ABSTRACT

EXPLORING THE EFFECTS OF POLYMER FUNCTIONALITY ON THE ACTIVITY AND STABILITY OF LYSOZYME AND CELLULASE CONJUGATES

by Melissa Eileen Dougherty

Biomacromolecules made by covalent attachment of polymers to the surfaces of proteins are an interesting area of research with significant applications in drug therapies and growing interest in energy and nanotechnology. Protein-polymer conjugates are typically synthesized by either the grafting-to or grafting-from approach. In grafting-to, polymer is separately synthesized and characterized prior to linkage to the protein. However, in grafting-from, an initiating group is attached to the protein, which allows for growth of the polymer directly from the protein surface. Herein, a hybrid approach is used, combining the advantages of both conjugation techniques. RAFT polymerization is used to grow water soluble polymers for grafting-to and subsequent chain extension from enzymes of interest. First, conjugates made from the well-studied, model enzyme lysozyme are discussed. Next, cellulase-polymer conjugates with potential applications in biofuel production are explored. In these studies, the impact of polymer size and functional groups on enzymatic activity, thermal and chemical stability are investigated. Important trends gleaned from this work contribute to the understanding of structure-property correlations in protein-polymer conjugates. These relationships are critical to the advancement of protein-polymer conjugates in efforts to produce engineered biomacromolecules with tunable behaviors for specific applications.
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EXPLORING THE EFFECTS OF POLYMER FUNCTIONALITY ON THE ACTIVITY AND STABILITY OF LYSOZYME AND CELLULASE CONJUGATES

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Introduction

Protein-polymer conjugates have been investigated for nearly 40 years, with the aim of imparting stability, functionality, and tunable properties to proteins. Over the course of many years of study, new heights have been achieved allowing for the use of diverse polymers and an array of proteins. Continued research has expanded the usefulness of these macromolecules from protein therapeutics to applications in medicine, catalysis, energy, nanotechnology, and materials science.

Bioconjugates exist at an interface between organic polymer chemistry and protein biochemistry. Both sides contribute to the characteristics observed in conjugates. Proteins, which are capable of catalysis, drug delivery and biorecognition, can be highly sensitive to proteases, organic solvents, light, heat, and to changes in pH and temperature. Synthetic polymers are robust and typically have greater tolerance to those stimuli which proteins are sensitive to. This threshold for stimuli makes polymers especially beneficial when attached to protein surfaces. Polymers can provide a handle to tune behaviors of the protein related to functionality, pH, temperature, and stability. Combining the unique properties of proteins and polymers into single entities opens doors to many applications of these interesting biomacromolecules.

The first successful attachment of polymer to protein was reported in 1977 when monomethoxy-poly(ethylene glycol) was coupled to bovine serum albumin (BSA). This and subsequent conjugations of PEG to proteins provided evidence that conjugates can increase protein circulation times compared to native proteins. Attaching PEG chains to proteins has shown to increase protein half-life and circulation times by increasing the size of the protein and adding steric bulk which makes removal from the body and degradation by other enzymes more difficult. Additionally, PEGylated drugs made from proteins with conjugated PEG chains have demonstrated longer half-lives than their native counterparts, which enables longer-lasting therapeutic behavior with fewer doses. These qualities make protein-polymer conjugates especially attractive for use as therapeutics. For example, in 1990 the first PEGylated protein gained FDA approval as the drug Adagen which treats severe combined immunodeficiency disease (SCID). The following decades produced a total of 10 FDA-approved protein-polymer conjugates as protein therapeutics, with the first site-specific conjugate gaining FDA approval in 2002.

The first polymer conjugated to proteins, PEG, remains one of the most widely used polymers in protein-polymer conjugations; however, the methods for attaching polymer chains to
protein surfaces have evolved since 1977. In the early years of conjugation studies, synthesis and purification of conjugates were often difficult, inefficient multistep processes.\(^2\) Derivatization of polymer end groups prior to attachment to the protein were challenging and yielded conjugates that were often weakly tethered, had poor attachment selectivity and required post-polymerization modifications to obtain desired functionality.\(^2\) These techniques progressed into reactions which allow for reliable conjugation of lysine, cysteine, glutamate, and aspartate, in addition to less common conjugations of tyrosine and tryptophan.\(^7\) Further, conjugation methods have advanced to include site-specific modification, “click” chemistry, enzymes as tools for conjugation, and introduction of non-natural amino acids to the protein backbone.\(^2, 7\) Improvements in conjugation techniques over the course of 40 years have allowed for the expansion of the field to conjugations of new enzymes and polymers.

PEGylation remains a popular method for producing protein therapeutics; however, drug delivery systems have also been developed based on self-assembling nanoparticles\(^8\) and “smart” polymers.\(^9\) Stimuli-responsive, or “smart”, polymers exhibit property changes in response to external stimuli.\(^9\)–\(^11\) A great deal of work has been done to show that these responsive polymers can be used to manipulate conjugates with pH and thermal controls.\(^10\) For example, pH-responsive conjugates have been developed for drug delivery because changes in hydrophobicity and surface interactions based on pH allow for controlled release of drugs in tablet and mucosal hydrogel forms.\(^9\) Specifically, responsive N-isopropylacrylamide (NiPAm) polymers\(^12, 13\) have been used to enhance the stability of conjugates\(^14\) and in thermoresponsive conjugates for recycling of cellulase for biocatalysis applications.\(^15\) Like pNiPAm, polymers of poly(2-(dimethylamino)ethyl methacrylate (pDMAEMA) have shown to provide a handle with which to tune enzymatic activity and stability.\(^16\) These conjugates exhibit both thermal and pH-responsiveness with changes in stimuli and demonstrate the ability to engineer conjugate behaviors around stimuli responses.\(^16\) Responsive polymers are interesting and versatile class of polymers that can impart stability and tunable behaviors to protein conjugates.

Glycopolymers make up another beneficial group of polymers that elicit intriguing effects when conjugated to proteins. Containing carbohydrate functional groups, glycopolymers mimic the structure of natural polysaccharides and exhibit similar stabilization properties.\(^17\) Maynard’s group has done a number of studies on trehalose-based glycopolymers for protein stabilization. In one such study, a series of glycopolymers was investigated for use as excipients, or additives, for
protein stability during storage. Activity studies following exposure to heat and lyophilization showed that samples containing glycopolymers additives impart enhanced stability over samples with no additive, with a stronger effect observed at high polymer concentrations. These principles were applied to novel protein-polymer conjugates containing RAFT-synthesized trehalose glycopolymers. Here, the conjugates exhibited greatly enhanced stability to heat and lyophilization compared to native lysozyme. The stabilization that glycopolymers provide proteins as excipients and in protein-polymer conjugates makes this class of polymers highly motivating for further protein-related studies.

As the technologies to produce protein-polymer conjugates have grown, the applications for which these biohybrids are used have also increased. What began as a means to stabilize proteins for medical treatments, has matured to advanced drug therapies, thermoresponsive macromolecules, biocatalysis, and nanotechnology uses as well. While work on applicable bioconjugate technologies advances, fundamental studies on the effects of polymers on model proteins are inherently valuable. Gaining a deeper understanding into the properties and interactions that govern protein-polymer conjugate behaviors will make engineering macromolecules for specific applications more feasible.

Polymerization Methods Suited for Synthesis of Conjugates

Radical polymerization techniques have been used in the commercial production of polymers with diverse compositions and architectures using an array of monomers and reaction conditions. All radical polymerizations (RP) consist of four key steps: initiation, propagation, transfer, and termination. In traditional radical polymerizations, a slow initiation step is followed by short, seconds-long chain growth lifetimes. Poor control over polymer molecular weights are caused by high concentrations of dead chains, control over radical concentration achieved through equivalent initiation and termination rates, and termination events caused by self-reacting combination of polymer chains. Radical polymerization is not a widely applicable technique where copolymerizations and advanced polymer architectures are concerned, thus leading to the development of more adaptable polymerization techniques.

The advent of controlled (living) radical polymerizations (CRP) allowed for increased control over molecular weight distributions of polymers produced. Rapid initiation, dynamic equilibrium between propagating and dormant chains, longer (at least 1 hour) chain growth lifetimes and typically less than 10% concentration of dead chains make the seemingly
simultaneous growth of polymer chains possible.\textsuperscript{20} Also referred to as reversible deactivation radical polymerization (RDRP), controlled radical polymerizations are characterized by the rapid initiation of all chains at the onset of the reaction followed by reversible transfer between actively propagating and dormant chains.\textsuperscript{21} All RDRP methods follow the same principal of reversible deactivation, but differ in the molecules that mediate the reversible process. The most common RDRP methods include nitroxide-mediated polymerization (NMP),\textsuperscript{22} atom transfer radical polymerization (ATRP),\textsuperscript{23} and reversible addition-fragmentation chain transfer (RAFT) polymerization.\textsuperscript{21, 24} Of these methods, ATRP and RAFT are particularly compatible for polymerizations of protein-polymer conjugates.\textsuperscript{10, 25-31}

In ATRP, the characteristic reversible deactivation process is achieved through a reversible redox reaction.\textsuperscript{2, 23, 32} Atom transfer is catalyzed by a transition-metal complex which breaks the alkyl halide bond of the initiator species resulting in oxidation of metal-halide complex.\textsuperscript{2, 20, 23} The reversible homolytic cleavage of the alkyl halide produces an alkyl radical which propagates to add monomer.\textsuperscript{20, 23} ATRP is a commonly used method for producing polymers of low molecular weight dispersion and has been used in protein-polymer bioconjugates.\textsuperscript{2} However, possible toxicity resulting from use of metal catalysts is a drawback of this method.\textsuperscript{2}

\textit{RAFT Polymerization}

One of the most commonly used and versatile RDRP method is RAFT polymerization. The mechanism of RAFT mirrors the reversible deactivation characteristic of all RDRP methods. This can be seen in the addition-fragmentation equilibrium shown in Scheme 1. Here, a chain transfer agent (CTA) serves as an intermediate species in the reversible chain transfer process between active and dormant chains.\textsuperscript{2, 20, 25, 33} Upon generation of a radical species by an initiator, the radical can either propagate monomer to give propagating polymer chain P\textsubscript{1}, or undergoes radical addition to the double bond of the chain transfer agent to give the radical intermediate shown.\textsuperscript{33} This intermediate species can either reversibly fragment back to radical P\textsubscript{1} and the original CTA species or eject another propagating radical species P\textsubscript{2}.\textsuperscript{33} This reversible addition-fragmentation reaction allows for the creation of polymer chains with a narrow molecular weight range. RAFT is widely applicable to various monomer classes, functional groups, solvents, and reaction conditions. In the interest of protein-polymer conjugates, the ability to produce polymers by RAFT in aqueous conditions, without use of potentially harmful metal catalysts is highly valuable.\textsuperscript{10, 25, 34}
Scheme 1. Reversible addition-fragmentation mechanism of RAFT polymerization.

