Macromolecules
ARTICLE

Macromolecules **2010**, *43*, 3721–3727 **3721**
DOI: 10.1021/ma100142w

Synthesis, Characterization, and Bioactivity of Mid-Functional PolyHPMA−Lysozyme Bioconjugates

Lei Tao,[†] Jiangtao Xu,[†] David Gell,[‡] and Thomas P. Davis*,[†]

[†]*Centre for Advanced Macromolecular Design (CAMD), School of Chemical Engineering, The University of New South Wales, Sydney, NSW 2052, Australia, and*
[‡]*School of Molecular and Microbial Biosciences, University of Sydney, NSW 2006, Australia*

Received January 19, 2010; Revised Manuscript Received March 7, 2010

ABSTRACT: A thiazolidine-2-thione mid-functionalized chain transfer agent (CTA) was synthesized and used as a reversible addition−fragmentation chain transfer (RAFT) polymerization agent to prepare poly(*N*-(2-hydroxypropyl)methacrylamide) (polyHPMA) with mid-chain thiazolidine-2-thione functionality. The synthesized polymers were fully analyzed by ^{1}H NMR and GPC, confirming well-defined structures (predesigned molecular weights, narrow polydispersities, and high functionalization efficiencies). A subsequent hydrolysis/analysis of the polymers was performed to verify their mid-functional structures. These mid-functionalized polymers were then incubated with a model protein (lysozyme) to generate branched polymer−protein bioconjugates. The bioactivity of the branched polymer−protein conjugate was tested and compared to similar molecular weight linear polyHPMA−protein bioconjugate; the branched polymer−protein conjugate remained much more protein activity, indicating the mid-chain-functional polyHPMA was more selective in its conjugation reaction on the lysozyme surface when compared with conjugation reactions involving terminal-functional polyHPMA. This straightforward methodology, described herein, for the synthesis of branched polymer−protein bioconjugates strikes a balance between protein protection by the attachment of polymer chains and the subsequent bioactivity retention of the bioconjugate.

Introduction

PEGylation, covalently linking poly(ethylene glycol) (PEG) to pharmaceutical proteins, imparts pharmacological enhancements to therapeutic proteins such as improved solubility and stability, thereby extending circulation times and reducing administration frequency by minimizing proteolytic degradation.[1,2] Since initial research in the 1970s,[3] PEGylation technology has developed rapidly, yielding significant therapeutic benefits and leading to market success in a number of areas.[4−6] First-generation PEGylation agents, such as PEG succinimidyl succinate (SS-PEG) and PEG succinimidy carbonate (SC-PEG), randomly modify the lysine residues on protein surfaces,[7] with an accompanying loss in bioactivity or receptor recognition of the protein, more than offset by an increased blood residence time, leading to improved therapeutic efficacies of the bioconjugates over equivalent native proteins.[5] Despite a simple synthetic approach and subsequent high yields, the first-generation PEGylation agents displayed serious disadvantages, such as diol contamination, unstable linkages, restriction to low molecular weight PEGs, and a lack of selectivity in substitution (bioconjugation reaction). To circumvent the problems displayed by first-generation PEGylated bioconjugates, second-generation PEGylation agents were developed. PEGs with mid-chain protein reactive functional groups (midfunctional PEGs) yielded as one of successful second-generation PEGylation agents, creating bioconjugates with unique properties. Compared to linear PEGs having similar molecular weights, the mid-functionalized PEGs were found to mask the protein surface more effectively using an "umbrella-like effect" (Scheme 1), enhancing protection leading to longer protein circulation half-life times.[8] Advantages of branched conjugates over linear conjugates have also been shown in analogous work on the protection of photochromic molecules by conjugation, where the branched structures have been proven to provide better encapsulation.[9] Another advantage of mid-chain polymer functionality can also be envisaged: steric hindrance of the mid-chain (polymer) functionality may potentially be used to enhance selectivity toward functionality on the protein surface, particularly at the active cleft sites, leading to higher bioactivity conservation (Scheme 1), thereby optimizing the benefit (circulation time)/loss (bioactivity) equation governing the successful design of bioconjugates.[10−14] However, mid-functional PEGylation agents do suffer from significant drawbacks as they are prepared using multistep organic reactions involving the hydroxyl groups of linear PEGs and branch agents such as lysine. The complex synthetic procedures necessitate onerous purification work-up processes resulting in increased costs.

Living radical polymerization (LRP) yields well-defined polymers with predetermined molecular weights (MWs) and narrow polydispersity indices (PDIs), both desirable properties for well-defined polymer−protein conjugates.[15] LRP is also tolerant to a large number of solvents[16−19] and protein reactive functional groups, such as maleimide,[20−22] *N*-hydroxysuccinimide (NHS),[23−25] pyridyl disulfide (PDS),[26−34] biotin,[35,36] aldehyde,[24,37,38] and thiazolidine-2-thione.[39,40] Thus, bioreactive initiators for atom transfer radical polymerization (ATRP) and chain transfer agents (CTAs) for reversible addition−fragmentation chain transfer (RAFT) have been prepared and utilized to synthesize semitelechelic bioreactive polymers.[41−44] The high interest in modern bioconjugation chemistry using LRP can be exemplified by a number of innovative approaches by a number of groups.[43−48] In a recent publication we disclosed a methodology for synthesizing branched polymer conjugates with bovine serum albumin (BSA)[49] exploiting pyridyl disulfide functionality (reaction with

*Corresponding author. E-mail: t.davis@unsw.edu.au.

Published on Web 03/23/2010

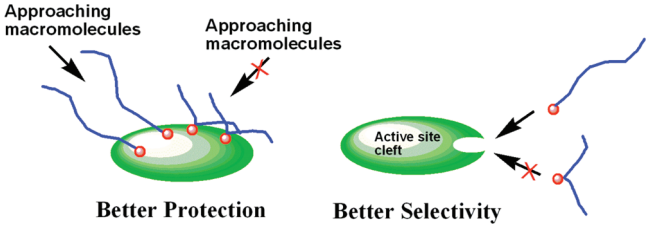
free thiol on the protein surface). Herein, we report the synthesis of poly(*N*-(2-hydroxypropyl)methacrylamide) (polyHPMA) with thiazolidine-2-thione, an amine reactive group, located in the middle of the polymer chain. This new synthetic approach, using thiazolidine-2-thione, is a general conjugation approach using lysine residues on protein surfaces to generate covalent amide linkages. In this paper, we use as an example, lysozyme, to demonstrate the versatility and effectiveness of our conjugation strategy.

