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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.

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 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].

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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, 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 (Fig. 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 s for small molecules and 10 s 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

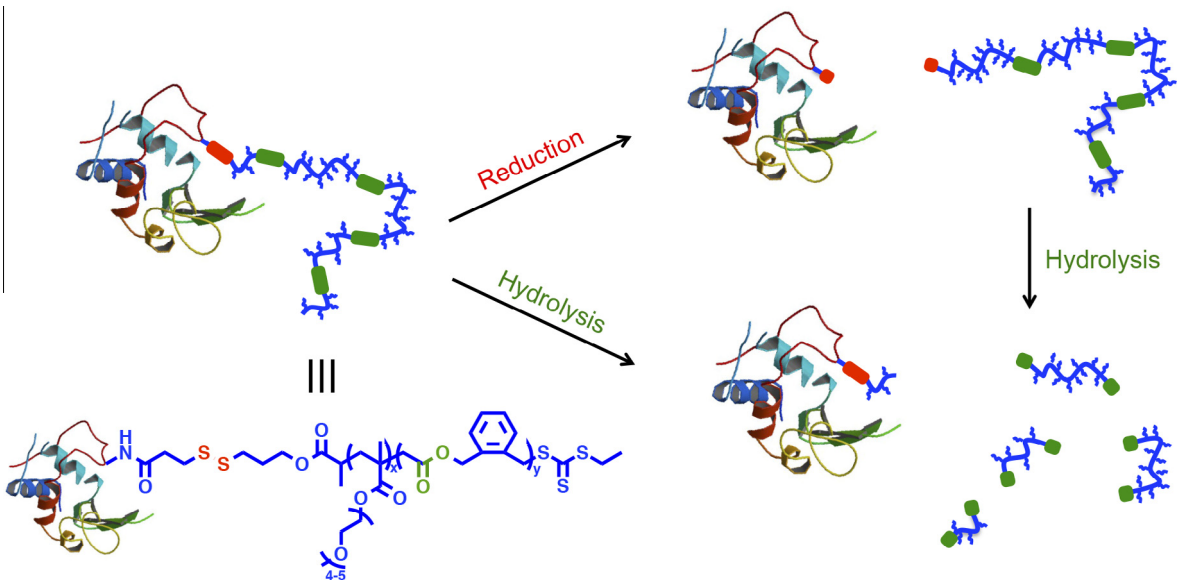

Fig. 1. PEGylated protein conjugate, released by either reduction or hydrolysis.