A large part of the efficiency of RAFT polymerization lies in selection of the chain transfer agent. Evolution from vinyl and allylic chain transfer agents to the more current agents occurred in 1998 when Moad, Rizzardo, and Thang used thiocarbonylthio compounds (shown below) in RAFT synthesis. Common classes of thiocarbonylthio RAFT agents including dithioesters, trithiocarbonates, dithiocarbamates, and xanthates are all achievable by varied substitution of the Z group. Modification of the Z group affects addition and fragmentation rates, serves to stabilize the intermediate radical species, and can contribute to solubility of the molecule. The R group also plays an important role such that it must act as both a good leaving group and as an efficient radical species efficient capable of reinitiating polymerization in the reverse direction.

While RAFT presents a highly advantageous method of polymerization, it too, has shortcomings. Polymerization reactions based on the RAFT technique are sensitive to oxygen, often require long reaction times, and struggle to achieve high molecular weight polymers. Radicals are easily quenched by oxygen, making this a universal challenge for radical polymerization reactions. However, the slower reactions and difficulty achieving high molecular weight polymers are two issues which have been addressed in effort to improve this reliable polymerization technique. Additionally, careful selection of chain transfer agent that agrees with the reactivity of the monomers of interest is a limitation of RAFT; mismatched monomers and CTAs can result in poor control over the polymerization. All synthetic approaches have advantages and disadvantages to consider when choosing a method to proceed with; controlled radical polymerizations are no exception. RAFT polymerization is a method for producing well-controlled polymers that, despite its drawbacks, is highly compatible with proteins in the synthesis of protein-polymer conjugates.

Approaches to Conjugation

Traditionally, protein-polymer conjugates are synthesized by one of two methods: grafting-to or grafting-from. With grafting-to, polymer is first synthesized in the absence of protein using RDRP polymerization methods. In this reaction, polymer is grown from a chain transfer agent
(CTA) or another RDRP mediating group which acts as an activating end group on the polymer chain, as seen in Scheme 2. As with any synthetic method there are advantages and disadvantages to the grafting-to approach. Due to the ability to synthesize under typical organic reaction conditions, straightforward synthesis is an advantage of the grafting-to method.\textsuperscript{37} Similarly, separate polymerization and conjugation reactions means there is no effect of polymerization conditions on the protein.\textsuperscript{37} The polymer can be purified and thoroughly characterized to accurately determine the molecular weight prior to conjugation to the enzyme.\textsuperscript{37} Preformed polymer chains have high molecular weights, compared to monomer, which proves challenging when attaching the large molecules to a larger biomolecule. These steric effects often lead to low graft density by the grafting-to method.\textsuperscript{27, 37} To address this challenge polymer must be added in excess to the conjugating entity on the protein.\textsuperscript{27} Another disadvantage of grafting-to is the difficulty associated with separation of unattached polymer from the newly formed conjugate after synthesis.\textsuperscript{27, 37}

\begin{figure}
  \centering
  \includegraphics[width=\textwidth]{scheme2.png}
  \caption{Scheme 2. Representations of grafting-to (TOP) and grafting-from (BOTTOM) conjugation approaches.}
\end{figure}

In the grafting-from approach, a small molecule activating group is covalently attached to the surface of the protein, as in Scheme 2. Using the living nature of RDRP polymerizations, initiator and monomer are added to the activated protein and the polymer chain is grown directly from the protein. In contrast to grafting-to, this method begins with attachment of a small molecule which faces fewer steric inhibitions and allows for higher graft density.\textsuperscript{27, 37} Additionally, the greater molecular weight difference between activating group and protein gives greater ease of
puriﬁcation. While polymerization in protein-compatible conditions is an advantage for maintaining protein function, challenges arise in matching conditions with those suitable for polymer synthesis. Each conjugation method has pros and cons, and these features must be considered with respect to the protein of interest and the polymerization goals when choosing a technique.

In effort to produce active, lysozyme-polymer conjugates, Falatach et. al. combined the familiar grafting-to and grafting-from methods. First, acrylamide oligomers of three different chain lengths were synthesized and grafted-to lysozyme through NHS/EDC coupling, wherein amide-bond formation links the polymer to the protein. Full characterization and activity studies were completed to show that conjugates retained activity after the reaction. Next, the shortest oligomer, DP5 Am, was chain extended from the surface of the protein with 200 units of oligo(ethylene oxide) methyl ether acrylate by the grafting-from method. The extended conjugate demonstrated negligible activity, likely due to steric hindrance of the large polymer near the active site. Regardless, development of this hybrid approach and its proof of concept gives researchers a method to circumvent some of the challenges associated with choosing either the grafting-to or grafting-from method.

While there have been great strides in developing active, stable protein-polymer conjugates there remains a need for deeper understanding of these fascinating biomacromolecules. Research has shown that the attachment of polymer chains to protein surfaces can impart stability to the protein, tune protein activity and solubility, and be used to produce responsive conjugates. However, much of the fundamental science of protein-polymer conjugates remains a mystery. Studies which probe polymer properties, interactions between polymer and protein, and protein conformational changes as a result of polymer attachment are highly valuable and necessary for the continued progression of protein-polymer bioconjugates.

Herein we investigate protein-polymer conjugates of lysozyme and cellulase to determine structure-function correlations which are key to producing biomaterials with properties tunable for each application. A collection of monomers is polymerized via RAFT for conjugation to enzymes using the hybrid grafting-to/grafting-from approach. A comprehensive study of the activity and stability of resulting lysozyme-polymer conjugates is presented. Using similar approaches, cellulase-polymer conjugates are studied as highly applicable bioconjugates of interest.
CHAPTER ONE
INVESTIGATING THE IMPACT OF POLYMER FUNCTIONAL GROUPS ON THE STABILITY AND ACTIVITY OF LYSOZYME-POLYMER CONJUGATES

All high “H” molecular weight and L-AGA experiments in this chapter were performed by Melissa Lucius Dougherty. Low “L” molecular weight polymerizations, conjugations, chain extensions, and subsequent activity and stability studies on those samples were performed by Rebecca Falatach and Dominik Konkolewicz. Melissa Lucius Dougherty contributed to the interpretation of all activity and stability data included herein.

Introduction

In studies of large macromolecules and proteins it is often useful to begin exploration on a less complex, better understood model molecule or protein. These model systems can provide insight into the characteristics of larger molecules, with simpler parameters due to the smaller size. One commonly used model enzyme is lysozyme. Lysozyme is an enzyme found in mucus, blood, saliva, and hen egg whites. The primary function of lysozyme is to hydrolyze β-glycosidic linkages that bind carbohydrate chains which form the bacterial cell wall. Through this bond cleavage, the antimicrobial enzyme breaks down bacterial cell walls to expose and subsequently destroy the bacterial cell membrane. While initially lysozyme seemed promising as an antibiotic, the enzyme was too large for use as a drug and to this day has few therapeutic and industrial applications. Regardless, the thermally stable 14.3 kDa protein has been extensively studied and characterized, making it a useful model for protein studies.

Maintaining the stability of proteins which are sensitive to changes in temperature, pH, and solvents is a challenge that has provided motivation for many protein-polymer bioconjugate studies. Model proteins like hen egg white lysozyme (HEWL) and chymotrypsin have been used in numerous studies of the stability of protein-polymer conjugates. The Maynard group investigated HEWL conjugates with trehalose side chain polymers in effort to mimic the stabilization that the disaccharide provides organisms in nature. The group reported that lysozyme-trehalose polymer conjugates showed greater stabilization to heat and lyophilization than native HEWL and demonstrated the importance of conjugating the polymer to the protein surface for stability purposes.
Additionally, pH- and temperature-dependence studies on chymotrypsin conjugates have contributed insight into the stabilization of proteins to environmental stressors.\textsuperscript{14, 16} In one such study with poly(dimethylamino) ethyl methacrylate, polymers were grown from the surface of chymotrypsin and showed pH- and temperature dependence on activity and stability.\textsuperscript{16} Results of these studies allowed for control over enzyme function through manipulation of pH and temperature.\textsuperscript{16} As the applicability of protein-polymer conjugates continues to grow, it is important to explore the relationships between synthetic polymers and the structure and function of the enzymes to which they are attached.

To date, there has been no comprehensive study to determine the impact of polymer chain lengths and functional groups on enzyme activity and structural stability. Protein-polymer conjugate stability is a multifaceted characteristic that is encompassed by a broad range of attributes including resistance and susceptibility to thermal and chemical denaturation separately, as well as reversible thermal stability. This study aims to address these unanswered questions and provide insight into the complex stability of protein-polymer bioconjugates. RAFT polymerization is utilized in a hybrid approach which combines grafting-to and grafting-from techniques to produce active conjugates. Addressing the unique stabilities of protein-polymer conjugates as separate entities is imperative to the understanding of these bioconjugates. By understanding the effects of polymer chain length and functionality on the conjugate’s properties, we can begin to design polymers to tune the properties as needed for various applications.

