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ARTICLE

Synthesis of Maleimide-End-Functionalized Star Polymers and Multimeric Protein−Polymer Conjugates

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ABSTRACT: Protein−polymer conjugates exhibit superior properties to unmodified proteins, generating a high demand for these materials in the fields of medicine, biotechnology, and nanotechnology. Multimeric conjugates are predicted to surpass the activity of monomeric conjugates. Herein, we report a straightforward method to synthesize multimeric polymer conjugates. Four-armed poly(*N*-isopropylacrylamide) (pNIPAAm) was synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization in the presence of a tetrafunctionalized trithiocarbonate chain transfer agent (CTA). The polymer molecular weight, architecture, and polydispersity index (PDI) were verified by gel permeation chromatography (GPC), dynamic light scattering gel permeation chromatography (DLS-GPC), and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. This approach afforded well-defined polymers (PDI's < 1.06) and the ability to target various molecular weights. Maleimide functional groups were introduced at the chain ends by heating the polymers in the presence of a furan-protected azo-initiator. This allowed for site-specific conjugation of V131C T4 lysozyme to the polymers to generate multimeric protein−polymer conjugates. MALDI-TOF mass spectrometry, electrospray ionization gas-phase electrophoretic mobility macromolecule analysis (ESI-GEMMA), gel electrophoresis, and liquid chromatography tandem mass spectrometry (LC-MS/MS) of the trypsin digests demonstrated that multimeric protein−polymer conjugates had formed. This simple strategy provides ready access to star protein−polymer conjugates for application in the fields of drug discovery, drug delivery, and nanotechnology.

Introduction

Recent advances in controlled radical polymerization (CRP) techniques have provided access to polymeric architectures containing a single branch point and three or more chains, called star polymers. Traditionally, these were synthesized by living anionic polymerization. CRPs are tolerant to a larger variety of monomers than anionic polymerization, thus allowing for a broader range of functional materials to be synthesized.[1−6] The most widely used techniques include nitroxide-mediated polymerization (NMP),[3,7] atom transfer radical polymerization (ATRP),[8−10] and reversible addition−fragmentation chain transfer (RAFT) polymerization.[5,11−21] Two distinct approaches for the synthesis of star polymers are (1) grafting of linear polymers to a core and (2) growth of the polymers from a core. The grafting approach can suffer from variable conjugation efficiencies of the arms to the core due to steric crowding.[5] Growth of the arms from a functionalized core is advantageous because it allows for precise control over the chain length and number of arms.[2−4,12]

Star polymers made by CRP techniques contain end groups that allow for additional modification.[2−4,12] High retention of the end group is essential for generating a variety of well-defined polymeric architectures. For example, Gao et al. demonstrated polymerization of styrene from a trifunctional ATRP initiator to generate well-defined, three-armed polystyrene (PS) with high retention of bromine on the arm termini postpolymerization. The bromines were displaced with azido groups and efficiently clicked to alkyne-functionalized poly(ethylene oxide) (PEG) to generate well-defined, PEG-*b*-PS three-armed stars.[22] Hao et al. utilized a triol core, first-generation dendrimer, and second-generation dendrimer to generate 3-, 6-, and 12-armed PS and poly(butyl acrylate) (pBA) by RAFT polymerization. The resulting polymers exhibited high retention of trithiocarbonates at the chain ends, thus allowing for multiarmed block copolymer stars of PS-*b*-pBA and pBA-*b*-PS to be synthesized from the macroinitiators.[23] In another example, Chen et al. synthesized a tetrafunctional chain transfer agent (CTA) to mediate the polymerization of styrene by RAFT, followed by polymerization of 2-(dimethylamino)ethyl methacrylate (DMAEMA), resulting in well-defined PS-*b*-PDMAEMA four-armed star polymers. These block copolymers self-assembled into spherical micelles and were utilized for the controlled release of chlorambucil, a chemotherapeutic.[24]

As demonstrated in the above examples, star polymers can provide access to a variety of architectures with interesting properties. Herein, we sought to utilize star polymers to generate scaffolds to produce multimeric protein conjugates. Protein−polymer conjugates are fundamentally interesting materials with far-reaching applications as therapeutics,[25] nanoassemblies,[26] and smart materials.[27] After linking the polymer to the protein, the properties of the biomolecule are altered advantageously. Because polymers with narrow molecular weight distributions are superior for many applications, CRPs[3,9,28−31] have emerged as excellent methods to produce biomolecule conjugates.[32,33] Polymers with protein-reactive end groups have been synthesized by both ATRP and RAFT polymerization.[34−42] Conjugates

