Aminooxy End-Functionalized Polymers Synthesized by ATRP for Chemoselective Conjugation to Proteins

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ABSTRACT: Chemoselective oxime formation between aminooxy end-functional polymers and levulinyl-modified proteins is an attractive method to prepare well-defined bioconjugates. We demonstrate the synthesis of Boc-protected aminooxy initiators for atom transfer radical polymerization (ATRP) of acrylamide and methacrylate monomers. Copper-mediated ATRP of *N*-isopropylacrylamide (NIPAAm), 2-hydroxyethyl methacrylate (HEMA), and poly(ethylene glycol) methacrylate (PEGMA) resulted in polymers with polydispersity indices (PDIs) as low as 1.11, 1.18, and 1.24, respectively. Kinetic analysis indicated that ATRP of HEMA was well-controlled. The polymer end groups of polyNIPAAm were deprotected with trifluoroacetic acid, exposing α-aminooxy moieties. Complete removal of the Boc groups was confirmed by ^{1}H NMR, and the ability to form oxime bonds was verified by conjugation to aldehyde fluorescent nanospheres. Chemospecific reaction with N^{ϵ}-levulinyl lysine-modified bovine serum albumin (BSA) to form "smart" polymer conjugates was demonstrated.

Introduction

Developing chemistry to prepare protein−polymer conjugates is an important area of research.[1−3] Bioconjugates offer many advantages over unmodified proteins, including increased stability and solubility, the ability to modulate protein activity, and increased bioavailability for therapeutic applications.[4−6] Techniques to prepare well-defined protein−polymer conjugates are important because they provide biomolecules with homogeneous structures and activities. Two methods are often used in tandem to accomplish this: (1) synthesis of polymers with defined architectures and molecular weights and (2) attachment of the polymers at specific locations on the protein. With the advent of controlled radical polymerization methods,[7,8] such as atom transfer radical polymerization (ATRP),[9,10] it is possible to prepare polymers containing many different functionalities with narrow molecular weight distributions. This unprecedented flexibility opens new avenues for the synthesis of a wide variety of polymer bioconjugates. This report describes a method to prepare reactive polymers by ATRP for chemospecific attachment to proteins via oxime bond formation.

Protein−polymer conjugates have traditionally been synthesized by coupling end-functionalized poly(ethylene glycol) (PEG) with the amine groups of lysine to form PEGylated proteins. This method has led to protein drugs with decreased immunogenicities and increased plasma lifetimes compared to their unmodified counterparts.[11] However, random and multiple polymer attachment to the various amines causes significant reductions in protein activities. This can be remedied by targeting the N-terminus by reaction at decreased pH.[12] The pK_a difference at the N-terminus vs the ϵ-amine side chains of lysine allows for better control over the conjugation site, although heterogeneity is often still observed.

Site-specific modification via ligand binding sites and free cysteines forms homogeneous bioconjugates. Polymers containing protein ligands, such as biotin for interaction with (strept)-avidin,[13,14] cofactor reconstitution,[15] or Ni^{2+} for interaction with polyhistidine-tagged recombinant proteins,[16] have been demonstrated for site-specific polymer attachment. Additionally, thiol side chains of cysteines are frequently targeted. Free cysteines are less common than other amino acids and can be exploited for site-specific attachment. Reversible disulfide bonds can be formed by modification of thiols with activated disulfides such as pyridyl,[17] alkoxycarbonyl, or *o*-nitrophenyl;[18] groups such as vinyl sulfone[19−25] and maleimide[26−28] result in thioether bonds formed via Michael addition. An alternative method to prepare site-specific polymers is to polymerize directly from modified biotin sites and free cysteines of protein macroinitiators to form the polymer conjugates in situ.[29,30]

Mild conditions and high coupling efficiencies of "click" reactions have recently been demonstrated for bioconjugate formation. Huisgen 1,3-dipolar cycloaddition between azides and alkynes has been employed to prepare bovine serum albumin (BSA)−polystyrene amphiphilic conjugates[31] and superoxide dismutase (SOD)−PEG conjugates.[32] The non-naturally occurring groups were incorporated by reaction of a maleimide functionalized alkyne with the free thiol of BSA and site-directed mutagenesis to install *p*-azidophenylalanine into SOD, respectively. Reaction between an aminooxy and a ketone/aldehyde is another "click" reaction that has the advantage that, other than the reacting partners, no other reagents are required. Therefore, oxime bond formation is an attractive method to form site-specific bioconjugates. Various routes have been implemented to install these groups into proteins. For example, solid-phase protein synthesis has been used to incorporate both *O*-hydroxylamine- and ketone-functionalized lysines using aminooxyacetic acid[33] and levulinic acid, respectivley.[34,35] The resultant *O*-hydroxylamine or ketone groups were targeted for site-specific PEGylation. Modification of tyrosine residues of tobacco mosaic virus (TMV) with diazonium salts has been demonstrated for the preparation of a ketone-modified TMV.[36] Alcohol side chains of serine have also been oxidized to aldehydes using sodium periodate.[37] Pyridoxal-5-phosphate (PLP)[38]-mediated N-terminal transamination has recently been used to form a site-specific PEGylated protein via oxime formation with an aminooxy end-functional PEG.[39] An alternate method to install the α-ketoamide moiety for conjugation is by

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transamination of the N-terminus with glyoxylic acid.[40−42] Yet, to our knowledge only PEG conjugates have been synthesized by oxime bond formation. Likely, other polymers have not been explored due to the lack of efficient methods to prepare the required end-functionalized polymers.

Polymerization from protein-reactive initiators circumvents postpolymerization reactions to install the desired moieties and guarantees that each polymer chain contains one reactive end group. These initiators enable facile synthesis of semitelechelic polymers for direct conjugation to proteins or after a simple deprotection step. Initiators for ATRP have been synthesized with protein-reactive groups resulting in α-functional polymers.[43] Pyridyl disulfide[44] and protected maleimide[45] ATRP initiators resulted in polymers for conjugation to thiol side chains, and biotinylated ATRP initiators[46−48] produced polymers that conjugated to (strept)avidin. Herein, we report the synthesis of Boc-protected aminooxy-functionalized initiators for ATRP of poly(ethylene glycol) methacrylate (PEGMA), 2-hydroxyethyl methacrylate (HEMA), and *N*-isopropylacrylamide (NIPAAm). To our knowledge, aminooxy-functionalized initiators for ATRP for chemoselective protein−polymer conjugates synthesis have not yet been demonstrated. The α-functionalized polyNIPAAm was subsequently deprotected and attached to N^{ϵ}-levulinyl lysine-modified bovine serum albumin (BSA) via oxime bond formation.

