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Degradable PEGylated Protein Conjugates Utilizing RAFT Polymerization

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Abstract

Poly(ethylene glycol) (PEG)-protein therapeutics exhibit enhanced pharmacokinetics, but have drawbacks including decreased protein activities and polymer accumulation in the body. Therefore a major aim for second-generation polymer therapeutics is to introduce degradability into the backbone. Herein we describe the synthesis of poly(poly(ethylene glycol methyl ether methacrylate)) (pPEGMA) degradable polymers with protein-reactive end-groups via reversible addition-fragmentation chain transfer (RAFT) polymerization, and the subsequent covalent attachment to lysozyme through a reducible disulfide linkage. RAFT copolymerization of cyclic ketene acetal (CKA) monomer 5,6-benzo-2-methylene-1,3-dioxepane (BMDO) with PEGMA yielded two polymers with number-average molecular weight (M_n) (GPC) of 10.9 and 20.9 kDa and molecular weight dispersities (Đ) of 1.34 and 1.71, respectively. Hydrolytic degradation of the polymers was analyzed by ^{1}H-NMR and GPC under basic and acidic conditions. The reversible covalent attachment of these polymers to lysozyme, as well as the hydrolytic and reductive cleavage of the polymer from the protein, was analyzed by gel electrophoresis and mass spectrometry. Following reductive cleavage of the polymer, an increase in activity was observed for both conjugates, with the released protein having full activity. This represents a method to prepare PEGylated proteins, where the polymer is readily cleaved from the protein and the main chain of the polymer is degradable.

Keywords

controlled radical polymerization (CRP); degradable; cyclic ketene acetal (CKA); polymer-protein conjugate; reversible addition-fragmentation chain transfer (RAFT); poly(ethylene glycol) (PEG)

1. Introduction

Covalent attachment of PEG-based polymers is known to improve the pharmacokinetics of protein therapeutics through stabilization and improved circulation time[1]. As a result there

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are many FDA-approved, PEGylated therapeutic agents on the market[2]. Protein conjugation to branched PEG-like polymers, such as pPEGMA, prepared by controlled radical polymerization (CRP), has also been shown to improve protein pharmacokinetics[3]. Despite these advantages, PEGylation has several drawbacks. Typically polymer attachment results in decreased activity of the protein[4], and long-term treatment with PEGylated therapeutics can result in PEG accumulation in the liver and spleen, hypersensitivity, the development of anti-PEG IgM antibodies, and lysozomal disease syndrome[5]. Therefore, PEG-like polymers, containing a degradable linkage and/or degradable moieties in the backbone are important to circumvent these issues[6,7].

Degradable linkages at the site of attachment between the polymer and protein are often installed so that the protein can be released (hydrolytically, enzymatically, or reductively) from the polymer *in-vivo*[8]. Such linkages include maleylamino peptide bonds[9], carbamate[10], ester[11], disulfide[12], hydrazone[13], and oxime[14] bonds. For instance, PEG-Intron® was designed with a degradable carbamate linkage to interferon alpha-2b[15]. Cleavage of the polymer results in regained protein activity. Roberts and Harris reported PEGylation of lysozyme (Lyz) through a degradable ester linkage; upon hydrolysis of the ester, the activity of Lyz was regained to 60% of the native activity[4]. However, in these cases the PEG backbone itself is non-degradable, and thus negative effects associated with polymer accumulation could persist. To prevent this, enzymatically or hydrolytically degradable moieties such as esters[16], vinyl ethers[17], acetals[18], oximes, or urethanes[19], as well as reduction sensitive disulfides[20] have been installed in the backbone of PEG. However, to our knowledge, main-chain degradable PEG-like polymers have not yet been conjugated to a protein. Several backbone degradable non-PEG polymer-protein conjugates have been developed. Most of these conjugates consist of sugar-based or sugar-derived polymers such as hydroxyethyl starch[21], polysialic acid[22], dextran derivitives[23] or dextrin[24]. In addition, recently, ring opening polymerization has been used to synthesize a poly(ε-caprolactone) which was covalently bound to bovine serum albumin[25]. Recently poly(l-glutamic acid) conjugates have also been reported[26]. Herein, we describe the combination of both a degradable linkage and a degradable backbone as an approach for next generation PEGylated protein therapeutics (Figure 1).

CRP offers easy end-group functionalization, well-defined polymer molecular weights, and compatibility with a wide variety of monomers. Therefore, much attention has been paid to the development of CRP techniques as a means to develop well-defined, PEG-like polymer-protein therapeutics[27]. Coupling of radical ring-opening polymerization (rROP) of cyclic ketene acetals (CKAs) with CRP techniques including atom transfer radical polymerization (ATRP)[28,29], nitroxide mediated polymerization (NMP)[30], RAFT polymerization and macromolecular design via interchange of xanthates (MADIX)[31–33] has led to polymer backbones that are degradable. These CKA polymers have been covalently conjugated to drugs[34]. However to our knowledge degradable CKA polymers prepared by CRP have not been covalently attached to proteins. In the work described here, we utilized RAFT polymerization to prepare polymers that are degradable, protein-reactive, and PEG-like. Furthermore, we demonstrate the conjugation of these degradable PEG-like polymers to a protein, specifically Lyz, through a reversible disulfide linkage.

