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Tailoring enzyme activity and stability using polymer-based protein engineering

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ABSTRACT

Polymer-based protein engineering (PBPE) offers an attractive method to predictably modify and enhance enzyme structure and function. Using polymers that respond to stimuli such as temperature and pH, enzyme activity and stability can be predictably modified without a dependence on molecular biology. Herein, we demonstrate that temperature responsive enzyme-polymer conjugates show increased stability while retaining bioactivity and substrate affinity. The bioconjugates were synthesized using a "grafting from" approach, where polymers were grown from a novel water-soluble initiator on the surface of a protein using atom transfer radical polymerization. Prior to polymer synthesis, the polymerization initiating molecule was covalently attached to surface accessible primary amines (lysine, N-terminal) of chymotrypsin, forming a macroinitiator. Poly(*N*-isopropylacrylamide) and poly[*N*,*N*'-dimethyl(methacryloylethyl) ammonium propane sulfonate] were grown separately from the initiator modified chymotrypsin. Both polymers were selected because of their temperature-dependent conformations. We observed that the enzyme-polymer conjugates retained temperature-dependent changes in conformation while still maintaining enzyme function. The conjugates exhibited dramatic increases in enzyme stability over a wide range of temperatures. We can now predictably manipulate enzyme kinetics and stability using polymer-based protein engineering without the need for molecular biology dependent mutagenesis.

1. Introduction

Protein-polymer conjugates have long been used to manipulate the native properties of proteins. Many bioconjugates have been synthesized with poly(ethylene glycol) (PEG), which helps to increase protein stability and the half-life of proteins in circulation in blood [1]. More recently, efforts have been devoted to attaching stimuli responsive polymers that create "smart" bioconjugates [2–4] that add functionality to enzymes. We have become interested in whether the attachment of temperature responsive polymers to proteins might impart unique properties on the enzyme. Two polymers that show temperature responsiveness are poly(*N*-isopropylacrylamide) (pNIPAm) and poly[*N*,*N*'-dimethyl(methacryloylethyl) ammonium propane sulfonate] (pDMAPS), though they respond to temperature in sharply distinct ways. pNIPAm exhibits lower critical solution temperature (LCST) behavior [5], where above ~32 °C the polymer experiences a reversible change in conformation, increasing its hydrophobicity and becoming immiscible in water. The same reversible change is seen for pDMAPS, except that the polymer is immiscible below the upper critical solution temperature (UCST). The UCST of pDMAPS has been shown to have strong dependence on polymer chain length and solution ionic strength [6], while the LCST of pNIPAm is less variable [7], but still can be affected by several factors, such as degree of chain branching and molecular weight [8].

The synthesis of protein-polymer bioconjugates can be accomplished using one of two methods: "grafting to" and "grafting from". In "grafting to", polymers are synthesized to a desired molecular weight or degree of polymerization (DP) prior to conjugation with the protein. Once synthesized, an end group functionality on the polymer is exploited to attach polymer chains, through covalent bonds [2–4], to specific amino acid residues, often serine or lysine, on the surface of the protein molecule. In the "grafting to" method, the achievable polymer density around the biomolecule is

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often limited due to steric hindrance between each bound long polymer molecules and subsequent polymer chains that could modify the protein. In addition, the specific polymer chain attachment site to the biomolecule is often unknown when using the "grafting to" approach. Conversely, in the "grafting from" approach, protein molecules themselves serve as the initiating site for controlled radical polymerization (CRP) reactions [9]. Several different CRP reactions, such as atom transfer radical polymerization (ATRP) or reversible addition-fragmentation chain transfer (RAFT) [10], can be used when synthesizing polymer conjugates using the "grafting from" approach. Previously our group utilized "grafting from" and ATRP to synthesize well defined chymotrypsin(CT)-polymer conjugates [11]. Using this method we were able to grow polymer from multiple ATRP initiating sites on the surface of each protein molecule but it was not possible to achieve a high density of polymer chains around each individual biomolecule because the efficiency of the protein-initiator reaction was low. In addition, one can vary ATRP reaction conditions and initial monomer-initiator concentration ratios in the "grafting from" approach to tune the desired chain length or molecular weight and the number of polymer chains per protein molecule. Lastly, it is relatively facile to control ATRP and thus to generate bioconjugates with a low polymer polydispersity index (PDI) and high uniformity.

