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Well-Defined Aminooxy Terminated *N*-(2-Hydroxypropyl) Methacrylamide Macromers for Site Specific Bioconjugation of Glycoproteins

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ABSTRACT: Syntheses and characterization of aminooxy terminated polymers of *N*-(2-hydroxyproyl) methacrylamide (HPMA) of controlled molecular weight and narrow molecular weight distribution are presented here. Design of a chain transfer agent (CTA) containing *N*-*tert*-butoxycarbonyl (*t*-Boc) protected aminooxy group enabled us to use reversible addition−fragmentation (RAFT) polymerization technique to polymerize the HPMA monomer. An amide bond was utilized to link the aminooxy group and the CTA through a triethylene glycol spacer. As a result, the aminooxy group is linked to the poly(HPMA) backbone through a hydrolytically stable amide bond. By varying the monomer to initiator ratios, polymers with targeted molecular weights were obtained. The molecular weights of the polymers were determined by gel permeation chromatography (GPC) and mass spectrometry (ESI and MALDI-TOF). The *t*-Boc protecting group was quantitatively removed to generate aminooxy terminated poly(HPMA) macromers. These macromers were converted to rhodamine B terminated poly(HPMA) by reacting *N*-hydroxysuccinimide (NHS) ester of the dye with the terminal aminooxy group to form a stable alkoxyamide bond. Utility of these dye-labeled polymers as molecular probes was evaluated by fluorescence microscopy by studying their intracellular uptake by renal epithelial cells. These aminooxy terminated poly(HPMA) were also tested as biocompatible carriers to prepare chemoselective bioconjugates of proteins using transferrin (Tf) as the protein. Oxidation of the sialic acid side chains of Tf generated aldehyde functionalized protein that was reacted with aminooxy terminated poly(HPMA), which resulted in protein−polymer bioconjugates carrying oxime linkages. These bioconjugates were characterized by gel electrophoresis and MALDI-TOF mass spectrometry.

INTRODUCTION

There has been significant interest in recent years in discovery and development of biocompatible polymers for numerous biomedical applications.[1−3] These polymers have shown promises as delivery vehicles for human therapeutics (i.e., proteins, antibodies, and nucleic acids), as imaging probes for medical diagnostics and biosensor applications, to mention a few.[4−7] For therapeutic applications, polymer conjugates of therapeutic proteins result in longer plasma circulation times and increased accumulation of the bioconjugates in targeted tissues. This leads to improvements in overall pharmacokinetic profiles and therapeutic indices of these pharmacological agents.

Poly(ethylene glycol) (PEG) derivatives with terminal functional groups have been the polymers of choice for therapeutic applications to prepare polymer−drug and polymer−protein conjugates.[8,9] Several conjugates of PEG with therapeutic proteins and small molecule drugs have been synthesized over the past decade including FDA approved products.[10−12] There has been continuing research interest in discovering a new generation of hydrophilic functional polymers with controlled molecular weight and well-defined architectures to expand the scope of polymer based therapies and biomaterials.[13−15] Polymers derived from functional vinyl monomers such as 2-hydroxyethyl methacrylate (HEMA) and *N*-(2-hydroxypropyl)-methacrylamide (HPMA) are two such systems. In particular, HPMA derived polymers are water-soluble, nonimmunogenic, and biocompatible. Therefore, these polymers have been studied quite extensively over the past several years as carriers for developing therapeutic agents.[16−18] For example, HPMA based polymeric drugs have exhibited interesting pharmacological properties as anticancer agents and some of these compounds have progressed to human clinical trials.[19−21]

In general, biomedical properties of HPMA based polymers have been studied using polymers that have been synthesized by classical free radical polymerization.[22−24] The major shortcomings of classical free radical polymerization include lack of control over polymer molecular weight and molecular weight distribution as well as poorly defined molecular architectures. The ill-defined molecular properties of polymers can lead to heterogeneity in pharmacokinetic properties of the resulting polymeric drugs that might present a major barrier toward clinical development of these polymer derived pharmacological agents. Stringent regulatory requirements for therapeutic agents for human use mandate controlling

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molecular properties of polymer based therapies in a reproducible manner.[25]

The living radical polymerization (LRP) technique has been utilized in recent years to synthesize (meth)acrylic polymers of controlled molecular weight with narrow polydispersity.[26−28] By optimizing the initiator systems appropriately, polymers of different architectures and polymers containing terminal functional groups have been synthesized.[29,30] Two of the most widely used LRP techniques are atom transfer radical polymerization (ATRP) and reversible addition−fragmentation chain transfer (RAFT) polymerization. These two polymerization techniques have been utilized to prepare a variety of biocompatible polymers, which have been evaluated as carriers for the syntheses of conjugates of polymers with various biologically active agents.[31−33] With regard to the synthesis of bioactive HPMA polymers of controlled molecular weight and narrow polydispersity for these bioconjugation applications, reported approaches involve synthesis of active ester precursor polymers such as poly(*N*-methacryoxy succinimide) and poly(pentafluorophenyl methacrylate) of controlled molecular weight by ATRP and their subsequent chemical modification to synthesize appropriate poly(HPMA) derivatives.[34,35] However, such a postpolymerization chemical modification method leads to incomplete reaction and/or side reactions, which results in polymers with structural and compositional heterogeneity.[36] RAFT polymerization technique has recently been optimized for direct controlled polymerization different (meth)acrylamide based monomers, including HPMA).[37−39]

In light of these earlier studies, we decided to design and synthesize appropriate functional chain transfer agents (CTA), which can be used to synthesize poly(HPMA) macromers (bearing terminal functional groups) of controlled molecular weight and narrow polydispersity. The aminooxy group has been considered as a versatile handle to synthesize protein−polymer bioconjugates via oxime bonds.[40] The oxime linkage between the aminooxy group and carbonyl group (aldehyde or ketone) is formed under broader pH range compared to other covalent bond like Schiff base and hydrazone linkages.[41−43] The conjugated ligand can be released in its intact form from the carrier at physiologically relevant conditions (e.g., within intracellular lysosomes and endosomes).[44] These attractive features of oxime linkage have been utilized to synthesize conjugates of proteins and antibodies with various carbohydrate based targeting ligands and polymers.[45−48] By using LRP technique, Maynard and co-workers have synthesized aminooxy terminated poly(*N*-isopropylmethacrylamide) and used this polymer to prepare protein bioconjugates by oxime linkage.[49,50] Esser-Kahn and Francis have synthesized random copolymer of HPMA containing pendant aminooxy group by classical free radical polymerization, and the resulting polymers were used to prepare protein−polymer hybrid materials through oxime ligation.[51]

In order to explore new generation of well-defined aminooxy functional polymers as novel carriers for oxime ligation of biomolecules, we set out synthesize aminoxy terminated poly(HPMA) of controlled molecular weight and narrow polydispersity by RAFT polymerization of HPMA monomer. In previously reported aminooxy terminated methacrylamide polymers, the aminooxy group was linked to the polymer chain through an ester linkage.[49,50] Relative instability of ester groups toward hydrolysis and their sensitivity toward non-specific esterase enzymes are potential shortcomings of these polymers to prepare bioconjugates of therapeutic agents. In order to overcome this liability, our design of aminoxy chain transfer agent (CTA) for RAFT polymerization involved replacement the ester bond with an amide bond. Aminooxy terminated HPMA polymers with varying molecular weights were synthesized and characterized by NMR, IR, GPC, and mass spectrometry. The reactivity of the terminal aminooxy group in these poly(HPMA) macromers was evaluated by synthesizing fluorescent dye labeled polymers (linked via amide bond), which produced well-defined polymeric fluorescent probes with a single dye molecule per polymer chain. Furthermore, oxime ligation mediated bioconjugation efficiency of these polymers with glycoproteins was studied using transferrin as the model protein.

EXPERIMENTAL SECTION

Materials. Unless mentioned otherwise, all chemical were obtained from Sigma Aldrich and were used as received. All solvents were anhydrous grade. 2,2′-Azobis(2-isobutyronitrile) (AIBN) was purified by recrystallization from ethanol twice and dried under reduced pressure and stored at −10 °C until use. 4,4′-Azobis(4-cyanopentanoic acid) (V-501) was purchased from Wako Pure Chemicals Industries (Tokyo, Japan). Deionized (DI) water was obtained using a Millipore Milli-Q reverse osmosis/filtration unit. HMPA monomer was synthesized by slight modification of the literature procedure.[52]

Briefly, to 10.0 g (137.7 mmol) of 1-amino-2-propanol dissolved in 100 mL of anhydrous dichloromethane was added a prechilled solution of 9.77 g (63.4 mmol) of freshly distilled methacrylic acid anhydride dissolved in 50 mL of dichloromethane in a dropwise manner over 30 min at 0 °C. After removal of the solvent under reduced pressure, the crude product was purified by silica gel column chromatography using a gradient of ethyl acetate:hexane as eluent. The column purified product was further recrystallized from acetone and stored at −20 °C.

Instrumentation. Flash column chromatography was performed on a Teledyne Isco Combiflash Companion system. ^{1}H NMR spectra were recorded on a Bruker Spectrometer operating at 400 MHz using either $CDCl_3$ or DMSO-d_6 as solvents. Infrared spectra were taken using a Bruker Tensor 27 FTIR spectrometer. UV−visible spectra were obtained using Varian Cary 50 Probe UV−visible spectrophotometer. Fluorescence spectra were recorded using a Varian Cary Eclipse fluorescence spectrophotometer. LC-MS measurements were carried out with Agilent 1100 series system fitted with a variable wavelength diode array detector operating in the wavelength range of 210−400 nm. X-bridge phenyl C-18 column and acetonitrile−water gradient as mobile phase were used for operation. GPC measurements were carried out using Agilent 1200 series HPLC using DMF containing 100 mM LiBr as the mobile phase. MALDI-TOF mass spectra were recorded using Applied Biosystems Voyager DE-STR MALDI-TOF instrument. For MALDI-TOF analysis, samples were prepared by diluting 1:1 in sinapinic acid matrix (supersaturated in 50% ACN, 1% TFA) and spotting 1 μL of the mixture per sample. MALDI-TOF data were collected using a laser intensity of 2800 over a mass range of 2000−200 000 amu in linear mode. An average of 500 shots per spectrum was used to give a complete picture of the sample with high intensity and the spectra were calibrated against the $[M+H]^+$ and $[M+2H]^{2+}$ ions of bovine serum albumin. Electron spray ionization mass spectrometry (ESI MS) was carried out using Orbitrap Velos instrument (Thermofisher Scientific, Bremen) adjusted to operate at 200