PolyHPMA was chosen as the synthetic polymer as polyHPMA is a neutral, nontoxic, biocompatible, and nonimmunogenic polymer (although in a very recent paper, we explain that some care is required in the judicious selection of RAFT functionality to minimize cytotoxicity of HPMA polymers[50]). Previous research on polyHPMA and its copolymers has focused on anticancer drug delivery,[51−54] tumor specific antisense oligonucleotides[55] and site-specific delivery to the gastrointestinal (GI) tract.[56,57] Multifunctionalized polyHPMA copolymers have previously been used for the production of protein conjugates displaying a dramatic increase in acetylcholinesterase survival in the bloodstream of mice and in the thermostability of modified enzymes when compared to native proteins.[58,59]

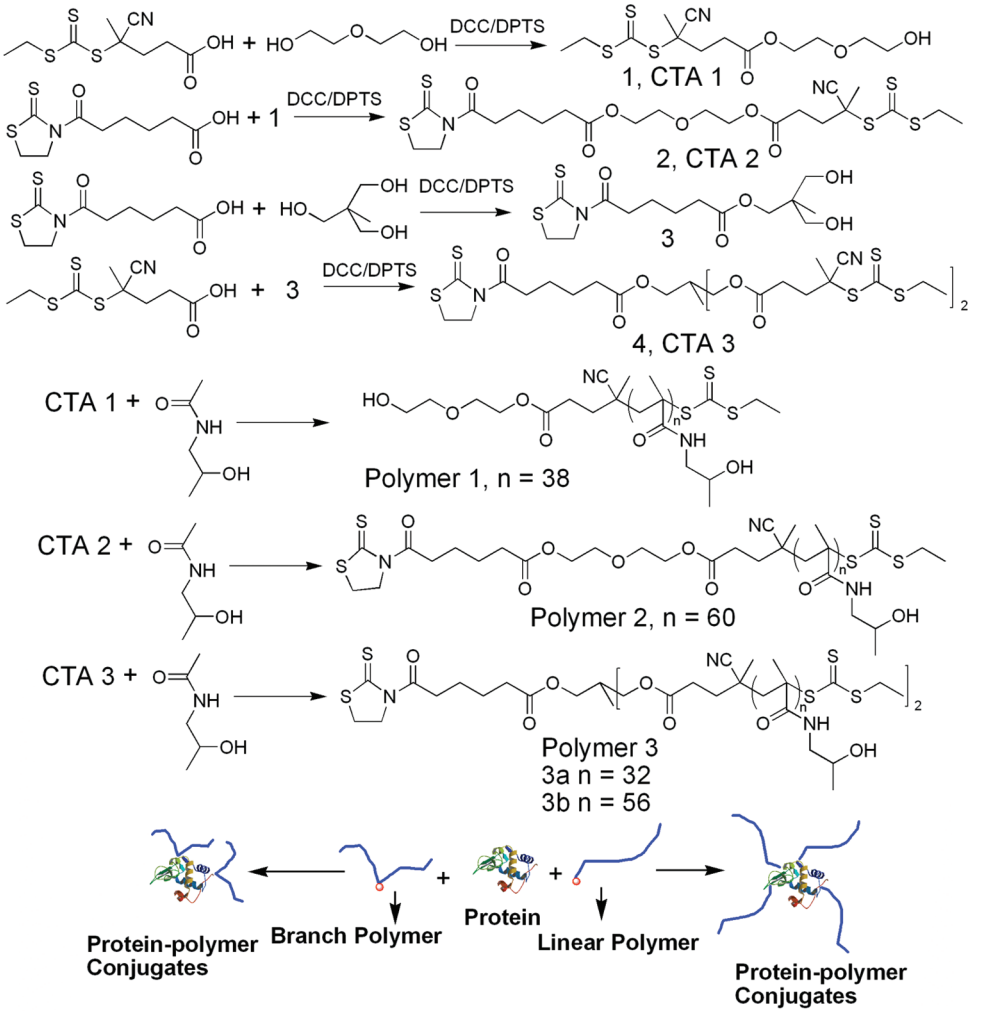
Semitelechelic polyHPMAs have also been synthesized using conventional free radical polymerization exploiting functional initiators or chain transfer agents (mercapto derivatives) to incorporate protein reactive groups at polymer chain ends. After purification using size exclusion chromatography to yield polymer fractions with narrow PDIs, the purified semitelechilic polyHPMAs were utilized for protein modification, exhibiting promising results.[59] The pioneering work reported by Kopecek and co-workers on polyHPMA occurred before the advent of LRP and thus was limited by the inevitable lack of (some) control in conventional radical polymerization. This early work was thus hampered by limited routes to semitelechelic polyHPMA, and therefore polyHPMA was not utilized as an alternative to PEG for protein bioconjugation. In 2005, well-defined polyHPMA was synthesized using the RAFT process (invented by a CSIRO team[60]) by McCormick's group,[61] enabling the development of well-defined polyHPMA as a new protein modification agent (analogous to PEGylation).

In this report, RAFT polymerization has been employed to synthesize branched polyHPMA with mid-chain functionality for conjugation to a model protein (lysozyme). The bioactivities of the resultant protein−polymer conjugates have been analyzed

Scheme 1. "Umbrella-like Effect" Masks Large Protein Surface and Increases the Selectivity of the Conjugation Reaction



Scheme 2. Synthesis of Thiazolidine-2-thione Mid-Functional PolyHPMA and the Subsequent Conjugation with Protein



using *Micrococcus lysodeikticus* (Ml) cells as substrates. To our knowledge, the data reported in this current paper represent the first disclosure of the use of mid-functional polyHPMA made by RAFT to conjugate amine residues on a protein surface. The synthetic strategy we have applied is summarized in Scheme 2.

Experimental Section

Materials. 2-Mercaptothiazoline (98%, Aldrich), 1,1,1-tris(hydroxymethyl)ethane (99%, Aldrich), *N,N′*-dicyclohexylcarbodiimide (DCC, 99%, Sigma), 4-(dimethylamino)pyridine (DMAP, 99%, Aldrich), diethylene glycol (≥99.0%, Aldrich), picrylsufonic acid solution (TNBS, 5% (w/v) in H_2O, Aldrich), *N*-(2-hydroxypropyl)methacrylamide (HPMA, PolyScience), hexylamine (99%, Aldrich), *Micrococcus lysodeikticus* (Ml cell, Sigma), and lysozyme (from chicken egg white, Sigma) were used as purchased. 2-(Dimethylamino)pyridinium *p*-tolenesulfonate (DPTS),[62] 4-cyano-4-(ethylthiocarbonothioylthio)pentanoic acid,[49,63] and 6-oxo-6-(2-thioxothiazolidin-3-yl)hexanoic acid[42] were synthesized as described previously. 2,2′-Azobis(isobutyronitrile) (AIBN, 98%, Sigma-Aldrich) was recrystallized twice from acetone. Dichloromethane (DCM, 99%, Ajax) was stored over calcium hydride and distilled before using.