Experimental Section

Materials. *N*-Boc-aminooxyacetic acid was purchased from Novabiochem. FluoSpheres aldehyde−sulfate spheres (20 nm, yellow-green) were purchased from Invitrogen. All other chemicals were purchased from Sigma-Aldrich or Fisher and used as received unless otherwise noted. HEMA and PEGMA were passed through a basic alumina column prior to use. NIPAAm was recrystallized twice from hexanes. Copper bromide (CuBr) and copper chloride (CuCl) were stirred in glacial acetic acid for 12 h at 22 °C, washed with ethanol and diethyl ether, and then dried under vacuum. Tetra(ethylene glycol) was dried over 4 Å molecular sieves for 24 h prior to use. Tris[2-(dimethylamino)ethyl]amine (Me_6TREN) was synthesized as previously described.[49] Abbreviations: 2,2′-bipyridine (bipy); 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide (EDC); 4-(dimethylamino)pyridine (DMAP); *tert*-butoxycarbonyl (Boc); dimethyl sulfoxide (DMSO); dichloromethane (DCM); methanol (MeOH); chloroform ($CHCl_3$); acetonitrile (ACN); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); *N*-hydroxysuccinimide (NHS); phosphate buffered saline (PBS); gel permeation chromatography (GPC); number-average molecular weight (M_n); polydispersity index (PDI).

Instrumentation. ^{1}H and ^{13}C NMR were performed on either a Bruker ARX or Avance DRX 500 MHz spectrometer. 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. Analysis of the FluoSphere Nanosphere was performed in THF (0.80 mL/min) at 22 °C and was monitored at 500 nm. SDS-PAGE was carried out using 4−20% precast gradient gels from Invitrogen, and samples were dissolved in TRIS buffer containing SDS, bromophenol blue, and glycerol.

Methods. *Synthesis of Tetra(ethylene glycol) 2-Bromoisobutyrate (**1**).* Tetra(ethylene glycol) (40 mL, 230 mmol) and triethylamine (1.93 mL, 13.9 mmol) were added via syringe into a dried round-bottom flask. The solution was cooled to 0 °C, and 2-bromoisobutyryl bromide (1.43 mL, 11.6 mmol) was added dropwise in 5 mL of dry DCM. The solution was warmed to 22 °C over 12 h with constant stirring. The reaction mixture was diluted with 100 mL of deionized water and then extracted with DCM (3 × 100 mL). The combined organic extracts were dried over magnesium sulfate, concentrated, and purified by silica gel chromatography (9:1 diethyl ether/methanol), yielding **1** as a colorless oil (71% yield). ^{1}H NMR 500 MHz ($CDCl_3$): δ 4.26 (t, 2H, J = 4.9), 3.67 (t, 2H, J = 4.9), 3.64 (t, 2H, J = 4.7), 3.61−3.58 (m, 8H), 3.51 (t, 2H, J = 4.7), 2.82 (s, 1H), 1.87 (s, 6H). ^{13}C NMR 500 MHz ($CDCl_3$): δ 171.38, 72.39, 70.48, 70.43, 70.35, 70.11, 68.54, 64.93, 62.7, 61.40, 57.79, 55.61, 30.55.

*Synthesis of N-Boc-(methylaminooxy)acetoxy Tetra(ethylene glycol) 2-Bromoisobutyrate (**2**).* **1** (820 mg, 2.39 mmol) and *N*-Boc-aminooxyacetic acid (456 mg, 2.39 mmol) were placed in a dried round-bottom flask and dissolved in dry DCM (25 mL). The mixture was cooled to 0 °C, and then EDC (550 mg, 2.87 mmol) and DMAP (29 mg, 0.24 mmol) were added in one portion. The solution was allowed to warm to 22 °C over 12 h. The crude product was concentrated and purified by silica gel chromatography (85:15 ethyl acetate/hexanes), yielding **2** as a colorless oil (75% yield). ^{1}H NMR 500 MHz ($CDCl_3$): δ 7.89 (s, 1H), 4.45 (s, 2H), 4.33−4.31 (m, 4H), 3.74−3.71 (m, 4H), 3.65−3.64 (m, 8H), 1.93 (s, 6H), 1.47 (s, 9H). ^{13}C NMR 500 MHz ($CDCl_3$): δ 171.49, 169.47, 156.04, 81.93, 72.43, 70.60, 70.51,70.45, 68.68, 68.62, 64.98, 63.98, 55.57, 30.61, 28.05.