Previous studies have described syntheses of pDMAPS and pNIPAm bioconjugates using various proteins [12–14]. These studies did not address the effects of the polymer on enzyme kinetics, stability, and substrate affinity or they utilized the "grafting to" approach. Recently, greater efforts have been applied toward developing aqueous based "grafting from" approaches to limit potential protein denaturation in organic solvents during conjugation. Averick et al. [15] described the synthesis of bovine serum albumin-oligo(ethylene oxide) methacrylate (BSA-OEOMA) conjugates in biologically relevant conditions. More recently, we have reported ([16]) on the synthesis of a novel water-soluble ATRP initiator molecule that was used to synthesize CT-poly(*N*,*N*-dimethylaminoethyl methacrylate) (CT-pDMAEMA) conjugates with pH-dependent enzyme kinetics and stability. This initiator enabled very high density growth of polymers from proteins that increased the molecular weight of conjugates by more than an order of magnitude.

In the study described herein, chymotrypsin (CT) was chosen as a model protein to modify with polymers that exhibit temperature-dependent changes in conformation. CT is a serine protease enzyme found in the small intestine that aids in digestion. CT can degrade itself via autolysis (self-digestion). The mechanism and kinetics of CT have been studied exhaustively over a wide temperature and pH range [17,18].

The goal of the study described herein was to predictably manipulate the kinetics and stability of CT-pDMAPS and CT-pNIPAm bioconjugates using temperature as the trigger for a change in enzyme function. Both pNIPAm and pDMAPS were chosen in order to examine changes in relative enzyme activity and stability at stimuli responsive temperatures both above and below room temperature. The contrasting temperature responsive behavior of the UCST and LCST bioconjugates provided an attractive approach to examine how polymer chain collapse at varying temperatures affects enzyme bioactivity, stability, and substrate affinity.

2. Materials and methods

2.1. Materials

α-Chymotrypsin (CT) from bovine pancreas (type II), copper (I) bromide, 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA), *N*-succinyl-L-Alanine-L-Alanine-L-Proline-L-Phenylalanine-*p*-nitoroanilide (Suc-AAPF-pNA), ([2-(Methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl) ammonium hydroxide) (DMAPS), were purchased from Sigma Aldrich (St Louis, MO) and used without further purification. *N*-isopropylacrylamide was purchased from Sigma Aldrich (St. Louis, MO) and purified by recrystallization using hexane. Me6TREN was synthesized as described previously by Ciampolini and Nardi [19]. Dialysis tubing (molecular weight cut off, 25-, 15- and 1.0-kDa, Spectra/Por®, Spectrum Laboratories Inc., CA) for conjugate isolation were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Reaction between the ATRP initiator and chymotrypsin

Synthesis of the ATRP initiating molecules was carried out as described previously [16]. Following synthesis, initiator molecule (469 mg, 1.4 mmol) and CT (1.0 g, 0.04 mmol protein, 0.56 mmol $-NH_2$ group in lysine residues) were dissolved in sodium phosphate buffer (100 mL of 0.1 M at pH 8.0). The solution was stirred at 4 °C for 3 h, then dialyzed against deionized water, using dialysis tubing with a molecular weight cut off of 15 kDa, for 24 h at 4 °C and then lyophilized.

