ABSTRACT

SYNTHESIS OF RANDOM AND SITE-SPECIFIC PROTEIN-POLYMER CONJUGATES BY RAFT POLYMERIZATION

by Rebecca Falatach

Proteins and enzymes have long been used in a wide variety of applications from laundry detergents to therapeutics; however, instability under conditions such as high temperature, extreme pH, organic solvents, and the presence of proteases limit new applications. Combining natural proteins with synthetic polymers to create protein-polymer conjugates offers the opportunity to create new functional materials with improved activity and stability. In this study, random and site-specifically modified protein-polymer conjugates were synthesized by RAFT polymerization using grafting-to and grafting-from approaches. Polymer conjugates of the model proteins, lysozyme and chymotrypsin, were synthesized by random modification and the enzyme activity, stability, and number of attachments were determined. Site-specific modification of green fluorescent protein was achieved using the LAP/Lipoic Acid Ligase system and RAFT polymerization. These bioconjugates were characterized to determine the number of CTA attachments and polymer length.
SYNTHESIS OF RANDOM AND SITE-SPECIFIC PROTEIN-POLYMER CONJUGATES BY RAFT POLYMERIZATION

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ABBREVIATIONS

RAFT  reversible addition fragmentation chain-transfer
ATRP  atom transfer radical polymerization
CTA  chain transfer agent
OEOA  oligo(ethylene oxide) methyl ether acrylate
CRP  controlled radical polymerization
LplA  lipoic acid ligase
LAP  LplA acceptor peptide
MALDI-MS  matrix-assisted laser deposition ionization-mass spectrometry
ESI-MS  electrospray ionization-mass spectrometry
GFP  green fluorescent protein
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CHAPTER I – Introduction to Protein-Polymer Conjugates

Rebecca Falatach

*References in APA format.
I.1. Uses and Limitations of Natural Proteins

Peptides and proteins are utilized in a wide variety of consumer and diagnostic products and industrial processes. Proteins have long been used as therapeutics to treat disease, as many proteins function naturally in the body to perform a wide variety of tasks. They are responsible for the mechanical integrity of hair and skin, facilitate important cellular functions, and have evolved to bind many pathogens, either naturally or through genetic engineering [1]. Enzymes have also been used as biocatalysts for industrial applications, including refining of pulp for the paper industry and biodiesel production, due to their fast catalytic rates and high specificities for their substrates [2]. Often using enzymes in industrial applications eliminates the need for toxic reagents, making them an environmental friendly alternative to many traditional chemical catalysts [3]. Many products used every day, such as laundry detergent and toothpaste, contain enzymes. A few examples of these proteins and their applications are shown in Table 1 [4].

Table 1. Table of industrially relevant enzymes, their function and applications.

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Function</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>Catalyzes the hydrolysis of starch into sugar</td>
<td>Toothpaste, textiles, paper production and recycling, fuel production, food industry</td>
</tr>
<tr>
<td>Cellulase</td>
<td>Catalyzes the decomposition of cellulose</td>
<td>Paper production and recycling, laundry detergent, textiles</td>
</tr>
<tr>
<td>Lipase</td>
<td>Catalyzes the hydrolysis of lipids</td>
<td>Laundry detergent, contact lens cleaner, paper production and recycling, textiles, biodiesel</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Catalyzes the hydrolysis of peptidoglycans</td>
<td>Antibacterials, laboratory use for disrupting cell membranes</td>
</tr>
<tr>
<td>Peroxidases</td>
<td>Catalyzes the oxidation of a variety of substrates using hydrogen peroxide</td>
<td>Diagnostics, biosensors, biobleaching, deodorization</td>
</tr>
<tr>
<td>Proteases</td>
<td>Catalyzes hydrolysis of peptide bonds in proteins</td>
<td>Laundry detergent, dish soap, contact lens cleaner, textiles, fuel production, food industry</td>
</tr>
<tr>
<td>(class of enzymes)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The application of enzymes and proteins in new commercial and industrial applications are often limited due to their sensitivity to environmental stresses. For therapeutics, these limitations include degradation by proteolytic enzymes, short circulation life, rapid kidney clearance, and tendency to produce an immune response. Limitations often associated with the production and distribution of protein products include enzyme inactivation during storage for long periods of time or under harsh conditions such as extreme temperature or pH, constant agitation, and exposure to surfactants or solvents [5]. The various limitations of enzymes yield a need for methods to improve enzyme stability in all phases of product development, from production to distribution to administration [6]. Much of the research currently being conducted to address these issues focus on the use of synthetic polymers to encapsulate enzymes in nanostructures, immobilize enzymes on surfaces, and create soluble protein-polymer conjugates [7][8]. Encapsulating and immobilizing via polymers allows for the retention of enzymes in industrial applications such as packed-bed bioreactors [9]. Immobilized enzymes can also be used for applications such as biosensors [9]. Creation of soluble protein-polymer conjugates typically consist of attaching one or more polymer chains to the peptide backbone of a protein that is free in solution. These bioconjugates have many applications including therapeutic proteins for in vivo use [10] and stabilized enzymes for storage [11] or thermal precipitation for recycling and reuse [12].