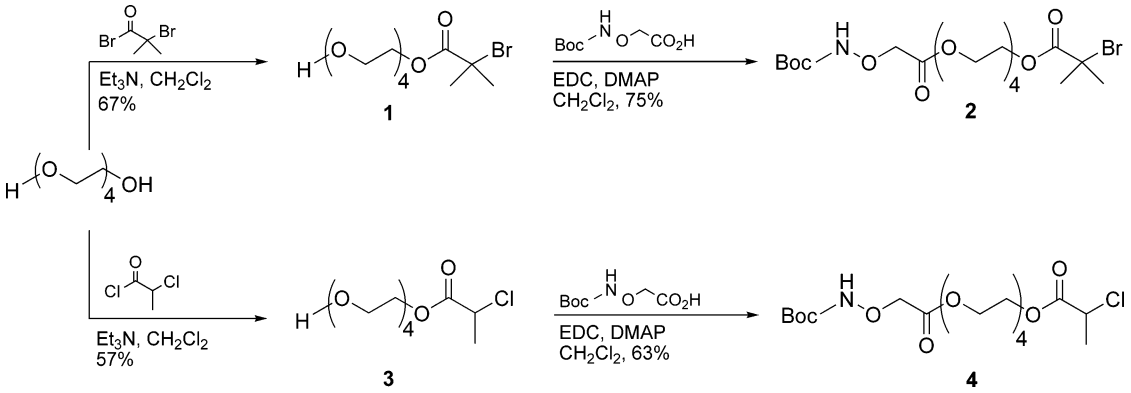
*Synthesis of Tetra(ethylene glycol) 2-Chloropropionate (**3**).* Tetra(ethylene glycol) (5.8 mL, 33.6 mmol) and triethylamine (930 μL, 6.7 mmol) were added to a dried round-bottom flask and then cooled to 0 °C. A solution of 2-chloropropionyl chloride (330 μL, 3.36 mmol) in dry DCM (5 mL) was added dropwise, and the reaction mixture was allowed to warm to 22 °C over 12 h with constant stirring. The solution was diluted in deionized water (25 mL) and extracted with DCM (3 × 50 mL). The organic extracts were dried over magnesium sulfate, concentrated, and purified by silica gel chromatography (9:1 diethyl ether/methanol), yielding **3** as a pale yellow oil (57% yield). ^{1}H NMR 500 MHz ($CDCl_3$): δ 4.41 (q, 1H, J = 6.9), 4.30 (t, 2H, J = 4.7), 3.71−3.68 (m, 4H), 3.63−3.62 (m, 8H), 3.57 (t, 2H, J = 4.5), 2.66 (s, 1H), 1.66 (d, 3H J = 3.5). ^{13}C NMR 500 MHz ($CDCl_3$): δ 169.99, 72.37, 70.50, 70.41, 70.20, 68.65, 64.87, 61.57, 52.27, 21.34.

*Synthesis of N-Boc-(methylaminooxy)acetoxy Tetra(ethylene glycol) 2-Chloropropionate (**4**).* **3** (1.48 g, 5.23 mmol) and *N*-Boc-aminooxyacetic acid (1.0 g, 5.23 mmol) were placed in a dried round-bottom flask and dissolved in dry DCM (30 mL). The solution was cooled to 0 °C, and then EDC (1.1 g, 5.75 mmol) and DMAP (63 mg, 0.52 mmol) were added in one portion. The solution was warmed to 22 °C over 12 h with constant stirring. The solvent was then removed under reduced pressure, and the crude product was purified by silica gel chromatography (80:20 ethyl acetate/hexanes), yielding **4** as a pale yellow oil (63% yield). ^{1}H NMR 500 MHz ($CDCl_3$): δ 7.97 (s, 1H), 4.40−4.35 (m, 3H), 4.29−4.26 (m, 4H), 3.68−3.60 (m, 12H), 1.64 (d, 3H, J = 3.4), 1.42 (s, 9H). ^{13}C NMR 500 MHz ($CDCl_3$): δ 169.96, 169.43, 156.13, 81.92, 72.42, 70.51, 70.47, 70.45, 70.42, 68.66, 68.64, 64.86, 63.97, 60.24.

General Polymerization of HEMA and PEGMA. Polymerization of HEMA was conducted using molar rations of 50:1:1:2 of monomer:**2**:CuBr:bipy and a 50:50 v/v ratio of methanol:HEMA. Polymerization of PEGMA was conducted using the same molar ratios with a 33:66 v/v ratio of PEGMA:methanol. CuBr (3.7 mg, 0.026 mmol) and bipy (8.1 mg, 0.052 mmol) were weighed into a Schlenk tube, and the tube was evacuated and argon-refilled five times. Degassed monomer (1.32 mmol) and methanol were added via syringe. When the solution was homogeneous, degassed **2** (10 μL, 0.026 mmol) was added via syringe to begin the polymerization. Aliquots were removed periodically and diluted into $MeOH_{d4}$ for NMR analysis. The solvent from the same samples was evaporated and diluted into DMF for GPC analysis. The final polymers were isolated by precipitation into cold diethyl ether.

General Polymerization of NIPAAm. Polymerization of NIPAAm was conducted using 1:50:2:2 molar ratios of **4**:NIPAAm:CuCl:Me_6TREN:NIPAAm (522 mg, 4.61 mmol), **4** (30 μL, 0.092 mmol), and DMSO (1.13 mL) were added to a Schlenk tube, and the

Scheme 1. Synthesis of Aminooxy-Functionalized ATRP Initiators



contents were subjected to four freeze−pump−thaw cycles. 400 μL of a catalyst stock solution (22.7 mg CuCl, 62.5 μL Me_6TREN in 500 μL of degassed DMSO) was added to the Schlenk tube to begin the polymerization. Aliquots were withdrawn periodically and diluted into $DMSO_{d6}$ for kinetic analysis. Conversions were determined using the following equation: $1 - M_t/M_0$ where M_t = monomer peak at 5.57−5.53 ppm and M_0 = monomer plus polymer overlap at 3.93−3.88 ppm. The solvent from the same samples was evaporated and diluted into DMF for GPC analysis. The final polymer was purified by dialysis (MWCO 1000) and then lyophilized to isolate the solid.

*Deprotection of **4**.* **4** (30 μL, 0.092 mmol) was dissolved in 1 mL of a 50% v/v solution of TFA/$CHCl_3$. After 30 min the solvent was removed by bubbling with argon, and the deprotected initiator was dried under vacuum. ^{1}H NMR 500 MHz ($CDCl_3$): δ 4.67 (s, 2H), 4.47−4.36 (m, 5H), 3.80−3.64 (m, 12H), 1.69 (d, 3H, J = 6.5).

*Model Oxime Formation of Deprotected **4** with Butyraldehyde.* Deprotected **4** (0.092 mmol) was dissolved in 500 μL of chloroform. Butyraldehyde (83 μL, 0.18 mmol) was added via syringe. After 10 min the solvent was removed by bubbling with argon. The oily residue was purified by silica gel chromatography (80:20 ethyl acetate/hexanes) to yield the desired oxime-linked initiator in 53% isolated yield. ^{1}H NMR 500 MHz ($CDCl_3$): δ 7.48, 6.70 (3:2 *syn*:*anti*, t, 1H J = 5.7) 4.62, 4.57 (2:3 *anti*:*syn*, s, 2H), 4.41 (q, 1H, J = 6.8), 4.32−4.29 (m, 4H), 3.73−3.63 (m, 12H), 2.39−2,35, 2.17−2.13 (2:3 *anti*:*syn*, m, 2H), 1.68 (d, 3H, J = 3.1), 1.53−1.48 (m, 2H), 0.96−0.93 (m, 3H). ^{13}C NMR 500 MHz ($CDCl_3$): δ 170.03, 153.47, 152.64, 70.54, 70.49, 70.47, 70.10, 69.86, 68.83, 68.64, 64.88, 63.72, 52.23, 31.07, 27.58, 21.34, 19.76, 19.36, 13.72, 13.42.