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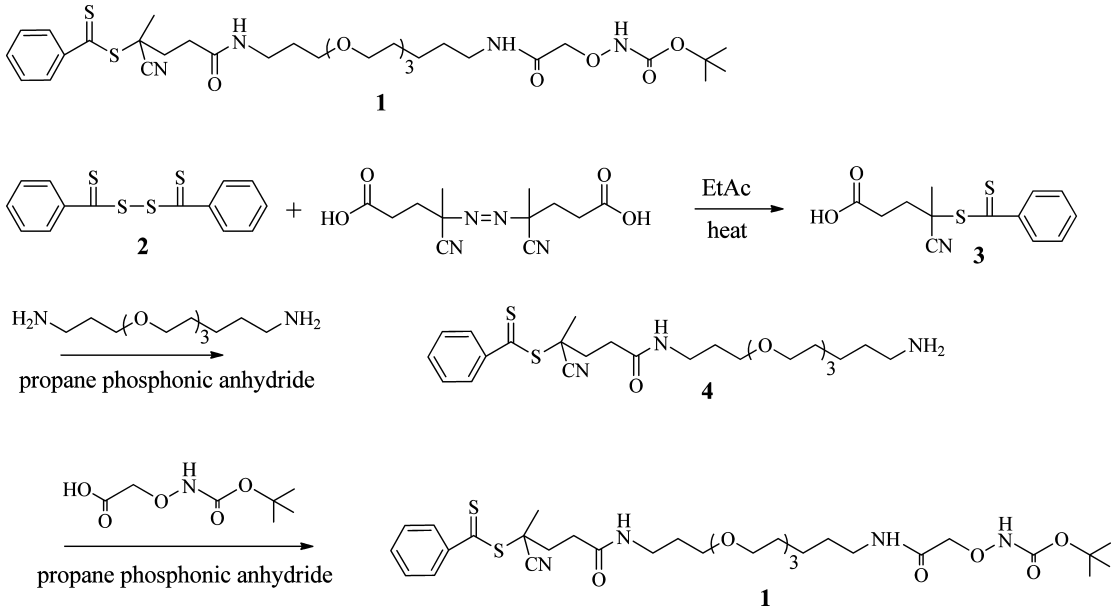
Scheme 1. Synthesis of Aminooxy Functional CTA Reagent for RAFT Polymerization



000 resolution at m/z of 400. Polymer samples were dissolved in water:acetonitrile (1:1, v/v) and were analyzed by direct infusion at 5 μL/minute. Deconvolution of the resulting isotope resolved spectra was performed by the Xtraxt algorithm (Thermofisher Scientific, Bremen). SDS-PAGE was carried out using 4−12% Bis-Tris Novex NuPAGE gradient gel (Invitrogen Life Technologies, Grand Island, New York). Protein contents of Transferrin−HPMA conjugates were determined using the Bio-Rad RC-DC assay (Bio-Rad Life Science, Hercules, California) according to the manufacturer's protocol. Protein (10 μg) was loaded to the gel and separated in NuPAGE MOPS-SDS running buffer (Invitrogen Life Technologies, Grand Island, New York) at 100 V for ∼4 h. The SeeBlue Plus2 Pre-Stained standard (Invitrogen Life Technologies, Grand Island, New York) was used for molecular weight determination. After separation gels were rinsed with DI water for 5 min, stained with LI-COR IRDye Blue (LI-COR Biosciences, Lincoln, Nebraska), and subsequently imaged using LiCor Odyssey Image Scanner (LI-COR Biosciences, Lincoln, Nebraska). Fluorescent microscopy to study cell uptake of the rhodamine B labeled polymers was carried out using Olympus IX71 microscope and Q-imaging RETIGA EXi camera (QImaging, British Columbia, Canada) run by *Image Pro Plus 6.2* software (MediaCybernetics, Bethesda, Maryland). Mouse renal epithelial cells were seeded on 2 Lab-Tek Permanox 8-Chamber Nunc slides (Thermo Fisher Scientific, Rochester, New York) at 1×10^5 cells/chamber and allowed to grow for 48 h. prior to adding the conjugates. At t = 0, cells were washed once with PBS and new medium containing increasing concentrations (3, 10, 30, and 100 μM) of the dye labeled polymers were added. At t = 30 min and t = 240 min, chamber slides were washed with PBS (3 × 5 mL) and subsequently fixed in 4% PFA/PBS for 20 min at RT. Slides where rinsed once more and incubated with DAPI/PBS for 5 min, washed three times in PBS prior to mounting in Prolong Gold antifade mounting medium (Invitrogen Life Technologies, Grand Island, New York), and allowed to cure overnight. Exposures were matched for all images at 118 ms (red) and 31 ms (blue) using an intensity filter. Background correction and collage were performed using Photoshop CS2 (Adobe, CA).

Synthesis of *tert*-Butoxylcarbonyl (*t*-Boc) Protected Aminooxy CTA 1. The RAFT polymerization reagent **1** was synthesized according to the synthetic procedure shown in Scheme 1. All the reactions were carried out under nitrogen atmosphere.

*Synthesis of Amine Terminated CTA **4**.* To a solution of 1 g (3.58 mmol) of 4-Cyanopentanoic acid Dithiobezoate **3**[53] and 0.796 g (7.876 mmol) of triethylamine in 10 mL of anhydrous tetrahydrofuran (THF) was added 2.27 g (7.16 mmol) of propane phosphonic acid anhydride (as 50% (w/w) solution in ethyl acetate). The reaction mixture was stirred at 0 °C for 10 min. Subsequently, 1.18 g (5.36 mmol) of 1,4,7,10-trioxa dodecadiamine dissolved in 15 mL of THF added to the above cooled solution. The resulting reaction mixture was stirred at 0 °C for 30 min and at RT for 5 h. After removing the solvent under reduced pressure, the residue was dissolved in 50 mL of ethyl acetate and extracted with DI water (2 × 50 mL) followed by brine (25 mL). After drying the organic layer over anhydrous magnesium sulfate, it was evaporated to dryness under reduced pressure. The residue was used in the next step without further purification.

*Synthesis of Aminooxy Terminated CTA **1**.* In an oven-dried 100 mL round bottomed flask were placed 10 mL of anhydrous THF, 0.19 g (0.997 mmol) of *N*-*t*-Boc aminooxy acetic acid, and 1.85 g (1.83 mmol) of triethylamine. To this reaction mixture was added 0.528 g (0.166 mmol) of ethyl acetate (50%) solution of propane phosphonic acid anhydride. After stirring the reaction mixture at 0 °C for 5 min, 0.4 g (0.83 mmol) of crude **4** was added. The resulting reaction mixture was stirred at 0 °C for 30 min and at RT for 6 h. After removing the solvent, the crude residue was purified by preparative HPLC using ethyl acetate:hexane as the mobile phase yielding 126 mg (26%) of **1**. Purity (HPLC) = 96.4%. ^{1}H NMR ($CDCl_3$): 10.2 (s, 1H, ONH), 8.05 (s, 2H, NH), 7.8 (2H, t, Ar-H), 7.6 (t, 1H, Ar-H), 7.4 (d, 2H, Ar-H), 4.2 (s, 2H, $COCH_2$-O), 3.6 (m, 8H, OCH_2), 3.4 (t, 4H, OCH_2-CH_2O), 3.2 (t, 4H, $CONHCH_2$), 2.5 (t, 2H, CN−C-CH_2), 2.3 (t, 2H,

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$COCH_2$-CH_2), 1.9 (s, 3H, CN-C-CH_3), 1.6 (m, 4H, CH_2-CH_2-CH_2), 1.4 (s, 9H, $C(CH_3)_3$). Mass spectrum (LC-MS) calcd. 581.8 *m/z*; observed 582.4 *m/z*.

Representative Example of RAFT Polymerization of HPMA Using 1 as the CTA. All manipulations for the polymerization reactions were carried out under ultra-high-purity argon atmosphere. In a 25 mL oven-dried round bottomed flask were placed 1.0 g (6.9 mmol) of HPMA monomer and 5 mL of anhydrous dimethyl sulfoxide (DMSO). To this solution were added a solution of 22.8 mg (0.035 mmol) of CTA **1** and 1.15 mg (0.007 mmol) of AIBN dissolved in 1 mL of DMSO. The reaction mixture was purged with argon for 30 min and it was subsequently stirred at 80 °C for 1 h. The polymerization reaction was quenched by freezing the reaction mixture below −40 °C with liquid nitrogen. The reaction mixture was poured into 100 mL of ethyl acetate and the resulting precipitate was isolated by filtration. The solid was redissolved in methanol and precipitated from ethyl acetate. The process was repeated twice and the resulting precipitate was dried under reduced pressure yielding 400 mg of the polymer as an off-white solid.

Removal of *t*-Boc Protecting Group. To a solution containing 16 mL of chloroform (16 mL), ethanol (2 mL), and trifluoroacetic acid (5 mL) was added the *t*-Boc protected aminooxy terminated poly(HPMA) (250 mg). The reaction mixture was allowed to stir at RT for 24 h under nitrogen. After removing the solvent under reduced pressure, the residue was dissolved in 5 mL of DI water and was dialyzed using a 2000 molecular weight cutoff membrane. After completion of dialysis, the polymer solution was lyophilized yielding 190 mg of the deprotected polymer as white solid. ^{1}H NMR and FTIR analysis showed complete removal of the *t*-Boc protecting group.

Synthesis of Rhodamine B Terminated Poly(HPMA). In a 10 mL, light-blocking glass vial were placed 20.3 mg of low free aminooxy group terminated low molecular weight polymer and 1 mL of anhydrous DMSO, followed by the addition of triethylamine (5 μL). While stirring, 5.2 mg of rhodamine B succinimidyl ester (Invitrogen, Eugene, Oregon) dissolved in 1 mL of anhydrous DMSO was added. The resulting reaction mixture was stirred in the dark at RT for 96 h. The reaction mixture was extensively dialyzed in DI water using a 3500 molecular weight cutoff membrane to remove any unreacted dye. The polymer solution was finally passed through a 0.2 μm syringe filter and the solution was lyophilized yielding 13.1 mg of a light pink colored solid. The dye labeled polymer was characterized by UV−visible and fluorescent spectrometry.

The corresponding rhodamine B terminated derivative of the high molecular weight aminooxy terminated poly(HPMA) was synthesized in an analogous manner by adjusting the stoichiometry based on the polymer's molecular weight.

Synthesis of poly(HPMA)−Transferrin (Tf) Conjugates. All polymer−protein conjugates were synthesized following a published procedure for the synthesis of polymer-Tf conjugate via hydrazine linker. [81] We have implemented modifications appropriate for our polymer and used polymer and Tf at different ratios. Thus, iron free human Tf (8 mg, 0.1×10^{-3} mmol) was dissolved in 4 mL of sodium acetate buffer (0.1 M, pH 5.5) and allowed to mix gently for 10 min at 4 °C. Sodium *meta*-periodate (1 mg, 4.7×10^{-3} mmol) was added to the Tf solution, the vial was wrapped with aluminum foil, and the solution mixed for an additional 30 min at 4 °C. The oxidized Tf solution was concentrated by centrifugal filtration (30 kDa molecular weight cutoff, Amicon Millipore, Billerica, MA) to 1 mL volume and buffer exchanged (3 volumes) with 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) buffer (0.1 M, pH 7.2). Concentrated Tf solution was transferred into a new vial and HEPES buffer was added to reach a final volume of 4 mL. To the protein solution was added 1.6 mg of the low molecular weight (5000 Da) aminooxy terminated poly(HPMA) as 5% (w/v) solution in HEPES buffer. The resulting reaction mixture was allowed to incubate at RT for 18 h with gentle mixing. Subsequently, the polymer-Tf conjugate was purified by centrifugal filtration of 30 kDa molecular weight cutoff using 3 volumes of HEPES buffer. Protein concentration was determined by UV spectroscopy (λ_{max} = 280 nm) and mass recovery was 90%. Conjugation of Tf to poly(HPMA) was analyzed by SDS-PAGE and MALDI-TOF mass spectrometry.