1.2. Synthesis of Soluble Protein-Polymer Materials

There are two primary approaches for creating soluble protein-polymer conjugates, grafting-to and grafting-from, as seen in Figure 1. Grafting-to refers to the synthesis of a polymer chain in solvent, then subsequently attaching the polymer to the desired macromolecule, often in a different solvent system. Grafting-from is a newer method that entails attaching an initiator to the protein then growing a polymer chain directly from this macromolecule initiator [13][14].
**Figure 1.** The top schematic shows the grafting-to technique and the bottom schematic shows the grafting-from method.

### I.2.1. Grafting-to Approaches for Synthesizing Protein-Polymer Conjugates

In grafting-to approaches, the polymer is first synthesized, purified, and functionalized with a chemical group, such as a maleimide or an NHS ester, that can then be used to attach the polymer to a protein. One of the oldest grafting-to methods for synthesizing protein-polymer conjugates is PEGylation. PEGylation involves covalently attaching long chains of polyethylene glycol (PEG) molecules to proteins or peptides [15]. The PEG polymers are typically synthesized by anionic polymerization and purified before being bound to a protein, as seen in Scheme 1.

**Scheme 1.** Scheme of PEGylation with an NHS ester PEG.

One benefit of this approach is the ability to easily characterize the polymer before it is bound to the protein or other target molecule. Binding large PEG chains to a protein or peptide for therapeutic uses has been shown to decrease immune response, reduce renal clearance, and increase resistance to proteolytic degradation. For this reason, there are currently multiple PEGylated therapeutics that are FDA approved and used clinically [16][17][18].

Although PEGylation has been shown to improve the stability of therapeutic proteins, it can result in a significant loss of enzyme activity due to steric hindrances associated with the
large, bulky polymer. Loss of activity is possible with the attachment of any polymer, especially if the polymer attaches near the active site of the enzyme. Steric hindrances associated with the attachment of a larger polymer can also make any grafting-to technique very inefficient, resulting in large amounts of waste and a high cost of production. Grafting-to is further complicated by the difficult separation of similarly sized products and unreacted polymer chains, making this process difficult to scale up to industrial production [19]. These potential issues have led to an increased interest in using grafting-from techniques for synthesizing conjugates with higher molecular weight polymer chains.

1.2.2. Grafting-from Approaches for Synthesizing Protein-Polymer Conjugates

Many grafting-from methods have been developed, but each with the same basic steps. The general approach involves attaching an initiator molecule to the protein or peptide of interest, to form a macro-initiator, then synthesizing the polymer directly from that molecule. This approach allows for the growth of a large polymer directly from the initiator attached to the protein. This reduces the steric hindrance and purification issues associated with trying to attach a large polymer directly [19][20]. One disadvantage to a grafting-from approach is the need for a protein-friendly method of synthesizing polymers. Typical methods of synthesizing a polymer involve high temperatures and solvent environments that are not suitable for dissolving proteins [13]. This challenge must be overcome to achieve successful production of protein-polymer conjugates.