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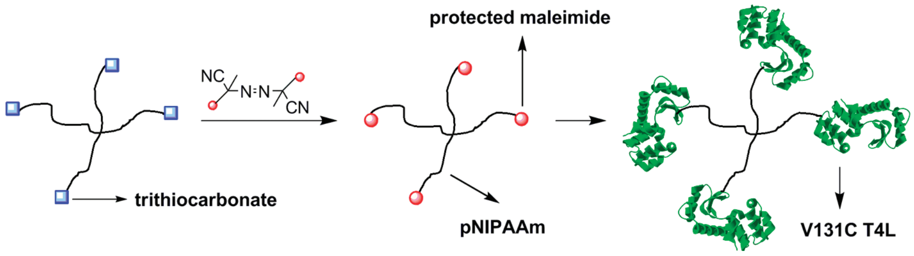
Scheme 1. Synthesis of Protein−Star Polymer Conjugates



have also been prepared by polymerizing directly from the proteins.[43−49] All of these examples consist of one protein attached to one or more polymer chains. Multimeric protein conjugates, where more than one protein is attached to a single polymer chain, can have superior properties for many applications. Enzymes often oligomerize in the active state in which homotetramers are highly prevalent.[50] Ion channels often consist of multiple proteins acting in concert. Antibodies are oligomers consisting of either 2, 4, 6, or 10 epitopes.[51] Thus, fixation of multiple proteins to a polymer scaffold can result in a conjugate that exhibits higher activities than the monomeric conjugate.[52] We disclose in this report for the first time a straightforward CRP strategy to access star protein−polymer conjugates.

We recently reported a strategy to access homodimeric conjugates.[53] We have also disclosed heterodimeric conjugates by CRP.[54] The key step involves exchange of the macroCTA chain ends with a protein-reactive azo-initiator. This radical coupling method was first disclosed to remove thiocarbonylthio moieties by Perrier.[55] Specifically in this report, we describe the synthesis of a star polymer by RAFT polymerization and the installment of maleimides at the chain ends (Scheme 1). Maleimides react with free cysteines, providing a means for site-specific functionalization of proteins.[56] Indeed, successful conjugation to a model protein containing a free cysteine, V131C T4 lysozyme (T4L), was performed, resulting in star protein−polymer conjugates (Scheme 1). Details of the synthesis, characterization of the polymers, and mass spectrometry analysis of the conjugates are discussed below.