\textbf{Materials and Methods}

\textit{Materials.}

Hen Egg White Lysozyme (HEWL) was purchased from MP Biomedicals. Acrylic acid (AA), dimethyl acrylamide (DMAm), and deuterated DMSO were obtained from Acros Organics. Acrylamide, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Thermo-Fisher. \textit{Micrococcus lysodeikticus}, 4-methylumbelliferyl \textbeta-D-N,N’,N”-triacetylchitotrioside, azobisisobutyronitrile (AIBN), ethanethiol, carbon disulfide, 2-bromopropionic acid, phosphoroylcholine methacrylate (PCMA), acryloyl chloride, and oligo(ethylene oxide) methyl ether acrylate (OEOA) were purchased from Sigma Aldrich. Sodium nitrite and D-glucosamine hydrochloride were from Alfa Aesar. Dimethylaminoethoxy Methacrylate (DMAEMA) was obtained from TCI, and potassium carbonate was purchased from
MCB. VA-044 was obtained from Wako chemicals. All materials were used without further purification. SDS-poly(acrylamide) gel electrophoresis was performed using Bio-Rad Mini-PROTEAN TGX 4-20% gradient gels. Gels were stained with GelCode Blue protein stain obtained from Thermo Scientific. The chain transfer agent 2-(((ethylthio)-carbonothioyl)thio)propanoic acid (PAETC) was synthesized as described in our previous work.\textsuperscript{38}

*Synthesis of N-acryloyl-D-glucosamine (AGA)*

The AGA monomer synthesis was adapted from the Matsuda et al procedure.\textsuperscript{42} D-glucosamine hydrochloride (8.6007g, 39.88mmol) and NaNO\textsubscript{2} (0.1468g, 2.13mmol) were mixed in 20 mL of 2 M aqueous K\textsubscript{2}CO\textsubscript{3} before being cooled to 0°C in a salt/ice bath. Acryloyl chloride (4.0208g, 44.42mmol) was added dropwise to the stirring reaction mixture, while the temperature was held below 5 °C. This temperature was maintained for three hours and then warmed to room temperature for 21 hours. The reaction mixture was poured into 200 mL of ethanol and refrigerated overnight. The mixture was filtered to remove the precipitated salts then concentrated under vacuum. This concentrated solution was recrystallized twice in methanol, followed by ethyl acetate at a ratio of 1:2. \textsuperscript{1}H NMR spectra showed peaks as described in literature, \textsuperscript{1}H-NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ ppm 8.00 (d, 1H), 6.50 (d, 1H), 6.39 (dd, 1H), 6.09 (dd, 1H), 5.56 (dd, 1H), 4.95 (d, 2H), 4.71 (d, 1H), 4.45 (t, 1H), and ESI-MS (256.1 m/z minus mass of sodium gives 233.1 m/z) confirmed the product.\textsuperscript{42, 43}

*RAFT Polymerization of Oligo-Acrylamide-CTA*

An oligo-acrylamide polymer chain was synthesized from the chain transfer agent PAETC using RAFT polymerization. AIBN (0.2345 g, 1.43 mmol), PAETC (3.0019 g, 14.28 mmol), and acrylamide (5.0853 g, 71.54 mmol), were added to a glass vial and mixed to dissolve in 10 mL of methanol. This mixture was transferred to a round bottom flask and combined with 10mL of water. To remove oxygen from the system, the reaction mixture and flask were purged with nitrogen gas for 10 minutes. The reaction was heated to 65 °C in an oil bath for 15 hours. Initial and final samples of the reaction mixture were analyzed by \textsuperscript{1}H NMR to confirm that the reaction had at least 95% conversion of monomer. To precipitate, the reaction mixture was added dropwise to an ice cold, stirring mixture of 200mL THF and 50mL ether. The resulting mixture was centrifuged at 6000 rpm for 2 minutes until all polymer precipitate was isolated. The collected polymer was left to dry in a fume hood.
Conjugation of Oligo-Acrylamide-CTA to lysozyme

Using the amine-coupling method N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (NHS/EDC) coupling, oligo-acrylamide-CTA was covalently attached to surface amines of lysozyme. In this reaction, final concentrations were kept at 1 mg/mL enzyme and 5 mM NHS, while molar ratios of 1:1 CTA:EDC were maintained. In a vial, EDC (0.5658 g, 2.95 mmol), NHS (0.0581 g, 0.50 mmol, 5.05 mM), lysozyme (0.1001 g, 7 μmol) and oligo-acrylamide-CTA (1.6618 g, 2.94 mmol) were dissolved in a small amount of 0.1 M phosphate buffer at pH 7.4 to dissolve. The reaction mixture was quantitatively transferred to a round bottom flask and buffer was added to reach a final volume of 100 mL. The reaction was stirred at room temperature for 2 hours then quenched with 0.5 mL of 0.2 M glycine buffer at pH 10.6 and stirred for an additional 30 minutes.

Purification of Conjugate

Ammonium sulfate precipitation was used to remove unreacted monomer, EDC, and NHS from the newly conjugated oligo-acrylamide-CTA lysozyme. The conjugation reaction mixture was transferred to a beaker and was stirred on ice. To this mixture, 63% (w/v) ammonium sulfate was slowly added to the mixture and allowed to stir. Once all the ammonium sulfate was added, the mixture was stirred for an additional 10 minutes to ensure all ammonium sulfate was dissolved. The resulting solution was centrifuged at 4 °C for 15 minutes at 11,500 rpm. The supernatant was poured off and the remaining protein was resuspended in 25 mM phosphate buffer at pH 6.2. For further purification, the conjugate was dialyzed with phosphate buffer in an Amicon stirred ultrafiltration cell dialysis unit using an Ultracel 10 kDa ultrafiltration disc. Following purification, the ultrafiltration cell was used to concentrate the conjugate to 10 mg/mL for further experiments.

Chain Extension of Polymers from Oligo-Acrylamide-CTA lysozyme

High-molecular weight polymers (DP 250) of AGA, Am, Am/Aa, Am/DMAEMA, and a low-molecular weight (DP 50) AGA polymer were grown from chains of oligo-acrylamide-CTA conjugated to lysozyme. Each RAFT chain extension reaction was set up following a similar procedure. For example, in the chain extension of high molecular weight acrylamide (H-Am), acrylamide monomer (24.4 mg, 343.3 μmol), VA-044 (2.2 mg, 6.8 μmol), and oligo-acrylamide-CTA lysozyme (0.95 mL, 0.62 μmol) were combined in a glass vial, mixed to dissolve, and transferred to a Schlenk flask. The system was deoxygenated for 10 minutes under a gentle flow.
of nitrogen gas. The reaction was heated in a 30 °C oil bath and proceeded for 14 hours. Initial and final samples were analyzed via $^1$H NMR to determine percent conversion.

**Activity Assays for Lysozyme and Conjugates**

To assess the activity of lysozyme and the resulting protein-polymer conjugates, two substrates of different sizes were used. Lyophilized *Micrococcus lysodeikticus* served as a large substrate, the cell wall of which is lysed by lysozyme. This reaction can be measured by the change in optical density over time at 450 nm. Each sample was subjected to *M. lysodeikticus* in 66 mM potassium phosphate buffer, pH 6.2 and optical density measurements were recorded every 15 seconds for 4 minutes in a BioTek Synergy H1 microplate reader. The activity, or rate of reaction, was given by the slope of optical density over time. Relative rates for each conjugate were compared to the native lysozyme to give percent activity retention.

The small substrate for lysozyme activity determination was 4-methylumbelliferyl β-D-N,N’,N”-triacetylchitotrioside ((NAG)$_3$-MUF), which fluoresces upon enzymatic hydrolysis. This assay was run in 50 mM acetate buffer, pH 5.5 at 37°C and was stopped at each time point using glycine buffer, pH 10.8. Glycine buffer serves to increase the pH of the reaction mixture, thus increasing the fluorescence intensity of the MUF product. Following the addition of glycine, fluorescence measurements were obtained on the BioTek Synergy H1 microplate reader using 360/460 as the excitation/emission setting.

**Differential Scanning Fluorimetry as a Measure of Thermal Stability**

Thermal stability of native lysozyme, polymer conjugates, and polymer control samples were studied using differential scanning fluorimetry. A solution of 11.44x SYPRO Orange was diluted from 5000x stock in 5 mL of 20 mM HEPES buffer, pH 7.5, 150 mM NaCl. Protein samples were diluted to final concentrations of 5 μM, and polymer controls were diluted to 12.5 μM using the SYPRO Orange solution. Samples of 50 μL volumes were analyzed in triplicate on a BioRad CFX96 RT-PCR. Fluorescence measurements were taken at 570 nm while the temperature increased from 25 to 95°C in 0.5°C increments. A 5 second equilibration was allowed at each temperature. Resulting DSF curves were processed using a Boltzmann fit to determine melting temperature of each sample.

**Chemical Stability as Determined by Guanidine Hydrochloride Denaturation**

The chemical stability of lysozyme conjugates was determined by using guanidine hydrochloride to chemically denature the protein and measuring the intrinsic fluorescence of
tryptophan residues. Twelve guanidine hydrochloride solutions with concentrations ranging from 0 M to 6 M were prepared in 20mM HEPES buffer, 150 mM NaCl, pH 7.5. Native lysozyme and conjugated samples were measured in triplicate at 27 μM final protein concentrations using a BioTek Synergy H1 microplate reader. Excitation wavelength parameters were set to 280 nm with emission scans being taken from 310 to 400 nm.

Results and Discussion

![Scheme 3](image)

Scheme 3. A) RAFT polymerization of oligo-acrylamide-CTA. B) Reaction scheme for the grafting-to conjugation and grafting-from chain extension of lysozyme. C) Monomers used for chain extension demonstrate a variety of functional groups, sizes, and charges.

RAFT Polymerizations for grafting-to and grafting-from

In this work, oligo-acrylamide was synthesized from 2-(propionic acid) ethyl trithiocarbonate (PAETC) chain transfer agent via RAFT polymerization according to Scheme 3A. This small molecule was characterized by ¹H NMR and ESI-MS prior to EDC/NHS conjugation to lysozyme. Grafting-to techniques allowed for the surface amines of lysozyme to be modified through amide-bond formation between the amine and the carboxylic acid of oligo-acrylamide-CTA. Because of the living nature of polymers produced by RAFT, initiator and monomer can be added to a solution of conjugated oligo-acrylamide-CTA lysozyme for growth of the polymer chain directly from the surface of the protein. This grafting-from technique, referred to as chain
extension, was used to produce high molecular weight polymers and copolymers with average chain lengths of 150 to 250 repeat units, based on monomer conversion and targeted molecular weight. Both the conjugation by grafting-to and chain extension by grafting-from can be seen in Scheme 3B.

Table 1. Conditions and results for RAFT polymerizations. All polymerizations except the synthesis of the short Am oligomer were performed at pH=6 at 30 °C at 10 mg/mL enzyme. All conversions estimated by NMR. Highlighted samples indicate synthesis by Melissa Lucius Dougherty.