The most widely used polymerization technique for the grafting-from approach is controlled radical polymerization (CRP) [14]. CRP, also called living radical polymerization or reversible-deactivation radical polymerization, is a type of free radical polymerization that offers good control over the polymerization process. Specifically, in CRP methods, many of the polymer chains formed are considered living because they are able to grow further upon the addition of more monomer to the reaction [21]. Although CRP techniques are also used to synthesize polymers for grafting-to techniques, they have been widely used in grafting-from approaches because of the good control over the polymer size and the ability to do many of these techniques in protein-friendly conditions. CRP methods have shown many improvements over other polymerization methods including the vast library of monomers that can be utilized and increased control over the size of the polymer attached [22].
Another advantage associated with using CRP techniques is the ability to synthesize polymers with functional end groups and complex architectures. Architectures such as branched polymers and block copolymers are more easily synthesized using CRP methods and can be used to impart novel functionalities to a protein. Functionalities such as stimuli responsive behavior and modified hydrophilic/hydrophobic properties can be imparted by copolymerizing monomers with these properties [22].

The two most widely used CRP techniques are atom-transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer polymerization (RAFT). ATRP is a widely used polymerization technique for creating complex polymer materials with well controlled polymer lengths and copolymer structures. Although ATRP has been shown to synthesize well controlled protein-polymer materials, it has a somewhat limited range of monomers and can display decreased control when done in water [19][23]. Additionally, the metal catalyst needed in this reaction can complicate the purification procedure and has the potential to affect enzyme activity by complexing with amide and carboxyl groups on amino acid side [24]. These disadvantages can make ATRP a less desirable method of modifying proteins with grafting-from polymerization.

RAFT proceeds through a degenerative transfer process with the help of a chain transfer agent (CTA), or RAFT agent. A CTA is a thiocarbonylthio moiety, such as a trithiocarbonate shown in Scheme 2, where the Z group activates the thiocarbonyl to participate in radical addition and stabilizes the resulting radical intermediate. The R group of the CTA initiates growth of the polymer chains. RAFT polymerization can be initiated using an initiator that can decompose into radicals through stimulation by ultraviolet light, gamma radiation, pulsed laser irradiation, or thermal decomposition. Thermal decomposition (initiation) is the most widely used method. In the initiation step, oligomeric radicals are formed, which then react with the CTA. Initiation results in the equilibrium shown below, where the radical intermediate can either fragment back to the original CTA or yield an oligomeric CTA. From there, the polymer chains continue to grow by adding monomers and they quickly exchange between the thiocarbonylthio group and growing radicals. In well controlled systems, equilibrium continues throughout the reaction [21].
Scheme 2. Proposed RAFT polymerization reaction. Step 1 initiator decomposition, step 2 initialization, step 3 propagation, and step 4 chain transfer.

RAFT has advantages over other CRP techniques such as a wide variety of reaction conditions and compatible chain transfer agents, which allows the use of the largest library of monomers [21][22][25]. Also, RAFT does not require a transition metal catalyst and can be done in water or buffer systems at relatively low temperatures while maintaining >90% monomer conversion [19][21]. However, many RAFT agents are hydrophobic and can cause the protein to become insoluble once attached [16].

I.3. Approaches for Conjugating Small Molecules and Polymers to Proteins

With RAFT polymerization, both grafting-to and grafting-from techniques can be used to synthesize protein-polymer conjugates. The most widely used method of attaching either the initiator molecule or polymer to a protein is through the terminal amine or side groups on naturally occurring lysine or cysteine residues of the protein. Two of the most commonly used chemistries to perform these attachments are shown in Table 2.
Maleimides or maleic acid imides are formed by reacting maleic acids with an amine containing compound. The double bond in maleimide compounds will undergo a Michael’s addition reaction with thiol groups in the pH range of 6.5 to 7.5 to form stable thioether bonds [26]. Maleimides can be used to create thioether bonds between initiators or polymers and cysteine residues in a protein’s backbone. The advantage to modifying the cysteine residue is the relatively low number of cysteine residues in a typical protein. This limits the number of attachment sites which is often desirable when studying the enzyme activity retention. However, the limited number of cysteine residues presents a disadvantage if the protein of study doesn’t contain any. When involved in disulfide bonding, cysteine residues can be very important to the folding of the protein in which case modifying them would be impractical. There are also many enzymes that require a free cysteine in their active site, so unintentional modification of that cysteine could result in enzyme inactivation [1].
Carbodiimides are compounds that promote the formation of bonds between carboxylic acids and amines, as well as phosphate groups and amines. One example of a water soluble carbodiimide is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) which is often used to create stable amide bonds between carboxylic acids on initiator molecules or polymers and primary amines in lysine residues in protein backbones. EDC coupling reactions are supplemented by the use of N-hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide (sulfo-NHS) [26]. Many products containing NHS esters are commercially available, making this approach relatively cost effective and simple. This approach is also a very general approach due to the high number of lysine residues contained in a majority of proteins, making this the quickest and most reliable method of producing a protein bioconjugate. However, due to the large number of lysines contained in a majority of proteins, the number and site of attachment cannot be controlled and is often difficult to determine [1]. Another disadvantage to modifying lysine side chains is the potential negative affects related to removing the positive charge associated with the primary amine. Changing the surface charge of a protein can result in the loss of water solubility or changes in structure [16].