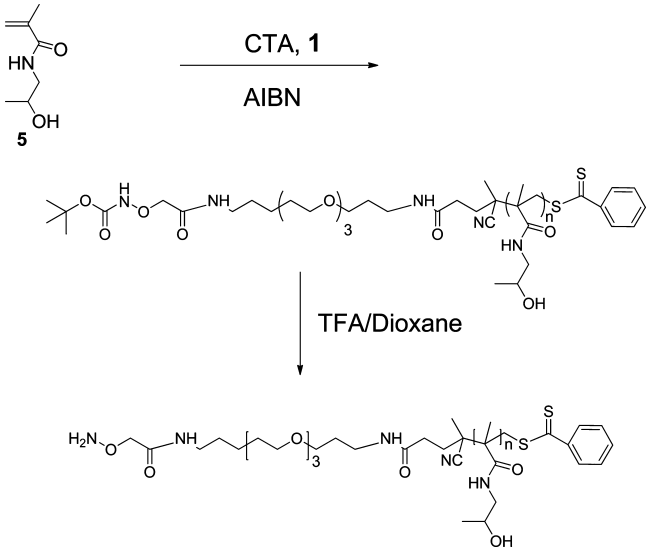
RESULTS AND DISCUSSION

Synthesis of Aminooxy Functional CTA for RAFT Polymerization. The key component for the synthesis of functional group terminated polymers by RAFT polymerization is the appropriate functional initiator (CTA). For the synthesis of aminooxy terminated poly(HPMA), design of the CTA structure includes the presence of aminooxy group in a protected form. The protecting group is selected so that it can be removed quantitatively under mild reaction conditions without affecting physical and chemical properties of the polymer. Furthermore, an appropriate spacer group between the aminooxy group and polymer chain is a requisite to overcome any potential steric hindrance to its chemical reactivity, in particular, during conjugation of large molecules such as proteins. Finally, the linker connecting the aminooxy moiety to the remaining portion of the CTA should be stable to hydrolysis in the circulation for a prolonged period of time. After taking these criteria into consideration, compound **1** was designed as the target CTA. The *t*-Boc protecting group is known to be stable during the RAFT polymerization conditions, yet it can be removed selectively and quantitatively from the polymer chain.[50,54] The hydrophilic triethylene glycol spacer will not affect the water solubility of the poly(HPMA). It provides adequate spacing between the polymer chain and aminooxy group so that reactivity of the latter toward various reagents including protein-bound carbonyl groups is not impaired. Finally, the amide bond linking the aminooxy and thioester moieties to triethylene glycol spacer is expected to be substantially more stable than an ester bond.

Scheme 1 shows the synthetic steps for the preparation of CTA reagent **1**. Reaction of the dithiobenzoic acid disulfide (**2**) with 4,4′-azobis-(cyanopentanoic acid) produced the carboxylic acid functionalized CTA, 4-cyanopentanoic acid dithiobezoate (**3**). Compound **3** was isolated with purity greater than 98% by column chromatography followed by recrystallization from benzene with 60% yield. Compound **3** was subsequently reacted with 1,4,7,10-trioxadodecadiamine using propane phosphonic acid anhydride as the coupling agent to produce the amino terminated CTA, **4**. Without any further purification, compound **4** was reacted with *t*-Boc protected aminooxyacetic acid in the presence of propane phosphonic acid anhydride and triethylamine to form the target aminooxy functional CTA, **1**. Attempts to synthesize compounds **4** and **1** by using other coupling agents (which have been used in peptide chemistry) did not yield the desired products.[55] The CTA, **1** was isolated in pure form by preparative HPLC and its structure was confirmed by ^{1}H NMR and mass spectrometry.

Synthesis and Characterization of Aminooxy Terminated Poly(HPMA) Macromers. Well-defined poly(HMPA) macromers of varying molecular weights bearing terminal aminooxy groups were synthesized by RAFT polymerization of the HPMA monomer (**5**) using the functional CTA, **1**. The polymerization reaction leading to formation of the aminooxy end functionalized poly(HPMA) macromers is shown in Scheme 2.

Scheme 2. Synthesis of Aminooxy Terminated Poly(HPMA) by RAFT Polymerization



Since commercially available HPMA monomer did not yield polymers of desired molecular weights and narrow polydispersity, high purity HPMA monomer was synthesized in-house. Polymerization reactions were carried out under argon atmosphere at 80 °C using DMSO as the solvent. Although RAFT polymerization can be carried out using a variety of thermally activated free radical initiators, we selected AIBN as the initiator. Reaction conditions were optimized to obtain polymers of the target molecular weight and narrow polydispersity. The RAFT initiator system consisting of AIBN and **1** at the molar ratio of 1:5 was found to be optimum for controlling the molecular weights and molecular weight distributions of these aminooxy terminated HPMA polymers. The living nature of RAFT polymerization enabled us to synthesize poly(HPMA) with different molecular using HPMA and the CTA **1** at different molar ratios. Incorporation of the aminooxy functional moiety of the CTA **1** in the polymer chain was established by ^{1}H NMR and FTIR spectral analysis of the polymers. Increase in molecular weight of the polymer as a function of polymerization time was attributed to the living nature of this polymerization process. The ^{1}H NMR spectra of polymers recorded at 1 and 2 h time points after polymerization of a representative polymerization system are shown in Figure 1.

Examination of these ^{1}H NMR spectra reveals that relative peak intensities of aromatic ring of the dithiobezoate group in comparison to HPMA associated peaks are smaller at the 2 h than the 1 h time point. This suggests an increase in polymer molecular weight as a function of time, which is characteristic of living polymerization process.[56] After optimizing reaction conditions, we set out to synthesize two polymers of different

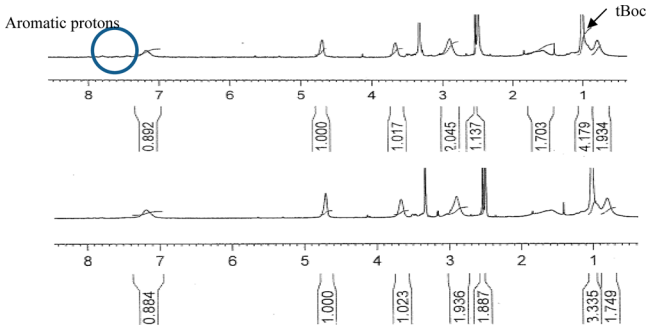


Figure 1. ^{1}H NMR spectra of poly(HPMA) at 1 h (top) and 2 h (bottom) synthesized by RAFT polymerization using **1** as the CTA.

molecular weights for further studies. The polymer samples were characterized by ^{1}H NMR spectroscopy, IR spectroscopy, GPC, and mass spectrometry (MALDI-TOF and ESI). ^{1}H NMR analyses of the polymers revealed the presence of appropriate peaks corresponding to structural features of the *t*-Boc protected aminooxy group at the terminus of the polymer chain. For example, the FTIR spectrum of a representative polymer sample (Figure 2a) showed the presence of amide N–H stretching band at 3327 cm^{-1} and C═O stretching bands due to amide I and amide II at 1523 cm^{-1} and 1630 cm^{-1}, respectively. More importantly, C═O stretching band due to carbamate group associated with *t*-Boc protecting group of the aminooxy moiety was present at 1720 cm^{-1}.

The molecular weights and molecular weight distributions of these polymers were determined by GPC using monodispersed poly(methyl methacrylate) as the molecular weight standards. The GPC chromatograms of the two polymers of different molecular weights are shown in Figure 3 and the molecular weight results of the two polymer samples are summarized in Table 1.

Both polymer samples exhibit narrow polydispersity (PDI < 1.3) and their molecular weights depend on the ratio of monomer to CTA used for polymerization. Since GPC measurement provides relative molecular weight, exact molecular weights of these two polymers were determined by ESI mass spectrometry. The mass spectra of the two polymers are shown in Figure 4. The main series of peaks in the ESI MS of both the polymers show an interval of 143.1 mass units, which correspond to HPMA repeat units. The center peak for the low molecular weight polymer is at 5299 amu and that of the high molecular weight polymer is at 9641 amu. This implies that average molecular weights of the polymers are ∼5000 and 10 000 kD, respectively.

There was a clear discrepancy in the molecular weight of the polymers determined by GPC and mass spectrometry. Mass spectrometry results revealed that polymers are of significantly lower molecular weight compared to the values obtained by GPC method. For the lower molecular weight polymer, the GPC gives a molecular weight of ∼14.4 kDa, and for the high molecular weight polymer, it was found to be 24.2 kDa. However, the mass spectral results showed the median molecular weights of these polymers to be ∼5 kDa and 10 kDa, respectively. These findings suggest that molecular weights of polymers obtained by GPC are not always accurate. This is because for polymers of similar molecular weights, their hydrodynamic volumes can be significantly different depending on their chemical natures (i.e., polarity, charge etc.) of the monomers used, which can result in polymers of different

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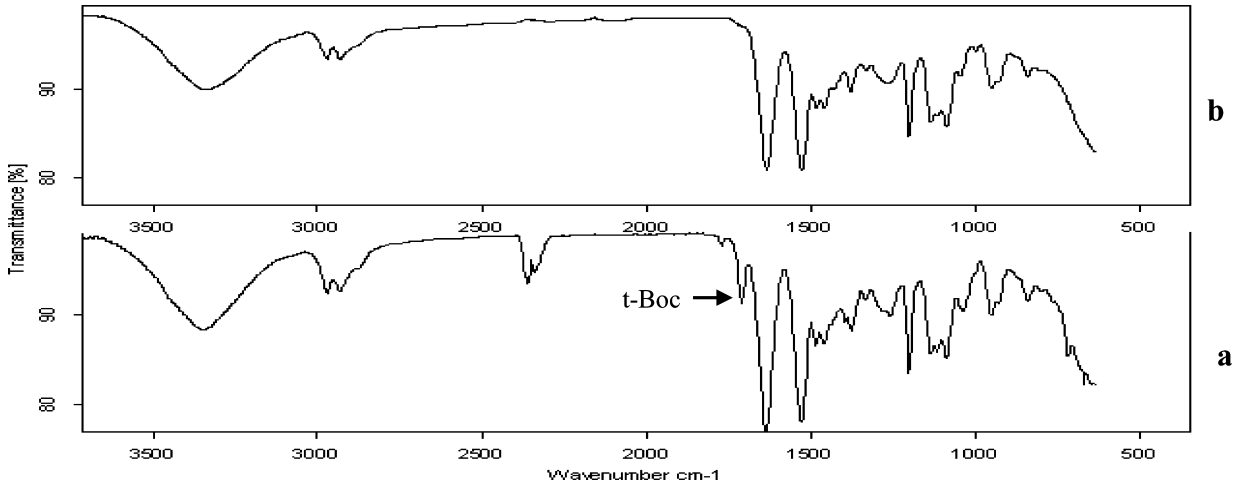

Figure 2. FTIR spectra of (a) *t*-Boc protected, and (b) free aminooxy terminated poly(HPMA) obtained by RAFT polymerization.