Analyses. Gel permeation chromatography (GPC) analyses of polymers was performed in *N,N*-dimethylacetamide (DMAc) (0.03% w/v LiBr, 0.05% BHT stabilizer) at 50 °C (flow rate: 0.85 mL min^{-1}) using a Shimadzu modular system comprised of a DGU-12A solvent degasser, an LC-10AT pump, a CTO-10A column oven, and an RID-10A refractive index detector. The system was equipped with a Polymer Laboratories 5.0 mm bead-size guard column (50 × 7.8 mm^2) followed by four 300 × 7.8 mm^2 linear PL columns (10^5, 10^4, 10^3, and 500). Calibration was performed with narrow polydisperse polystyrene standards ranging from 500 to 10^6 g mol^{-1}.

^{1}H NMR spectra were obtained using a Bruker AC300F (300 MHz) spectrometer or a Bruker DPX300 (300 MHz) spectrometer. Multiplicities are reported as singlet (s), broad singlet (bs), doublet (d), triplet (t), quad (q), and multiplet (m). FT-IR spectra were obtained using a Bruker Spectrum BX FT-IR system using diffusing reflectance sampling accessories. UV−vis analyses were performed on a Varian Cary 300scan spectroscope. Mass spectra were obtained on a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an atmospheric pressure ionization source operating in the nebulizer-assisted electrospray mode. The instrument was calibrated in the *m*/*z* range 195−1822 Da using a standard containing caffeine, Met-Arg-Phe-Ala acetate salt (MRFA), and a mixture of fluorinated phosphazenes (Ultramark 1621) (all from Aldrich). SDS-PAGE was carried out with 4−20% Tris-HCl gels (Biorad, 1.0 mm × 10 well).

Methods. *2-(2-Hydroxyethoxy)ethyl 4-Cyano-4-(ethylthiocarbonothioylthio)pentanoate* (***1****, CTA* ***1***). 4-Cyano-4-(ethylthiocarbonothioylthio)pentanoic acid (1.50 g, 5.7 mmol), diethylene glycol (3.63 g, 34.0 mmol), and DPTS (0.05 g) were dissolved in dry DCM (75 mL), and DCC (1.30 g, 6.3 mmol) was added under a nitrogen atmosphere. The system was stirred at 18 °C for 4 h, the solid was filtered, and the solution was washed with distilled water (3 × 50 mL). The organic layer was separated, dried over magnesium sulfate, and evaporated to remove volatile solvents. The crude mixture was purified by column chromatography on silica gel (2% methanol in DCM) to yield the product as a yellow oil (1.30 g, 65.0%). ^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.30−4.26 (m, 2H, $COOCH_2$), 3.75−3.70 (m, 4H, CH_2OCH_2), 3.62−3.59 (m, 2H, CH_2OH), 3.34 (q, 2H, J = 7.4 *Hz*, SCH_2CH_3), 2.69−2.33 (m, 4H, CH_2CH_2C), 1.88 (s, 3H, CCH_3), 1.35 (t, 3H, J = 7.4 *Hz*, SCH_2CH_3). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 217.11, 171.77, 119.41, 72.80, 69.28, 64.50, 62.26, 46.62, 34.16, 31.61, 30.01, 25.22, 13.09. IR (cm^{-1}): 3320, 2928, 2852, 1733, 1626, 1571, 1448, 1381, 1292, 1185, 1128, 1074, 1032. ESI-MS: M+Na^+ expected (observed): 374.05 (374.07).

6-Cyano-6-methyl-9-oxo-4-thioxo-10,13-dioxa-3,5-dithiapentadecan-15-yl 6-oxo-6-(2-thioxothiazolidin-3-yl)hexanoate (***2****, CTA* ***2***). Compound **1** (0.60 g, 1.7 mmol), 6-oxo-6-(2-thioxothiazolidin-3-yl)hexanoic acid (0.46 g, 1.9 mmol), and DPTS (0.10 g) were dissolved in dry DCM (30 mL). DCC (0.42 g, 2.0 mmol) was added under a nitrogen atmosphere. The mixture was stirred at 18 °C for 6 h; the solid was filtered, and the solvent was evaporated under reduced pressure. The crude mixture was purified by column chromatography on silica gel (ethyl acetate/hexane: 1/4 to 1/1) to yield the product as a yellow oil (0.90 g, 91.3%). ^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.57 (t, J = 7.5 *Hz*, 2H, NCH_2), 4.27−4.21 (m, 4H, $CH_2CH_2OCH_2CH_2$), 3.71−3.67 (m, 4H, CH_2OCH_2), 3.38−3.24 (m, 6H, SCH_2CH_2, NC═OCH_2, SCH_2CH_3), 2.69−2.33 (m, 6H, $CH_2CH_2CH_2CH_2COO$, CCH_2CH_2-COO), 1.87 (s, 3H, CCH_3), 1.73−1.61 (m, 4H, $CH_2CH_2CH_2CH_2$-COO). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 237.95, 201.74, 174.43, 173.40, 171.56, 119.11, 69.28, 69.01, 64.14, 63.41, 56.15, 46.47, 38.21, 33.98, 33.92, 31.51, 29.83, 28.43, 24.98, 24.32, 24.25, 12.88. IR (cm^{-1}): 2932, 1732, 1698, 1368, 1281, 1138, 1049. ESI-MS: M + Na^+ expected (observed): 603.08 (603.05).

3-Hydroxy-2-(hydroxymethyl)-2-methylpropyl 6-Oxo-6-(2-thioxothiazolidin-3-yl)hexanoate (***3***). 6-Oxo-6-(2-thioxothiazolidin-3-yl)-hexanoic acid (1.00 g, 4.0 mmol), 1,1,1-tris(hydroxymethyl)ethane (2.50 g, 20.8 mmol), and DPTS (0.10 g) were suspended in dry DCM/THF (1:1, 100 mL). DCC (1.24 g, 6.0 mmol) was added under a nitrogen atmosphere. The mixture was stirred at 18 °C for 16 h; the solid was filtered, and the solvent was evaporated under reduced pressure. The residue was redissolved in ethyl acetate (100 mL) and washed with water (3 × 50 mL); the organic layer was separated, dried over magnesium sulfate, and evaporated to remove solvent. The crude was purified by column chromatography on silica gel (ethyl acetate/hexane: 1/1 to ethyl acetate/hexane: 4/1) to yield the product as a yellow oil (0.94 g, 67.2%). ^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.57 (t, 2H, J = 7.5 *Hz*, NCH_2), 4.19 (s, 2H, CH_2C), 3.55 (dd, 4H, 2 × CH_2OH), 3.31−3.25 (m, 4H, CH_2C═ON, SCH_2), 2.40 (t, 2H, J = 7.0 *Hz*, CH_2COO), 1.78−1.69 (m, 4H, CH_2CH_2-CH_2CH_2), 0.84 (s, 3H, CCH_3). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 201.92, 185.36, 174.77, 67.86, 66.53, 56.27, 41.04, 38.40, 34.09, 28.47, 24.50, 24.42, 17.21. IR (cm^{-1}): 3395, 2935, 1699, 1463, 1366, 1279, 1225, 1148, 1047, 884. ESI-MS: M + Na^+ expected (observed): 372.09 (372.10).