Deprotection of Boc-aminooxy PolyNIPAAm. Boc-protected polyNIPAAm (10 mg, M_n = 5820, PDI = 1.12) was dissolved in 1 mL of a 50% v/v TFA/$CHCl_3$ solution. After 30 min the solvent was removed by bubbling through with argon and then dried under vacuum. The polymer was used for conjugation without any further purification. ^{1}H NMR 500 MHz ($CDCl_3$): δ 6.93 (1H), 4.75 ($NOCH_2$ end group), 4.44 ($COOCH_2$ end group), 4.25 (s, $COOCH_2$ end group) 3.93 (1H), 3.76−3.64 (CH_2OCH_2 end group), 2.17 (1H), 1.78−1.57 (2H), 1.11 (6H).

Conjugation to FluoSphere Aldehyde Nanosphere. Deprotected polyNIPAAm (10 mg) was dissolved in 100 μL of ACN and added to 100 μL of green fluorescent FluoSpheres aldehyde−sulfate spheres (20 mg/mL) in water. The sample was placed at 4 °C overnight in the dark. The solution was lyophilized and redissolved in 200 μL of THF for GPC analysis.

*Synthesis of N-Hydroxysuccinimidyl Levulinate (**5**).* Levulinic acid (100 mg, 0.86 mmol) was weighed into a dried round-bottom flask and dissolved in 3 mL of dry DCM. NHS (109 mg, 0.95 mmol) was added, and the mixture was cooled to 0 °C. EDC (182 mg, 0.95mmol) was added, and the reaction was allowed to slowly warm to 23 °C over 5 h. The solvent was removed in vacuo, and the crude product was purified by silica gel chromatography (DCM), yielding **5** as a white solid (80% yield). ^{1}H NMR 500 MHz ($CDCl_3$): δ 2.91−2.86 (m, 4H), 2.83 (s, 4H), 2.21 (s, 3H). ^{13}C NMR 500 MHz ($CDCl_3$): δ 204.91, 169.04, 168.33, 37.73, 29.81, 25.71, 25.22.

Synthesis of N^{ϵ}-Levulinyl Lysine-Modified Bovine Serum Albumin (BSA). BSA (464 mg, 0.007 mmol) was dissolved in 10 mL of PBS (pH 8.0). **5** (15 mg, 0.07 mmol) was added in 1 mL of DMSO/MeOH (1:1). After 60 min the protein was purified by dialysis (MWCO 50 000) and lyophilized to isolate the N^{ϵ}-levulinyl lysine-modified BSA.

Conjugation of AminooxyPolyNIPAAm to Levulinyl Lysine-Modified BSA. Levulinyl lysine-modified BSA (1 mg) and deprotected polyNIPAAm (5 mg) were dissolved in 300 μL of 50/50 v/v ACN/Milli-Q H_2O. After 30 min the solvent was removed by lyophilization. The protein was then redissolved in 200 μL of H_2O for SDS-PAGE analysis. To isolate the polyNIPAAm−BSA conjugate, 150 μL of this solution was warmed above 32 °C to precipitate the polymer and then centrifuged at 15 000 rpm to isolate the solids. The pellet was redissolved in 50 μL of water for SDS-PAGE analysis.

BSA Control Experiment 1. Levulinyl lysine-modified BSA (1 mg) and protected polyNIPAAm (5 mg) were dissolved in 300 μL of 50/50 v/v ACN/Milli-Q H_2O. After 30 min the solvent was removed by lyophilization, and the protein was redissolved in 200 μL of H_2O for SDS-PAGE analysis.

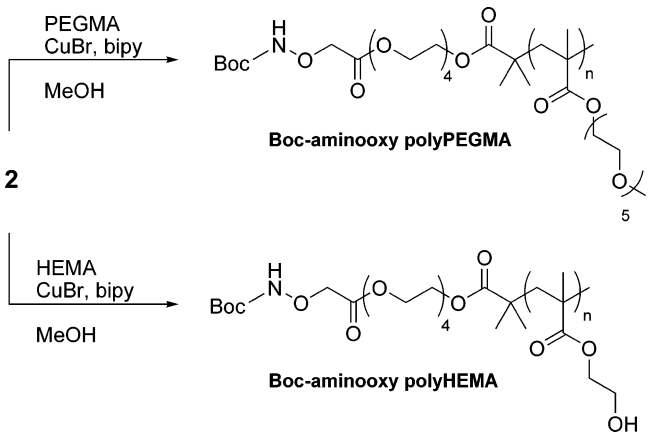
BSA Control Experiment 2. Unmodified BSA (1 mg) and deprotected polyNIPAAm (5 mg) were dissolved in 300 μL of 50/50 v/v ACN/ Milli-Q H_2O. After 30 min the solvent was removed by lyophilization, and the protein was redissolved in 200 μL of H_2O for SDS-PAGE analysis.

Results and Discussion

Initiator Synthesis. We envisioned polymerization from aminooxy-modified ATRP initiators would result in α-functionalized polymers for oxime bond formation with ketone-modified proteins. We anticipated that the chemoselective nature of this "click" reaction, coupled with ATRP to prepare well-defined polymers, would provide a straightforward route to site-specific and well-defined protein−polymer conjugates. Two initiators for ATRP were synthesized, each containing a Boc-protected aminooxy group (Scheme 1). Initiator **2** contained a 2-bromoisobutyrate group for polymerization of methacrylates, while initiator **4** had a 2-chloropropionate for polymerization of acrylamides.[9] In each case, esterification with either 2-bromoisobutyryl bromide or 2-chloropropionyl chloride was conducted using a large excess of tetra(ethylene glycol), forming the tetra(ethylene glycol)-functionalized ATRP initiators **1** and **3** in 67% and 57% yields, respectively. An excess of tetraethylene glycol prevented bis-functionalization of the diol. Esterification of alcohols **1** and **3** with Boc-aminooxyacetic acid using EDC and DMAP yielded the protected aminooxy-functionalized initiators **2** and **4** in 75% and 63% yield, respectively.