2. Experimental

2.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. The chain transfer agent (CTA), 3-(pyridine-2-yldisulfanyl)propyl-2-(ethylthiocarbonothioylthio) propanoate was synthesized as previously described[35]. 2,2-azobis(2-methylpropionitrile) (AIBN) was recrystallized from acetone.

2.2 Analytical Techniques

NMR spectra were obtained on an Avance 500 MHz DRX spectrometer. Proton NMR spectra were acquired with a relaxation delay time of 2 seconds for small molecules and 10 seconds for all polymers. MALDI-TOF mass spectrometry was performed on an Applied Biosystems Voyager-DE STR and operated in linear mode with an external calibration. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μm mixed D columns. LiBr (0.1 M) in dimethylformamide (DMF) at 40 °C was used as an eluent (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) (PMMA) standards from Polymer Laboratories. SDS-PAGE was performed using Bio-Rad Any kD Mini-PROTEAN-TGX gels. SDS-PAGE protein standards were obtained from Bio-Rad (Precision Plus Protein Pre-stained Standards). For SDS-PAGE analysis, approximately 5 μg of protein was loaded into each lane. Samples were incubated with 0.65 M DTT in Laemmli buffer (20 μL total) at 95 °C for 6 minutes for reducing lanes. Fast protein liquid chromatography (FPLC) was performed on a Bio-Rad BioLogic DuoFlow chromatography system equipped with a GE Healthcare Life Sciences Superdex 75 10/300 column. For Lyz and Lyz conjugates, Dulbecco's Phosphate-Buffered Saline (D-PBS) at 4 °C was used as the solvent (flow rate: 0.5 mL/min). Protein and conjugate concentrations were determined using the Thermo Scientific Pierce Coomassie Plus (Bradford) Protein Assay. Lyz activity was determined using the EnzChek® Lysozyme Assay Kit (E-22013) from Molecular Probes.

2.3 Synthesis of 5,6-benzo-2-methylene-1,3-dioxepane (BMDO)

BMDO was synthesized following literature procedures[36,37], taking extra precaution to prevent hydrolysis of the product. All glassware was oven-dried overnight. 20 mL of *tert*-butanol was distilled into a round bottom flask. 5,6-benzo-2-(bromomethyl)-1,3-dioxepane (4.5 g, 18.6 mmol) and potassium *tert*-butoxide (2.1 g, 18.6 mmol) were then added to the flask with stirring, under argon. The solution was brought to reflux and then heated at 100 °C for 12 hours, with a water-cooled condenser. The solvent was removed by rotatory evaporation and immediately placed back under inert atmosphere. 25 mL ether, dried by passing through an activated alumina column, was added to the crude mixture, which was then immediately filtered over celite to remove any remaining solids, into an oven-dried collection flask. Ether was then removed from the filtrate via rotatory evaporation. The crude product (a yellow oil) was then distilled under vacuum. The product was collected at 200 atm, 80 °C as a hard, white solid. Some fluffy white crystals were collected at 70 °C, but contained impurities, so a second vacuum distillation was performed. Yield: 1.87 g

(62%). δ ^{1}H-NMR 500 MHz ($CDCl_3$): 7.41-6.95 (m, 4H), 5.07 (s, 4H), 3.73 (s, 2H). δ ^{13}C-NMR 500 MHz ($CDCl_3$): 164.27, 135.85, 127.46, 126.21, 72.10, 69.54.