<table>
<thead>
<tr>
<th>Sample</th>
<th>M 1</th>
<th>M 2</th>
<th>[M1]: [M2]</th>
<th>DP [M 1]: [M2]</th>
<th>$M_n$ Polymer (Th.)</th>
<th>$M_n$ Conjugate (Th.)</th>
<th>$M_p$ Conjugate (MALDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEWL</td>
<td>-</td>
<td>-</td>
<td>0:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-Am</td>
<td>Am</td>
<td>-</td>
<td>5:0</td>
<td>5:0</td>
<td>565</td>
<td>15678</td>
<td>15000</td>
</tr>
<tr>
<td>L-Am</td>
<td>Am</td>
<td>-</td>
<td>50:0</td>
<td>50:0</td>
<td>4239</td>
<td>22778</td>
<td>21000</td>
</tr>
<tr>
<td>L-DMAm</td>
<td>DMAm</td>
<td>-</td>
<td>50:0</td>
<td>34:0</td>
<td>4055</td>
<td>22410</td>
<td></td>
</tr>
<tr>
<td>L-OEOA</td>
<td>OEOA</td>
<td>-</td>
<td>50:0</td>
<td>50:0</td>
<td>24689</td>
<td>63678</td>
<td></td>
</tr>
<tr>
<td>L-Am/PCMA</td>
<td>Am</td>
<td>PCMA</td>
<td>40:10</td>
<td>24:9</td>
<td>5048</td>
<td>24396</td>
<td>18000</td>
</tr>
<tr>
<td>L-Am/DMAEMA</td>
<td>Am</td>
<td>DMAEMA</td>
<td>40:10</td>
<td>26:10</td>
<td>4105</td>
<td>22510</td>
<td>21000</td>
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<tr>
<td>L-Am/DMAEMA*</td>
<td>Am</td>
<td>DMAEMA</td>
<td>40:20</td>
<td>20:20</td>
<td>5249</td>
<td>24798</td>
<td>21000</td>
</tr>
<tr>
<td>L-Am/AA</td>
<td>Am</td>
<td>AA</td>
<td>40:10</td>
<td>20:5</td>
<td>2469</td>
<td>19238</td>
<td>18000</td>
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<tr>
<td>L-Am/AA*</td>
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<td>AA</td>
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<td>30:14</td>
<td>3827</td>
<td>21954</td>
<td>20000</td>
</tr>
<tr>
<td>L-AGA</td>
<td>AGA</td>
<td>-</td>
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<td>47.5:0</td>
<td>11756.5</td>
<td>37813</td>
<td></td>
</tr>
<tr>
<td>H-Am</td>
<td>Am</td>
<td>-</td>
<td>250:0</td>
<td>237.5:0</td>
<td>17551.5</td>
<td>49403</td>
<td></td>
</tr>
<tr>
<td>H-Am/DMAEMA</td>
<td>Am</td>
<td>DMAEMA</td>
<td>200:50</td>
<td>100:50</td>
<td>15639</td>
<td>45578</td>
<td></td>
</tr>
<tr>
<td>H-Am/AA</td>
<td>Am</td>
<td>AA</td>
<td>200:50</td>
<td>160:37.5</td>
<td>14749</td>
<td>43798</td>
<td></td>
</tr>
<tr>
<td>H-AGA</td>
<td>AGA</td>
<td>-</td>
<td>250:0</td>
<td>225:0</td>
<td>53114</td>
<td>120528</td>
<td></td>
</tr>
</tbody>
</table>
RAFT polymerization is highly tolerant to a variety of monomers. Scheme 3C displays monomers containing different functional groups that were used in chain extension of oligo-acrylamide-CTA lysozyme. At the pH of the chain extension reaction, acrylic acid (AA) (pKa 4.75)\textsuperscript{45} is expected to be anionic due to deprotonation of the carboxylic acid, while the dimethylaminoethoxy methacrylate (DMAEMA) (pKa 8.0)\textsuperscript{46} is cationic due to protonation of the tertiary amine.\textsuperscript{47} A summary of all polymerization data including monomer ratios, chain lengths, and molecular weights can be seen in Table 1. Samples designated as “L” represent conjugation with relatively low molecular weight polymers with chain lengths ranging from 30 to 50 repeat units. Conversely, “H” samples are those conjugates with high molecular weight polymers of 150 to 250 repeat units attached. Classification based on molecular weight allows for comparisons to be made based on the relative length of polymer chain attached.

Proof of chain extension can be confirmed through the use of PAGE gels. Figure 1 shows SDS-PAGE data for native lysozyme and all conjugates. SDS-PAGE data becomes increasingly complex to analyze with high molecular weights and charged molecules present as are studied here. Despite these challenges, qualitative trends can be made from the data presented in Figure 1. Unmodified, native lysozyme has a known molecular weight of 14.3 kDa,\textsuperscript{41} which agrees well with the corresponding PAGE band at ~15 kDa. As O-Am-CTA is conjugated, a shift to slightly higher molecular weight is observed. Conjugates that were chain extended with low molecular weight polymers (Figure 1A) show further shift in molecular weight from the O-Am-CTA sample. This trend is also observed for the high molecular weight samples (Figure 1B). Samples of similar molecular weights residing in distinct regions of the PAGE gel give rise to proof of chain extension. Likewise, differences in properties emerge between the native, O-Am, L-molecular weight, and H-molecular weight classes of conjugates.
To provide insight into polymer steric effects on the enzymatic activity of native lysozyme and conjugates, two different sized substrates were assayed. *Micrococcus lysodeikticus* served as a large substrate for optical density measurements, while 4-methylumbelliferyl β-D-N,N’,N”-triacetylchitotrioside ((NAG)₃-MUF) was the small substrate of interest for activity studies.
Figure 2. Activity data of lysozyme-polymer conjugates. A) Activity of Am conjugates. B) Activity of low molecular weight (L) conjugates with different functional groups. C) Activity of high molecular weight (H) conjugates.

Activity trends based on molecular weight can be gleaned from Figure 2A which shows activity data for acrylamide conjugates of increasing size from native lysozyme to conjugates with DP5, 50 or 250 acrylamide chains attached. The small fluorogenic substrate shows slight losses in activity with each increase in polymer chain size. Overall, a 40% loss in activity suggests that the small substrate remains accessible to the active site with little regard for the size of polymer attached to the protein surface.
The large micrococcus substrate demonstrates a much more substantial loss in activity with increasing polymer chain length. From native to O-Am, approximately 40% of activity is lost. This same drop in activity is observed from O-Am to L-Am until there is essentially no retention of activity for the H-Am sample. While the small (NAG)₃-MUF substrate shows little effect of increasing polymer size on activity retention in Figure 2A, the large *M. lysodeikticus* appears to be much more sensitive to the steric hindrance from large polymer chains at or near the enzyme active site.

The role of functional group variation on activity of low molecular weight (L-) conjugates is studied in Figure 2B. Here, polymers chains of 30 to 50 repeat units are attached to lysozyme resulting in relatively low molecular weight conjugates. The activity of these conjugates was measured by both small and large substrates. The small (NAG)₃-MUF substrate exhibits only slight differences in activity retention from one functional group to the next. The *M. lysodeikticus* activity shows vast differences based on functional group character. In general, a significant loss in activity is observed for these conjugates, once again suggesting steric inhibition with the 1 micron substrate near the active site. Specifically, AGA retains nearly no activity as it is expected to experience hydrogen-bonding interactions with the active site, therefore blocking any lysis of the substrate. It is likely that polymers of AA are deprotonated at pH 6.2, thus leading to repulsion between the polymer-protein conjugate and the negatively charged surface of *M. lysodeikticus*. In cationic polymer conjugates containing DMAEMA, significant activity retention is observed. Unlike AA, at pH 6.2 amines on the DMAEMA polymer will predominantly be protonated resulting in a positive charge with electrostatic attraction for the negatively charged substrate. It appears that the electrostatic attraction offsets the steric hindrance thus allowing nearly 80% retention of activity.

Figure 2C shows the effects of functional group variation on high molecular weight (H-) conjugates. In these conjugates, large steric effects are observed resulting in significant losses of activity for all conjugates across both substrates. With fluorogenic (NAG)₃-MUF, nearly 40% retention of activity is observed for both anionic and cationic polymer conjugates. There is no activity retained for H-AGA for either small or large substrate. Hydrogen bonding of AGA with the active site blocks both substrates from exposure to the active site and being digested by HEWL. In the large substrate assay, steric bulk and active site interactions results in no activity for H-AGA, while AA activity is lost due to electrostatic repulsion between polymer and substrate. As with the L-Am/DMAEMA conjugates, the protonated high molecular weight conjugate of
DMAEMA exhibited higher retention of activity than other functional groups due to attraction to the oppositely charged substrate.

The effects of polymer molecular weight and functional group on activity retention were examined in Figure 2. From these results it is clear that increasing chain lengths of polymers attached to the protein surface decreases activity in both small and large substrate assays due to steric hindrance. Additionally, polymers of anionic nature or those capable of interactions within the active site, namely AA and AGA, lead to decreases in activity with both substrates, though these trends are less significant in the small substrate assays. Low and high molecular weight polymers with positively charged regions have electrostatic attraction for the negatively charged *M. lysodeikticus* which counteracts the steric effects resulting in significantly higher retention of activity than other polymers examined.

*Multifaceted Examination of Lysozyme Stability*

In addition to activity studies, this work has investigated the stability of lysozyme after thermal and chemical denaturation. Protein stability is often described as circulation lifetime, resistance to proteases, or functional thermal stability. In circulation and proteolytic studies, steric bulk resulting from conjugated polymers offers stability for the enzyme by increasing circulation times and blocking enzyme surfaces from proteolytic cleavage. Functional thermal stability is a measure of activity retention as a function of heating time. Maynard et. al. demonstrated increased functional thermal stability when trehalose polymers were conjugated to lysozyme. Trehalose, a disaccharide, is thought to interact strongly with the surface of lysozyme to improve stability. While each of these measures is useful, direct approaches of measuring thermal stability and intrinsic chemical stability are explored in this study.

Differential scanning fluorimetry using SYPRO Orange dye was used to measure an increase in fluorescence as a function of enzyme unfolding. Alone, the fluorescence of SYPRO Orange dye is weak. As the protein thermally unfolds, core hydrophobic residues are exposed and interact with SYPRO Orange to increase fluorescence signal. Fluorescence is collected as a function of temperature and resulting DSF curves are fitted with a Boltzmann equation to determine the melting temperature (Tm) for native lysozyme and conjugates.