With both of these methods the polymer can attach to any solvent-exposed lysine or cysteine on the protein backbone. Depending on the solvent accessibility and pKa of the side group and the frequency, this could result in a heterogeneous population of conjugates with different numbers and sites of attachments. The site of attachment can be very important; if a large polymer attaches near the active site of an enzyme, the activity of the enzyme could be greatly reduced [27]. Also, if the targeted side chain is vital to the folding of the protein, there could be a large impact on the structure. These potential issues can be resolved by developing site specific attachment techniques where the location of the polymer on the protein can be carefully chosen to avoid active sites and perturbations in the structure.

I.4. Methods of Site Specific Attachment of Polymers to Proteins

With site specific attachment methods, the location and number of polymer attachments can be carefully chosen to avoid any loss in enzyme activity or disturbances in the structure of the protein. Choosing the sites of attachment also allows for homogeneous and well-defined conjugates. Site specific protein modification can be utilized to study post-translational modifications, to achieve oriented protein immobilization, and to study protein properties such as
folding, protein-surface interactions, and protein-protein interactions [28]. There are many methods for achieving site specific attachment, each with their own advantages and disadvantages.

### 1.4.1. Currently used Methods of Site Specific Polymer Attachment

One method of site specifically attaching a polymer or initiator is targeting either the N-terminus or C-terminus of the protein. For example, the N-terminus can be specifically targeted through serine oxidation or transamination [1]. This method can be used without genetically modifying the protein, allowing for the use of commercially available proteins. Although this can be an effective way of attaching a molecule in a specific location, these methods are limited to attaching one polymer to a protein. An N-terminus that has undergone post-translational acylation would not be available for modification using these methods [1]. Also, modification of the terminus may be undesirable due to proximity to the active site or other regions important for protein function [2][29].

One somewhat simple method of choosing the number of attachments and the site of attachments is by adding or subtracting natural amino acids such as lysine’s and cysteine’s in the protein backbone. If there are multiple of these natural amino acids in a protein’s structure, certain amino acids can be blocked or replaced by another similar amino acid [30]. With this method, reactions utilizing natural amino acids can be used in a site specific manner. Blocking a natural amino acid to prevent it from being modified has the advantage of using a wild type protein without the need for genetic engineering. Alternatively, a lysine or cysteine can be added in a desired location on the protein backbone using genetic modification. For example, a cysteine can replace a serine or threonine with very little to no effect on the protein [31]. The advantage to this method is the ability to use well known chemistries such as maleimide or EDC/NHS coupling, as well as being able to use naturally occurring amino acids to help avoid any structural disruptions. The disadvantage to replacing or adding amino acids to the protein structure is the potential of protein misfolding due to adding or subtracting cysteine residues involved in disulfide bonding or changing protein solubility by changing the surface charge when adding or subtracting lysine residues [1][15].

Another common method of site specifically attaching molecules to proteins is by genetically incorporating non-canonical or unnatural amino acids into the protein backbone
A majority of these unnatural amino acids include an azide or alkyne functional group from which alkyne-azide copper-catalyzed cycloaddition can be performed. These “click” reactions are efficient reactions and naturally site selective since neither azide nor alkyne functional groups occur naturally in proteins [34]. Recently, copper-free variants of this reaction have been developed to overcome the cytotoxicity of the copper catalyst. In the copper-free versions, the reaction is driven by strain relief in functionalized cyclooctanes [9]. However, copper-catalyzed cycloaddition proceeds at a much faster rate than copper-free versions [35]. Other non-natural amino acids have been developed to include polymerization initiators, allowing the direct genetic incorporation of the initiator molecule. This method allows the subsequent step of attaching the initiator molecule to be skipped [36].