2-Methyl-2-((6-oxo-6-(2-thioxothiazolidin-3-yl)hexanoyloxy)-methyl)propane-1,3-diyl Bis(4-cyano-4-(ethylthiocarbonothioylthio)-pentanoate (***4****, CTA* ***3***). Compound **3** (0.57 g, 1.6 mmol), 4-cyano-4-(ethylthiocarbonothioylthio)pentanoic acid (0.95 g, 3.6 mmol), and DPTS (0.10 g) were dissolved in dry DCM, and DCC (0.83 g, 4.0 mmol) was added under a nitrogen atmosphere. The system was stirred at 20 °C for 16 h. The solid was filtered, and the solvent was removed under reduced pressure. The residue was purified by column charomatography on silica gel (ethyl acetate/hexane: 1/4 to 1/2) to yield the product as a yellow oil (1.21 g, 90.0%). ^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.58 (t, J = 7.5 *Hz*, 2H, NCH_2), 4.03−4.00 (m, 6H, 3 × $COOCH_2C$), 3.38−3.24 (m, 8H, SCH_2CH_2N, 2 × SCH_2-CH_3, CH_2CON), 2.67−2.32 (m, 10H, $CH_2CH_2CH_2CH_2COO$, 2 × CCH_2CH_2COO), 1.89 (s, 6H, 2 × CCH_3), 1.79−1.62 (m, 4H, $CH_2CH_2CH_2CH_2COO$), 1.36 (t, J = 7.4 *Hz*, 6H, 2 × SCH_2CH_3), 1.03 (s, 3H, CCH_3). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 217.11, 174.64, 173.33, 171.55, 119.35, 66.52, 65.81, 60.81, 56.44, 46.68, 38.82, 38.45, 34.16, 31.80, 30.02, 28.71, 25.30, 24.52, 21.46, 17.58, 14.60, 13.15. IR (cm^{-1}): 2930, 1733, 1698, 1447, 1371, 1281, 1155, 1076, 1047, 875. ESI-MS: M + Na^+ expected (observed): 862.09 (862.10).

Synthesis of Mid-Functional PolyHPMA. A typical polymerization procedure is described as follows: HPMA (0.50 g, 3.48 mmol), CTA 3 (29 mg, 0.058 mmol), and AIBN (2.8 mg, 0.017 mmol) were dissolved in DMAc/methanol (1/1, 3.0 mL). Aliquots were transferred to six different vials, which were then

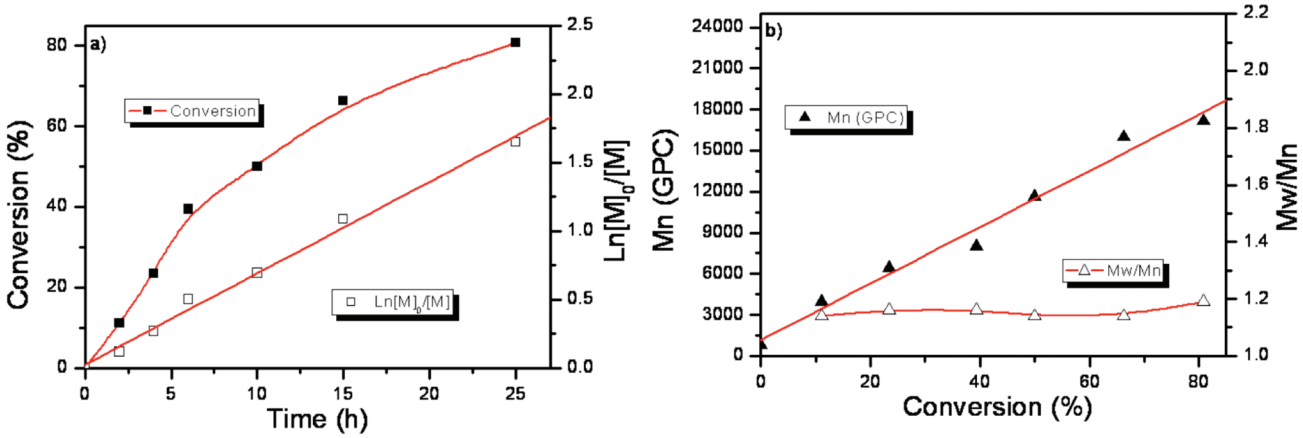


Figure 1. Polymerization of HPMA using CTA **3** in DMAc−methanol (1:1) at 65 °C ($[M]_0$:[CTA **3**]:[AIBN] = 60:1:0.3): (a) monomer conversion and the first-order kinetic curve versus polymerization time; (b) molecular weights and PDIs of polyHPMA versus monomer conversion.

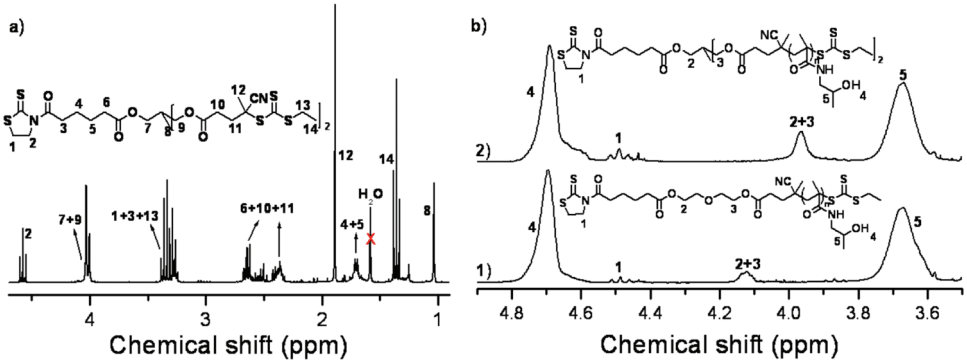


Figure 2. ^{1}H NMR spectra of (a) CTA **3** ($CDCl_3$) and (b) (1) linear polyHPMA (polymer **2**) and (2) branched polyHPMA (polymer **3**) (d_6-DMSO).

sealed with rubber septa. Each vial was deoxygenated by purging with nitrogen for 30 min prior to placement in a preheated oil bath at 65 °C. The vials were taken out at 2, 4, 6, 10, 15, and 25 h. Immediate cooling with an ice/water bath and exposure to air quenched the polymerizations. The monomer conversion for each sample was measured by ^{1}H NMR directly. After removal of volatiles, the residue was redissolved in DMAc for GPC analysis. The final polymers were collected after precipitation (twice) from methanol to diethyl ether and then dried under vacuum. CTA **1** and CTA **2** were utilized to prepare linear hydroxyl- and thiazolidine-2-thine-terminated polyHPMAs using the same procedure.

Aminolysis of Mid-Functional PolyHPMA. Mid-functionalized polyHPMA (10 mg) and hexylamine (0.1 mL) were dissolved in methanol (5.0 mL). The mixture was refluxed for 15 h, and the volatiles were removed under vacuum. The residue was redissolved in DMAc for direct GPC analysis.