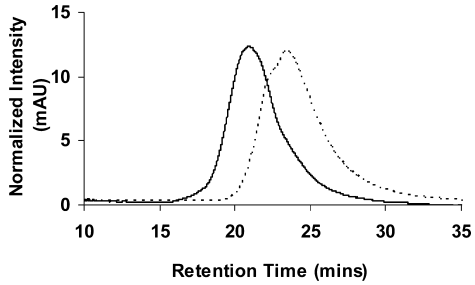

Figure 3. Gel permeation chromatograms of low molecular weight (solid line) and high molecular weight (dotted line) *t*-Boc protected aminooxy terminated poly(HPMA) obtained by RAFT polymerization.

solution conformations (globular vs extended rigid rod).[57] In the present case, the hydrophilic methacrylamide based hydrophilic polymer might possess larger hydrodynamic volume compared to poly(methyl methacrylate). On the other hand, mass spectrometry is a valuable tool to determine molecular weights of polymers with higher precision.[58] The accuracy of the molecular weights of these polymers determined by mass spectrometry is further evident in the polymer–protein conjugate study (see below).

Removal of *t*-Boc Protecting Group. Reactivity of terminal aminooxy groups in these poly(HPMA) compounds was at first assessed by evaluating the ease of removal of the *t*-Boc protecting group to generate free aminooxy moiety. Incubation of the polymers in methanolic HCl solution for 1 h led to removal of the *t*-Boc group (Scheme 3). After removal of the solvent, the polymers were dialyzed (to remove excess reagents and low molecular weight components) and lyophilized. The FTIR spectrum of the polymer (Figure 2b) reveals the absence of the peak at 1720 cm^{-1} in the FTIR spectrum (Figure 2a) corresponding to the carbamate stretching band of the *t*-Boc group. This confirms the complete removal of the *t*-Boc protecting group. ^{1}H NMR spectral analysis of the polymer showed the absence of the methyl proton signals due to the tertiary butyl group further confirming the complete removal of the *t*-Boc group. GPC analysis of the polymers did not show any change in the molecular weight or molecular weight distribution patterns (Figure 5). Thus, the deprotection reaction condition employed was selective toward the *t*-Boc group and does not appear to affect the polymer chain.

Fluorescent Dye Terminated Poly(HPMA) Macromers. In order to ascertain the chemical reactivity of terminal aminooxy group of the poly(HPMA), covalent conjugation of a fluorescent dye molecule was carried out. In addition to providing information on chemical reactivity of the aminooxy group, these dye labeled polymers may be used as molecular probes for biological studies. For example, labeled polymers with appropriate reporter probe have been explored in recent years to study *in vitro* cell uptake as well as *in vivo* biodistribution and pharmacokinetic properties of therapeutic polymers and polymer–drug conjugates.[59−61] For example, radioactive-isotope labeled polymers, including poly(HPMA) have been synthesized to study biodistribution properties of these polymers by using imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT).[62,63] Biocompatible polymers labeled with fluorescent dyes (including near-infrared fluorescence (NIRF) dyes) have been found to be very valuable imaging probes to study cellular biology, pharmacology, and *in vivo* biodistribution of polymers in a noninvasive manner without resorting to the use of radioisotopes that are required for SPECT and PET techniques.[64,65] Fluorescent dyes with appropriate functional groups are commercially available to prepare dye labeled biocompatible polymers.

Table 1. RAFT Polymerization of HPMA Monomer Using 1 as the CTA[a]

entry	HPMA (g)	CTA 1 (g)	AIBN (mg)	DMSO (mL)	polymn. temp. (°C)	polymn. time (min.)	target mol. wt × 10^{-4}	M_n × 10^{-4}	M_w × 10^{-4}	M_w/M_n
1	10	0.228	11.0	60	80	60	1.0	1.39	1.53	1.10
2	20	0.456	22.0	120	80	180	2.0	2.42	2.73	1.12

[a]Polymer molecular weights were determined by GPC method using monodispersed poly(methyl methacrylate) as molecular weight standard.

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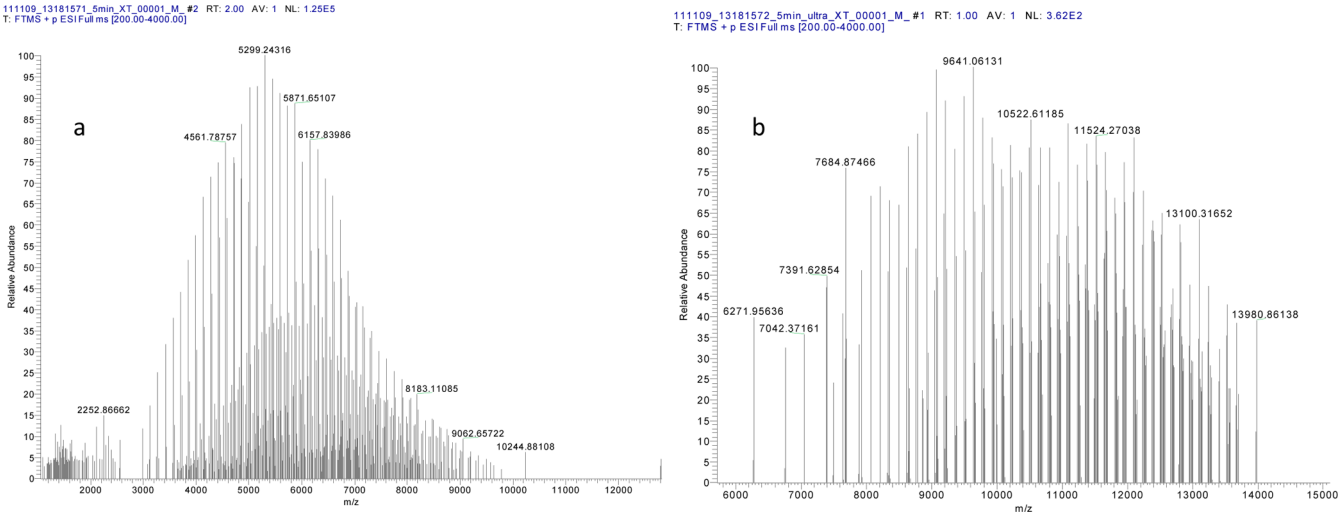
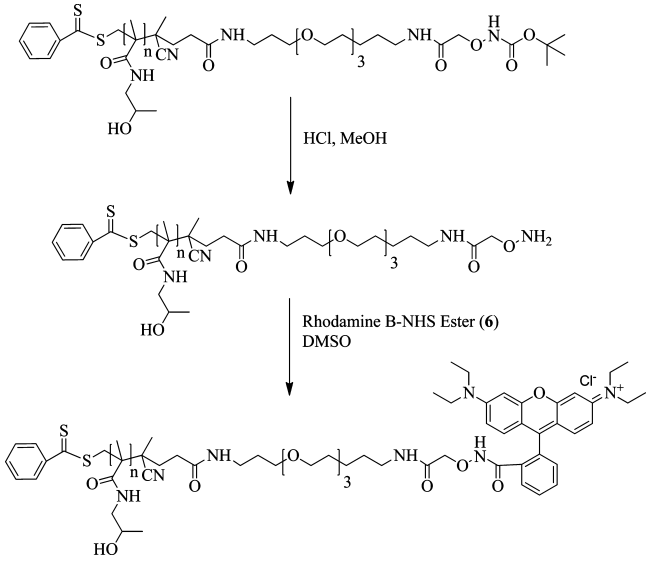


Figure 4. ESI mass spectra of (a) low molecular weight, and (b) high molecular weight aminooxy terminated poly(HPMA) obtained by RAFT polymerization.

Scheme 3. Deprotection and Conjugation of Rhodamine B to Aminooxy-Terminated Poly(HPMA)



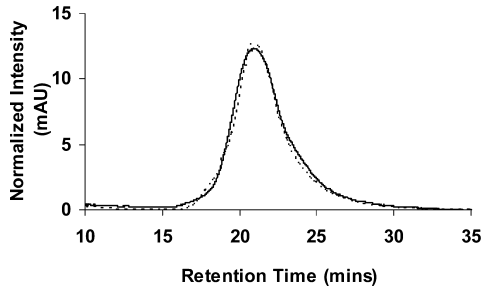


Figure 5. Gel permeation chromatogram of *t*-Boc protected (solid line) and deprotected (dotted line) aminooxy terminated poly(HPMA).

We chose rhodamine B as the fluorescent dye to prepare dye labeled poly(HPMA). In the published literature dye labeled polymers are largely synthesized with multiple copies of dye molecules linked to polymers. In the current approach, by utilizing terminal aminooxy group as the linking site, it was possible to prepare dye labeled polymers bearing a single dye molecule per polymer chain. The aminooxy group is known to behave like the amino group and reacts with carboxylic acid groups to form amide bonds.[66] *N*-Hydroxysuccinimide (NHS) derivative of rhodamine B was used as the reagents to produce fluorescent dye labeled poly(HPMA). Thus, rhodamine B NHS ester (**6**) was allowed to react with aminooxy terminated poly(HPMA) in anhydrous DMSO (Scheme 3). In this manner, both samples of poly(HPMA) of two different molecular weights were converted to their corresponding rhodamine B terminated macromers. To achieve complete conversion, 5-fold molar excess of the NHS dye with respect to aminooxy group was used. After completion of the reaction, the dye-labeled polymers were purified by extensive dialysis followed by membrane filtration to remove excess free rhodamine B.

Figure 6 shows the UV spectra of one of the labeled polymers and the free rhodamine B in methanol. The

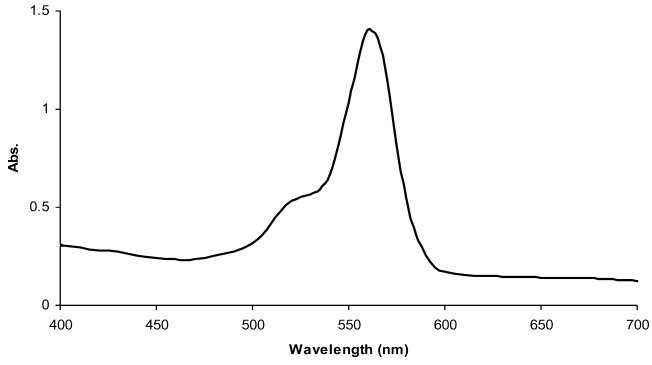


Figure 6. UV−vis spectrum of rhodamine B terminated low molecular weight poly(HPMA).

absorption maxima (λ_{max}) of the free dye and the dye labeled polymer were found to be at 560 nm. On the other hand, the dye labeled polymer exhibited a λ_{max} at 575 nm in water, which is ascribed to expected solvatochromic shift. Also, the fluorescent spectra of these polymers were measured along with that of the free dye for comparison purposes. Fluorescence

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emission behavior of the polymer bound rhodamine B was essentially similar to that of the free dye (Figure 7).