Scheme 3. Copper-catalyzed alkyne-azide cycloaddition reaction mechanism.

These non-canonical amino acids can be placed anywhere in the secondary structure of a protein allowing a very careful choice to be made taking into account proximity to active sites and other important regions, as well as solvent accessibility of the site. The main disadvantage of unnatural amino acids is the decrease in protein expression levels, especially with multiple insertion sites. [15][37]. Adding unnatural amino acids can also disturb protein solubility and protein folding. Proteins functionalized with an azide must be stored in a manner to avoid light exposure, otherwise the azide can become reduced to an amine and lose its ability to react with alkyne groups [8][38].
I.4.2. Methods of Protein Ligation

A relatively recently explored method of site specifically modifying proteins is through enzyme-mediated ligation reactions. This approach adopts the enzymes that are normally used by cells perform post-translational modifications on proteins. The primary function of these enzymes is to recognize a specific amino acid sequence on a target protein and ligate or attach a highly specific substrate to a sidechain in that amino acid sequence. An example of this would be the ATP dependent covalent attachment of lipoic acid to the E2p subunit of the pyruvate dehydrogenase complex by the enzyme lipoic acid ligase [39]. In order to use this method for site-specific protein modification, these enzymes must be engineered to accept novel substrates and are often modified to recognize different amino acid sequences in the target protein. Table 3 includes a list of enzymes used in ligation reactions and their associated peptide sequences [28].

Figure 2. General method for using enzyme mediated ligation for attaching small molecules.
Table 3. Enzymes used in ligation reactions and their associated peptide sequences.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognized Peptide Sequence</th>
<th>Site of Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formylglycine generating enzyme</td>
<td>CXPXR or LCTPSRGSLFTGR</td>
<td>C or N Terminus</td>
</tr>
</tbody>
</table>
| Phosphopantetheinyl transferase     | ACP or PCP or DSLEFIASKLA or VLDSLEFIASKLA or GSQDVLD
|                                    | LEFIASKLA                                            | C or N Terminus or flexible loops    |
| Sortase                            | LPXTG                                               | Any section                          |
| Farnesyltransferase                | CaaX                                                | C terminus                           |
| Biotin Ligase                      | GLNDIFEAQIEWHE                                      | C or N Terminus                      |
| Lipoic Acid Ligase                 | GFEIDKVVYDLDHA                                      | C or N Terminus or flexible loops    |

Each of the enzymes listed above have been modified to attach a non-native substrate using the enzyme’s natural mechanism. Formylglycine generating enzyme works by converting the cysteine within the amino acid sequence CXPXR, where the X represents a variety of amino acids, to formylglycine. The natural use of this enzyme is to facilitate the post translational modification of type I sulfatases. Formylglycine is an aldehyde-bearing residue from which covalent modification can be performed using complementary aminooxy- or hydrazide-functionalized reagents. Phosphopantetheinyltransferase (PPTase) performs critical post-translational modifications on two small folding domains, peptide carrier protein (PCP) and acyl carrier protein (ACP). PCP and ACP domains are not active until they are post-translationally modified by PPTase. PPTase adds a phosphopantetheinyl (Ppant) prosthetic group to a hydroxyl group on a serine residue through a phosphodiester bond. This Ppant group serves as an anchoring point for a growing peptide, fatty acyl chains, or polyketides. This enzyme has also been used to label proteins with biotin, which can then be used to immobilize the modified protein, or fluorophores. Sortagging is the use of the enzyme sortase for site specifically labeling or modifying proteins. Sortase will recognize a specific amino acid sequence and form a covalent acyl-enzyme intermediate through a thioester bond. That thioester bond is then attacked by the N-terminal of a glycine-containing probe to form an amide bond. Protein farnesyltransferase (PFTase) performs post-translational modification by transferring a farnesyl group using farnesyl...
pyrophosphate (FPP) to the cysteine in a “CaaX-box” tetrapeptide sequence, where C is cysteine, a is an aliphatic amino acid, and X is a variety of amino acids. PFTase has been shown to accept other isoprenoid molecules containing azide, alkyne, aldehyde, biotin, or aryl groups. All of these functional groups can be modified further using a variety of chemistries, such as azide-alkyne cycloaddition. Biotin ligase mediates the binding of biotin groups to the lysine residue within a specific peptide sequence. Once the biotin is attached avidin can then be used to produce a very strong noncovalent bond [28].