Preparation of Protein−Polymer Conjugates. A typical synthesis procedure for the protein−polymer conjugates (pH 6.5) is described as follows: polymer **1** (M_n (NMR): 5800, M_n (GPC): 11 600, PDI: 1.18) (8.1 mg, 0.0014 mmol), polymer **2** (M_n (NMR): 9200, M_n (GPC): 16 000, PDI: 1.15) (12.9 mg, 0.0014 mmol), polymer **3a** ((M_n (NMR): 5400, M_n (GPC): 8600, PDI: 1.16) (7.6 mg, 0.0014 mmol), and polymer **3b** (M_n (NMR): 8900, M_n (GPC): 14 800, PDI: 1.16) (12.5 mg, 0.0014 mmol) were placed in four different small plastic vials. Freshly prepared lysozyme solution (0.5 mL, 3.42×10^{-5} mmol, 1.0 mg/mL in PBS buffer, pH 6.5) was added to the vials. The vials were kept at 16 °C, with gentle shaking for 18 h. The conjugation solution was used for bioactivity testing and TNBS analysis directly. After removing the salt in the solution by centrifugation filtration (MWCO: 10 000), the concentrated solution was used for SDS-PAGE analysis.

Evaluation of the Number of Polymer Chains on Protein Surface. The number of primary amines on the protein surface, after conjugation, was analyzed via a TNBS assay.[64] Briefly, protein conjugation solution [P] (20 μL) was added with 1% TNBS solution (20 μL) and 4% sodium bicarbonate solution (pH 8.5, 20 μL). The mixture was incubated at 40 °C for 2 h. The reaction was quenched by the addition of 10% SDS solution (20 μL) and 1 N HCl solution (20 μL). Water (0.9 mL) was added to yield a final solution for UV analysis (absorption at 420 nm). Unmodified protein solution [N] and buffer solution (blank, [B]) were also tested. The number of amines substituted was calculated as follows: $\{1 - [\mathrm{Abs(P)} - \mathrm{Abs(B)}]/[\mathrm{Abs(N)} - \mathrm{Abs(B)}]\} \times 7$ (lysozyme has 7 free amines, 6 lysines, and 1 terminal amine).

Bioactivity Tests on the Protein−Polymer Conjugates. The bioactivities of the conjugates were tested using Ml cell as substrates.

Ml cell (17.0 mg) was suspended in PBS buffer solution (45 mL, pH 7.0). An aliquot of suspension (3.0 mL) was transferred to a cuvette. The initial absorbance, at wavelength 450 nm, was defined as the baseline. Subsequently, lysozyme solution (5.0 μL of 1.0 mg/mL in PBS buffer, pH 6.5) was added, and the absorbance was measured every 15 s for 3 min. The bioactivity was calculated from the equation A (unit/mL) = $-K/(0.001VD)$, where A is defined as relative lysozyme concentration, K is the slope of the graph, V is the volume (mL) of sample solution, and D is the dilution coefficient. The data were used as a control in the calculation of the retention of bioactivity of protein conjugates, as shown in Figure 5. The bioactivities of the protein−polymer conjugates were tested using the same procedure as that used for native lysozyme, that is, adding the conjugation solution (5.0 μL) to the Ml cell suspension (3.0 mL), followed by absorbance analysis (450 nm) as detailed above.

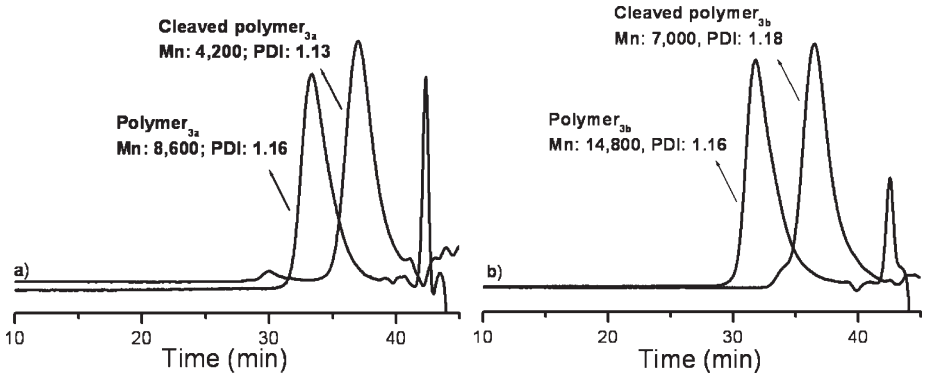


Figure 3. GPC curves of mid-chain functional polymer before and after aminolysis: (a) polymer **3a**; (b) polymer **3b**.

Results and Discussion

Polymer Synthesis. Thiazolidine-2-thione-functionalized terminal and mid-chain polyHPMA were prepared via RAFT polymerization using different CTAs. CTA **3** exerted control over the polymerization of HPMA, as expected, with a linear pseudo-first-order kinetic plot and a linear growth of molecular weights with monomer conversion (Figure 1). All polymers had narrow PDIs (≤1.2), indicating a well-controlled RAFT polymerization.

The polymer structures were analyzed by ^{1}H NMR (Figure 2). The spectra of polymers **2** and **3** both yielded signals at 4.50 ppm (Figure 2b) resulting from the methylene group (CH_2N) on the thiazolidine-2-thione moiety. In the spectrum of polymer **2**, a signal corresponding to the two ester groups in CTA **2** appeared at 4.12 ppm (Figure 2b-1). The integration ratio between the signal at 4.50 and that at 4.12 is 2/4.1, close to the expected value (2/4). The spectrum of polymer **3** yielded a signal of the three ester groups in CTA **3** at 3.97 ppm (Figure 2b-2), and the integration ratio of the signal at 4.50 to that at 3.97 ppm is 2/5.8, closing to the expected value (2/6). The NMR results confirmed the integrity of the thiazolidine-2-thione group after polymerization.

Aminolysis of the Mid-Chain-Functional Polymers. The mid-chain functionality of the polymer is critical in this study; thus, the aminolysis reaction of the mid-functional polyHPMAs was carried out. The ester linkage in the CTA **3** structure was thus cleaved, and the resultant polymers were analyzed by GPC (Figure 3). By comparison with the original polymers, the molecular weights of the cleaved polymers reduced significantly, while the PDIs of the cleaved polymers remained narrow (Figure 3). These aminolysis results confirmed the predesigned midfunctional structure of the polymers.