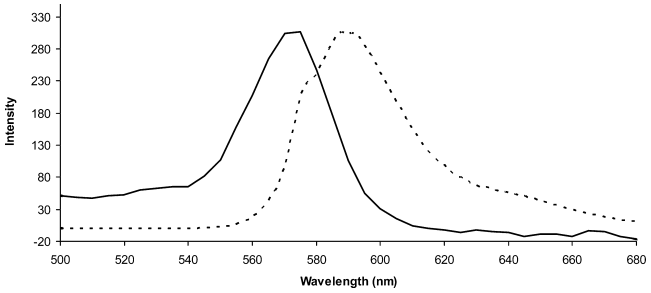

Figure 7. Excitation (solid line) and emission (dotted line) spectra of rhodamine B terminated low molecular weight poly(HPMA).

Thus, the emission maximum (λ_{em}) of the free dye is 580 nm (excitation wavelength (λ_{em}) = 560 nm) and λ_{em} of the corresponding polymer appeared at 581 nm. These results indicate that since only one dye molecule was present per polymer chain, any dye−dye interaction leading to fluorescent quenching was minimized.

These rhodamine B labeled HPMA polymers were used as molecular probes to study intracellular uptake of HPMA polymers in renal epithelial cells. Epithelial cells are known to take up foreign molecules from their environment through a variety of processes, such as endo-, pino-, and phagocytosis.[67] To determine intracellular uptake of polymers, the cultured cells were incubated with increasing amounts (3, 10, 30, and 100 μM) of the dye labeled polymer (both low molecular weight and high molecular polymers) at 37 °C for 30 min and 4 h. At the end of the incubation time, the cells were washed multiple times to remove any free dye labeled polymers. Cells were subsequently fixed and costained with DAPI to highlight cell nuclei that allowed the ability to focus on an intracellular plane as opposed to only looking at the cell surface.

Figure 8 shows the fluorescence microscopy images of the polymer incubated cells at 30 min. A steady increase in polymer uptake by the cells in a concentration dependent manner was observed for both polymers. However, the low molecular weight polymer was found to be located both in the cytoplasm and on the membrane in a concentration dependent manner. On the other hand, the high molecular polymer showed similar concentration dependence uptake, but was present predominantly inside the cytoplasm of the cell. This is probably due to a size dependent endocytosis that is more efficient for larger-sized polymers. Interestingly, after 4 h both polymers are present inside the cell (Figure 9). This suggests that these soluble HPMA-conjugates can be taken up by live cells in both concentration and time dependent manner, most likely through nonspecific pinocytosis, or fluid phase endocytosis.[68] Thus, these well-defined dye labeled polymers can be used as novel reporter molecules to study cellular uptake of therapeutic polymers. Efforts are currently underway in our laboratory to conjugate ligands for specific cell surface receptors together with fluorescent dyes to the same polymer chain to study receptor mediated intracellular uptake of polymers.

Synthesis of Transferrin−poly(HPMA) Conjugate. Site specific and chemoselective conjugation of aminooxy functional polymers with aldehyde containing proteins offers a novel approach to prepare stable oxime linked protein−polymer bioconjugates of defined composition.[69] Typically, aldehyde and ketone groups have been introduced into proteins by chemical modification of specific amino acid residues or by site directed mutagenesis using carbonyl group containing unnatural amino acids.[70−72] A large number of therapeutic proteins and antibodies are glycoproteins and glycosylation is needed to suppress the immunogenicity of these recombinant biotherapeutics.[73,74] Carbohydrate components (also called glycans) of glycoproteins are located on the surfaces of these proteins and are usually remote from the active site. Therefore, glycan-directed synthesis of protein−polymer bioconjugates can avoid any potential interference with the biological activities of the therapeutic proteins and antibodies. These glycan moieties generally contain terminal sialic acid residues that can be oxidized to generate aldehyde groups, which are reactive with aminooxy functional polymers.[75,76]

To demonstrate the ability of these aminooxy terminated poly(HPMA) macromers to form oxime-mediated protein−polymer conjugates, serum Tf was used as the test glycoprotein. Tf is responsible for intracellular uptake of iron from the blood, which is mediated by cell surface receptor mediated endocytosis of an iron-Tf complex.[77] Tf receptors are overexpressed in various tumor cells and this phenomenon has been used as a strategy for targeted therapy to selectively deliver drugs and nucleic acids to cancer cells as Tf-conjugates.[78,79] Tf is an 80 kDa glycoprotein bearing two glycan chains attached to aspargines 413 and 611 in the C-terminal domain.[80] Although Tf is microheterogeneous in terms of sialic acid contents, nearly

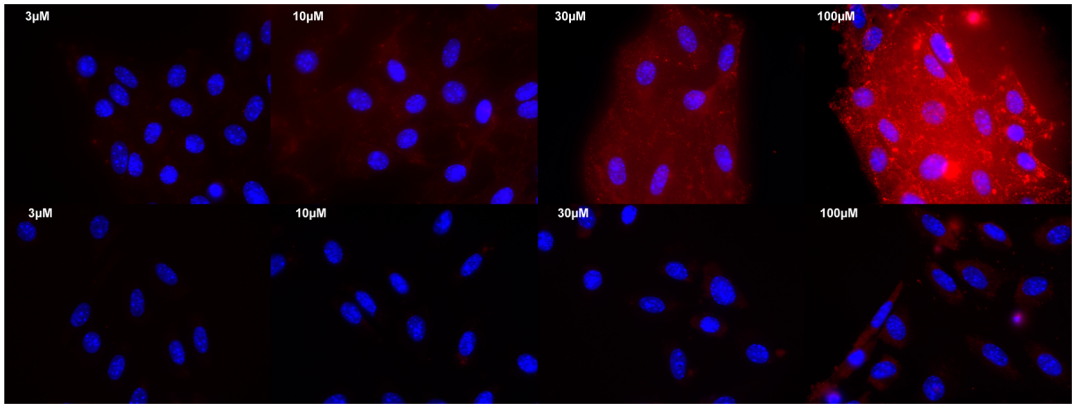

Figure 8. Fluorescence microscopy images of live renal epithelial cells incubated with varying concentrations of Rhodamine B terminated low molecular weight (top panel) and high molecular weight (bottom panel) poly(HPMA) for 30 min.

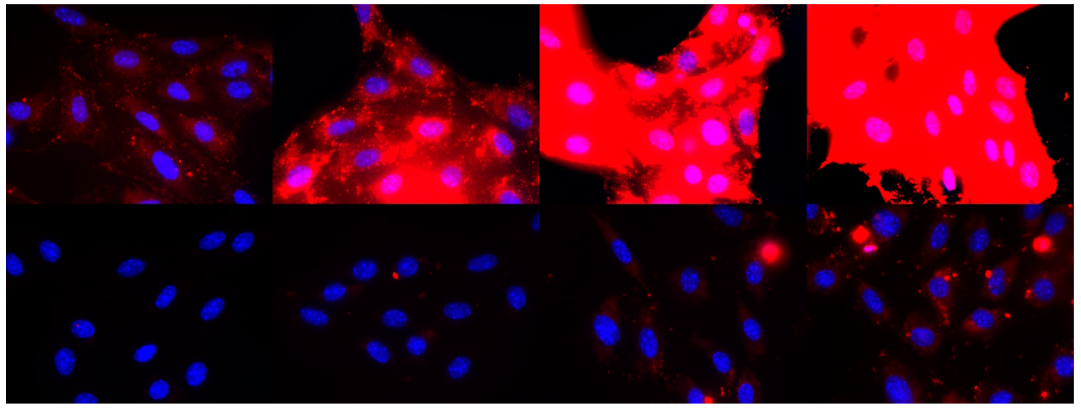

Figure 9. Fluorescence microscopy images of live renal epithelial cells incubated with varying concentrations of Rhodamine B terminated low molecular weight (top panel) and high molecular weight (bottom panel) poly(HPMA) for 4 h.

80% of the isoforms contain four sialic acid units (tetrasialo-Tf). Remaining isoforms consist of triasialo-Tf and pentasialo-Tf. Since the glycan moieties of Tf neither participate in receptor interaction nor metal binding, the side chain diols of the sialic acid residues of the glycan moieties have been used as anchors to prepare (bio)conjugates of Tf.

Sodium periodate mediated oxidation of the sialic acid residues in Tf generated aldehyde group containing Tf. The oxidizing agent was found to be mild and selective toward side chain diol of sialic acid.[81] Optimization of the oxidation condition was needed to avoid unwanted protein oxidation as well as limit the oxidation to the side chain diol of sialic residues. Thus, while typical literature procedures involve use of 4 mM of sodium periodate followed by coupling at pH 5.6, we were able to achieve oxidation using 1 mM of sodium periodate and coupling pH at 7.2. After completion of the reaction, excess reagents and low molecular weight side products were removed by dialysis using a 35 000 molecular weight cutoff membrane. Subsequently Tf-polymer conjugates were synthesized by reacting the oxidized protein with aminooxy terminated poly(HPMA) macromers. After the conjugation reaction was completed, the reaction mixture was subjected to dialysis (35 000 molecular weight cutoff) followed by gel filtration using sephadex column to remove any unreacted polymers. Synthetic steps used for the preparation of these oxime linked Tf bioconjugates are shown in Scheme 4. Polymer to protein ratio was varied to evaluate the reactivity of the polymer bound aminooxy group toward the aldehydes groups in Tf. Table 2 summarizes these experimental conditions.