Although each of these ligation methods work in slightly different manners, each can be used, either naturally or after being genetically modified, to site specifically modify a protein. Since the likelihood of a very specific amino acid sequence to appear naturally in a protein is low, the peptide sequence associated with the desired enzyme can be genetically added to the protein in a desired location. The enzyme used needs to be chosen carefully based on the desired location of attachment and the type of molecule being attached. A few of the enzymes can only be used to attach molecules at the C or N terminus and each enzyme can only attach certain types of molecules, which can limit their use. Some enzymes in this class also function by cleaving the protein’s backbone to attach a small molecule to the C or N terminus of that cleaved amino acid [28][37]. However, the enzymes can be genetically modified to recognize modified amino acid sequences, as well as modified to accept substrates that are not native to their function increasing the applications for this method [40].

1.4.3. Lipoic Acid Ligase Method of Site Specific Attachment

Among the enzyme mediated ligation reactions listed in Table 3, lipoic acid ligase (LplA) was used in this study to site specifically attach an alkyl azide acid in multiple locations on green fluorescent protein (GFP). LplA is an enzyme that recognizes a specific amino acid sequence and subsequently attaches a lipoic acid to the lysine residue within this amino acid sequence. This natural activity has been slightly modified to recognize a 13 amino acid sequence called a LAP (LplA Acceptor Peptide) sequence and attach an azide-containing alkyl acid [37][40].
Figure 3. (a) Scheme of the natural function of lipoic acid ligase. (b) Scheme of modified lipoic acid ligase with an alkyl azide as the substrate.

This LAP sequence can then be introduced to the N or C terminus or a flexible loop in the protein’s structure through mutagenesis. The modified lipoic acid ligase can then ligate the azide acid which can then be modified with many different molecules through azide-alkyne cycloaddition [28]. With the method described here, the 13 amino acid sequence can be added without causing protein misfolding or a decrease in water solubility. Also, the LAP sequence can be added in almost any location in the enzyme backbone so that a location can be carefully chosen to avoid the active site and other regions important to the function and structure of the protein. The main advantage of using this site-specific method is the ability to have more than one LAP sequence incorporated into the protein structure while retaining high expression levels and protein folding [37][40]. This allows polymers to be attached in multiple locations without greatly affecting the activity or structure of the enzyme.
1.5 Approaches for Protein Immobilization

A natural extension of soluble protein-polymer materials is the insoluble protein containing materials. There are a number of ways to incorporate or immobilize enzyme within solid support materials. This includes passive adsorption to a solid support facilitated by electrostatic or hydrophobic/hydrophilic interactions, covalent attachment through reactions with an activated support material, entrapment with cross-linked polymer systems, and multipoint covalent cross-linking directly between polymers. There are many specific applications for immobilized enzymes including packed-bed bioreactors, biosensors, drug delivery systems [6], and tissue engineered scaffolds. Due to the large number of applications, there have been many methods researched and developed to attach enzymes to a variety of surfaces. Most of the early approaches of immobilization were considered non-specific because the location of attachment and orientation of the protein could not be controlled. Since the protein is attached using functional groups native to the protein, the attachment site can vary from protein to protein. This can also result in a heterogeneous population due to the possibility of multiple, different sites of attachment. If the targeted functional group appears at multiple locations within the protein’s structure, any and/or multiple of these locations can become bound to the surface.

Non-specific methods of enzyme binding can be divided into two categories, non-covalent and covalent binding. Non-covalent attachment or absorption of proteins to surfaces are often employed for protein purification techniques such as in chromatography. These types of attachments often involve either electrostatic interactions or passive adsorption caused by hydrophobic and hydrophilic interactions. The advantage of using non-covalent attachment techniques for immobilization is the relative simplicity of absorbing a protein without modifying it first or using coupling reagents. However, non-covalent bonds are typically weak and reversible leading to gradual loss of the protein from the support material by desorption [2][3]. In addition, passive binding to surfaces can often cause conformational changes and inactivation of the protein [9].