2.4 Synthesis of PDS-p(PEGMA-co-BMDO) (1)

Reversible addition-fragmentation chain transfer (RAFT) polymerization was employed to copolymerize poly(ethylene glycol) methyl ether methacrylate (PEGMA) and 5,6-benzo-2-methylene-1,3-dioxepane (BMDO). An initial feed ratio of 0.5:1:50:50 for AIBN:CTA:PEGMA:BMDO was used. AIBN (2.0 mg, 12.3 μmol), the CTA (9.7 mg, 24.7 μmol), PEGMA (0.35 mL, 1.23 mmol), BMDO (200.0 mg, 1.23 mmol), and 2.0 mL of dry DMF were placed into a 100 mL schlenk tube and subjected to five freeze-pump-thaw cycles before immersion in an oil bath at 70 °C. Aliquots were taken for time points and diluted in DMF and CD_3CN for analysis by GPC and ^{1}H-NMR, respectively. Percent conversion was calculated by comparing the sum of the integrations of vinylic protons from PEGMA (6.18-5.96 ppm) in the ^{1}H-NMR spectrum to the integration of regions where both PEGMA and the growing polymer chain overlap (4.46-3.99 ppm). BMDO conversion was not calculated due to the close proximity of the monomer peaks and those of the growing polymer chain. The polymerization was stopped at 71% PEGMA conversion after 4.62 hours by exposing the reaction mixture to atmosphere, and cooling with liquid nitrogen. The polymer was purified by extensive dialysis (Spectra/Por® Regenerated Cellulose Dialysis Membrane, MWCO 6-8000) against MeOH followed by 1:1 MeOH : MilliQ water, and then MilliQ water alone before lyophilization to remove water. The final molecular weight was determined by comparing the integrations for the aryl BMDO units (subtracting one proton for the PDS end-group) from 7.67-6.89 ppm and the PEGMA side-chain protons from 4.49-3.17 ppm to the PDS end-group proton at 8.44 ppm. Using this analysis, the final polymer was found to contain 5.3 BMDO units and 45.3 PEGMA units, resulting in an M_n of 14.8 kDa, and a BMDO content of 10.5%. δ ^{1}H-NMR 500 MHz (CD_3CN): 8.41 ppm (1H, PDS end-group NC**H**), 7.75 ppm (2H, PDS end-group NCCHC**H** and NCHC**H**), 7.57-6.90 ppm (22 H, PDS end-group NCC**H**CH and BMDO aryl C**H**), 5.26-4.87 ppm (10 H, backbone BMDO ester COOC**H**$_2$CCCH$_2$), 4.68 ppm (2H, Z end-group after BMDO unit, CC**H**$_2$S), 4.39-3.17 ppm (PEGMA side-chains), 3.00-0.27 ppm (polymer backbone). M_n = 10.9 kDa by GPC, Đ = 1.34 by GPC.

2.5 Synthesis of PDS-p(PEGMA-co-BMDO) (2)

An initial feed ratio of 0.5:1:200:200 for AIBN:CTA:PEGMA:BMDO was used. AIBN (1.0 mg, 6.2 μmol), the CTA (4.9 mg, 12.3 μmol), PEGMA (0.70 mL, 2.47 mmol), BMDO (400.0 mg, 2.47 mmol), and 4.3 mL of dry DMF were placed in a 100 mL schlenk tube and subjected to five freeze-pump-thaw cycles before immersion in an oil bath at 70 °C. Aliquots were taken for time points and diluted in DMF or CD_3CN for analysis by GPC and ^{1}H-NMR, respectively. Percent conversion was calculated as described above for Polymer **1**. The polymerization was stopped at 59% PEGMA conversion after 4.75 hours, by exposing the reaction mixture to atmosphere, and cooling with liquid nitrogen, and the polymer was purified as described above. The polymer chain was found to contain 141.1 PEGMA units and 13.9 BMDO units, with a final M_n of 45.0 kDa, and BMDO content of 9%. δ ^{1}H-NMR 500 MHz (CD_3CN): 8.44 ppm (1H, PDS end-group NC**H**), 7.79 ppm (2H, PDS end-group NCCHC**H** and NCHC**H**), 7.67-6.89 ppm (57 H, PDS end-group NCC**H**CH

and BMDO aryl C**H**), 5.31-4.93 ppm (26 H, backbone BMDO ester COOC**H**$_2$CCCH$_2$), 4.73 ppm (2H, Z end-group after BMDO unit, CC**H**$_2$S), 4.49-3.17 ppm (PEGMA side-chains), 3.04-0.29 ppm (polymer backbone). M_n (GPC) = 20.9 kDa, Đ (GPC) = 1.71.

2.6 Hydrolytic Degradation of 1 and 2

7.8 mg of either polymer (0.7 μmol for **1** and 0.4 μmol for **2**) was weighed into each of five 1.5 mL eppendorf tubes. 1 mL of either: 5% KOH, 0.5 M tosic acid in MilliQ water, MilliQ water acidified to pH 4 with HCl, D-PBS (pH 7.4), or 100 mM Carbonate/Bicarbonate (pH 10) was added to a tube and the sample placed on a rotating plate at 4 °C. Degradation was analyzed over one week, with timepoints taken at 1, 3, and 7 days. For **1** in D-PBS, day 4 was analyzed instead of day 3. Timepoints at 1 and 7 months for polymer **2** were also taken for samples diluted in D-PBS and 100 mM Carbonate/Bicarbonate (pH 10) at 4 °C to assess long-term stability under these conditions. 250 μL of each sample was lyophilized, dissolved in DMF, 0.1 M LiBr, filtered through a 0.2 μ filter, and analyzed by GPC.

2.7 Typical Conjugation of Thiolated Lysozyme 1 or 2 (Lyz-1, Lyz-2)

Lyz from hen egg-white was thiolated as previously described[38,39], and an average of 0.7 thiols/protein (verified by Ellman's assay) were installed. 2.83 mg (0.20 μmol) of thiolated Lyz (stored on TCEP resin at 4 °C), dissolved in 500 μL of D-PBS was placed in a LoBind eppendorf tube. 10 equivalents (based on end-group determined molecular weight by ^{1}H-NMR) of either polymer was then dissolved in 1 mL of D-PBS and added to the eppendorf tube. For the 10.9 kDa polymer (14.8 kDa by ^{1}H-NMR), 29.2 mg (1.98 μmol) was added. The solution was then placed on a rotating plate at room temperature for 4 hours, followed by concentration by ultracentrafugation (10 kDa MWCO Centriprep®, Millipore). This solution was then purified by FPLC. Unmodified Lyz eluted around 35 minutes, while Lyz-**1** and Lyz-**2** eluted between 20–31 minutes. Conjugates were characterized by SDS-PAGE, the concentration was determined by Bradford assay, and the activity was analyzed using the EnzChek® Lysozyme Assay Kit.