Protein−Polymer Conjugation. Thiazolidine-2-thione has been studied previously as an efficient protein-reactive group.[39,40,65] When hydroxyl-terminated polyHPMA (polymer **1**) was mixed with lysozyme, there was no conjugate observed using PAGE analysis (lane B), confirming that thiazolidine-2-thione is the sole reactive group to protein. Terminal-functional polyHPMA (polymer **2**, M_n(NMR): 9200, M_n(GPC): 16 000, PDI: 1.15) and mid-chain functional polyHPMA (polymer **3b**, M_n(NMR): 8900, M_n(GPC): 14 800, PDI: 1.16) with similar molecular weights were used to conjugate a model protein (lysozyme). The reactions were carried out at pH 6.5 and pH 7.0, respectively, with excess polymer concentration (polymer/protein = 40/1). SDS-PAGE analysis of the conjugates confirmed that the lysozyme starting material completely disappeared while new bands corresponding to protein−polymer conjugates appeared at higher molecular weight positions (Figure 4, lanes C, C′, E, E′), confirming both polymers conjugated successfully to protein. The conjugate obtained at pH 7.0 (lanes C′, E′) has a slightly higher molecular weight than the conjugate formed at pH 6.5 (lanes C, E), indicating more polymer chains attached to the protein surface. This can be explained by the fact that at higher pH values free amine group concentration is higher (altered ratio between free and protonated amine groups), leading to more effective attachment to the polymer. Since the number of attached polymer chains is crucial in the bioactivity retention of the conjugate, the pH provides a possible design parameter to control the number of polymer chains linked to the protein.

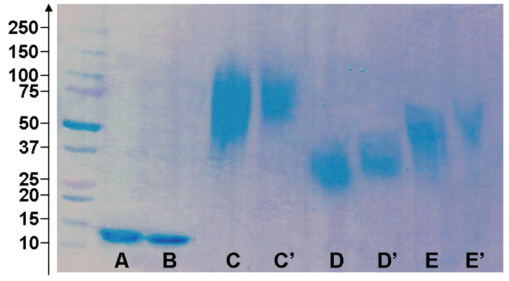


Figure 4. SDS-PAGE of polyHPMA−lysozyme conjugates: (A) native lysozyme; (B) polymer **1**−lysozyme, pH 6.5; (C) polymer **2**−lysozyme, pH 6.5; (C′) polymer **2**−lysozyme, pH 7.0; (D) polymer **3a**−lysozyme, pH 6.5; (D′) polymer **3a**−lysozyme, pH 7.0; (E) polymer **3b**−lysozyme, pH 6.5; (E′) polymer **3b**−lysozyme, pH 7.0.

Table 1. Evaluation of Modified Primary Amine Groups on Lysozyme

	UV−vis absorption (420 nm)[d]		number of conjugated amines[e]	
sample	pH 6.5	pH 7.0	pH 6.5	pH 7.0
buffer[a]	1.0872	1.1440		
unmodified protein[b]	1.3704	1.3752	0	0
polymer **2** + lysozyme[c]	1.2437	1.2119	3.13	4.94
polymer **3a** + lysozyme[c]	1.2742	1.2706	2.38	3.17
Polymer **3b** + lysozyme[c]	1.3063	1.3041	1.58	2.15

[a] The absorption was recorded as [B]. [b] The absorption was recorded as [N]. [c] The absorption was recorded as [P]. [d] The data were collected three times and reported as a mean (SD < 0.001). [e] The number of conjugated amine was calculated as $[1 - ([P] - [B])/([N] - [B])] \times 7$.

When mid-chain-functional polyHPMA was used for the conjugation, the branched conjugate displayed a lower molecular weight when compared to the analogous linear polyHPMA−protein conjugate despite the similar molecular weights of the initial polymers, suggesting the conjugation of less branched polyHPMA attached on the protein surface. This experimental result was in accord with expectation and supports the hypothesis that the bulk structure of the

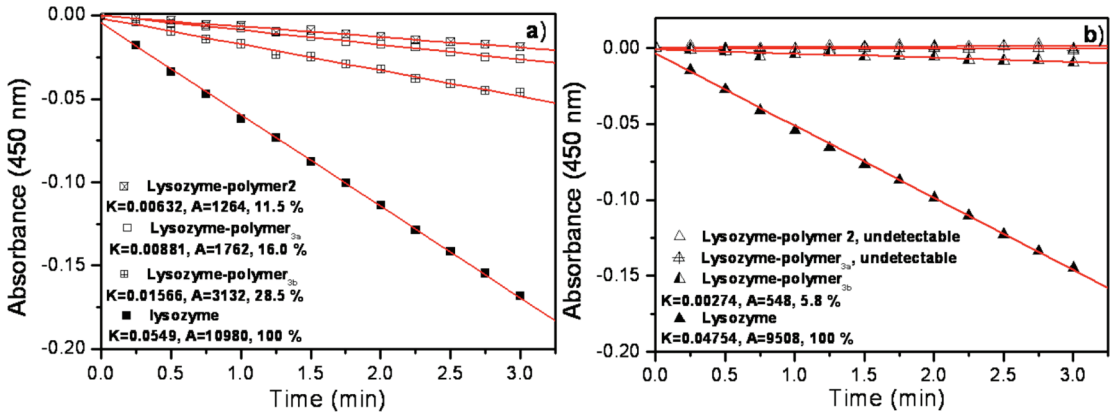


Figure 5. Bioactivity analysis of native lysozyme and polyHPMA−lysozyme conjugates: (a) pH 6.5; (b) pH 7.0.

polymer is important in achieving a selective reaction of the polymer to the protein surface.

Evaluation of Degree of Amine Modfication. The TNBS assay assesses the concentration of unconjugated primary amines in the reaction mixture. The assay results are shown in Table 1. The TNBS assay confirms that more polymer chains attach to the protein surface at pH 7.0 than at pH 6.5, supporting the SDS-PAGE analysis of the conjugates. At constant pH, the number of linear polymer chains conjugated to the protein is higher than equivalent branched polymers (despite their similar molecular weights, polymers **2** and **3b**), confirming the hypothesis that an "umbrella-like" structure of the mid-functional polymers hinders their approach to the protein surface, resulting in a degree of selectivity to the free amino acid residues. The molecular weight of the polymer also influences the efficiency of conjugation. As expected, lower molecular weight favors conjugation efficiency (polymers **3a** and **3b**), confirming the importance of steric effects in conjugation reactions.

Protein−Polymer Bioactivity Analyses. Polymer structure and molecular weight affect the bioactivity of corresponding polymer−protein conjugates. When different structural polymers with similar molecular weights (polymers **2** and **3b**) were employed to conjugate with lysozyme (Figure 4, lanes C, C′; E, E′), all the polymer−lysozyme conjugates showed reduced bioactivity compared to native lysozyme (Figure 5; pH 6.5, polymer **2**−lysozyme: 11.5%, polymer **3b**−lysozyme: 28.5%; pH 7.0, polymer **2**−lysozyme: undetectable, polymer **3b**−lysozyme: 5.8%). However, conjugates obtained from mid-functional polyHPMAs displayed better bioactivity retention when compared to the counterparts prepared using terminal-functional polyHPMA (yielding linear polymer bioconjugates). When conjugates were prepared at pH 6.5, the bioactivity of polymer **3b**−lysozyme was ~2.5 times higher than that of polymer **2**−lysozyme. This difference can be attributed (at least in part) to the structure of the branched polymer conjugate, with polymer chains only attached to the more accessible lysine residues on the protein surface, restricting bioactivity loss. A further experiment on conjugates prepared at pH 7.0 yielded similar results; the bioactivity of polymer **2**−lysozyme is almost undetectable while polymer **3b**−lysozyme still displayed 5.8% bioactivity.