Experimental Section

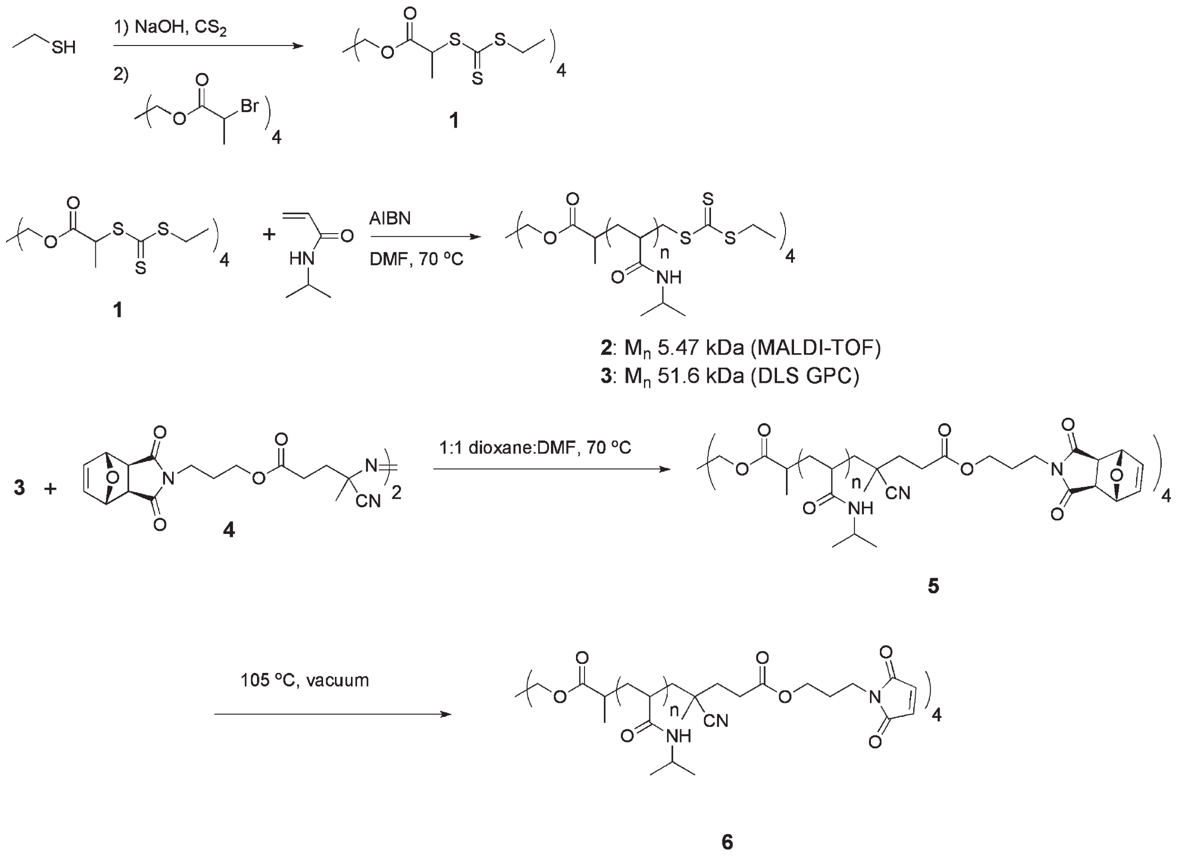
Materials. 2,2′-Azobis(isobutyronitrile) (AIBN) (Aldrich, 98%) was recrystallized twice from acetone. *N*-Isopropylacrylamide (NIPAAm, Acros, 99%) was recrystallized twice from hexane. The V131C T4-lysozyme (T4L) mutant expression vector was kindly provided by Professor Wayne Hubbell (UCLA), and the protein was expressed from *E. coli* and purified as previously described.[46,57,58] Pentaerythritol tetrakis-(2-bromopropionate)[59] and the furan-protected maleimide azo-initiator **4**[54] were synthesized as previously described. All other solvents were obtained at the highest purity available from Aldrich Chemical Co. and used without further purification.

Methods. Polymerization and radical coupling reactions were carried out using standard Schlenk techniques under an inert atmosphere of argon. TLC was performed using precoated silica gel 60 F254 and developed in the solvent system indicated. Compounds were visualized by use of 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 with the EZStart 7.2 chromatography software. 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 internal standard. The number-average molecular weight (M_n) by dynamic light scattering (DLS) was collected from a Viscotek GPCmax VE2001 GPC solvent/sample module with 270 dual detector and VE 3580 RI detector. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 4−20% Tris-Glycine gels (Invitrogen, 1.0 mm × 12 well). Matrix-assisted laser desorption/ionization time-of-flight (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 *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DTCB) matrix (40 mg/mL in THF) to potassium trifluoroacetate (5 mg/mL in THF) to polymer (1 mg/mL in THF). Infrared absorption spectra were recorded on a PerkinElmer FT-IR instrument equipped with an ATR accessory.

Synthesis of Tetrameric CTA (1). A solution of ethanethiol (4.0 mL, 54.0 mol) in 8.0 mL of water was kept at 0 °C for 10 min. A 50% NaOH aqueous solution (4.32 g, containing 2.16 g, 54.0 mol of NaOH) was then added followed by 3.0 mL of acetone. The clear colorless solution was stirred for 30 min and treated with carbon disulfide (4.45 g, 59.0 mol) to give a bright orange solution. The system was stirred at 23 °C for 30 min and cooled down to 0 °C. Then 20 mL of dichloromethane containing pentaerythritol tetrakis(2-bromopropionate) (6.0 g, 8.9 mmol) was added slowly, and the mixture was stirred at 23 °C for 48 h. After filtration to remove the suspended solid, the solvent was distilled under vacuum. The remaining crude was purified by column chromatography on silica gel (diethyl ether: hexane = 1:4) to give the product as a yellow oil (2.7 g, yield: 33.6%). ^{1}H NMR (400.13 MHz, $CDCl_3$)/ppm: 4.82 (q, $J = 7.5$ Hz, 4H, CHS), 4.11−4.00 (m, 8H, CCH_2), 3.41−3.31 (m, 8H, SCH_2), 1.58 (d, $J = 7.5$ Hz, 12H, $CHCH_3$), 1.35 (t, $J = 7.6$ Hz, 12H, CH_2CH_3). ^{13}C NMR (100.61 MHz, $CDCl_3$)/ppm: 221.76 (C=S), 170.75 (C=O), 62.92 (CCH_2), 47.40 ($CHCH_3$), 42.41 (CCH_2), 31.88 (SCH_2), 16.63 ($CHCH_3$), 12.75 (CH_2CH_3). IR (cm^{-1}): 2929, 1736, 1448, 1374, 1147, 1028, 807. MALDI-TOF MS: M+Na$^+$ expected (observed): 926.96 (926.98).