2.8 Typical Reduction of 3 and 4 with Dithiothreitol

1 to 5 μg of Lyz-**1** or Lyz-**2** were diluted in 20 μL of Laemmli buffer with 0.65 M DTT. The samples were incubated at 95 °C for 6 minutes before loading into a gel lane for SDS-PAGE analysis.

2.9 Typical Hydrolytic Cleavage of Lys-1 and Lys-2 with 5% KOH

Lyz-polymer conjugates (about 58 μg of Lyz-**1** or Lyz-**2**) were diluted in 200 μL of degassed MilliQ water, 5% KOH (final concentration 0.29 mg/mL) and allowed to incubate on a rotating plate at 4 °C for 24 hours. The solution was then neutralized by ultracentrafugation (10 kDa MWCO Centriprep®, Millipore) with D-PBS for four ten-minute cycles at 12 rpm to a final volume of 40 μL, to be used for SDS-PAGE analysis.

2.10 Reduction of Lyz-1 and Lyz-2 with Glutathione

Lyz-polymer conjugates (0.71 mg/mL for Lyz-**1** and 0.51 mg/mL for Lyz-**2**) were incubated with 5 mM glutathione (GSH) on a rotating plate at 4 °C for 6 hours. The polymer was not

released under these conditions for Lyz-**2**, and therefore treatment with 10 mM GSH on a rotating plate at room temperature over 4 days was analyzed. FPLC purification of Lyz-**2** after reduction separated unmodified Lyz (33–39 minutes) from uncleaved conjugates (20–31 minutes). Concentrations were determined by Bradford assay and activity analyzed using the EnzChek® Lysozyme Assay Kit.

3. Results

3.1 Synthesis of PDS-p(PEGMA-co-BMDO)s 1 and 2

We chose to install the cysteine reactive chain-end by using a PDS-modified chain transfer agent (CTA), 3-(pyridine-2-yldisulfanyl)propyl-2-(ethylthiocarbonothioylthio) propanoate. While this CTA is not ideal for the polymerization of methacrylates, Junkers found that the addition of BMDO as a comonomer resulted in well-defined polymers with a similar CTA, and BMDO has been shown to polymerize with methacrylates under typical CRP conditions[33,40]. Two copolymers, PDS-p(PEGMA-*co*-BMDO) (**1** and **2**) were synthesized by RAFT polymerization, with M_n (^{1}H-NMR) of 14.8 kDa and 45.0 kDa and M_n (GPC) of 10.9 kDa and 20.9 kDa, (as compared to poly(methyl methacrylate) (PMMA) standards) and molecular weight dispersities of 1.34 and 1.71. The molecular weight dispersities are broad, as observed for other CKA polymers[28,31]. Yet, the resulting polymers clearly contained the pyridyl disulfide end group for conjugation to free cysteines on proteins (see NMR spectra, Figure S1a and Figure S2a). The polymers had an average of 10.5% and 9% BMDO units per polymer chain, respectively, determined through analysis of ^{1}H-NMR peak ratios. This incorporation is much lower than the feed ratios of 50:50 and 200:200 BMDO:PEGMA for **1** and **2**, respectively. Reactivity ratios for BMDO and MMA have been reported under ATRP conditions as $r_1 = 0.53$ and $r_2 = 1.96$ by Wickel[41]; however Junkers assessed reactivity ratios under free radical conditions at low conversions to avoid the affects of composition drift, and determined the ratios to be $r_1 = 0.33$ and $r_2 = 6$. Although in this research, PEGMA was utilized instead of MMA, the incorporation of BMDO observed is consistent with a large difference in reactivity ratios as described by Junkers for MMA and BMDO.

3.2 Hydrolytic Degradation of 1 and 2

We next investigated the polymer degradation kinetics under acidic, basic, and physiological conditions. Stability at neutral and mildly basic conditions would be ideal for storage and delivery to the blood stream while acidic degradation would be advantageous for release upon cell uptake in the acidic endosome, for tumor therapy or for oral drug delivery. Complete degradation has been shown for similar polymers incubated in complete cell medium for one week[32] In addition, p(PEGMA-co-BMDO), synthesized by ATRP, was shown to completely degrade within 24 hours after incubation with 5% KOH, and partially degrade under acidic conditions (pH 3–5)[28,29. 32]Therefore, to test the degradation of our polymers, we applied the following conditions: 5% KOH, 0.5 M tosic acid, 100 mM carbonate/bicarbonate buffer (pH 10), MilliQ water acidified to pH 4 with HCl, and D-PBS, pH 7.4.