2.3. Surface initiated ATRP from CT-Initiator

To synthesize the CT-pDMAPS conjugates, the CT-Initiator complex (50 mg, 0.024 mmol initiator) and DMAPS (335 mg, 1.2 mmol) were dissolved in sodium phosphate buffer (20 mL, pH 6.0). In a separate flask, HMTETA (33 μL, 0.12 mmol) was dissolved in deionized water (10 mL) and bubbled with Argon for 10 min. Cu(I)Br (17 mg, 0.12 mmol) was added to the HMTETA solution and Argon was bubbled for an additional 50 min prior to addition of the copper catalyst solution. The solution was then stirred for 18 h at 4 °C. Lastly, the solution was purified using dialysis tubing with a molecular weight cut off of 25 kDa for 48 h against deionized water at 4 °C, and then lyophilized.

For CT-pNIPAM synthesis, CT-Initiator conjugate (50 mg, 0.024 mmol initiator) and NIPAm (271 mg, 2.4 mmol) were dissolved in deionized water (20 mL). In a separate flask, Me6TREN (32 μL, 0.12 mmol) was dissolved in deionized water (10 mL) and bubbled with Argon for 10 min. Cu(I)Br (17 mg, 0.12 mmol) was added to the Me6TREN solution and Argon was bubbled for an additional 10 min. The procedure for CT-pNIPAM synthesis from this point forward was the same as described above for CT-pDMAPS synthesis.

2.4. Polymer cleavage from CT surface

Both pDMAPS and pNIPAm were cleaved from the surface of CT using acid hydrolysis. CT-pDMAPS conjugates were incubated (15 mg/mL) in 6 N HCl at 110 °C under vacuum for 24 h. CT-pNIPAm (20 mg/mL) conjugates were incubated in 4.5 N *p*-toluene sulfonic acid at 80 °C under vacuum for 72 h. Following incubation, samples were isolated from CT using dialysis tubing (MWCo 1 kDa) for 48 h, and then lyophilized. Lastly, polymer molecular weight was determined using GPC.

2.5. Gel permeation chromatography

Number and weight average molecular weights (M_n and M_w) and the polydispersity index (M_w/M_n) were estimated by gel permeation chromatography (GPC). For pDMAPS, analysis was conducted on a Waters 2695 Series with a data processor, using 80% 100 mM sodium phosphate buffer(pH = 9.0)/20% Acetonitrile with 0.01 volume % NaN_3 as an eluant at a flow rate 1 mL/min, with detection by a refractive index (RI) detector. Polystyrene sulfonate standards were used for calibration. For pNIPAm, analysis was conducted using dimethylformamide (DMF) with 50 mM LiBr at a flow rate of 1 mL/min and 50 °C, with detection by an RI detector. Poly(ethylene oxide) standards were used for calibration and diphenylethylene was used as a flow marker.

2.6. LCST/UCST determination

CT-pDMAPS and CT-pNIPAm (2–3 mg polymer/mL each) were dissolved in 0.1 M phosphate buffer (pH 8.0). CT-pNIPAm samples were heated from 20 to 35 °C and CT-pDMAPS samples were cooled from 30 to 5 °C at ±1 °C/min. The absorbance at 490 nm was measured and LCST/UCST temperature was calculated from the inflection point on the temperature versus absorbance curves.

2.7. Dynamic light scattering

CT-pDMAPS (3 mg/mL) and CT-pNIPAm (0.5 mg/mL) samples were dissolved in 0.1 M phosphate buffer (pH 8.0) and then filtered using a 0.45 μM cellulose filter. A Malvern Zetasizer nano-ZS was used to measure hydrodynamic radius (R_h). Each sample was measured in triplicate or greater at each specified temperature.

2.8. CT and CT-conjugate biocatalytic activity

N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide was used as a substrate for enzyme bioactivity assays. In a 1 mL cuvette, 0.1 M sodium phosphate buffer (810–990 μL, pH 8.0, incubated at 25 °C), substrate (0–180 μL, 6 mg/mL in DMSO (0–1.2×10^{-3} M)), and enzyme (10 μL, 0.1 mg enzyme/mL 0.1 M pH 8.0 sodium phosphate buffer (4×10^{-8} M)) were mixed. The rate of the hydrolysis was determined by recording the increase in absorbance at 412 nm for the first 30 s after mixing. K_M and