ATRP from Aminooxy-Functionalized Initiators. The three monomers chosen for investigation were HEMA, PEGMA, and

Scheme 2. ATRP of PEGMA and HEMA



NIPAAm. PolyHEMA is a well-known biocompatible, hydrogel-forming polymer and is used in contact lens manufacturing.[50] PolyPEGMA should have similar biocompatibility to PEG for therapeutic applications. The thermoresponsive nature of polyNIPAAm is often exploited for applications in biotechnology.[51] Copper-mediated ATRP of PEGMA and HEMA were conducted using initial molar ratios of 1:1:2 of **2**:CuBr:bipy in methanol at 22 °C with monomer-to-initiator ratios of 50:1 (Scheme 2). Progression of the polymerizations was monitored by ^{1}H NMR and conversions of 74% and 84% were observed for PEGMA (Figure 1a) and HEMA (Figure 1b), respectively. The pseudo-first-order kinetic plot of the polymerization of PEGMA was slightly curved, indicating termination reactions as evident by the slight high molecular weight shoulder observed in the GPC traces at higher conversions (Figure 1a). Commercially available PEGMA is a heterogeneous mixture often containing bis-methacrylate species that are difficult to remove; these impurities were likely a factor in the observed kinetics. The pseudo-first-order kinetic plot for the polymerization of HEMA was linear with respect to time, indicating a controlled polymerization with a constant radical concentration throughout the reaction (Figure 1b). In both systems, molecular weights

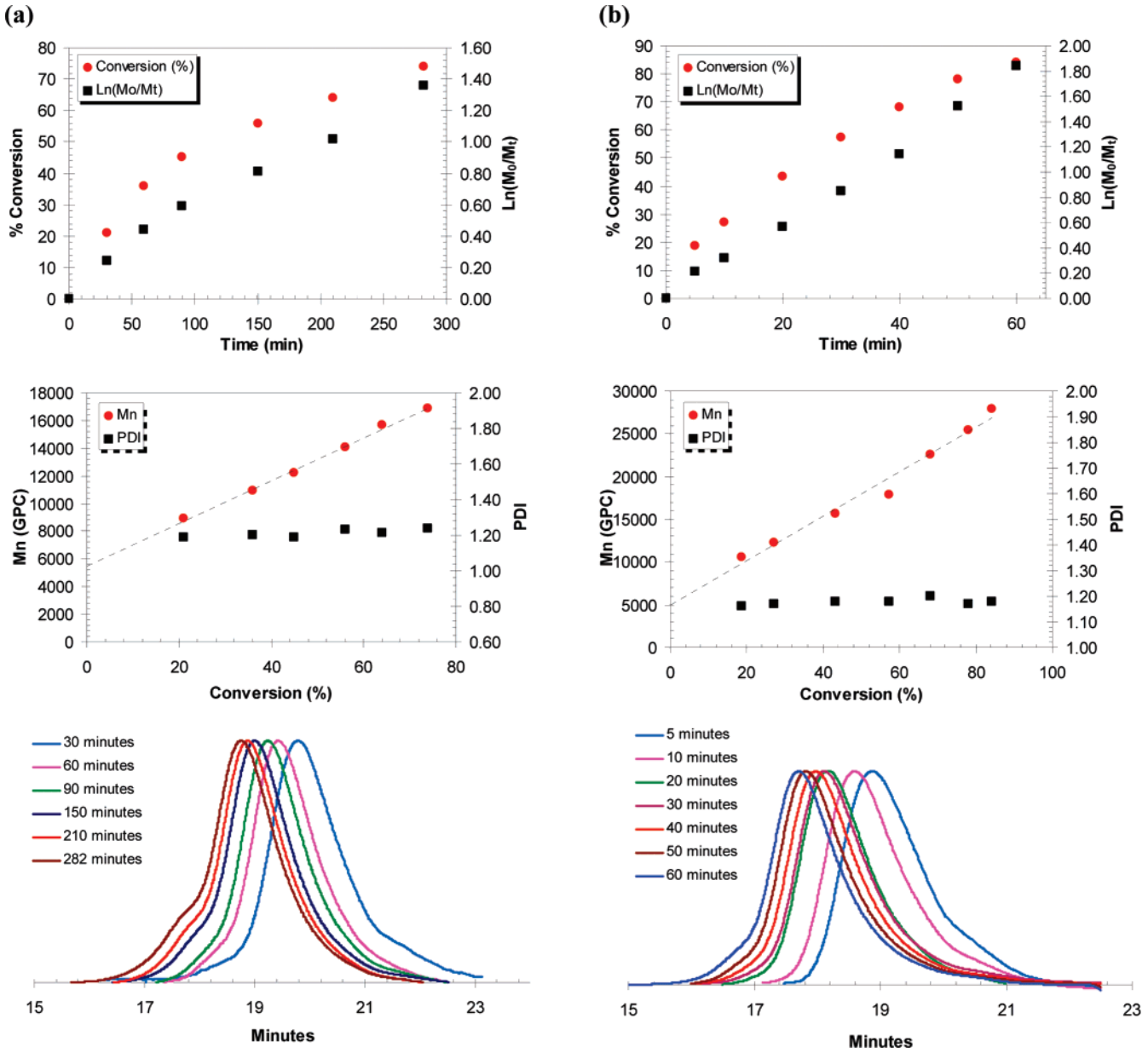
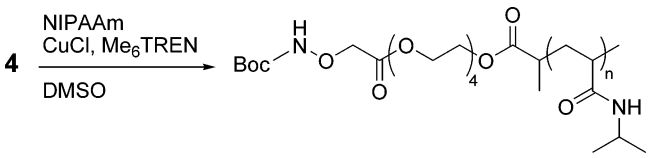


Figure 1. ATRP of (a) PEGMA and (b) HEMA. Kinetic plot determined from ^{1}H NMR in $MEOH_{d4}$ (top); evolution of molecular weight plot determined from GPC (middle); GPC traces (bottom). Reaction conditions: monomer:**2**:CuBr:bipy = 50:1:1:2, methanol, 22 °C.