Preparation of Four-Armed Poly(NIPAAm) (3). NIPAAm (2.22 g, 20.0 mmol), **1** (22.6 mg, 2.5×10^{-2} mmol), and AIBN (0.82 mg, 5.0×10^{-3} mmol) were dissolved in 5 mL of DMF; the Schlenk tube was subjected to three freeze−pump−thaw cycles and placed into a 70 °C oil bath. Samples were removed periodically using a degassed syringe for conversion analysis by ^{1}H NMR. After the conversion reached 52%, the polymerization was halted by cooling the reaction mixture in an ice−water bath. Most of the DMF was removed under high vacuum at 40 °C, and the polymer was purified by dialysis against methanol (MWCO: 6−8000). ^{1}H NMR (400.13 MHz, $CDCl_3$)/ppm: 7.10−5.62 (main chain, NH), 3.99 (main chain, CH), 3.34 (chain end, SCH_2), 2.07 (main chain, CH), 1.83−1.60 (main chain, CH_2), 1.12 (main chain, $CH(CH_3)_2$). Using the M_n obtained by DLS and the calculated molar extinction coefficient

Scheme 2. Synthesis of Four-Armed Maleimide Poly(NIPAAm)



of 54 804 M^{-1} cm^{-1} the percent of chain-end trithiocarbonate was calculated as 95% by UV–vis analysis in 1,2-dichloroethane (see Supporting Information (SI) for details).

Aminolysis of Core. Four-armed poly(NIPAAm) (**2**) (15 mg, 3.00×10^{-4} mmol) was dissolved in 5 mL of methanol, 0.10 mL of butylamine was added, and the solution was refluxed for 20 h. The volatile organics were removed by vacuum distillation. The residue was redissolved in DMF and employed for GPC analysis directly.

Radical Coupling of Polymer with Functionalized Azo-Initiator (4). Four-armed poly(NIPAAm) (**3**) (50 mg, $M_n \sim 50\,000$, 10^{-3} mmol) and **4** (69 mg, 0.10 mmol) were dissolved in 2 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, and the crude was dissolved in 10 mL of methanol. The insoluble portion was removed by centrifugation. The methanol solution was then dialyzed against ethyl acetate/methanol (1:1, MWCO: 6–8000) for 72 h to give the clean furan-protected maleimide functionalized four-armed polyNIPAAm (**5**). ^{1}H NMR (400.13 MHz, $CDCl_3$)/ppm: 7.10–5.66 (main chain, NH), 6.48 (chain end, CH=CH), 5.23 (chain end, CHOCH), 3.97 (main chain, CH), 3.54 (chain end, NCH_2), 2.82 (chain end, CHOCH) CHC=O), 2.07 (main chain, CH), 1.79–1.59 (main chain, CH_2), 1.10 (main chain, $CH(CH_3)_2$).

Retro-Diels–Alder Reaction (6). The four-armed poly(NIPAAm) with furan-protected maleimide end group (**5**) was heated at 105 °C under vacuum for 2 h to give four-armed poly(NIPAAm) terminated with maleimide (**6**). ^{1}H NMR (400.13 MHz, $CDCl_3$)/ppm: 6.96–5.66 (main chain, NH), 6.72 (chain end, CH=CH), 4.00 (main chain, CH), 3.63–3.49 (chain end, NCH_2), 2.07 (main chain, CH), 1.81–1.60 (main chain, CH_2), 1.13 (main chain, $CH(CH_3)_2$).