After incubation in 5% KOH, GPC indicated polymer degradation by a shift in molecular weights from 10.9 kDa to 4.4 kDa fragments for **1** and 20.9 to 3.0 kDa fragments for **2** with final dispersities of 1.2 and 1.4, respectively (Figure 2). ^{1}H-NMR analysis of the polymers indicated that after incubation for one day in 5% KOH complete hydrolysis was achieved as visualized by the disappearance of BMDO methylene proton signals at 5 ppm for both polymers (see supporting information Figure S3 and S4). These results confirm that both polymers can be degraded into smaller fragments for rapid clearance from the body. For **1** tosic acid and 100 mM carbonate/bicarbonate (pH 10) buffer partially degraded the polymer backbone, while D-PBS (pH 7.4) and MilliQ water acidified to pH 4.0 did not significantly degrade the polymer within one week (Figure S5a). For the longer polymer **2**, tosic acid partially degraded the polymer backbone, but 100 mM carbonate/bicarbonate (pH 10) buffer, D-PBS (pH 7.4), and MilliQ water acidified to pH 4.0 did not significantly degrade the polymer (Figure S5b). To investigate long-term degradation, timepoints at 1 and 7 months for polymer **2** in either D-PBS or 100 mM carbonate/bicarbonate (pH 10) were assessed. The polymer was stable at pH 7.4 over the 7-month time period while at pH 10 the polymer degraded to 18.3 kDa (Đ = 1.7) after 1 month and to 8.2 kDa (Đ =1.7) after 7 months. Therefore the polymers could be expected to remain intact at physiological pH while a very gradual degradation of the polymer could be expected under mildly basic conditions.

3.3 Conjugation of 1 and 2 to Thiolated Lysozyme

Lyz was chosen as a model protein for polymer conjugation. Thiols are often added to the lysine side-chains of proteins to allow for single polymer-chain conjugation. Therefore thiolated Lyz was conjugated to polymer **1** and **2** (Scheme 1b) resulting in Lyz-**1** and Lyz-**2** conjugates visualized by gel electrophoresis as smeared bands (Figure 3 lanes 7 and 8). Such smears are expected due to variation in polymer molecular weight, and are commonly seen with proteins conjugated to non-monodisperse polymers. Indeed, the polymers alone result in smear bands when stained with iodine (data not shown).

3.4 Hydrolytic or Reductive Cleavage of 1 or 2 from Lyz-1 or Lyz-2

Enzymatic or hydrolytic degradation of the polymer chain from the conjugates should occur *in-vivo*. Similar polymers have been shown to degrade partially via enzymes, for example lipases[28], or completely in cell culture medium such as Dulbecco's Modified Eagle Medium (DMEM)[32]. However, due to the difficulty of separating the released Lyz from other enzymes and proteins in medium, we chose to visualize the release of Lyz under basic conditions by gel electrophoresis. Since the polymer alone degraded to the greatest extent with 5% KOH, this condition was chosen to quickly visualize the hydrolytic degradation of the polymer chain from Lyz (Scheme 2). The purified conjugates were incubated with 5% KOH for 24 hours and complete release of Lyz in both cases was observed (Figure 3 lanes 4, 5 reducing and 9, 10 nonreducing). ^{1}H-NMR analysis of polymer **1** indicated an average of 5 BMDO units, and therefore complete cleavage of the backbone should result in 6 fragments. The expected M_n of the polymer fragments after complete hydrolysis is 10.9 kDa/6 = 1.8 kDa. For **2**, the original polymer contained 14 BMDO units/polymer chain, which would result in an average of 15 fragments, with M_n = 1.4 kDa. Therefore, the degradation products were also analyzed by mass spec, resulting in fragments with m/z = 1.9 kDa for

both size conjugates indicating complete degradation. As expected some degradation of Lyz itself was observed under such harsh conditions (seen as a smear below the normal Lyz band in Figure 3 lanes 4, 5). Yet, these results serve as a proof of concept, indicating that the polymer backbone can be hydrolytically cleaved from the conjugates.

3.5 Activity of Lyz-1 and Lyz-2 before and after reductive treatment with glutathione

In the body, the polymer chain may either hydrolytically degrade off of the conjugates, or reductively cleave prior to hydrolytic degradation[5,42]. To visualize the reductive cleavage of the polymer chain from Lyz, the conjugates were incubated with 0.65 M DTT at 95 °C for 6 minutes (Scheme 2). Complete reduction of the disulfide bond and release of Lyz was observed (Figure 3, lanes 2 and 3).