Figure 3A shows the results of differential scanning fluorimetry fitted with a Boltzmann function for native lysozyme, O-Am, L-Am, and H-Am samples. All remaining conjugates produce similar DSF curves to the one shown here. Conjugates of all molecular weight and
functional groups exhibit lower $T_m$ than the native (shown in black). Melting temperature data is tabulated for all conjugates and the difference in $T_m$ from native lysozyme is given in Table 2. As seen by a decrease in $T_m$ compared to native lysozyme, all conjugates exhibit lower thermal stability than the unmodified enzyme. High molecular weight conjugates (Figure 3B) have lower $T_m$ values than native HEWL (black), as well as the lower molecular weight conjugates. Additionally, as seen in Table 2, these high molecular weight conjugates have lower melting temperatures than corresponding low molecular weight samples. In each case a difference of approximately 6°C is observed, except AGA conjugates which have nearly identical thermal stability regardless of polymer size. Additionally, DSF of polymer only controls assessed fluorescence signal of the polymer backbone to rule out interference with SYPRO Orange dye. Subsequent baseline signals shown in Figure 3C corroborate that interaction between SYPRO Orange and polymer is negligible, and signal arises as a result of protein unfolding.

**Figure 3.** Analyses of thermal stability. (A) Differential scanning fluorimetry was used to examine thermal stability of each sample. A Boltzmann fit to each sample (dotted lines) enabled determination of $T_m$ values reported in Table #. (B) DSF of high-molecular weight conjugates. (C) DSF of polymer only controls.
Chemical stability was determined by measuring the concentration of guanidine hydrochloride required to unfold lysozyme. As the enzyme unfolds, fluorescent tryptophan residues become exposed resulting in a change of $\lambda_{\text{max}}$ of fluorescence. Emission around 330 nm is consistent with the enzyme in the fully folded state, and as unfolding occurs the $\lambda_{\text{max}}$ shifts to around 342 nm representative of a fully unfolded state. The $\lambda_{\text{max}}$ of fluorescence was measured at increasing concentrations of guanidine hydrochloride to produce a two-state model of unfolding. The mid-point of resultant curves gives the $D_{50}$ value, the concentration of guanidine hydrochloride required to unfold 50% of the enzyme, as a degree of chemical stability. The data have been summarized in Table 2.

Figure 4 shows experimental data from chemical stability studies using guanidine hydrochloride as chemical denaturant and a fitted two-state model based on the assumption that the system is fully folded at low $\lambda_{\text{max}}$ and fully unfolded at high $\lambda_{\text{max}}$. In Figure 4A, the chemical stability of native lysozyme (black) is rivaled only by that of the H-Am (blue) sample, while smaller polymers lead to poorer chemical stability. As with Am, high molecular weight conjugates demonstrate larger $D_{50}$ values than native lysozyme, thus exhibiting greater chemical stability. This can be seen in Figure 4B which depicts a shift to higher concentrations of guanidine needed to denature the protein for high molecular weight polymers. Conversely, low molecular weight conjugates destabilize the enzyme regardless of polymer functional groups, data which is presented in Table 2. The only exception is L-AGA with a $D_{50}$ value nearly identical to the H-AGA counterpart, both of which are higher than the native. AGA polymers offer the best overall stabilization against guanidine hydrochloride, likely due to hydrogen bonding interactions at or near the active site which impede denaturation.
Figure 4. Tryptophan fluorescence versus concentration of guanidine as a measure of chemical stability. (A) Conjugates with increasing acrylamide polymer size. (B) Conjugates made with high molecular weight polymers.

Data presented in Table 2 make it possible to draw conclusions about the effects of polymer size and functional group on the thermal and chemical stability of lysozyme.

Table 2. Thermal and Chemical stability data for all lysozyme polymer conjugates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tm (°C)</th>
<th>ΔTm (°C)</th>
<th>D50 (M)</th>
<th>ΔD50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEWL</td>
<td>67.5±0.2</td>
<td>0</td>
<td>4.1±0.5</td>
<td>0</td>
</tr>
<tr>
<td>O-Am</td>
<td>62±0.2</td>
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<td>3±0.7</td>
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<tr>
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<td>-1.6</td>
</tr>
<tr>
<td>H-Am</td>
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<td>-10.1</td>
<td>4.6±0.4</td>
<td>0.5</td>
</tr>
<tr>
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<td>2.8±0.5</td>
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</tr>
<tr>
<td>L-Am/PCMA</td>
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<td>2.9±0.5</td>
<td>-1.2</td>
</tr>
<tr>
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<td>3.7±0.4</td>
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<tr>
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<td>3.3±0.6</td>
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<tr>
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<td>59±0.2</td>
<td>-8.5</td>
<td>5±0.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Low molecular weight conjugates show destabilizing effects in both thermal and chemical denaturation. On the contrary, high molecular weight conjugates show decreased thermal stability, yet are stabilizing toward chemical denaturation. These trends suggest that there is a tradeoff between stabilizing and destabilizing effects against thermal and chemical denaturants. While
there is a reduction in thermal stability of high molecular weight polymers, stabilizing effects are seen when exposed to chemical denaturant. Steric hinderance, electrostatic interactions, hydrogen bonding effects, and surface interactions all contribute to the complex activity and stability trends observed in this study.

**Conclusions**

In this work, a short, oligo-acrylamide polymer was synthesized via RAFT polymerization and attached to hen egg white lysozyme. Polymer chains of low- and high-molecular weights were extended from the enzyme using a variety of monomers with various functional groups. Activity assays using both small and large substrates were used to investigate steric effects and active site changes caused by the attached polymer. Differential scanning fluorimetry was used to assess thermal stability, while denaturation by guanidine hydrochloride was used to measure chemical stability of the resulting conjugates.

These studies provide valuable insight into the effects that polymer chain lengths and functional groups have on enzymatic function. As size of the polymer chain increased, a decrease in activity in both small and large substrates was observed. Steric hindrance likely plays a role in loss of activity that was most prevalent for high molecular weight polymers. Variation in functional group also affects enzyme activity; namely, anionic polymers containing DMAEMA saw significant retention of activity with the negatively charged *M. lysodeikticus* substrate. Thermal and chemical stability were also impacted by changes in polymer size and functionality. Specifically, low molecular weight polymers demonstrate destabilization of the enzyme in both chemical and thermal denaturation. Conversely, high molecular weight polymers destabilize when the enzyme is thermally denatured, but present improved stability against chemical denaturation. This work has provided detailed structure-property correlations based on the effects of polymer characteristics on enzyme activity and stability. Understanding these relationships makes it possible to engineer protein-polymer conjugates and tune their properties for specific applications.
CHAPTER TWO
EXPLORING THE ACTIVITY AND STABILITY OF CELLULASE-POLYMER CONJUGATES

Introduction

Over the course of recent decades there has been a growing need to reduce fossil fuel consumption due to rising global carbon emissions. In 2005, the United States Congress put into place the Renewable Fuel Standard (RFS) as part of the Energy Policy Act of 2005 to address growing emissions and related energy challenges. This program seeks to decrease the dependence on traditional non-renewable energy sources and increase production of renewable energy solutions. As a result, production of renewable fuels such as ethanol and biodiesel have increased. The Environmental Protection Agency projects that total renewable fuel volumes will reach 18.8 billion gallons in 2017, up from 16.28 billion gallons in 2014. Traditionally, bioethanol has been produced from corn by breaking down starch within the corn kernel into sugars that are then fermented into ethanol. However, this decreases the amount of grain available for human and animal consumption. In recent years, cellulose has received significant interest as a source for biofuels due to its high availability without imposition on food supply materials such as corn.

Cellulose is a linear biopolymer of glucose containing β-1-4 glycosidic linkages. Since cellulose can be found in all plants, it is a promising source of biomass because it can be collected from agricultural waste products like grasses, straw, and cornstalls. Upon decomposition, cellulose becomes simple sugars which can be fermented into fuels. The fermentation process has been well-developed; however, the depolymerization of cellulose into simple sugars remains a challenge. Cellulases are efficient enzymes with the primary function of decomposing cellulose by hydrolyzing glycosidic bonds. However, the isolation of cellulase enzymes is expensive, and the efficiency of enzymatic hydrolysis alone is not cost effective on an industrial scale; this is a problem that must be addressed if biofuels are to become a competitive fuel option.

Industrial hydrolytic conversion of cellulotic biomass to biofuels occurs at high temperatures and can require long reaction times, conditions which are not favorable for many enzymes. Protein-polymer conjugates have been shown to affect the activity and stability of
proteins making them a promising alternative to traditional industrial enzymes.\textsuperscript{14, 16, 17, 37, 49} This advancing technology can be applied to cellulas with the aim of synthesizing more active, stable bioconjugates for the efficient breakdown of cellulose for biofuel production.\textsuperscript{7, 15, 56} For example, studies of recyclable, thermoresponsive cellulase bioconjugates with poly(N-isopropylacrylamide) (NiPAm) suggest that enzyme activity\textsuperscript{56} and enzyme recovery can be tuned by the lower critical solution temperature (LCST) behaviors of the conjugated polymer.\textsuperscript{15} In one particular study, Mackenzie and Francis produced bioconjugates using LCST NiPAm copolymers and a thermophilic family 5 cellulase, EGPh.\textsuperscript{15} Their resulting conjugates exceeded native activity following multiple heating cycles which utilized the controllable solubility of the attached LCST polymers to collect and re-solubilize the enzyme.\textsuperscript{15} However, the LCST character of NiPAm causes the polymer to precipitate out of solution at 32 °C which is problematic for industrial cellulase conjugates which will precipitate at cellulose hydrolysis at temperatures greater than 32 °C.\textsuperscript{15} While these studies have enhanced the field of protein-polymer bioconjugates and contributed to the quest for industrially practical cellulases, there is still a need for greater understanding of the effects of polymer characteristics on enzyme function at a more fundamental level.

In this study, a family of water-soluble polymers was synthesized via RAFT polymerization and attached to \textit{Fervidobacterium nodosum} Cel5a (\textit{Fn}Cel5a) to investigate the effects of attaching functional polymers to cellulase. The thermophilic \textit{Fn}Cel5a is a family 5 cellulase shown to exhibit high thermal stability and enhanced activity on a variety of substrates.\textsuperscript{55} Structural components including shortened loops and increased aromatic and charged residues may contribute to greater intramolecular forces which can help to maintain enzyme conformation and increase stability to thermal denaturation.\textsuperscript{55} Additionally, the large pocket-like active site of \textit{Fn}Cel5a is lined with polar and aromatic residues making substrate binding and housing of glucose subunits especially attractive.\textsuperscript{55} These structural features provide insight into \textit{Fn}Cel5a for protein-polymer conjugates studies. Polymers of acrylamide (Am) and dimethylacrylamide (DMAm) were produced using RAFT polymerization and were attached to \textit{Fn}Cel5a through amine conjugation. The activity of \textit{Fn}Cel5a-polymer bioconjugates was measured using a colorimetric assay for the breakdown of carboxymethylcellulose substrate. As a dual assessment of stability, both the thermal stability and functional chemical stability were studied to provide insight into the behavior of cellulase bioconjugates. This work provides an introductory view into the
characteristics of *Fn*Cel5a as a function of polymer functionality when attached to the enzyme surface.