Scheme 3. ATRP of NIPAAm



increased linearly with conversion and the PDIs remained low. The final PDI of polyPEGMA was 1.24 and for polyHEMA was 1.18.

Copper-mediated ATRP of NIPAAm was performed using a 1:2:2 molar ratio of **4**:CuCl:Me_6TREN in DMSO at 22 °C with a 1:50 molar ratio of **4**:NIPAAm (Scheme 3). Aliquots were withdrawn periodically for kinetic analysis by ^{1}H NMR. As is typically observed for ATRP of acrylamides,[47,52−54] curvature of the pseudo-first-order kinetic trace was seen (Figure 2a). Catalyst inactivation due to monomer coordination, low values of equilibrium constants, and displacement of the terminal halide are factors that complicate the ATRP of acrylamides. Various ratios of copper to initiator were tested in order to improve the linearity of the kinetic plot; 2 equiv of catalyst to initiator proved to be the most effective, providing conversions greater than 80%. We had previously observed that addition of copper(II) was beneficial for the polymerization of polyNIPAAm from a biotinylated initiator;[47] however in this case, copper(II) slowed the rate of reaction but did not improve the linearity of kinetic trace. The molecular weight increased linearly with conversion, and the GPC traces of the resultant polymers were symmetrical (Figure 2b). These conditions provided polyNIPAAm with remarkably narrow molecular weight distributions; the resulting polymer had a PDI of 1.11.

The ability to produce different molecular weights of the well-defined polymers was explored next. Each monomer was polymerized utilizing monomer-to-initiator ratios of 25 to 1, 50 to 1, and 100 to 1. In this way, increasing M_n values were obtained (Table 1). The molecular weights were consistently larger than theoretically expected for all of the polymers. The discrepancy could have been due in part to comparison to nonauthentic polyMMA standards to calculate the M_n values. Initiator inefficiency, particularly for the HEMA polymerization, could also have been a factor. The polymers formed in each case had narrow molecular weight distributions (Table 1), demonstrating that well-defined polymers were prepared by this method. With the exception of PEGMA (M/I = 100/1) the conversions were also high.

Deprotection of Initiator 4 and Oxime Formation. As a model system for protein−polymer conjugation, the Boc of initiator **4** was removed and oxime bond formation with a small molecule aldehyde was verified. Deprotection of **4** proceeded for 30 min in a 50% solution of trifluoroacetic acid in chloroform. After removal of the solvent, ^{1}H NMR analysis (Figure 3a,b) showed complete deprotection of the end group as evident by the disappearance of the *tert*-butyl protons at 1.42 ppm. Further, the methylene protons α to the aminooxy group shifted from 4.40 to 4.67 ppm. Coupling of the deprotected initiator to butyraldehdye was undertaken. ^{1}H NMR analysis of the product confirmed oxime formation by the characteristic peaks of the N=CH protons at 7.48 and 6.70 ppm in a 3:2 ratio of *syn* to *anti*. The model system demonstrated that deprotection was straightforward and that conjugation to aldehydes was facile.

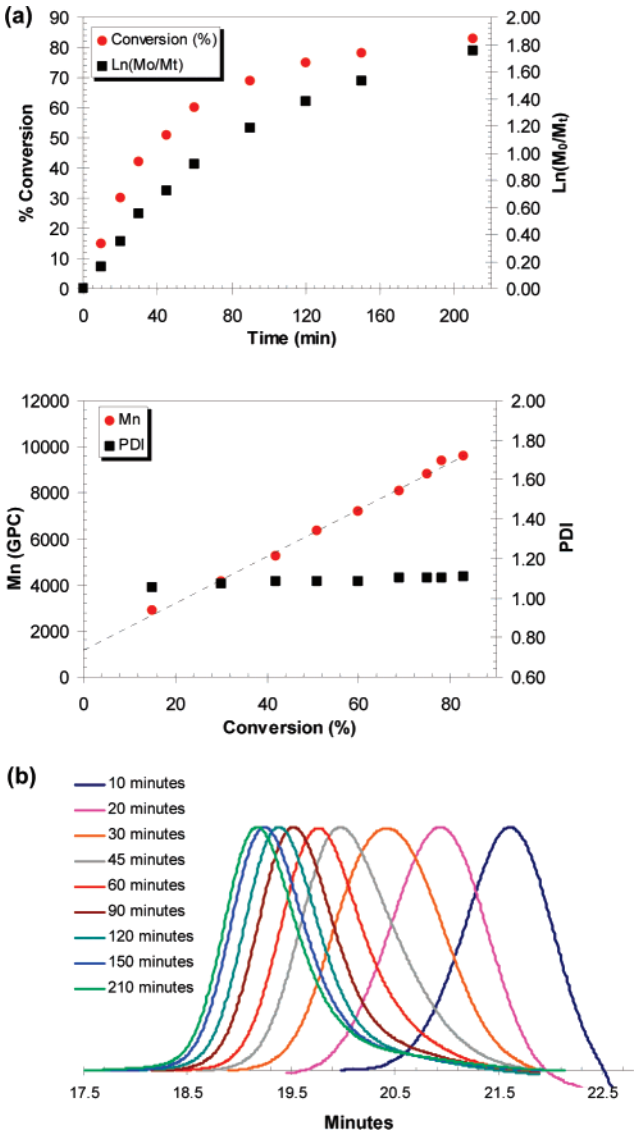


Figure 2. ATRP of NIPAAm. (a) Kinetic plot determined from ^{1}H NMR in $DMSO_{d6}$ (top) and evolution of molecular weight plot determined from GPC (bottom). (b) GPC traces. Reaction conditions: NIPAAm:**4**:CuCl:Me_6TREN = 50:1:2:2, DMSO, 22 °C.

Table 1. Targeted Molecular Weights of Polymers Using Initiators 2 and 4

entry	monomer[a]	monomer/initiator	time (h)	conversion (%)	$M_{n(theory)}$	$M_{n(SEC)}$[b]	PDI
1	PEGMA	25:1	4	77	5770	12340	1.26
2	PEGMA	50:1	4.3	74	11100	16880	1.24
3	PEGMA	100:1	7	50	15000	23340	1.26
4	HEMA	25:1	1	95	3090	16 700	1.18
5	HEMA	50:1	1	84	5920	27900	1.18
6	HEMA	100:1	4	91	11840	39900	1.24
7	NIPAAm	25:1	4.6	83	2630	6260	1.11
8	NIPAAm	50:1	3.5	83	4530	9600	1.11
9	NIPAAm	100:1	6	82	9280	16580	1.12

[a] Entries 1−6 employed initiator **2** using the catalyst system CuBr/bipy in MeOH and entries 7−9 employed initiator **4** using the catalyst system CuCl/Me_6TREN in DMSO. [b] Molecular weight was determined by GPC using polyMMA standards (0.1 M LiBr in DMF, 0.8 mL/min).