Characterization of these protein−polymer conjugates was carried out by gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectroscopy. After running the samples on the gel for 4 h, protein bands were stained using Li-Cor IR Dye Blue and were imaged on a Li-Cor scanner at 700 nm. The HPMA polymers do not stain under these conditions. Electropherograms of the bioconjugates along with those of the native and oxidized Tf are shown in Figure 10. The electropherogram of native Tf shows that the protein was predominantly present as the monomeric species with molecular weight ~80 kDa. However, the presence of small amounts of dimeric and trimeric forms of the protein was evident in the electropherogram (lane 4). Periodate mediated oxidation of Tf affects neither the protein's molecular weight nor its monomer:-dimer:trimer distribution ratio (lane 5). Syntheses of poly-(HPMA)-Tf conjugates were studied by using different molar

Scheme 4. Chemoselective Bioconjugation of Transferrin with Aminooxy Terminated Poly(HPMA) via Oxime Ligation

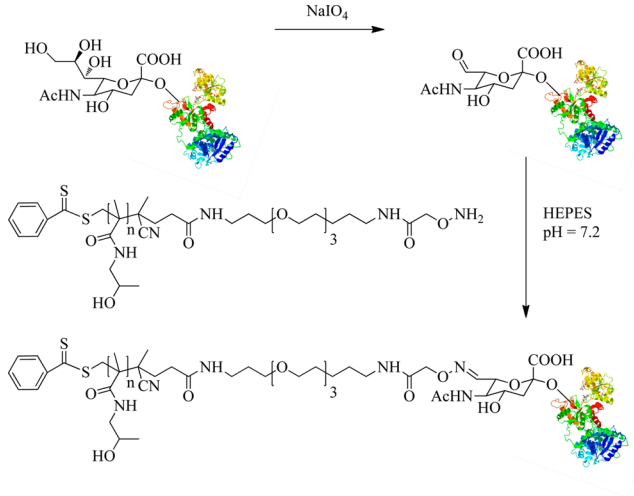

Table 2. Synthesis of Poly(HPMA)−Transferrin Conjugates *via* Oxime Linkage by Varying Protein to Polymer Ratio[a]

Entry	Polymer Used	Amount of Transferrin used (mg)	Amount Polymer used (mg)
1	5 kDa poly(HPMA)	8	1.6
2	5 kDa poly(HPMA)	8	6.4
3	5 kDa poly(HPMA)	8	14.6
4	5 kDa. poly(HPMA)	8	29.0
5	10 kDa poly(HPMA)	4	1.3
6	10 kDa poly(HPMA)	4	4.4
7	10 kDa poly(HPMA)	4	12.1
8	10 kDa poly(HPMA)	4	24.2

[a]For details on conjugation conditions, please see Experimental Section

equivalents of the polymer for a fixed amount of the protein. This enabled us to evaluate the reactivity of the protein bound

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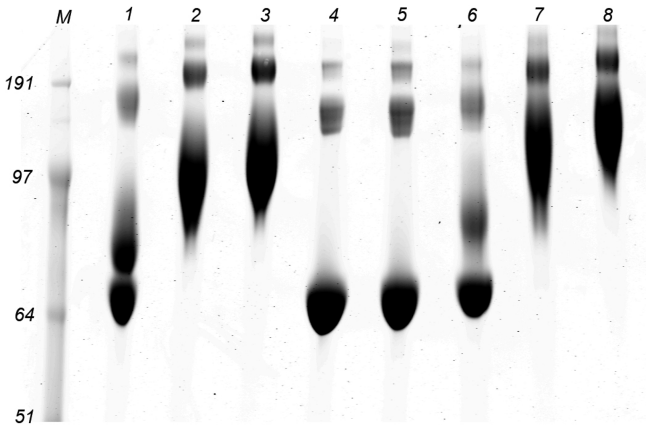

Figure 10. SDS-PAGE electropherogram of conjugates of transferrin with aminooxy terminated poly(HPMA): **band 4** -- Native Tf; **band 5** -- periodate oxidized Tf; **bands 1, 2, and 3** -- conjugates prepared using 1.1, 4.4, and 10 mol equiv of the 5 kDa poly(HPMA); **bands 6, 7, and 8** -- conjugates prepared using 1.1, 4.4, and 10 mol equiv of the 10 kDa poly(HPMA).

aldehyde groups toward the polymeric aminooxy reagents and to optimize reaction parameters for complete conjugation of Tf. As can be seen in Figure 10, when 1.1 mol equiv of the polymer was used, a heterogeneous population of the conjugates were formed and a significant amount of the protein remains unconjugated for both the low molecular weight and high molecular weight poly(HPMA) macromers (lanes 1 and 6). By increasing the polymer amount to 4.4 equiv, conjugate formation appears to be completed (lanes 2 and 7). When the polymer amount is increased to 10 equiv, the electropherogram does not appear to change any further (lanes 3 and 8). However, a close examination suggests that with 4.4 equiv of the polymers, some conjugates with three polymer chains per protein are present. On the other hand, when 10 equiv of the polymer were used, all the four aldehyde groups of Tf have been involved in oxime formation. Furthermore, shift in the bands of dimeric and trimeric proteins suggests that all forms of protein are conjugated. Relative positions of the bands of the conjugates for the two sets of polymers are attributed to the difference in their molecular weights. The smear in electropherograms of the conjugates is attributed to the fact that the aminooxy terminated poly(HPMA) macromers used are not single molecular entities. These narrowly dispersed polymers contain chains of different molecular weights within a narrow range. As a result, a relatively small population of protein–polymer conjugates of varying molecular weights was formed.

Figure 11 shows the MALDI-TOF mass spectra of the naked and periodate oxidized Tf are shown in Figure 11. For the native protein (Figure 11a), the major peak at m/z = 79 379 is attributed to the monomeric Tf. The peak at m/z = 158 772 is due to the Tf dimer. These results agree with those obtained by SDS-PAGE analysis. However, a peak at 39 749 amu (approximately half of the Tf molecular ion peak) was observed in the mass spectra of the protein, which is absent in the SDS-PAGE. Being a soft ionization method, dominant species in MALDI-TOF are generally single protonated molecular ions. However, some times, a doubly charged species at approximately half the m/z value can appear.[82] Thus, the peak at 39 379 amu is most likely due to this phenomenon. The mass spectral profile of the periodate oxidized Tf is similar to that of the native protein (Figure 11b), except with a slightly lower

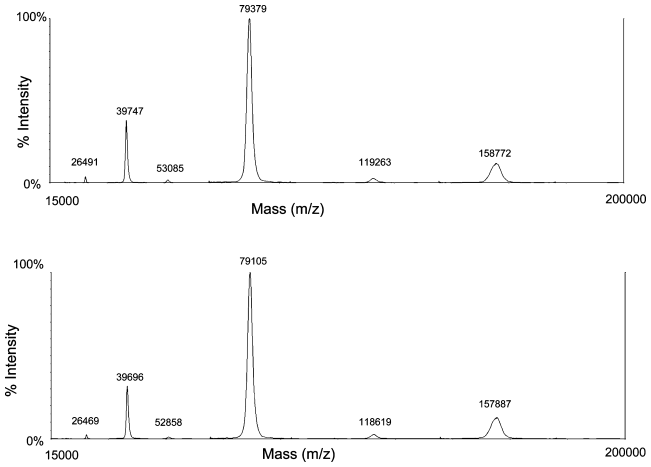

Figure 11. MALDI-TOF mass spectra of conjugates of native transferrin (**a**) and periodate oxidized transferrin (**b**).

molecular weight of the individual peaks due to modification of terminal sialic acid groups of the glycan components to generate aldehyde groups.

MALDI-TOF mass spectra of the polymer bioconjugates of Tf by using low and high molecular weight poly(HPMA) macromers are shown in Figures 12 and 13, respectively. For

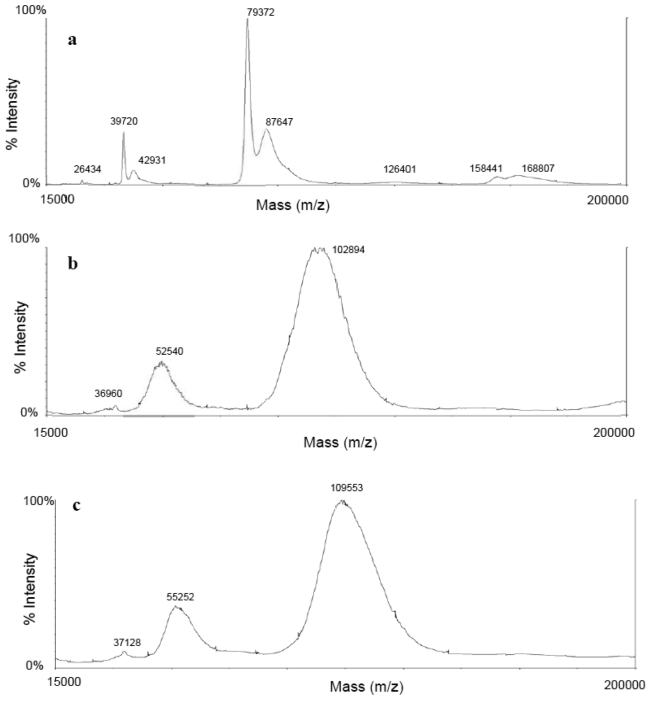

Figure 12. MALDI-TOF mass spectra of conjugates of transferrin with low molecular weight (5.5 kDa) aminooxy terminated Poly(HPMA) at polymer to protein ratio of 1.1 (**a**); 4.4 (**b**); and 10 (**c**).

the conjugates with low molecular weight polymer using 1.1 mol equiv of the polymer, the mass spectra (Figure 12a) shows two key peaks; a peak at 79 372 amu corresponding to the free protein and a peak at 87 647 amu corresponding to the conjugate carrying mostly one polymer chain per protein. In the mass spectra of the conjugates obtained with 4.4 and 10 mol equiv of the polymer (Figure 12b,c), the peak corresponding to the free protein has completely disappeared

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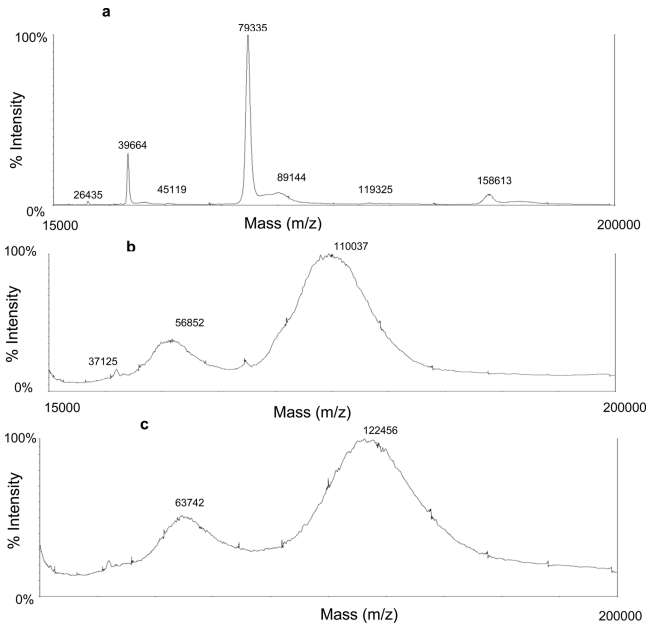

Figure 13. MALDI-TOF mass spectra of conjugates of transferrin with high molecular weight (10 kDa) aminooxy terminated Poly(HPMA) at polymer to protein ratio of 1.1 (**a**); 4.4 (**b**); and 10 (**c**).

and the major peaks are centered at 102 894 and 109 553 amu, respectively. Since the polymer used in this case has a molecular weight ~5500 Da, these peaks correspond to attachment of four to five polymer chains per protein. The broadening of the mass spectra of the conjugates compared to the native protein might be due to polydispersity of the poly(HPMA) macromers, which results in multiple species of close molecular weights. In other words, since the polymer is composed of chains of varying lengths, the conjugates thus obtained, even with the same degree of modification, are of varying molecular weights. The protein conjugates obtained using the high molecular weight poly(HPMA) macromer showed a parallel pattern in mass spectra. Mass spectrum of the conjugates obtained with 1.1 mol equiv of this polymer contains a peak at 89 144 amu (in addition to the peak due to the naked protein at 79 195 amu), which is ascribed to conjugate containing mostly one polymer chain attached to the protein (Figure 13a). Although mass spectra of the conjugates obtained with excess polymers are broad, with 4.4 equiv of the polymer, the mean peak centered around 110 037 amu (Figure 13b) suggests that the majority of the conjugates carry three polymer chains per protein. On the other hand, with 10-fold excess of polymer, the main peak centered around 122 456 amu (Figure 13c) suggests that all the four aldehyde groups have been reacted with the aminooxy macromer. Thus, the polymer chain length appears to have imparted some steric effect on conjugation reaction and excess of reagent is needed to achieve complete conjugation. Such a phenomenon is not uncommon in polymeric systems.