**Materials and Methods**

*Materials:*

Acrylic acid (AA), dimethyl acrylamide (DMAm), deuterated DMSO, and Dinitrosalicylic acid were obtained from Acros Organics. Acrylamide (Am), N-hydroxysuccinimide (NHS), and D-glucose were purchased from Thermo-Fisher and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Carbosynth. Azobisisobutyronitrile (AIBN), ethanethiol, carbon disulfide, and 2-bromopropionic acid were purchased from Sigma Aldrich. Dimethylaminoethoxy Methacrylate (DMAEMA) was obtained from TCI. All materials were used without further purification. SDS-poly(acrylamide) gel electrophoresis was performed using Bio-Rad Mini-PROTEAN TGX 4-20% gradient gels. Gels were stained with GelCode Blue protein stain obtained from Thermo Scientific. Carboxymethylcellulose (CMC) was purchased from Millipore Corp and SYPRO Orange was obtained from Life Technologies.

*Methods:*

**Synthesis of Chain Transfer Agent: PAETC**

The synthesis and purification of RAFT chain transfer agent (CTA) 2-(((ethylthio)-carbonothioyl)thio)propanoic acid (PAETC) was adapted from our previous synthesis.\(^3^\)\(^8\) Potassium hydroxide (14.6 g, 0.26 mol) was dissolved in distilled water (15.0 mL) and was added dropwise to a solution of ethanethiol (18.6 mL, 0.26 mol) in acetone (150 mL) with stirring on an ice bath. Carbon disulfide (16.1 mL, 0.27 mol) was added to the reaction mixture and stirred for 30 minutes while still on ice. The mixture was removed from the ice bath and 2-bromopropionic acid (23.0 mL, 0.26 mol) was added dropwise. The reaction mixture was left to stir overnight at room temperature. Solvent was removed via rotary evaporation and the residue was dissolved into 200 mL of ether, followed by 200 mL of water. The ether layer was added onto the aqueous layer and shaken. The yellow ether layer was collected, and subsequently washed with water (6 × 200 mL), and once with 200 mL brine. Solvent was removed by rotary evaporation to give 32.7 g of PAETC (0.155 mol, 59%) as a viscous yellow liquid, which solidifies upon freezing. The product was characterized as being at least 94% pure by \(^1\)H NMR. \(^1\)H-NMR (300 MHz, CDCl\(_3\)) \(\delta\) ppm
4.87 (1H, q, J = 7.4 Hz, CH₃CH(S)COOH), 3.38 (2H, q, J = 7.4 Hz, CH₃CH₂S), 1.63 (3H, d, J = 7.4 Hz, CH₃CH(S)COOH), 1.36 (3H, t, J = 7.4 Hz, CH₃CH₂S).

**RAFT Polymerization**

A family of polymers was synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization for conjugation to cellulase. Polymers with target chain lengths of 30 repeat units were synthesized in molar ratios of [M]:[CTA]:[I] equal to 30:1:0.2. Typical polymerization of dimethylacrylamide containing polymers is outlined (Scheme 4A). In a glass vial, dimethylacrylamide (1.4903 g, 15.03 mmol), PAETC (0.1082 g, 0.51 mmol), and AIBN (0.0172 g, 0.10 mmol) were dissolved in 2.1 mL of ethanol. The mixture was transferred to a round bottom flask for deoxygenation and was heated in a 65 °C oil bath. With stirring, the reaction was allowed to run for 21-22 hours. Initial and final ¹H NMR were taken on a 300 MHz Bruker NMR to determine conversion. The resulting polymer solution was added dropwise to hexanes to precipitate. Solutions were centrifuged for 2 minutes at 6000 rpm and the supernatant was removed. Remaining polymer was collected and left in the vacuum oven until dry. Once dry, a spectrum of the polymer was taken on a 500 MHz Bruker NMR (in D₂O, 32 scans) to determine the actual chain length of polymer by taking the ratio of protons on the CTA region of the polymer to protons given by each monomer unit. All remaining polymers were produced under similar procedures.
Scheme 4. Scheme of Polymerization and Conjugation of Cellulase. A) Typical RAFT polymerization of DMAm. B) Monomers used to produce polymers and copolymers. C) Grafting-to cellulase using polymers synthesized.

Matrix-Assisted Laser Desorption/Ionization (MALDI) of Polymers

Polymer molecular weight information was obtained by MALDI-TOF for pAM and pDMAm. Each target spot was prepared by mixing 0.5 μL (~100 μM) of polymer with 0.5 μL of 2,5-dihydroxybenzoic acid (DHB) directly on the target plate. Spots were allowed to dry at room temperature. Samples were analyzed in positive ion linear mode.

Gel Permeation Chromatography (GPC)

Additional molecular weight data was collected by GPC for pDMAm, pDMAm/AA and pDMAm/DMAEMA samples. Each polymer sample was dissolved in ~2 mL of DMF with 2 drops of toluene in a glass vial. Once dissolved, solutions were filtered through a 0.2 μm PTFE filter into a smaller GPC vial for analysis.

Optimization of Conjugation Conditions

To determine the optimal conditions for attaching polymers to any of the 27 amine groups on FnCel5a (cellulase) via N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (NHS/EDC) coupling, conjugation studies were completed. All coupling reactions
were set up with final concentrations of 1 mg/mL enzyme and 5 mM NHS. Molar ratios of 1:1 CTA: EDC were maintained for all reactions while varying ratios of amine:polymer-CTA from 1:3, 1:8, 1:14, and 1:20. A representation of the grafting-to conjugation reaction is shown in Scheme 4C.

For example, in the 1:3 reaction with polyacrylamide (pAm), FnCel5a (0.52 mL of 1.92 mg/mL stock), pAm-CTA (6.2 mg, 2.43 μmol), EDC (0.1 mL of 4.3 mg/mL stock, 2.24 μmol), and NHS (0.1 mL of 5.8 mg/mL stock, 5.04 μmol, 5.04 mM) were combined in 0.28 mL of 0.1 M potassium phosphate buffer, pH 9 for a total volume of 1 mL. The mixture was stirred for 2 hours at room temperature after which a 500 μL sample was taken and quenched with 0.2 M glycine buffer (2.5 μL, final concentration 1 mM glycine) and vortexed to mix. The remaining mixture was left to stir for an additional 4 hours then quenched with 0.2 M glycine buffer. The samples were added to 5 mL 10,000 MWCO PES Centricon tubes and diluted to the 5 mL mark with buffer (25 mM phosphate, pH 8), then centrifuged in a 4 °C rotor at 6000 rpm for 10 minutes. This process was repeated until 15 mL of buffer had been dialyzed through each sample. Each sample was collected and concentration was estimated based on volume. Confirmation of polymer attachment was determined by SDS PAGE gel.

*Colorimetric Determination of Cellulase Activity*

The activity of native FnCel5a and corresponding bioconjugates was studied using a colorimetric analysis of the degradation of carboxymethylcellulose by cellulase. Solutions of carboxymethylcellulose (CMC), 3,5-dinitrosalicylic acid (DNSA) stop solution, and glucose standard stock solution were produced following protocol outlined by Konig et al. All standards and samples were prepared in sodium acetate buffer (100 mM sodium acetate, 20 mM calcium chloride, 10% Tween 20, pH 5). Enzyme samples were diluted to 32 nM and 6 μL of each sample was pipetted into 0.2 mL PCR tubes for seven time points, to be run in triplicate. Additionally, glucose standards were prepared at concentrations of 0, 1, 5, 10, 20, 30, 40 and 50 mM and 6 μL of each concentration were pipetted into PCR tubes, in duplicate. The glucose standards, enzyme samples and CMC substrate were pre-incubated at 81 °C for 5 minutes in a Proflex 3x32-Well PCR System (Life Technologies). Following pre-incubation, 60 μL of substrate was added to each PCR tube and 30 μL of DNSA stop solution was added to the time zero tubes which were subsequently transferred to a 90 °C block on the same PCR instrument. At 2.5-minute time intervals, 30 μL of DNSA stop solution was added to corresponding tubes which were then
transferred to 90 °C to incubate for 10 minutes. Following this incubation samples were cooled on ice for 5 minutes, centrifuged for 10s and 40 μL were plated on a 384-well plate. Absorbance scans were obtained on a BioTek Synergy H1 microplate reader.

**Thermal Denaturation by Differential Scanning Fluorimetry**

Enzyme and conjugate thermal stabilities were determined by measuring the fluorescence of SYPRO Orange dye as a function of temperature. SYPRO Orange 5000x stock was diluted to 11.44x in 5 mL of HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.8). Enzyme samples were diluted to 5 μM final concentrations in PCR tubes with the SYPRO Orange buffer solution. Samples were pipetted in 50 μL volumes and analyzed in triplicate. DSF curves were collected on a BioRad CFX96 RT-PCR, which measured fluorescence at 570 nm as a function of increasing temperature by 0.5 °C from 25 to 95 °C. At each temperature, a 5 second equilibration was allowed. Data was processed by fitting a Boltzmann equation to each sample curve to give the melting temperature (Tm) of each conjugate sample.

**Functional Chemical Stability via Dimethylformamide (DMF)**

An analysis of chemical stability was determined by measuring enzyme activity following chemical denaturation by dimethylformamide. First, conditions for chemical denaturation were optimized on triplicate samples of native cellulase. In PCR tubes, 5 μL of 20 μM FnCel5a was combined with sodium acetate buffer (100 mM sodium acetate, 20 mM calcium chloride, 10% Tween 20, pH 5) and either 0% or 76% DMF (v/v%) for a total volume of 100 μL per tube and final concentration of 1 μM protein in solution. These solutions were vortexed to mix and allowed to sit for one hour at room temperature. Following denaturation, samples were diluted to 32 nM to be used for the colorimetric assay with carboxymethylcellulose. The assay was completed according to protocol outlined above, with 0, 6, and 12-minute time points. The process was repeated for conjugate samples using a solution of 76% DMF (v/v%) in sodium acetate buffer (100 mM sodium acetate, 20 mM calcium chloride, 10% Tween 20, pH 5) with 1 μM final enzyme concentrations. Absorbance values were measured on a BioTek Synergy H1 microplate reader.