More physiologically relevant reducing conditions (5 or 10 mM GSH) were used to analyze the activity of Lyz released from the polymers (Figure 4). Original activity of Lyz-**1** was 63 +/− 3 % of native Lyz, which is fairly high. For comparison, Roberts and Harris observed 60% activity after cleavage of the polymer[4]. This high activity is likely due to the smaller size of the polymer as well as the addition of a single polymer chain per Lyz. After treatment with 5 mM GSH for 6 hours at 4 °C, the activity of Lyz-**1** increased to 77 +/− 2% indicating incomplete release of Lyz within that time period. The activity of conjugate Lyz-**2** was 19.6 +/− 1.9%. This lower activity was likely due to the large size of the polymer. However, after treatment with 5 mM GSH at 4 °C for 6 hours, the activity did not significantly increase and incomplete cleavage of the polymer chain from Lyz as visualized by gel electrophoresis and FPLC (data not shown). The large size of polymer **2** may shield Lyz from the reducing agents. After treatment with 10 mM GSH at room temperature for 4 days, the activity increased to 39.4 +/− 2.0%. To ensure that the released Lyz was active, we purified the reduced conjugate Lyz-**2** by FPLC, and separated the released Lyz from the uncleaved conjugates. This released Lyz exhibited 100.7 +/− 3.7% native activity, indicating that the released Lyz is completely active. It should be noted that adding **1** or **2** did not result in decreased activity, demonstrating that the observed decreases were a result of covalent conjugation.

4. Discussion

Overall, these results indicate that the initial activity of a protein therapeutic can be adjusted by varying the length of the polymer chain, and that the protein activity increases under reducing conditions that are similar to those found in the endosome or lysosome. There are some cases when high initial activity of a protein-polymer conjugate would be desired. Polymer **1** is a good choice in this case. However, there are other cases when it is desirable to mask the activity of the protein until a triggered release in order to avoid off-target effects. In this case, polymer **2** is a good choice because the activity is reduced considerably, yet 100% active protein is obtained upon cleavage of the polymer.

It is the degradability of the polymer main chain that is the significant advantage of these polymers for biomedical use. Small polymers (less than 30 kDa for PEG) are eliminated from the body[5]. Yet current data on the observation of PEG aggregates indicates that this is a significant challenge, particularly for replacement therapies where the protein conjugate

would be administered over a long period of time. For instance, PEGs as small as 20 kDa have been found to cause vacuolization with repeated administration or with high dosage[5]. Furthermore, it is known that larger polymers result in better pharmacokinetics. Yet, polymers that are too large cannot be used because they are not cleared from the body. With the approach reported herein, it is expected that the polymer will be released from the protein and subsequently degraded, avoiding polymer accumulation for any size polymer and allowing secretion of even larger polymers. Thus, by using a dually degradable polymer (through backbone hydrolysis and reductive cleavage) as described herein, the resulting conjugates should exhibit the advantage of enhanced pharmacokinetics, with enhanced activities and reduced accumulation in the body.

5 Conclusions

In this report we described the synthesis of two protein-reactive, PEGylated, backbone degradable polymers by RAFT polymerization and the conjugation of these polymers to Lyz. Hydrolytic degradation of both polymers from Lyz with base as well as the reductive cleavage of the polymer chains from Lyz with DTT was observed. The smaller 10.9 kDa polymer-Lyz conjugate retained 63% activity and the larger 20.9 kDa polymer-Lyz conjugate retained only 19.6% native activity, yet regained 100% activity when slowly released by reduction with GSH. These results indicate that such degradable polymers should be explored in the field of polymer-protein therapeutics both with PEG-like and tailored polymers, and these studies are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Degradable PEG-like, protein-reactive polymers were synthesized by RAFT polymerization
- p(PEGMA-*co*-BMDO)-lysozyme conjugates were prepared with or without masked activity
- Degradation of the polymers alone and from lysozyme was assessed.
- The activity of lysozyme reductively released from the polymer(s) was analyzed

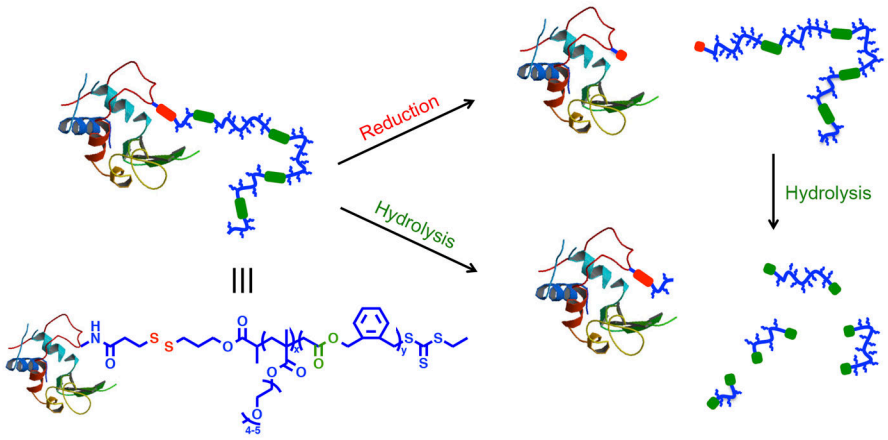


Figure 1.
PEGylated protein conjugate, released by either reduction or hydrolysis.