Conjugation of T4L with 6. A typical conjugation was carried out as follows. Star polymer **6** (0.33 mg, 4.3 mg/mL) in phosphate buffered saline (PBS), pH 7.5, with 10 mM of ethylenediamine tetraacetic acid (EDTA) and 10 mM of tris(2-carboxyethyl) phosphine (TCEP) was added to a T4L solution (1.69 mg/mL) in Tris-buffer at pH 7.4. The mixture was maintained at 4 °C with gentle stirring for 16 h. After concentration by centrifugal filtration (Amicon Ultra, MWCO 10 000) the solution was used directly for SDS PAGE analysis.

Electrospray Ionization–Gas-Phase Electrophoretic Mobility Macromolecule Analysis (ESI-GEMMA). Samples were prepared 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 sample buffer exchange. The ESI-GEMMA device[60] (TSI Inc.) consists of an electrospray aerosol generator 3480, electrostatic classifier 3080 with Nano-DMA (differential mobility analyzer) 3085, and ultrafine condensation particle counter 3025A. The DMA separates the particles based on their electrophoretic mobility in air. Sample solutions were introduced in the ESI source through a fused silica capillary tube at a flow rate of 70 nL/min and voltage of 2–3 kV. To promote particle charge reduction, the gases present inside the ESI neutralizing chamber were ionized by a ^{210}Po α-radiation source. The DMA sheath flow was set to 20 L/min, and the voltage was scanned −10 to −10 000 V for 120 s to sample the electrophoretic mobility diameter (EMD) range of 2–56 nm. The DMA voltage was scanned, and data were recorded by Aerosol Instrument Manager Software (TSI Inc.). PeakFit Version 4.12 (SeaSolve Software Inc.) was used to centroid EMD peaks of interest. Data points were smoothed using Igor Pro 4.0.8.0 (Wavemetrics, Inc.). Molecular weights were calculated using the formula for density and the volume of a sphere: $MW = VdN_a$, where d is the effective density, N_a is Avogadro's number, and the volume $V = (EMD)^3\pi/6$.

Results and Discussion

The four-armed CTA (**1**) was synthesized by displacement of the bromine on pentaerythritol tetrakis(2-bromopropionate) with the trithiocarbonate anion (Scheme 2) in 34% yield.

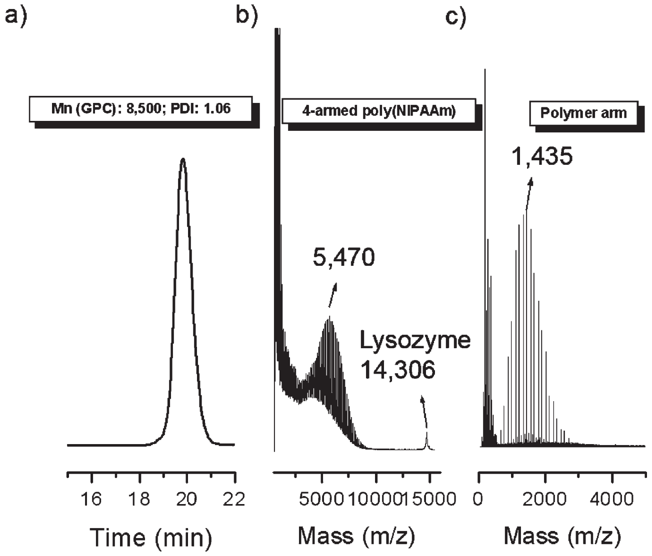


Figure 1. GPC and mass spectra of four-armed poly(NIPAAm) (**2**). GPC trace of the four-armed poly(NIPAAm) (left); MALDI-TOF spectra before (middle) and after (right) aminolysis.

A polyNIPAAm star polymer was then prepared by RAFT polymerization using **1**. Initially, a low molecular weight polymer (**2**) was targeted in order to be able to analyze the efficiency of the reaction by mass spectrometry. Ratios of [NIPAAm]:[**1**]:[AIBN] of 100:1:0.2 were used. To avoid unwanted cross-linking reactions, the polymerization was stopped at 45%. The number-average molecular weight (M_n) by GPC (trace shown as Figure 1a) was 8500 Da, and the polydispersity index (PDI) was 1.06, demonstrating that a well-defined star had been synthesized. To further characterize the star, polymer **2** was analyzed by MALDI-TOF mass spectrometry. This gave a MW of 5470 Da (Figure 1b), which was close to the theoretical target (6000 Da). Aminolysis with butylamine was utilized to cleave the arms from the core to confirm that the polymer contained four arms of similar molecular weights. The mass of the cleaved arms was 1435 Da (Figure 1c). Because of the heterogeneity intrinsic to a polymer sample, this result was considered within the uncertainty of the expected value (1351 Da) and suggested that the desired four-arm star had been synthesized.