**Results and Discussion**

A family of four hydrophilic acrylamide (Am) and dimethylacrylamide (DMAm) polymers were produced from PAETC using RAFT polymerization. A summary of the polymers produced and their corresponding degree of polymerization (DP) can be seen in Table 3. In this table, degree
of polymerization (DP) is determined by comparing the ratio of CTA protons to monomer protons in $^1$H NMR spectra. Estimating the corresponding molecular weight by NMR is generally close to the anticipated theoretical molecular weight, except for pDMAm/DMAEMA in Table 3. This discrepancy may be caused by poor initiation from DMAm to the methacrylic DMAEMA monomer and because the chosen RAFT agent PAETC is not ideal for the DMAEMA monomer.

Table 3. Monomer ratios and polymerization results. *As determined by NMR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monomer 1</th>
<th>Monomer 2</th>
<th>[M1]:[M2]</th>
<th>DP* [M1]:[M2]</th>
<th>$M_n$ polymer (Th)</th>
<th>$M_n$ polymer (NMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAm</td>
<td>Am</td>
<td>-</td>
<td>30:0</td>
<td>33:0</td>
<td>2342.4</td>
<td>2600</td>
</tr>
<tr>
<td>pDMAm</td>
<td>DMAm</td>
<td>-</td>
<td>30:0</td>
<td>35:0</td>
<td>3183.9</td>
<td>3700</td>
</tr>
<tr>
<td>pDMAm/AA</td>
<td>DMAm</td>
<td>AA</td>
<td>24:6</td>
<td>27:7</td>
<td>3021.5</td>
<td>3400</td>
</tr>
<tr>
<td>pDMAm/DMAEMA</td>
<td>DMAm</td>
<td>DMAEMA</td>
<td>24:6</td>
<td>34:7</td>
<td>3532.4</td>
<td>4700</td>
</tr>
</tbody>
</table>

Additionally, molecular weight information can be determined from MALDI-TOF mass spectrometry and gel permeation chromatography (GPC). MALDI spectra were collected for pAm and pDMAm as seen in Figure 5. The spectra for pAm (Figure 5A) shows a characteristic polymer distribution beginning at ~2400 m/z with a peak at 2619.8 m/z corresponding to 34 repeat units. Each peak is separated by 71 m/z which corresponds to one unit of Am monomer (71.08 g/mol). These data agree with the DP derived from NMR data in Table 3 for pAm. Figure 5B shows the spectra for pDMAm with a broad polymer distribution throughout. Peaks are separated by 100 m/z which agrees well with the molecular weight of DMAm monomer (99.13 g/mol). The peak at 2597.6 m/z suggests 24 repeat units on the polymer which was lower than the NMR predicted DP in Table 3.
An advantage of the grafting-to-conjugation approach is that polymers can be characterized by a variety of methods prior to attachment to the protein. A collection of all molecular weight data collected for the family of hydrophilic polymers can be seen in Table 4. The pAm sample was not compatible with the non-aqueous GPC, so no molecular weight information was collected from that source. The data from NMR and MALDI agree well for pAm, with the difference in molecular weights equal to less than one unit of Am. There is a larger discrepancy for the pDMAm molecular...
weights. The $M_n$ by MALDI estimates a difference of nearly 11 DMAm units compared to NMR. Additionally, GPC molecular weight data for all polymers are less than predicted by other methods. This discrepancy can be attributed to calibration of the GPC column. The GPC system used is calibrated with poly(methyl methacrylate) standards which can cause discrepancies when analyzing other polymers. An advantage of RAFT polymerization is the ability to produce well-defined polymers. The low polydispersities ($M_w/M_n$) obtained by GPC for the polymers described in Table 4 suggest narrow molecular weight range samples, adhering to good polymer control.

**Table 4.** Comparison of polymer molecular weight data collected from NMR, MALDI-MS, and GPC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$M_n$ polymer (Th)</th>
<th>$M_n$ polymer (NMR)</th>
<th>$M_n$ polymer (MALDI)</th>
<th>$M_n$ polymer (GPC)</th>
<th>$M_w$ polymer (GPC)</th>
<th>$M_w/M_n$ (GPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAm</td>
<td>2342.4</td>
<td>2555.6</td>
<td>2619.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pDMAm</td>
<td>3183.9</td>
<td>3679.6</td>
<td>2597.6</td>
<td>2027</td>
<td>2342</td>
<td>1.16</td>
</tr>
<tr>
<td>pDMAm/AA</td>
<td>3021.5</td>
<td>3390.9</td>
<td>-</td>
<td>985</td>
<td>1372</td>
<td>1.39</td>
</tr>
<tr>
<td>pDMAm/DMAEMA</td>
<td>3532.4</td>
<td>4680.9</td>
<td>-</td>
<td>2559</td>
<td>3344</td>
<td>1.31</td>
</tr>
</tbody>
</table>

Following characterization, polymers were grafted-to *FnCel5a* via EDC/NHS coupling. Poly(acrylamide) gel electrophoresis (PAGE) gels were run to show proof of conjugation. By attaching polymer chains of approximately 30 repeat units, molecular weight increases between 2-3 kDa should be seen for each polymer attachment to the protein surface. Figure 6 shows that molecular weight increases as a result of conjugation are visible by PAGE gels. The gel in panel A contains conjugates of pAm and pDMAm at varying amine: polymer-CTA ratios at both the 2- and 6-hour time points. The results of this gel suggest that the 1:3 amine:polymer-CTA ratios for both polymers do not provide sufficient conjugation to the protein as evidenced by the strong bands equivalent to native protein, while only a faint bands appear at higher molecular weights indicative of modification. Similarly, the 1:8 ratios show strongly prevalent native protein bands, though there is stronger evidence of conjugation in the darker, higher molecular weight bands. In lanes showing 1:20 samples for both polymers, little to no native sample remains, while long, dark bands at higher molecular weights suggest significant polymer modification of the protein surface. It is important to note that the results of these PAGE gels can only be used qualitatively, as the gels become increasingly more complex to analyze with increasing molecular weights, heterogeneous
attachment, etc. The second gel, panel B, shows samples of 1:14 and 1:20 amine:polymer-CTA ratios for pAm and pDMAm. In both the 1:14 pAm and pDMAm samples, essentially complete polymer attachment is confirmed as the unmodified native band is faint or absent from these lanes. Interestingly, the 1:14 and 1:20 samples appear identical for both the pDMAm/AA and pDMAm/DMAEMA polymers. As mentioned previously, PAGE gel analysis becomes much more involved with increasing molecular weight and polymer composition. In both the pDMAm/AA and pDMAm/DMAEMA samples, charged polymers add to the complexity of the PAGE gel. The similarity between different ratios of the pDMAm/AA and pDMAm/DMAEMA samples, respectively, may also suggest that there is no difference in polymer concentration attached to the enzyme. For the remainder of the data, only 1:14 and 1:20 samples will be regarded, since essentially complete conjugation of the enzyme is necessary to prevent skewed results from unmodified enzyme.

Figure 6. PAGE gels comparing native FnCel5a to conjugates with polymers attached. A positive shift in molecular weight confirms attachment to the protein surface.

A: Lanes

B: Lanes

The activity of each conjugate was determined by calculating the rate of production of glucose as a function of time. The slope of each sample, in triplicate, was averaged and compared
to native, which was set relative to 100% activity. Interestingly, in all cases, conjugate activity exceeded that of the native enzyme as seen in Figure 7A and B. Most notably, the 1:14 conjugates of both pAm and pDMAm produced activities at least 50% greater than native FnCel5a. The pAm and pDMAm 1:20 conjugates showed lower activity retention than the 1:14 samples which suggests that more polymer chains on the enzyme surface results in additional steric bulk at or near the active site which can hinder activity. The data presented in Figure 7A would suggest that not only is the 1:14 ratio superior to the 1:20 ratio in terms of activity capabilities for neutral polymers, but also that there may be some positive interaction between the neutral polymers attached to the enzyme surface and the substrate which would allow for greater activity. For example, the neutral pAm and pDMAm may hydrogen bond with the carboxymethylcellulose thus effectively tethering the substrate to the surface of the enzyme making it less difficult to digest the cellulose into glucose subunits.

Figure 7. Activity data for native FnCel5a and polymer conjugates. A) Percent relative activity for native FnCel5a and neutral conjugates. B) Percent relative activity for native and ionic conjugates.
Figure 7B demonstrates that in addition to the neutral polymers, a 1:20 copolymer of pDMAm/DMAEMA yields the highest activity of all samples, native and conjugates alike. With 60% greater activity than native, the behavior of the DMAEMA copolymer suggests increased interaction with the substrate due to a cationic polymer and negatively charged carboxymethylcellulose. Also in Figure 7B is data for the pDMAm/AA bioconjugate. Both the 1:14 and 1:20 conjugates containing anionic AA have the same activity, which agrees with the PAGE data suggesting that the concentration of polymer attached to the enzyme may be similar for both samples. Both pDMAm/AA conjugates have lower retention of activity than the majority of other conjugates studied. Here, the opposite effect is seen with the anionic polymer which results in a decreased interaction between the copolymer chain and the negatively charged substrate. Overall, these activity data show promise for the production of highly active cellulase-polymer conjugates.

High temperatures are often a staple of industrial processes and is therefore a standard that industrially relevant protein-polymer bioconjugates must be able to meet or exceed. Differential scanning fluorimetry (DSF) was used to determine thermal stability of protein-polymer conjugates by measuring the fluorescence of SYPRO Orange dye as a function of temperature. The inherent fluorescence of SYPRO Orange dye is weak. However, as thermal denaturation unfolds the protein and exposes core hydrophobic groups, these residues interact with SYPRO Orange resulting in a measurable increase in fluorescence. The observed fluorescence signal as a function of increasing temperature is plotted to give melting point data. Analyzed DSF curves show SYPRO Orange fluorescence as a function of temperature, fitted with a Boltzmann sigmoid function\textsuperscript{44} to determine the melting point of each conjugate. Figure 8 shows the DSF curves and Boltzmann fits for native $Fn$Cel5a and polymer conjugates, many of which overlap, suggesting little to no difference in melting temperature from native $Fn$Cel5a.
Figure 8. Thermal stability results obtained from differential scanning fluorimetry. (Data processed by Thai Wright)

It is well-documented that \textit{FnCel5a} is a thermostable enzyme.\textsuperscript{55} This study draws the same conclusion; the data can be seen in Table 5 which provides melting temperature (\(T_m\)) data for native \textit{FnCel5a} and conjugates. The DSF derived \(T_m\) of native \textit{FnCel5a} was 84.88 °C and all conjugate samples gave similar results. The greatest difference in melting temperature comes from the 1:20 pDMAm/AA sample, whose \(T_m\) ca. 2 °C below the native. From these data it is difficult to determine which polymers if any play a significant role in stabilizing or destabilizing the enzyme against thermal denaturation. However, these data confirm that protein-polymer conjugates of \textit{FnCel5a} retain the intrinsically high thermal stability of the native, an important characteristic for conjugates of industrial interest.