The role of polymer molecular weight was analyzed using mid-functional polyHPMAs at different molecular weights (polymer **3a**, M_n(NMR): 5400, M_n(GPC): 8600, PDI: 1.16; polymer **3b**, M_n(NMR): 8900, M_n(GPC): 14 800, PDI: 1.16) conjugated to lysozyme. An interesting result was elicited showing that the protein conjugate obtained from higher molecular weight polymer exhibited better bioactivity conservation (pH 6.5, polymer **3a**−lysozyme: 16.0%, polymer **3b**−lysozyme: 28.5%; pH 7.0, polymer **3a**−lysozyme: undetectable, polymer **3b**−lysozyme: 5.8%). This counterintuitive result has also been reported in a previous research publication[39] and is explained using a hypothesis that polymers with higher molecular weight are sterically hindered, reducing their coupling efficiency to protein−surface amines, resulting in reduced coupling efficiency (supported by the TNBS results). This result indicates that the more efficient coupling of lower molecular weight polymer chains dominates over molecular weight in reducing the protein activity of bioconjugates.

Conclusions

In summary, we have described a straightforward methodology to synthesize thiazolidine-2-thione mid-functional polymers by RAFT polymerization for protein modification. Subsequent conjugation to a model protein (lysozyme) demonstrated effective coupling of the mid-functionality of polyHPMA and some free amine residues on the protein surface. The conjugates obtained from mid-functional polyHPMA displayed higher bioactivity compared with conjugates prepared with similar molecular weight terminal-functional polyHPMA. The synthetic methodology we describe strives to seek a balance between protein protection via linked polymer and protein bioactivity retention after conjugation. The synthesis approach we describe is quite general and represents a new, versatile synthetic method to prepare synthetic polymers for protein conjugation, broadening significantly the PEGylation methodology.

Acknowledgment. T.P.D. thanks the Australian Research Council for the award of a Federation Fellowship.

References and Notes

(1) Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54* (4), 459–476.
(2) Veronese, F. M.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54* (4), 453–456.
(3) Abuchowski, A.; Van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252* (11), 3578–81.
(4) Veronese, F. M.; Mero, A. *BioDrugs* **2008**, *22* (5), 315–329.
(5) Fishburn, C. S. *J. Pharm. Sci.* **2008**, *97* (10), 4167–4183.
(6) Lanthier, M.; Behrman, R.; Nardinelli, C. *Nat. Rev. Drug Discovery* **2008**, *7* (9), 733–737.
(7) Kozlowski, A.; Milton Harris, J. *J. Controlled Release* **2001**, *72* (1−3), 217–224.
(8) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003**, *2* (3), 214–221.