These data indicated that the designed tetrafunctional CTA was facilitating homogeneous growth of the arms; thus, a larger molecular weight polymer was next pursued. This polymer was synthesized with [NIPAAm]:[**1**]:[AIBN] ratios of 800:1:0.2 to 52% conversion and purified by dialysis to give **3**. DLS GPC indicated a M_n of 51 600 Da and a PDI of 1.05. ^{1}H NMR indicated a M_n of 50 000 Da. Both were close to the targeted molecular weight of 48 900 Da. Hydrolysis of **3** produced linear polymers with a M_n of 14 000 Da by DLS GPC. This was slightly larger than the expected mass (12 900 Da), and the PDI broadened to some extent to 1.09 (Figure 2). This result suggested that the star contained 3.7 arms, indicating that the larger polymer was a mixture of three- and four-armed stars.

Fortunately, the end groups of the star polymer were available for further elaboration; UV−vis analysis of the star polymer (see Supporting Information) demonstrated retention of the trithiocarbonate CTA end group of ~95%. After heating in the presence of functionalized azoinitiator **4** and performing the retro-Diels−Alder reaction, the desired star polymer with a maleimide chain end was obtained as observed by the maleimide peak at 6.8 ppm (a, Figure 3c) and the disappearance of the protected maleimide peaks (a, c, and e, Figure 3b). Analysis by GPC indicated that postpolymerization (Figure 4a), after radical coupling (Figure 4b), and after maleimide deprotection (Figure 4c) the polymer remained well-defined (PDI = 1.05−1.06).

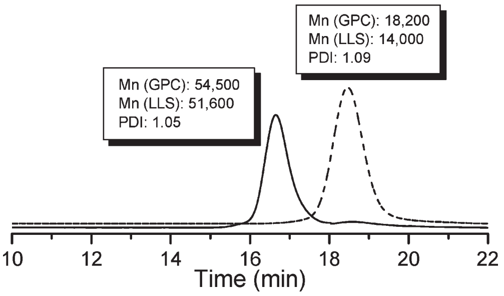


Figure 2. GPC traces of four-armed poly(NIPAAm) and polymer arm after aminolysis.

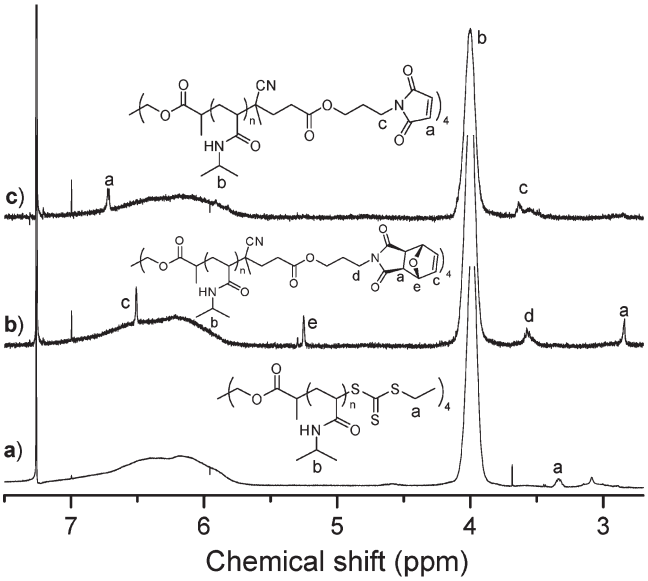


Figure 3. ^{1}H NMR (CDCl$_3$) spectra of (a) four-armed polyNIPAAm **3**, (b) four-armed furan-protected polyNIPAAm **5**, and (c) four-armed maleimide polyNIPAAm **6**.