Functional chemical stability provides a measure of retained enzymatic activity following unfolding by a chemical denaturant. N,N-dimethylformamide (DMF) is often used as a solvent in cellulose derivitization reactions and in efforts to produce soluble cellulose intermediates.\textsuperscript{58, 59} Due to the frequent use of DMF with cellulose, it is important to understand how the organic solvent will effect cellulases such as \textit{FnCel5a}. In this study, native \textit{FnCel5a} was subjected to a solution of either 0% or 76% DMF (v/v%) to determine the effects of the solvent on the enzyme's activity. The results of this study are shown in Figure 9. It is apparent that incubation in DMF results in a loss of activity for native \textit{FnCel5a}.
Figure 9. Functional chemical stability data as determined by incubation in DMF solvent and colorimetric activity assay.

The functional chemical stability of native FnCel5a and conjugates was determined by incubating 1 mM samples in a solution of 76% DMF (v/v%) and sodium acetate buffer (100 mM sodium acetate, 20 mM calcium chloride, 10% Tween 20, pH 5) for one hour. Following incubation, activity data was collected and analyzed using the colorimetric assay previously discussed. The results of the functional chemical stability assay are shown in Figure 10A as percent relative activity, where each DMF-denatured sample is set relative to its undenatured counterpart (Figure 7). Figure 10A shows the results of functional chemical stability for native cellulase and conjugates with neutral polymers attached. In this figure, the 1-14Am and both pDMAm samples show decreased functional stability following DMF incubation. These samples are statistically the same, and only 1-20DMAm is outside the error of native cellulase. The 1-20Am conjugate shows improved functional chemical stability over all over conjugates, including native. These data suggest a positive correlation between increased neutral polymer modification and chemical stability. Hydrogen bonding interactions between the neutral polymer and the protein surface likely contribute resistance to unfolding, resulting in improved chemical stability for 1-20Am.
Figure 10. Functional chemical stability data as determined by incubation in DMF solvent and colorimetric activity assay. A) Percent relative activity for native FnCel5a and neutral conjugates. B) Percent relative activity for native and ionic conjugates.

As seen in Figure 10B, conjugates with ionic polymers attached also lose functional chemical stability when compared to native cellulase. However, the 1:20 samples for both the pDMAm/AA and pDMAm/DMAEMA show greater retained activity than 1:14 samples. These increasing activity data correlate to increases in polymer modification of the enzyme, unlike trends observed for traditional enzyme activity where enzyme activity decreased as a result of increasing polymer modification. Improved activity for higher polymer concentrations in Figure 10B suggests that enzyme unfolding is reduced as a result of greater polymer modification. These trends provide evidence of hydrogen bonding interactions between the conjugated polymer and the protein surface resulting in resistance to chemical denaturation.
Table 5. Summary of thermal and functional chemical stability studies. Table provides melting temperatures and % relative activities for native FnCel5a and all conjugates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_m$ (°C)</th>
<th>$\Delta T_m$ (°C)</th>
<th>%Relative Activity</th>
<th>$\Delta$% Rel. Act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>84.88 ± 0.06</td>
<td>0</td>
<td>32 ± 7</td>
<td>0.00</td>
</tr>
<tr>
<td>1:14 pAm</td>
<td>85.12 ± 0.06</td>
<td>0.24</td>
<td>25 ± 5</td>
<td>-7</td>
</tr>
<tr>
<td>1:20 pAm</td>
<td>84.14 ± 0.08</td>
<td>-0.74</td>
<td>41 ± 5</td>
<td>10</td>
</tr>
<tr>
<td>1:14 pDMAm</td>
<td>84.66 ± 0.08</td>
<td>-0.22</td>
<td>23 ± 7</td>
<td>-9</td>
</tr>
<tr>
<td>1:20 pDMAm</td>
<td>84.2 ± 0.1</td>
<td>-0.7</td>
<td>19 ± 2</td>
<td>-13</td>
</tr>
<tr>
<td>1:14 pDMAm/AA</td>
<td>84.22 ± 0.05</td>
<td>-0.66</td>
<td>22 ± 3</td>
<td>-10</td>
</tr>
<tr>
<td>1:20 pDMAm/AA</td>
<td>83.59 ± 0.05</td>
<td>-1.29</td>
<td>30 ± 10</td>
<td>-1</td>
</tr>
<tr>
<td>1:14 pDMAm/DMAEMA</td>
<td>85.05 ± 0.06</td>
<td>0.17</td>
<td>19 ± 3</td>
<td>-13</td>
</tr>
<tr>
<td>1:20 pDMAm/DMAEMA</td>
<td>84.83 ± 0.05</td>
<td>-0.05</td>
<td>23 ± 2</td>
<td>-9</td>
</tr>
</tbody>
</table>

A comprehensive look at the thermal and functional chemical stability data is provided in Table 5. From these data, broad conclusions about which polymers and concentrations optimize enzyme characteristics can be made. While all conjugates maintain thermal stability close to that of the native, functional chemical stability is decreased in all conjugates, except 1:20Am. When comparing stability of 1:14 and 1:20 samples, slight trends emerge. With increasing polymer modification, small losses in thermal stability and increases in chemical stability result. This trend proves true for all conjugates, except in the chemical stability of DMAm, suggesting that there may be a compensation between thermal and functional chemical stability. Similar trends are observed in work with lysozyme conjugates.

Conclusions

Water-soluble synthetic polymers from the acrylamide and dimethylacrylamide families were synthesized by RAFT polymerization techniques and characterized by $^1$H NMR. Polymers of approximately DP 30 chain lengths were conjugated to FnCel5a cellulase through NHS/EDC coupling reactions. The resulting conjugates were subjected to a colorimetric carboxymethylcellulose activity assay, differential scanning fluorimetry, and chemical denaturation by DMF followed by activity determination.
Based on improved enzymatic activity, retention of thermal stability and increased functional chemical stability in the 1:20 sample, pAm polymers emerge as front runners for highly functioning cellulase bioconjugates. Additionally, a lower polymer concentration pDMAm conjugate also contributes increased relative activity, with a small decrease in functional chemical stability compared to the native enzyme. Results of studies completed herein allow for preliminary conclusions regarding the effects of hydrophilic polymers on the behaviors of cellulase.

In an effort to confirm these findings, further experiments may be completed. First, aqueous GPC and MALDI-TOF mass spectrometry can be optimized as techniques with which to experimentally determine the molecular weights of the synthetic polymers prior to attachment to the protein. MALDI-TOF MS can also be a useful tool to show proof of conjugation and in determining molecular weight of the resultant conjugates. Second, confirmation of polymer attachment sites via trypsin digest is important in suggesting where polymers may be interacting with the protein surface and helpful in explaining behaviors like activity and denaturation. Lastly, while this study looked at the functional chemical stability, perhaps a more useful measure is of the intrinsic chemical stability which can be determined through circular dichroism experiments. These additional experiments would offer more evidence and insight into the protein and polymer interactions and would allow for more concrete conclusions to be made about the types of polymers that improve cellulase bioconjugates. The studies completed in this work and recommended additional studies aim to provide important information about the ability to tune cellulase-polymer conjugates for real-world applications.
Closing Remarks

RAFT polymerization is a suitable technique for producing polymers with good control over molecular weight and can be completed in aqueous conditions. In this work, RAFT polymerization has been used to synthesize a variety of well-defined polymers for attachment to proteins. Water-soluble, neutral and ionic polymers of low and high molecular weights have been conjugated to lysozyme and cellulase through a hybrid grafting-to/grafting-from approach via EDC/NHS amine coupling. Subsequently, conjugates were characterized and subjected to activity and stability studies.

Enzyme activity was analyzed to determine the impact of polymer size and functionality on enzyme function. For lysozyme, activity was investigated using both a small and large substrate to illustrate that polymer steric hindrance negatively effects enzymatic activity; this trend was more evident in the larger substrate studies. However, in lysozyme studies, all conjugates resulted in decreased activity. In contrast, all cellulase-polymer conjugates demonstrate comparable or improved activity over native enzyme. Like lysozyme, increased polymer modification also negatively impacts the enzymatic activity of cellulase, where low concentration samples have greater activity retention than higher concentration samples. Exceptions occur with the anionic cellulase samples which had equivalent activity and the cationic sample in which high concentration had greater activity than the lower concentration sample. From activity data trends in both studies, it can be concluded that increasing polymer modification increases steric hindrance on the polymer surface and results in decreased activity.

Differential scanning fluorimetry studies provide insight into the thermal stability of lysozyme and cellulase conjugates. The analyses for both proteins suggest that thermal stability is maintained or decreases upon conjugation of polymer. Specifically, lysozyme samples demonstrate that increasing polymer concentration on the enzyme results in decreasing thermal stability. The melting temperatures of cellulase conjugates differed very little from the native cellulase suggesting nearly equivalent thermal stability in all cases. As with lysozyme, nearly all samples demonstrate decreased thermal stability with increased polymer modification.

Distinct measures of chemical stability were studied for each enzyme. Inherent chemical stability of lysozyme was measured as a function of guanidine concentration while DMF was used to denature cellulase as a measure of functional chemical stability. For lysozyme conjugates, increasing polymer modification on the enzyme imparts greater chemical stability to denaturant.
Conversely, with cellulase samples, functional chemical stability is less than native in nearly all samples except for one conjugate modified with neutral polymer (1:20Am). Many of the polymers studied herein are relatively short which makes any observable trends promising.

This work contributes important information about the effects of polymers on enzyme behaviors to the field of protein-polymer conjugates. Trends in polymer size and functionality have begun to take form following these studies; however, more research is needed to make definitive, global conclusions. Continued studies that strive to answer questions about the reasons behind observed activity and stability trends are needed in hopes of understanding the structure and function of enzymes as a result of polymer attachment. Future fundamental studies and progress in the field of protein-polymer conjugates promises to take these fascinating and valuable biomacromolecules to new heights.
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