CONCLUSIONS

A novel functional RAFT polymerization reagent to synthesize molecular weight controlled poly(HPMA) macromers of narrow molecular weight distribution with a-terminal aminooxy group has been developed. By direct polymerization of the HPMA monomer using an amide bond containing RAFT reagent, polymers with chemical compositional homogeneity and with greater hydrolytic stability of the linker arm were obtained. It enabled us to overcome the structural heterogeneity issues associated with synthesis of the poly(HPMA) by chemical modification of active ester precursor polymers. These aminooxy end terminated polymers were systematically characterized. In particular, ESI and MALDI-TOF mass spectral analyses have been helpful in establishing the precise molecular weights of these polymers. The terminal aminooxy group has been found to participate in various chemical reactions, thus making these polymers novel macromolecular reagents for various biological studies. Fluorescent dye labeled macromer was found to be useful as a molecular probe to study cell uptake characteristics of this class of biocompatible polymers. Ability of these aminoxy end-functionalized poly(HPMA) macromers to conjugate glycoproteins by chemoselective oxime ligation makes them promising carriers for the development of bioconjugates of therapeutic proteins and antibodies for various biomedical applications. Facile synthesis of the functional RAFT reagents with stable linkage makes it a modular approach to prepare tailored functional initiators. Thus, beyond the examples presented in this article, the approach presented here may expand the scope of the functional groups that can be introduced at the chain terminus and polymer structures that may be achieved.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Duncan, R. (2003) The dawning era of polymer therapeutics. *Nat. Rev. Drug Discovery 2* (5), 347−360.

(2) Dhal, P. K., Polomoscanik, S. C., Avila, L. Z., Holmes-Farley, S. R., and Miller, R. J. (2009) Functional polymers as therapeutic agents: concept to marketplace. *Adv. Drug Delivery Rev. 61* (13), 1121−1130.

(3) Liu, S., Maheshwari, R., and Kiick, K. L. (2008) Polymer-based therapeutics. *Macromolecules 42* (1), 3−13.

(4) Vicent, M. J., Diuedonne, L., Carbajo, L. J., and Pineda-Lucena, A. (2008) Polymer conjugates as therapeutics: future trends, challenges, and opportunities. *Expert Opin. Drug Delivery 5* (5), 593−614.

(5) Haag, R., and Kratz, F. (2006) Polymer therapeutics: concepts and applications. *Angew. Chem., Int. Ed. Engl. 45*, 1198−1215.

(6) Almutairi, A., Guillaudeu, S. J., Berezin, M. Y., Achilefu, S., and Frechet, J. M. J. (2008) Biodegradable pH-sensing dendritic nanoprobes for near-infrared fluorescence lifetime and intensity imaging. *J. Am. Chem. Soc. 130* (2), 444−445.

(7) Teles, F. R. R., and Fonseca, L. P. (2008) Applications of polymers for biomolecule immobilization in electrochemical biosensors. *Mater. Sci. Eng. C: Mater. Biol. Appl. 28* (8), 1530−1543.

(8) Harris, J. M., and Chess, R. B. (2003) Effect of pegylation of pharmaceuticals. *Nat. Rev. Drug Discovery 2* (3), 214−221.

(9) Pascut, G., and Veronese, F. M. (2007) Polymer-drug conjugations: recent achievements and general strategies. *Prog. Polym. Sci. 32*, 933−961.

(10) Alconcel, S. N. S., Baas, A. S., and Maynard, H. D. (2011) FDA-approved poly(ethylene glycol)-protein conjugated drugs. *Polym. Chem. 2*, 1442−1448.

(11) Fishburn, C. S. (2008) The pharmacology of pegylation: balancing PD with PK to generate novel therapeutics. *J. Pharm. Sci. 97* (10), 4167−4183.

(12) Kang, J. S., DeLuca, P. P., and Lee, K. C. (2009) Emerging pegylated drugs. *Expert Opin. Emerging Drugs 14* (2), 363−380.

(13) Barz, M., Luxenhofer, R., Zentel, R., and Vicent, M. J. (2011) Overcoming the PEG-addiction: well-defined alternatives to PEG,

from structure-property relationships to better defined therapeutics. *Polym. Chem. 2* (9), 1900−1918.
(14) Megan, E., Fox, M. E., Szoka, F. C., and Frechet, J. M. J. (2009) Soluble polymer carriers for the treatment of cancer: the importance of molecular architecture. *Acc. Chem. Res. 42* (8), 1141−1151.
(15) Adams, N., and Schubert, U. S. (2007) Poly(2-oxazolines) in biological and biomedical application contexts. *Adv. Drug Delivery Rev. 59* (15), 1504−1520.
(16) Kopecek, J., and Kopeckova, P. (2010) HPMA copolymers: Origins, early developments, present, and future. *Adv. Drug Delivery Rev. 62* (2), 122−149.
(17) Duncan, R., and Vicent, M. J. (2010) Do HPMA copolymer conjugates have a future as clinically useful nanomedicines? A critical overview of current status and future opportunities. *Adv. Drug Delivery Rev. 62* (2), 272−282.
(18) Rihova, B. (2007) Biocompatibility and immunocompatibility of water-soluble polymers based on HPMA. *Composites, Part B: Engineering 38B* (3), 386−397.
(19) Kopeček, J., Kopečková, P., Minko, T., and Lu, Z. (2000) HPMA copolymer−anticancer drug conjugates: design, activity, and mechanism of action. *Eur. J. Pharm. Biopharm. 50* (1), 61−81.
(20) Nowotnik, D. P. (2011) AP5346 (ProLindac), a DACH platinum polymer conjugate in phase II trials against ovarian cancer. *Curr. Bioactive Comp. 7* (1), 21−26.
(21) Seymour, L. W., et al. (2009) Phase II studies of polymer−doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. *Int. J. Oncol. 34* (6), 1629−1636.
(22) Pan, H., Sima, M., Kopečková, P., Wu, K., Gao, S., Liu, J., Wang, D., Miller, S. C., and Kopeček, J. (2008) Biodistribution and pharmacokinetic studies of bone-targeting N-(2-hydroxypropyl)-methacrylamide copolymer-alendronate conjugates. *Mol. Pharmacol. 5* (4), 548−558.
(23) Wang, D., Kopečková, P., Minko, T., Nanayakkara, V., and Kopeček, J. (2000) Synthesis of starlike N-(2-hydroxypropyl)-methacrylamide copolymers: Potential drug carriers. *Biomacromolecules 1* (3), 313−319.
(24) Johnson, R. N., Kopečková, P., and Kopeček, J. (2009) Synthesis and evaluation of multivalent branched HPMA copolymer-Fab' conjugates targeted to the B-Cell antigen CD2. *Bioconjugate Chem. 20* (1), 129−137.
(25) Gasper, R., and Duncan, R. (2009) Polymeric carriers: Preclinical safety and the regulatory implications for design and development of polymer therapeutics. *Adv. Drug Delivery Rev. 61* (13), 1220−1231.
(26) Ouchi, M., Terashima, T., and Sawamoto, M. (2009) Transition metal-catalyzed Living radical polymerization: Toward perfection in catalysis and precision polymer synthesis. *Chem. Rev. 109* (11), 4963−5050.
(27) Moad, G., Rizzardo, E., and Thang, S. H. (2009) Living radical Polymerization by the RAFT process - A second update. *Aust. J. Chem. 62* (11), 1402−1472.
(28) Braunecker, W. A., and Matyjaszewski, K. (2008) Controlled/living radical polymerization: Features, developments, and perspectives. *Prog. Polym. Sci. 32* (1), 93−146.
(29) Ouchi, M., Terashima, T., and Sawamoto, M. (2008) Precision control of radical polymerization via transition metal catalysis: From dormant species to designed catalysts for precision functional polymers. *Acc. Chem. Res. 41* (9), 1120−1132.
(30) Bays, E., Tao, L., Chang, C.-W., and Maynard, H. D. (2009) Synthesis of semitelechelic maleimide poly(PEGA) for protein conjugation by RAFT polymerization. *Biomacromolecules 10* (7), 1777−1781.
(31) Droumaguet, B. L, and Nicolas, J. (2010) Recent advances in the design of bioconjugates from controlled/living radical polymerization. *Polym. Chem. 1* (5), 563−598.
(32) Grover, G. N, and Maynard, H. D. (2010) Protein-polymer conjugates: synthetic approaches by controlled radical polymerizations and interesting applications. *Curr. Opin. Chem. Biol. 14* (6), 818−827.
(33) Jones, M. W., Mantovani, G., C. A., Blindauer, C. A., Ryan, S. M., Wang, X., Brayden, D. J., and Haddleton, D. M. (2012) Direct peptide bioconjugation/PEGylation at tyrosine with linear and branched polymeric diazonium salts. *J. Am. Chem. Soc. 134* (17), 7406−7013.
(34) Godwin, A., Hartenstein, M., Muller, A. H. E., and Brocchini, S. (2001) Narrow molecular weight distribution precursors for polymer-drug conjugates. *Angew. Chem., Int. Ed. 40* (3), 594−597.
(35) Barz, M., Canal, F., Koynov, K., Zentel, R., and Vicent, M. J. (2010) Synthesis and in vitro evaluation of defined HPMA folate conjugates: influence of aggregation on folate receptor (FR) mediated cellular uptake. *Biomacromolecules 11* (9), 2274−22282.
(36) Yong, S. Y., and Putnam, D. (2007) Overcoming limiting side reactions associated with an NHS-activated precursor of polymethacrylamide-based polymers. *Bioconjugate Chem. 18* (3), 970−982.
(37) Scales, C. W., Vasilieva, Y. A., Convertine, A. J., Lowe, A. B., and McCormick, C. L. (2005) Direct, controlled synthesis of the nonimmunogenic, hydrophilic polymer, poly(N-(2-hydroxypropyl)-methacrylamide) via RAFT in aqueous media. *Biomacromolecules 6* (4), 1846−1850.
(38) York, A. W., Huang, F., and McCormick, C. L. (2010) Rational design of targeted cancer therapeutics through the multiconjugation of folate and cleavable siRNA to RAFT-synthesized (HPMA-s-APMA) copolymers. *Biomacromolecules 11* (2), 505−514.
(39) Pan, H., Yang, J., Kopeckova, P., and Kopecek, J. (2011) Backbone degradable multiblock N-(2-hydroxypropyl) methacrylamide copolymer conjugates via reversible addition-fragmentation chain transfer polymerization and thiol-ene coupling reaction. *Biomacromolecules 12* (1), 47−252.
(40) Lemieux, G. A., and Bertozzi, C. R. (1998) Chemoselective ligation reactions with proteins, oligosaccharides and cells. *Trends Biotechnol. 16* (12), 506−513.
(41) Peri, F., and Nicotra, F. (2004) Chemoselective ligation in glycochemistry. *Chem. Commun. 6*, 627−627.
(42) Dirksen, A., Hackeng, T. M., and Dawson, P. E. (2006) Nucleophilic catalysis of oxime ligation. *Angew. Chem., Int. Ed. 45* (45), 7581−7584.
(43) Kalia, J., and Raines, R. T. (2008) Hydrolytic stability of hydrazones and oximes. *Angew. Chem., Int. Ed. 47* (39), 7523−7526.
(44) Jones, D. S., Branks, M. J., Campbell, M.-A., Cockerill, K. A., Hammaker, J. R., Kessler, C. A., Smith, E. M., Tao, A., Ton-Nu, H.-T., and Xu, T. (2003) Multivalent poly(ethylene glycol)-containing conjugates for *in vivo* antibody suppression. *Bioconjugate Chem. 14* (6), 1067−1076.
(45) Zhou, Q., Stefano, J. E., Harrahy, J., Finn, P., Avila, L., Kyazike, J., Wei, R., Van Patten, S. M., Gotschall, R., Zheng, X., Zhu, Y., Edmunds, T., and Pan, C. Q. (2011) Strategies for neoglycan conjugation to human acid α-glucosidase. *Bioconjugate Chem. 14* (6), 1067−1076.
(46) Henderson, G. E., Isett, K. D., and Gerngross, T. U. (2011) Site-specific modification of recombinant proteins: a novel platform for modifying glycoproteins expressed in *E. coli*. *Bioconjugate Chem. 22* (5), 903−912.
(47) Werlen, R. C., Lankinen, M., Offord, R. E., Schubiger, P. A., Smith, A., and Rose, K. (1996) Preparation of a trivalent antigen-binding construct using polyoxime chemistry: Improved biodistribution and potential for therapeutic application. *Cancer Res. 56* (4), 809−815.
(48) Zatsepin, T. S., Stetsenko, D. A., Gait, M. J., and Oretskaya, T. S. (2005) Use of carbonyl group addition-elimination reactions for synthesis of nucleic acid conjugates. *Bioconjugate Chem. 16* (3), 471−489.
(49) Maynard, H. D., Heredia, K. L., Li, R. C., Parra, D. P., and Vazquez-Dorbatt, V. (2007) Thermoresponsive biohybrid materials synthesized by ATRP. *J. Mater. Chem. 17* (38), 4015−4017.
(50) Vazquez-Dorbatt, V., Tolstyka, Z. P., and Maynard, H. D. (2009) Synthesis of aminooxy end-functionalized pNIPAAm by raft polymerization for protein and polysaccharide conjugation. *Macromolecules 42* (20), 7650−7656.