(9) Ercole, F.; Malic, N.; Harrisson, S.; Davis, T. P.; Evans, R. A. *Macromolecules* **2010**, *43* (1), 249–261.
(10) Ramon, J.; Saez, V.; Baez, R.; Aldana, R.; Hardy, E. *Pharm. Res.* **2005**, *22* (8), 1374–1386.
(11) Veronese, F. M. *Biomaterials* **2001**, *22* (5), 405–417.
(12) Guiotto, A.; Canevari, M.; Pozzobon, M.; Moro, S.; Orsolini, P.; Veronese, F. M. *Bioorg. Med. Chem.* **2004**, *12* (19), 5031–5037.
(13) Nojima, Y.; Suzuki, Y.; Iguchi, K.; Shiga, T.; Iwata, A.; Fujimoto, T.; Yoshida, K.; Shimizu, H.; Takeuchi, T.; Sato, A. *Bioconjugate Chem.* **2008**, *19* (11), 2253–2259.
(14) Fee, C. J. *Biotechnol. Bioeng.* **2007**, *98* (4), 725–731.
(15) Boyer, C.; Bulmus, V.; Davis, T. P.; Ladmiral, V.; Liu, J.; Perrier, S. *Chem. Rev.* (*Washington, DC, U. S.*) **2009**, *109* (11), 5402–5436.
(16) Lowe, A. B.; McCormick, C. L. *Handb. RAFT Polymerization* **2008**, 235–284.
(17) Pintauer, T.; Matyjaszewski, K. *Chem. Soc. Rev.* **2008**, *37* (6), 1087–1097.
(18) Rizzardo, E.; Moad, G.; Thang, S. H. *Handb. RAFT Polymerization* **2008**, 189–234.
(19) Stenzel, M. H. *Chem. Commun.* (*Cambridge, U.K.*) **2008**, *30*, 3486–3503.
(20) Mantovani, G.; Lecolley, F.; Tao, L.; Haddleton, D. M.; Clerx, J.; Cornelissen, J. J. L. M.; Velonia, K. *J. Am. Chem. Soc.* **2005**, *127* (9), 2966–2973.
(21) Bays, E.; Tao, L.; Chang, C.-W.; Maynard, H. D. *Biomacromolecules* **2009**, *10* (7), 1777–1781.
(22) Geng, J.; Mantovani, G.; Tao, L.; Nicolas, J.; Chen, G.; Wallis, R.; Mitchell, D. A.; Johnson, B. R. G.; Evans, S. D.; Haddleton, D. M. *J. Am. Chem. Soc.* **2007**, *129* (49), 15156–15163.
(23) Lecolley, F.; Tao, L.; Mantovani, G.; Durkin, I.; Lautru, S.; Haddleton, D. M. *Chem. Commun.* (*Cambridge, U.K.*) **2004**, *18*, 2026–2027.
(24) Samanta, D.; McRae, S.; Cooper, B.; Hu, Y.; Emrick, T.; Pratt, J.; Charles, S. A. *Biomacromolecules* **2008**, *9* (10), 2891–2897.
(25) McDowall, L.; Chen, G.; Stenzel, M. H. *Macromol. Rapid Commun.* **2008**, *29* (20), 1666–1671.
(26) Boyer, C.; Bulmus, V.; Davis, T. P. *Macromol. Rapid Commun.* **2009**, *30* (7), 493–497.
(27) Boyer, C.; Bulmus, V.; Liu, J.; Davis, T. P.; Stenzel, M. H.; Barner-Kowollik, C. *J. Am. Chem. Soc.* **2007**, *129* (22), 7145–7154.
(28) Boyer, C.; Liu, J.; Bulmus, V.; Davis, T. P. *Aust. J. Chem.* **2009**, *62* (8), 830–847.
(29) Boyer, C.; Liu, J.; Bulmus, V.; Davis, T. P.; Barner-Kowollik, C.; Stenzel, M. H. *Macromolecules* **2008**, *41* (15), 5641–5650.
(30) Jia, Z.; Liu, J.; Boyer, C.; Davis, T. P.; Bulmus, V. *Biomacromolecules* **2009**, *10* (12), 3253–3258.
(31) Liu, J.; Bulmus, V.; Herlambang, D. L.; Barner-Kowollik, C.; Stenzel, M. H.; Davis, T. P. *Angew. Chem., Int. Ed.* **2007**, *46* (17), 3099–3103.
(32) Bontempo, D.; Heredia, K. L.; Fish, B. A.; Maynard, H. D. *J. Am. Chem. Soc.* **2004**, *126* (47), 15372–15373.
(33) Heredia, K. L.; Nguyen, T. H.; Chang, C.-W.; Bulmus, V.; Davis, T. P.; Maynard, H. D. *Chem. Commun.* (*Cambridge, U.K.*) **2008**, *28*, 3245–3247.
(34) Vazquez-Dorbatt, V.; Tolstyka, Z. P.; Chang, C.-W.; Maynard, H. D. *Biomacromolecules* **2009**, *10* (8), 2207–2212.
(35) Bontempo, D.; Li, R. C.; Ly, T.; Brubaker, C. E.; Maynard, H. D. *Chem. Commun.* (*Cambridge, U.K.*) **2005**, *37*, 4702–4704.
(36) Hong, C.-Y.; Pan, C.-Y. *Macromolecules* **2006**, *39* (10), 3517–3524.
(37) Tao, L.; Mantovani, G.; Lecolley, F.; Haddleton, D. M. *J. Am. Chem. Soc.* **2004**, *126* (41), 13220–13221.
(38) Pound, G.; McKenzie, J. M.; Lange, R. F. M.; Klumperman, B. *Chem. Commun.* (*Cambridge, U.K.*) **2008**, *27*, 3193–3195.
(39) Tao, L.; Liu, J.; Xu, J.; Davis, T. P. *Org. Biomol. Chem.* **2009**, *7* (17), 3481–3485.
(40) Tao, L.; Liu, J.; Xu, J.; Davis, T. P. *Chem. Commun.* (*Cambridge, U.K.*) **2009**, *43*, 6560–6562.
(41) Liu, J.; Bulmus, V.; Barner-Kowollik, C.; Stenzel, M. H.; Davis, T. P. *Macromol. Rapid Commun.* **2007**, *28* (3), 305–314.
(42) Xu, J.; Boyer, C.; Bulmus, V.; Davis, T. P. *J. Polym. Sci., Part A: Polym. Chem.* **2009**, *47* (17), 4302–4313.
(43) Heredia, K. L.; Maynard, H. D. *Org. Biomol. Chem.* **2007**, *5* (1), 45–53.
(44) Nicolas, J.; Mantovani, G.; Haddleton, D. M. *Macromol. Rapid Commun.* **2007**, *28* (10), 1083–1111.
(45) De, P.; Li, M.; Gondi, S. R.; Sumerlin, B. S. *J. Am. Chem. Soc.* **2008**, *130* (34), 11288–11289.
(46) Heredia, K. L.; Grover, G. N.; Tao, L.; Maynard, H. D. *Macromolecules* **2009**, *42* (7), 2360–2367.
(47) Tao, L.; Kaddis, C. S.; Loo, R. R. O.; Grover, G. N.; Loo, J. A.; Maynard, H. D. *Chem. Commun.* (*Cambridge, U.K.*) **2009**, *16*, 2148–2150.
(48) Tao, L.; Kaddis, C. S.; Loo, R. R. V.; Grover, G. N.; Loo, J. A.; Maynard, H. D. *Macromolecules* **2009**, *42* (21), 8028–8033.
(49) Tao, L.; Liu, J.; Davis, T. P. *Biomacromolecules* **2009**, *10* (10), 2847–2851.
(50) Pissuwan, D.; Boyer, C.; Gunasekaran, K.; Davis, T. P.; Bulmus, V. *Biomacromolecules*, **2010**, *11*, 412−420.
(51) Duncan, R.; Kopecek, J. *Adv. Polym. Sci.* **1984**, *57* (Polym. Med.), 51−101.
(52) Kopecek, J.; Kopeckova, P.; Minko, T.; Lu, Z. R.; Peterson, C. M. *J. Controlled Release* **2001**, *74* (1−3), 147–158.
(53) Lu, Z. R.; Kopeckova, P.; Kopecek, J. *Nat. Biotechnol.* **1999**, *17* (11), 1101–4.
(54) Lu, Z.-R.; Shiah, J.-G.; Sakuma, S.; Kopeckova, P.; Kopecek, J. *J. Controlled Release* **2002**, *78* (1−3), 165–173.
(55) Whiteman, K. R.; Subr, V.; Ulbrich, K.; Torchilin, V. P. *J. Liposome Res.* **2001**, *11* (2 & 3), 153–164.
(56) Kopeckova, P.; Rathi, R.; Takada, S.; Rihova, B.; Berenson, M. M.; Kopecek, J. *J. Controlled Release* **1994**, *28* (1−3), 211–22.
(57) Rihova, B.; Jelinkova, M.; Strohalm, J.; St'astny, M.; Hovorka, O.; Plocova, D.; Kovar, M.; Draberova, L.; Ulbrich, K. *Bioconjugate Chem.* **2000**, *11* (5), 664–673.
(58) Chytry, V.; Kopecek, J.; Sikk, P.; Sinijarv, R.; Aaviksaar, A. *Makromol. Chem., Rapid Commun.* **1982**, *3* (1), 11–15.
(59) Lu, Z.-R.; Kopeckova, P.; Wu, Z.; Kopecek, J. *Bioconjugate Chem.* **1998**, *9* (6), 793–804.
(60) Chiefari, J.; Chong, Y. K.; Ercole, F.; Krstina, J.; Jeffery, J.; Le, T. P. T.; Mayadunne, R. T. A.; Meijs, G. F.; Moad, C. L.; Moad, G.; Rizzardo, E.; Thang, S. H. *Macromolecules* **1998**, *31* (16), 5559–5562.
(61) Scales, C. W.; Vasilieva, Y. A.; Convertine, A. J.; Lowe, A. B.; McCormick, C. L. *Biomacromolecules* **2005**, *6* (4), 1846–1850.
(62) Ihre, H.; Hult, A.; Frechet, J. M. J.; Gitsov, I. *Macromolecules* **1998**, *31* (13), 4061–4068.
(63) Convertine, A. J.; Benoit, D. S. W.; Duvall, C. L.; Hoffman, A. S.; Stayton, P. S. *J. Controlled Release* **2009**, *133* (3), 221–229.
(64) Habeeb, A. F. S. A. *Anal. Biochem.* **1966**, *14* (3), 328–36.
(65) Greenwald, R. B.; Pendri, A.; Martinez, A.; Gilbert, C.; Bradley, P. *Bioconjugate Chem.* **1996**, *7* (6), 638–641.