out at 4 °C in 100 mM PB, pH 6.5 whereas bis-maleimide pNIPAAm **5** was incubated with T4L in 100 mM PB, pH 7.5 with 10 mM TCEP and 10 mM EDTA to prevent disulfide formation and oxidation of the cysteine (Scheme 2c). Conjugations were run at the same final protein concentration, each using 2 equivalents of protein to polymer. The temperature was maintained at 4 °C to ensure solubility of the temperature-responsive pNIPAAm. Homodimer was formed in 37% and 38% respectively, for both the bis-tetrazine pNIPAAm **4** and bis-tetrazine PEG **6** after one hour (Figure 2, lane 3 and 4). Michael addition to bis-maleimide pNIPAAm **5**, however, gave less than 1% homodimer after one hour (Figure 2, lane 5) and only 5% after 24 hours (Figure 2, lane 8). These results indicate that the end group reactivity, not the polymer structure dictates protein dimer yields.

pNIPAAm is known to exhibit a lower critical solution temperature (LCST) near 32 °C.⁴⁶ To study differences in LCST of the pNIPAAm tether and the T4L-pNIPAAm-T4L dimer, UV-Vis turbidity studies were performed. At 0.2 mg/mL in DPBS pH 7.4 the polymer exhibited a sharp transition with a LCST at 32.8 °C and as expected, T4L did not display any LCST up to 60 °C. Upon dimerization of T4L-TCO with Tz-pNIPAAm-Tz, the transition was broadened and LCST increased to 39.5 °C (Figure 3). This was expected as previous reports have demonstrated an increase in the LCST of pNIPAAm when conjugated to proteins.⁴⁷ The results also confirmed that the polymer was conjugated to the T4L.

We have previously shown maleimide functionalized pNIPAAm to yield homodimers up to 21%,^{2,4} but the polymers used for those conjugations were much longer (15.5 kDa). The shorter tether may be the factor that drastically reduced overall yield of Michael addition dimer in this case. The yield is similar to what has been reported with homodimerization of proteins using small molecule bis maleimide linkers.²⁷ Thus, it could be a result of charge as reported for the small molecule linker or steric repulsion. Since T4L forms a natural dimer quite readily, it is more likely steric repulsion. In the case of the slower Michael addition chemistry, there was quite a bit of untethered protein dimer formed, which may be a result of the reaction time (i.e. the dimerization of the protein was competitive with dimerization with the polymer via the tether). Further, the small molecule Mal-TCO **7** extends the reactive moiety out from the protein, potentially facilitating tetrazine conjugation compared to maleimide conjugation to the native cysteine. To investigate this last possibility further, reactions were run with 30–50 equivalents of polymer to protein in order to target monoconjugates. For each conjugation, moderate to high yields of monoconjugate were formed after 24 hours (Figure 4). Specifically, the tetrazine ligations with pNIPAAm and PEG were both 56% yield, while the maleimide conjugation yield was 81%. This result

demonstrates that maleimide functionalized pNIPAAm does react with the T4L to form monoconjugate in significantly greater yield than does the tetrazine polymer, and that access to the reactive site on the protein is not likely an issue.

Higher polymer concentrations may have facilitated the high yields of monoconjugate, and tens to hundreds of equivalents of polymer are commonly used for monoconjugations. However, homodimerization requires exact equivalents or excess protein and as a result, lower polymer concentrations. Thus, the faster, more robust tetrazine ligation is ideal for higher yielding homodimerizations. The length of polymer tethers is often imperative to protein activity, and access to shorter linker lengths are often required.^{4,49} Therefore, the tetrazine ligation is a better choice for polymers prepared by CRP especially when the polymer length is short. However, we expect that the tetrazine ligation will result in even higher yields with longer polymers.

Interestingly, lysozyme had significantly reduced activity after dimerization with pNIPAAm, both below and at the LCST (see Supporting Information). While modification with the cyclooctene moiety alone did not affect the activity, dimerization of the protein with pNIPAAm resulted in a 75% loss in activity. We have previously shown that the conjugation of pNIPAAm chains to T4L at this conjugation site does not reduce activity.⁵⁰ Thus we speculate that the presence of the second protein conjugated by a short tether is the cause of the activity loss, particularly because the substrate for the enzyme is large. The effect could be lower or non-existent for proteins with smaller substrates. In addition, a reversible linkage within the polymer could allow for lysozyme activity to be turned on by an outside trigger.

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 free cysteine. Dimerization of the polymers was conducted and yields were assessed after 1 hour. Higher yields with both bis-tetrazine pNIPAAm (38%) and bis-tetrazine-PEG dimerization (37%) were observed compared to less than 1% with bis-maleimide pNIPAAm. 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 Michael addition syntheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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expression vector for T4L(V131C) as well as Dr. Julian Whitelegge (David Geffen School of Medicine, UCLA) for ESI-MS analysis of the modified protein.

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Figure 1.

Final ^1H -NMR of homotelechelic polymers a) bis-tetrazine pNIPAAm **4** b) bis-tetrazine PEG **6** and c) bis-maleimide pNIPAAm **5** (all in CDCl_3 , 500 MHz). See the supporting information for peak assignments.

Figure 2. SDS-PAGE of T4L homodimerization using 2 eq. protein to polymer incubated for 1 hour (Lanes 3–5) or 24 hours (Lanes 6–8). Lane 1: protein ladder; Lane 2: T4L; Lane 3 and 6: 2:1 T4L-TCO:Tz-pNIPAAm-Tz; Lane 4 and 7: 2:1 T4L-TCO:Tz-PEG-Tz; Lane 5 and 8: 2:1 T4L:Mal-pNIPAAm-Mal.

Figure 3.
UV-Vis turbidity experiments of pNIPAAm, T4L-pNIPAAm-T4L and T4L.

Figure 4. SDS-PAGE of monoconjugations after 24 hour incubation. Lane 1: protein ladder; Lane 2: 1:30 T4L-TCO:Tz-pNIPAAm-Tz; Lane 3: 1:30 T4L-TCO:Tz-PEG-Tz; Lane 4: 1:50 T4L:Mal-pNIPAAm-Mal.

Scheme 1.

Synthesis of homotelechelic polymers a) bis-tetrazine and bis-maleimide pNIPAAm (**4** and **5**) with 94% and 93% end group conversion, respectively and b) bis-tetrazine PEG **6** with 92% end group conversion. Note that step 1 of the bis-maleimide reaction was undertaken twice to increase the end group conversion.

Scheme 2.

Homodimerization of T4L with homotelechelic polymers a) Modification with TCO b) tetrazine ligation with **4** or **6** c) Michael addition with **5**. PDB 2HUK (cysteine 131 represented in yellow in space-filling mode)