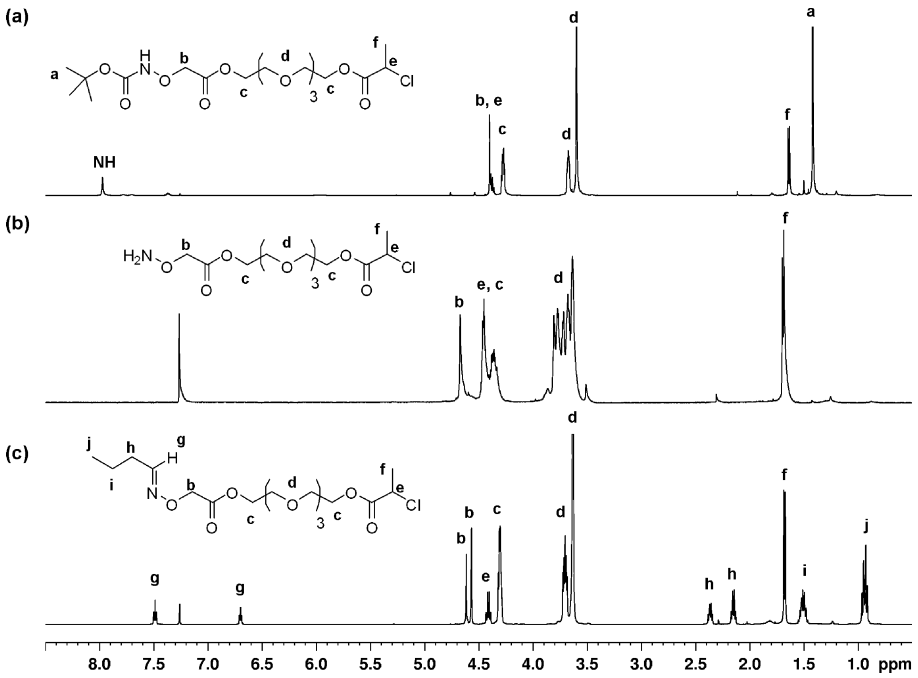


Figure 3. ^{1}H NMR in $CDCl_3$ of (a) Boc-protected **4**, (b) deprotected **4**, and (c) product of butyraldehdye and deprotected **4**.

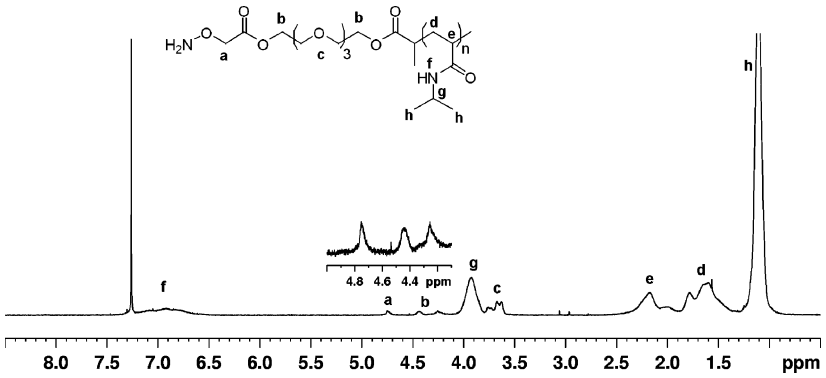


Figure 4. ^{1}H NMR in $CDCl_3$ of deprotected α-aminooxypolyNIPAAm.

Bioconjugates via Oxime Ligation. Bioconjugate formation was demonstrated with polyNIPAAm. This "smart" polymer undergoes a reversible hydrophobic collapse above its lower critical solution temperature (LCST) of 32 °C. As a result, polyNIPAAm conjugates are incredibly useful in biotechnology for applications including protein switches, affinity separations, and in microfluidic devices.[51,55−57] Trifluoroacetic acid deprotection of the polymer end group was accomplished in the same manner as for the model initiator system. Complete removal of the Boc group was evident by the disappearance of the *tert*-butyl protons at 1.42 ppm and the characteristic shift of the α-aminooxy protons to 4.75 ppm in the ^{1}H NMR spectrum (Figure 4). In order to verify end-group reactivity, the polymer was then coupled to an aldehyde-functionalized fluorescent nanosphere. The polymer was dissolved in acetonitrile and mixed with an aqueous solution of the FluoSpheres aldehyde−sulfate spheres (20 nm, yellow-green) for 12 h in the dark. After lyophilization, the crude product was analyzed by GPC. A significant shift of the nanosphere to shorter retention times (higher molecular weights) was observed after incubating with the polymer (Figure 5) which indicated formation of a polyNIPAAm−nanosphere conjugate. Taken together these data illustrate that after the Boc group was removed the aminooxy end-functionalized polymer was available and able to react with aldehydes via oxime bond formation. Thus, bioconjugate formation was pursued next.