Immobilization of V131C T4L on **6** was pursued at 4 °C. The conjugation was investigated using different ratios of protein to polymer (1:2, 2:1, and 8:1). In all three systems a shift to higher molecular weight was observed by SDS-PAGE (Figure 5, lanes C, D, and E, respectively). Importantly, when the maleimides were protected and not available for conjugation, the observed band was exactly the same as for unmodified T4L (Figure 5, lanes B and A, respectively). LC-MS/MS of the trypsin digests of some of the fractions verified that these bands contained T4L and were likely the polymer conjugate (see Supporting Information for details, Table S1). A ratio of T4L to polymer of 8 to 1 gave the highest molecular weight band in the gel. Although gels of polymer conjugates cannot be expected to provide accurate molecular weight values when compared to the protein standards, the mass determined by comparison to protein markers of known molecular weight was reasonably close to the expected molecular weight of a tetramer at ~124 kDa. In addition, the band was broad, suggesting that a mixture of conjugates was obtained.

MALDI-TOF mass spectrometry failed to reveal the molecular mass of the conjugates, possibly because of the large size of the expected macromolecule. Thus, to determine the actual number of proteins conjugated to the polymer, characterization by ESI-GEMMA was pursued. First, the conjugate was prepared using the optimized conjugation conditions and purified by Sephadex column (G-100). The SDS PAGE of the product (Figure 6) displayed a higher molecular weight band shown in lane C; once

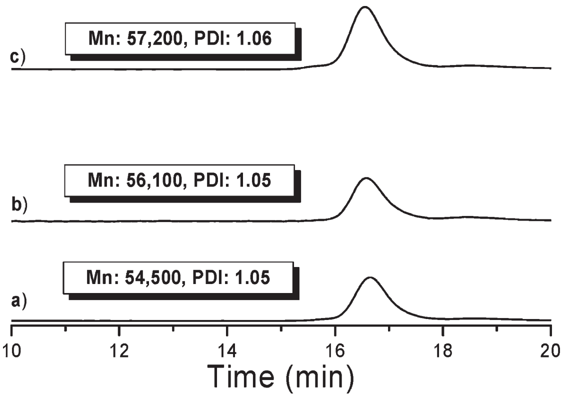


Figure 4. GPC traces of (a) four-armed polyNIPAAm **3**, (b) four-armed furan-protected polyNIPAAm **5**, and (c) four-armed maleimide polyNIPAAm **6**.

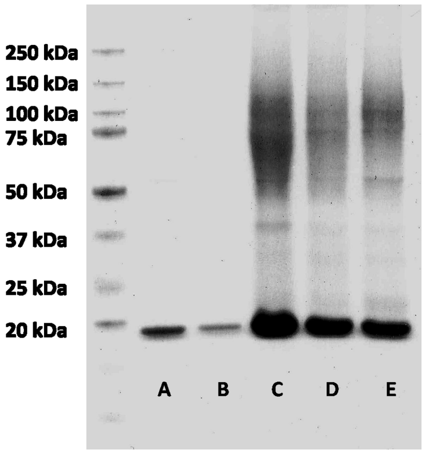


Figure 5. SDS-page analysis: (A) native T4L; (B) T4L+furan protected polymer **5**; (C) T4L + maleimide poymer **6** 1:2; (D) T4L/**6** 2:1; (E) T4L/**6** 8:1.

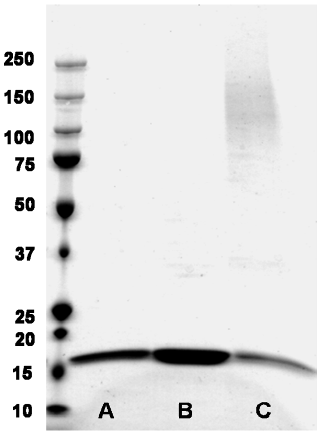


Figure 6. SDS-PAGE analysis: (A) native T4L; (B) T4L + **5**; (C) T4L + **6** (ratio of T4L/**6** = 1:8).

Table 1. Calculated Molecular Weights of Protein−Polymer Conjugate (Four-Armed Polymer) Determined from ESI-GEMMA Data

EMD (nm)	apparent MW (Da, $d = 0.67$ g/cm^3)[a,b,c]	assignment[d]
3.9		P (hydrolyzed)
6.1		P_4
8.0	11×10^4	$P_4 + 3$ L
10.1	21×10^4	$2\,(P_4 + 3$ L)