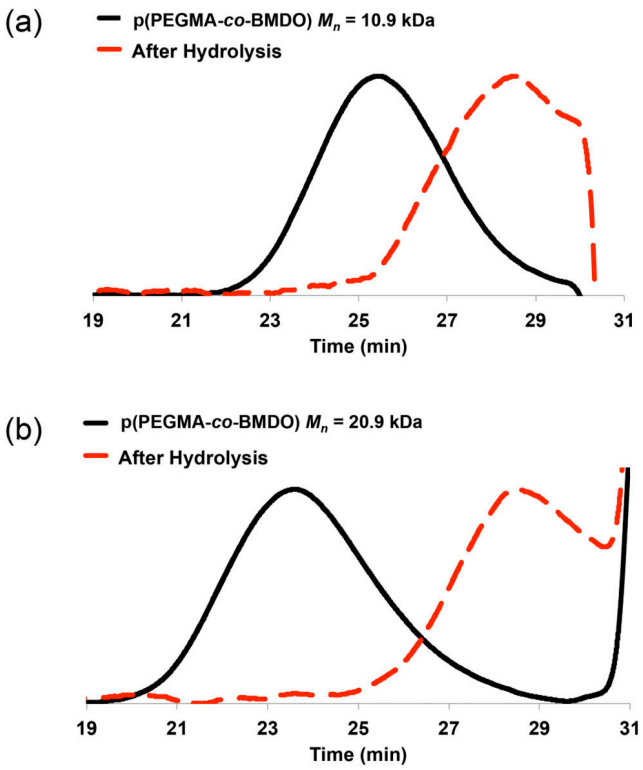


Figure 2.
GPC analysis of polymer before and after incubation in 5% KOH of a) **1** for 7 days and b) **2** for 1 day.

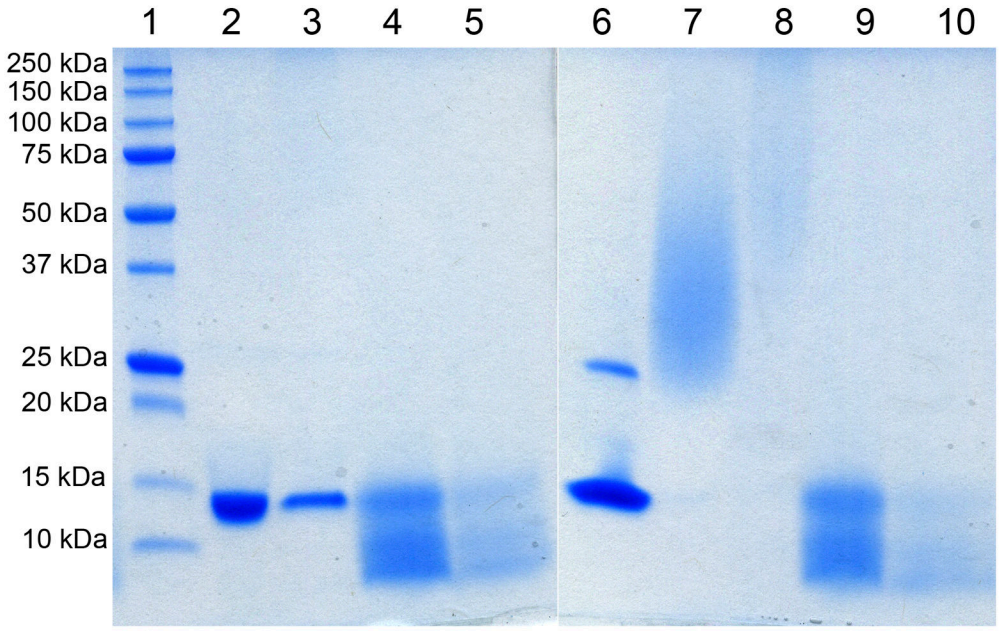


Figure 3.

SDS-PAGE gel electrophoresis, visualized by Coomassie blue staining, of Lyz-**1** and Lyz-**2** before and after treatment with reducing agent and/or base (lane 1: protein marker; lane 2: Lyz-**1** reducing conditions; lane 3: Lyz-**2** reducing conditions; lane 4: Lyz-**1** KOH treated, reducing conditions; lane 5: Lyz-**2** KOH treated, reducing conditions; lane 6: Lyz; lane 7: Lyz-**1**; lane 8: Lyz-**2**; lane 9: Lyz-**1** KOH treated; lane 10: Lyz-**2** KOH treated).