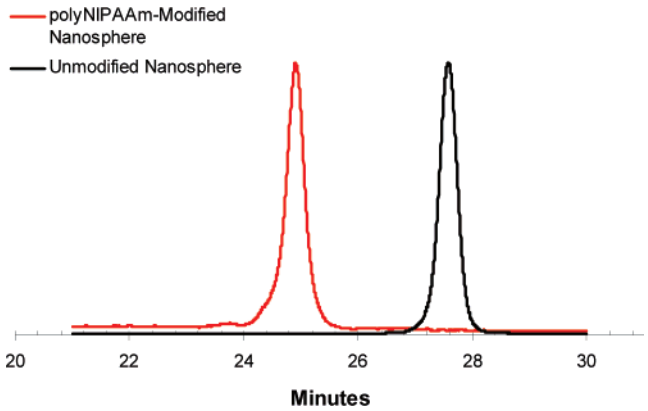
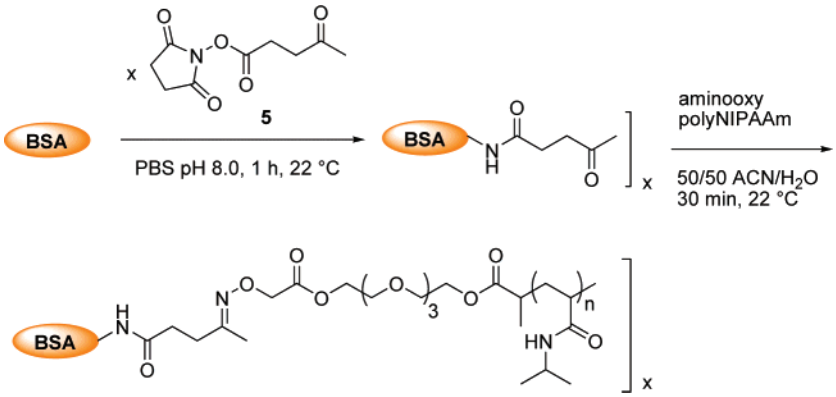


Figure 5. GPC trace of aminooxypolyNIPAAm conjugated to an aldehyde-functionalized fluorescent nanosphere. Black line: unmodified FluoSphere aldehyde−sulfate spheres (20 nm, yellow-green); Red line: oxime-linked polyNIPAAm−FluoSphere nanosphere. GPC performed in THF (0.8 mL/min) and monitored at 500 nm.

Bioconjugate formation by conjugation of the aminooxy polymer to a ketone-modified protein was explored. Ketones can be installed into proteins by modification of lysine

Scheme 4. Synthesis of BSA−PolyNIPAAm Conjugate



residues.[43] This approach can be site-specific by incorporating N^{ϵ}-levulinyl lysines by native chemical ligation. Kochendoerfer and co-workers employed this method to produce a PEGylated synthetic erythropoiesis protein (SEP) therapeutic.[34] In this case, α-functional *O*-hydroxylamine branched PEGs were conjugated to SEP with two N^{ϵ}-levulinyl residues, and the result was a site-specific and well-defined conjugate. Unlike typical PEGylated therapeutics, this homogeneous conjugate exhibited equivalent bioactivity to native erythropoietin. Thus, conjugation to levulinyl-modified proteins is an excellent way to form bioactive conjugates.

For ease of synthesis, we prepared a levulinyl-modified bovine serum albumin (BSA) by coupling *N*-hydroxysuccinimidyl ester levulinate (**5**) with BSA. Although this particular approach would result in heterogeneous modification of BSA, what is learned should be readily translatable to homogeneous conjugates. *N*-Hydroxysuccinimidyl levulinate **5** was prepared in one step using EDC-mediated coupling of levulinic acid with *N*-hydroxysuccinimide in 80% yield. Coupling of **5** with BSA in phosphate buffer at pH 8.0 formed the levulinyl lysine-modified BSA (Scheme 4). The attached levulinyl groups were targeted for bioconjugate synthesis via oxime bond formation.

Bioconjugation was performed by mixing deprotected aminooxy polyNIPAAm with the levulinyl-modified BSA in a 50/50 v/v solution of acetonitrile/water (Scheme 4). Analysis by SDS-PAGE (Figure 6) clearly showed a shift to high molecular weight after conjugation with polyNIPAAm (lane 1 vs lane 5). To confirm that the polymer was covalently attached to the protein, the reaction mixture was subjected to thermal precipitation. Gentle heating above 32 °C caused the polymer to phase separate from solution, and SDS-PAGE of the precipitated product (lane 6) was similar to before precipitation (lane 5). This demonstrated that the hydrophobic collapse of the polyNIPAAm when heated above the LCST also caused the protein to precipitate, indicating covalent attachment. Two control studies were also performed. In the first experiment, levulinyl-modified BSA was mixed with protected polyNIPAAm, and in the second experiment unmodified BSA was mixed with deprotected polymer. In both cases the SDS-PAGE bands were exactly the same as unmodified BSA (lanes 2 and 3, respectively, vs lane 4). These results demonstrated that the presence of both the ketone and the aminooxy was necessary, suggesting that conjugate formation is chemospecific and via oxime bond formation.

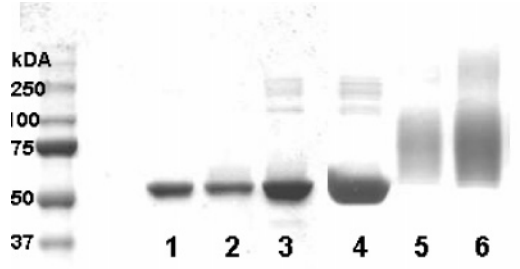


Figure 6. SDS-PAGE of BSA−polyNIPAAm conjugates and control studies: lane 1, levulinyl-modified BSA; lane 2, levulinyl-modified BSA mixed with Boc-protected polyNIPAAm; lane 3, unmodified BSA mixed with deprotected aminooxy polyNIPAAm; lane 4, unmodified BSA; lane 5, polyNIPAAm−BSA crude; lane 6, polyNIPAAm−BSA isolated by thermal precipitation.

Conclusions

In this report we describe a straightforward approach to prepare polymers with narrow polydispersity and α-aminooxy functionality. PolyPEGMA, polyHEMA, and polyNIPAAm were prepared in a range of molecular weights with narrow polydispersities utilizing Boc-aminooxy ATRP initiators. Oxime bond formation between levulinyl-modified BSA and aminooxy end-functionalized polyNIPAAm was demonstrated. The "click" reaction occurred rapidly and without additional reagents. The resulting conjugate exhibited thermal sensitivity characteristic of the attached polyNIPAAm. In this study we demonstrated conjugation to N^{ϵ}-levulinyl lysines. Yet there exists a variety of other ways to modify proteins with reactive carbonyls, and we are currently exploring these for conjugate formation. ATRP is an excellent technique to synthesize a wide range of well-defined semitelechelic polymers, and oxime bond formation is a chemoselective reaction. We anticipate that this method will be generally useful to prepare a large variety of well-defined polymer−protein conjugates for applications in medicine, biotechnology, and nanotechnology.

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