(51) Esser-Kahn, A. P., and Francis, M. B. (2008) Protein-cross-linked polymeric materials through site-selective bioconjugation. *Angew. Chem., Int. Ed. 47* (20), 3751−3754.
(52) Chytil, P., Etrych, T., Kriz, J., Subr, V., and Ulbrich, K. (2010) N-(2-Hydroxypropyl)methacrylamide-based polymer conjugates with pH-controlled activation of doxorubicin for cell-specific or passive tumor targeting. Synthesis by RAFT polymerization and physicochemical characterization. *Eur. J. Pharm. Sci. 41* (3−4), 473−482.
(53) Mitsukami, Y., Donovan, M. S., Lowe, A. B., and McCormick, C. L. (2001) Water-soluble polymers. 81. Direct synthesis of hydrophilic styrenic-based homopolymers and block copolymers in aqueous solution via RAFT. *Macromolecules 34* (7), 2248−2256.
(54) Kumar, S., Roy, S. G., and De, P. (2012) Cationic methacrylate polymers containing chiral amino acid moieties: controlled synthesis via RAFT polymerization. *Polym. Chem. 3* (5), 1239−1248.
(55) El- Faham, A., and Fernando Albericio, F. (2011) Peptide-coupling reagents. In *Amino Acids, Peptides and Proteins in Organic Chemistry: Building Blocks, Catalysis and Coupling Chemistry* (Hughes, A. B., Ed.) pp 407−444, Vol. 3, Wiley-VCH, Weinheim.
(56) Yanjarappa, M. J., Gujraty, K. V., Joshi, A., Saraph, A., and Kane, R. S. (2006) Synthesis of copolymers containing an active ester of methacrylic acid by raft: controlled molecular weight scaffolds for biofunctionalization. *Biomacromolecules 7* (5), 1665−1670.
(57) Nicolas, J., Khoshdel, E., and Haddleton, D. M. (2007) Bioconjugation onto biological surfaces with fluorescently labeled polymers. *Chem. Commun. 17*, 1722−1724.
(58) Hart-Smith, G., and Barner-Kowollik, C. (2010) Contemporary mass spectrometry and the analysis of synthetic polymers: trends, techniques and untapped potential. *Macromol. Chem. Phys. 211* (14), 1507−1529.
(59) Kim, J.-H., Park, K., Nam, H. Y., Lee, S., Kim, K., and Kwon, I. C. (2007) Polymers for bioimaging. *Prog. Polym. Sci. 32*, 1031−1053.
(60) Lorenz, M. R., Holzapfel, V., Musyanovych, A., Nothelfer, K., Walther, P., Frank, H., Katharina Landfester, K., Schrezenmeier, H., and Mailander, V. (2006) Uptake of functionalized, fluorescent-labeled polymeric particles in different cell lines and stem cells. *Biomaterials 27*, 2820−2828.
(61) Saad, M., Ola B. Garbuzenko, O. B., Ber, E., Chandna, P., Khandare, J. J., Pozharov, V. P., and Minko, T. (2008) Receptor targeted polymers, dendrimers, liposomes: Which nanocarrier is the most efficient for tumor-specific treatment and imaging? *J. Controlled Release 130*, 107−114.
(62) Almutairi, A., Rossin, R., Shokeen, M., Hagooly, A., Ananth, A., Capoccia, B., Guillaudeu, S., Abendschein, D., Anderson, C. J., Welch, M. J., and Frechet, J. M. J. (2008) Biodegradable dendritic positron-emitting nanoprobes for the noninvasive imaging of angiogenesis. *Proc. Natl. Acad. Sci. U.S.A. 106* (3), 685−690.
(63) Patil, R. R., Yu, J., Banerjee, S. R., Ren, Y., Leong, D., Jiang, X., Pomper, M., Tsui, B., Kraitchman, D. L., and Mao, H.-Q. (2011) Probing in vivo trafficking of polymer /DNA micellar nanoparticles using SPECT /CT imaging. *Mol. Therapy 19* (9), 1626−1635.
(64) Weissleder, R., Tung, C.-H., Mahmood, U., and Bogdanov, A. (1999) In vivo imaging of tumors with protease activated near-infrared fluorescent probes. *Nat. Biotechnol. 17* (4), 375−378.
(65) Almutairi, A., Akers, W. J., Mikhail, Y., Berezin, M. Y., Achilefu, S., and Frechet, J. M. J. (2008) Monitoring the biodegradation of dendritic near-infrared nanoprobes by *in vivo* fluorescence imaging. *Mol. Pharmaceutics 5* (6), 1103−1110.
(66) Li, X., and Yang, D. (2006) Peptides of aminooxy acids as foldamers. *Chem. Commun. 31*, 3367−3379.
(67) Meier, O., and Greber, U. F. (2004) Adenovirus endocytosis. *J. Gene Med. 6* (S1), S152−S163.
(68) Gump, J. M., and Dowdy, S. F. (2007) TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol. Med. 13* (10), 443−448.
(69) Kochendoerfer, G. G., et al. (2003) Design and chemical synthesis of a homogeneous polymer -modified erythropoiesis protein. *Science 299* (5608), 884−887.
(70) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.
(71) Scheck, R. A., Dedeo, M. T., Iavarone, A. T., and Francis, M. B (2008) Optimization of a biomimetic transamination reaction. *J. Am. Chem. Soc. 130* (35), 11762−11770.
(72) Wu, P., Shui, W., Carlson, B. L., Hu, N., Rabuka, D., Lee, J., and Bertozzi, C. R. (2009) Site-specific chemical modification of recombinant proteins produced in mammalian cells by using the genetically encoded aldehyde tag. *Proc. Natl. Acad. Sci. U.S.A. 106* (9), 3000−3005.
(73) Beck, A., Cochet, O., and Wurch, T. (2010) GlycoFi's technology to control the glycosylation of recombinant therapeutic proteins. *Expert Opin. Drug Discovery 5* (1), 95−111.
(74) Ho, R., and McConkey, B. J. (2011) Modulating glycosylation of recombinant monoclonal antibodies for therapeutic applications. *Curr. Top. Biotechnol. 6*, 57−69.
(75) Zeng, Y., Ramya, T. N. C., Dirksen, A., Dawson, P. E., and Paulson, J. C. (2009) High-efficiency labeling of sialylated glycoproteins on living cells. *Nat. Methods 6* (3), 207−209.
(76) Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2009) Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol. 21* (6), 660−666.
(77) Baker, E. N. (1994) Structure and reactivity of transferrins. *Adv. Inorg. Chem. 41*, 389−463.
(78) Qian, Z. M., Li, H., Sun, H., and Ho, K. (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharm. Rev. 54* (4), 561−587.
(79) Bellocq, N. C., Pun, S. H., Gregory S. Jensen, G. S., and Davis, M. E. (2003) Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjugate Chem. 14* (6), 1122−1132.
(80) Sun, H., Li, H., and Sadler, P. J. (1999) Transferrin as a metal ion mediator. *Chem. Rev. 99* (9), 2817−2842.
(81) Pones, B., Mouhoubi, L., Adib, A., Godzina, P., Behr, J.-P., and Zuber, G. (2006) ω-Hydrazino linear polyethylenimine: A mono-conjugation building block for nucleic acid delivery. *ChemBioChem 7* (2), 303−309.
(82) Dell, A., and Morris, H. R. (2001) Glycoprotein structure determination by mass spectrometry. *Science 291* (5512), 2351−2356.