[a] Based on the standard deviation of the effective density; a mass accuracy of ±10% is estimated. [b] Molecular weight was calculated using the formula MW = VdN_a where d is the effective density, N_a is Avogadro's number, and the volume, $V = (\text{EMD})^3\pi/6$. [c] Density of the polymer−protein conjugate is based on the predicted estimate that a polymer with three lysozymes would be 53% lysozyme and 47% polymer. Density of protein and protein complexes is from ref 60. Density of the polymer is based on mass estimated from NMR of 50 000 and the apparent EMD using the formula d = MW/V. [d] Abbreviations are defined as follows: L is T4 lysozyme; P (hydrolyzed) is the four-armed polymer after hydrolysis; P_4 is the four-armed polymer; P_4 + 3L is the trimer-linked four-armed polymer; 2 (P_4 + 3L) is the dimer of the trimer-linked four-armed polymer.

again the furan protected polymer did not react, and only T4L was observed as shown in lane B. This conjugate, the star polymer, and the star polymer posthydrolysis (arms) were analyzed by ESI-GEMMA. Table 1 lists the EMD of the peaks identified in the ESI-GEMMA spectra (provided as Figure S3) of the protein−polymer conjugate sample.

The EMD of the star polymer and star polymer posthydrolysis was 6.0 and 3.8 nm, respectively (data not shown). Given the structure of the four-armed polymer, the density of the star polymer was expected to exhibit a different density than that of a linear polymer. Based on the EMD and the mass estimated by NMR (~50 000 Da), the density (d = MW/V) of the star polymer was calculated to be 0.73 g/cm^3.[60,61] Peaks consistent with the size of the star polymer (6.1 nm) and star polymer posthydrolysis (3.9 nm) were identified (Figure S3). The 8.0 and 10 nm peaks were assigned to the protein−polymer conjugate. Based on ESI-GEMMA measurements, the average effective density of proteins and protein complexes is 0.58 g/cm^3.[60] The density of the polymer−protein conjugate was calculated to be 0.67 g/cm^3 based on the prediction that a polymer with three lysozymes would be 53% lysozyme and 47% polymer by weight. The EMD and calculated molecular weights for the protein−polymer conjugate of three lysozymes (8.0 nm, 11 × 10^4 Da) were consistent with what was observed by SDS-PAGE. A dimer of this protein−polymer conjugate was also observed (10 nm, 21 × 10^4 Da).

Using ESI-GEMMA, it was determined that an average of three proteins was attached to each star polymer. From the GPC data of the cleaved arms, the star was likely a mixture of three and four arms. Hydrolysis of the maleimide over the course of the conjugation reaction or steric crowding around the star polymer core could be additional factors as to why tetrameric conjugates were not observed. For the former, use of a more stable Michael acceptor, such as a vinyl sulfone, may prove to be advantageous. For the latter, stars with longer polymer arms could be explored.

Yet, these data do demonstrate that multimeric protein−polymer conjugates are readily accessible by this technique. Site-selective binding of proteins to polymers is important for bioactivity, and maleimides provide this. In addition, a wide variety of end groups can be envisioned as a number of azo-initiators could be synthesized. Moreover, this approach should be amenable to the range of architectures and polymer identities accessible by RAFT polymerization. For many biological applications, tethering multiple proteins together onto a single polymer chain is expected to provide constructs with superior activities than simple protein−polymer conjugates. This approach immediately provides access to trimer conjugates. It also offers a synthetic strategy to target different valency constructs, depending on the desired application.

Conclusions

A multimeric star poly(NIPAAm)−lysozyme conjugate was successfully synthesized utilizing a combination of RAFT, radical coupling with a designed initiator, and site-specific bioconjugation. Different molecular weights were readily accessed. Mass spectrometry analysis of the star and cleaved arms indicated that the CTA was efficient and that the desired four-armed star polymers had been prepared when the molecular weight was low. With a larger molecular weight star, an average of 3.7-armed stars was prepared. ^{1}H NMR analysis and GPC indicated that the maleimide was readily incorporated utilizing a radical reaction with an azo-initiator, followed by heating. Using SDS PAGE the reaction conditions, specifically the optimal ratios for conjugating protein to polymer, were explored. A conjugate prepared using an 8 to 1 ratio of T4L to polymer was further analyzed by ESI-GEMMA. This measurement revealed that three proteins were attached to each conjugate. We anticipate that this method to produce site-selective multimeric conjugates will be generally useful for preparing materials for applications in nanotechnology and medicine.

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Supporting Information Available: UV−vis analysis, LC-MS/MS data, and ESI-GEMMA spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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