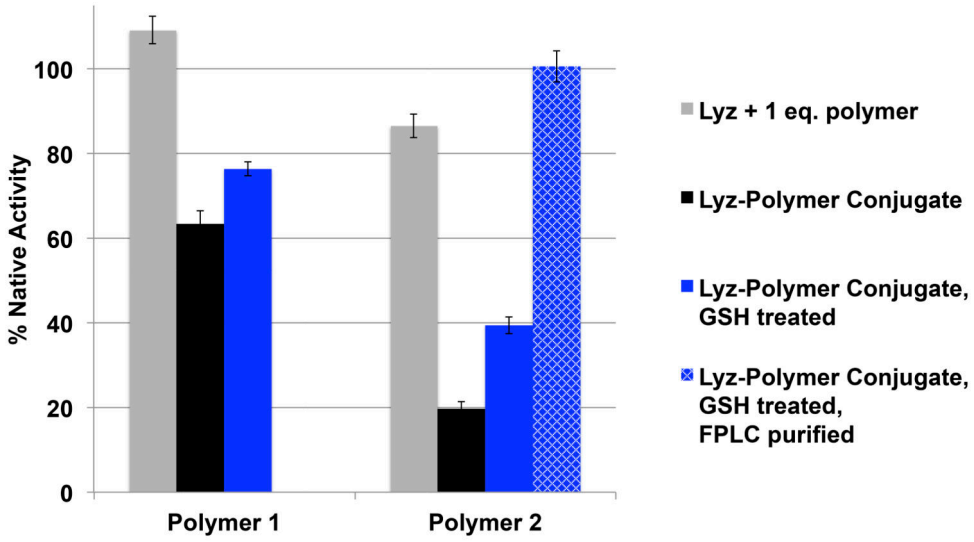
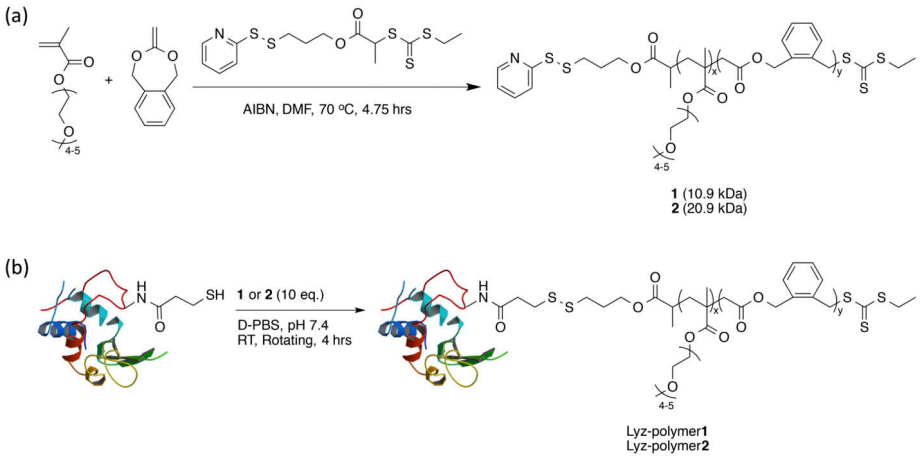


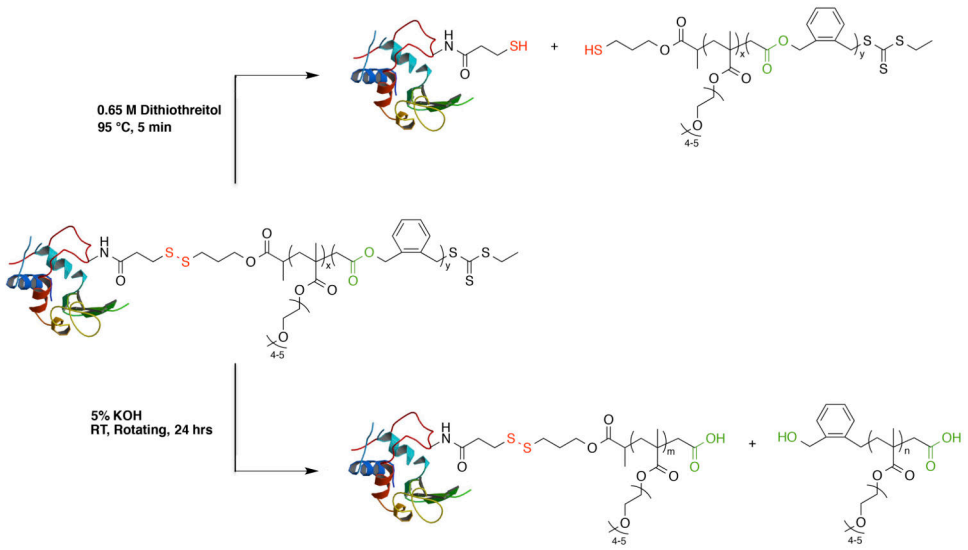
Figure 4.

Activity of native lysozyme + one equivalent of (non attached) polymer in solution, Lyz-polymer conjugates before and after treatment with GSH, and Lyz after cleavage from Lyz-**2**. Lyz-**1** was treated with 5 mM GSH and Lyz-**2** was treated with 10 mM GSH.



Scheme 1.

(a) RAFT polymerization of PEGMA and BMDO (b) Covalent modification of thiolated Lyz with polymer **1** or **2**.



Scheme 2.

Reductive or hydrolytic cleavage of polymer **1** or **2** from Lyz