To prevent gradual loss of protein from the support material, covalent attachment approaches have been utilized. Covalent binding techniques can provide much stronger attachments, preventing or reducing enzyme loss from detachment. Covalent immobilization in the form of polymeric cross-linking has also shown improved thermal stability of enzymes [7]. There are many methods for covalently binding proteins to surfaces, but traditional routes
involve reactions with functional groups already present on the protein’s backbone such as thiols and amines. An advantage to non-specific covalent binding approaches is that proteins can be used without modification through the use of side groups contained in the protein’s natural amino acid sequence [9].

Although both methods of non-specifically attaching proteins to surfaces allow the use of unmodified proteins, the site of attachment is random which leads to a heterogeneous population where the orientation of the proteins is non-uniform and often unknown. This could greatly affect the activity of an enzyme, such as in cases where the targeted attachment site is next to or inside the active site or binding site of the enzyme [2][3][31]. Also, if the attachment site is important in the movement of the protein, such as hinged motion or conformational changes necessary to the function of the protein, the structure could be negatively affected which can lead to a decrease in activity [9].

With these issues in mind, research has shifted to the development of approaches for site-specific modification. The ability to choose the binding site on a protein allows for the creation of a homogeneous population in which the orientation can be carefully selected to maximize activity and structure retention. This is often achieved through genetic engineering of the protein structure to include reactive handles that are not naturally present in the protein’s structure at the desired location. Examples of this type of protein engineering include the addition of a cysteine or lysine to the desired location, a specific amino acid sequence that can be used for enzyme-mediated ligation reactions, or the addition of functional groups such as azides or alkynes that are not naturally present in proteins. There are also non-covalent and covalent methods of site specifically attaching a protein to a support structure. These methods are most often used for affinity chromatography but have been repurposed for immobilization techniques. Affinity chromatography often involves the insertion of a peptide tag into a protein that does not naturally occur in the amino acid sequence of the protein. A tag can usually be placed at the C or N terminus or a flexible loop. This allows the site to be chosen to avoid disturbing the structure or activity of the protein while allowing for a defined and homogeneous orientation. Non-covalent methods of site specified attachment still have the issue of weak binding and result in protein loss when stored for a long period of time. Also, the tags used in this approach are often bulky which can negatively affect protein function and structure, especially when placed somewhere other than the C or N terminus [2][31].
To create more permanent immobilized protein structures, a vast library of covalent methods for site specific attachment have been developed. Some of these methods involve adding or subtracting naturally occurring amino acids from a protein’s secondary structure and using the traditional methods of covalent attachment through thiols and amines. There are numerous other techniques for inserting functional groups to allow for site specific attachments through a variety of chemical reactions. A few examples include the insertion of unnatural amino acids and peptide sequences that are recognized by enzymes that can then ligate a new functional group to that peptide sequence. Both methods are capable of introducing many functional groups such as alkynes and azides from which cycloaddition reactions can take place. Although immobilization can be an effective way to modify a protein for a wide variety of uses, it is not an appropriate approach for all applications. There are many cases in which proteins need to be free in solution, while also being modified to effect stability, solubility, and/or activity [9].

I.6. Hypothesis
The activity and stability of protein-polymer conjugates can be limited by the size and type of polymer attached and the site of conjugation. RAFT polymerization is a type of controlled radical polymerization that is ideally suited for synthesis of protein-polymer conjugates since it can tolerate numerous monomers of different functionalities. The hypothesis of this study was that RAFT polymerization could be used to create well defined protein-polymer conjugates.

I.6.1. Objective 1
Develop an approach to create randomly modified protein-polymer conjugates by the grafting-from approach using RAFT. Demonstrate this approach on the enzyme lysozyme. Characterize the activity of the resulting protein-polymer conjugates.

I.6.2. Objective 2
Develop an approach to create site-specific protein polymer conjugates by grafting-from approach using RAFT. Use Lipoic Acid Ligase mediated attachment of azide groups to site specifically attach a polymer. Demonstrate approach on GFP and characterize.

I.6.3. Objective 3
Controlled radical polymerization methods and enzyme mediated ligation approach can be utilized to synthesize well-defined protein-polymer conjugates with improved activity.

**1.7 Thesis Outline**

Chapter 1 presents an introduction to the uses and methods of synthesizing protein-polymer conjugates. I wrote this chapter solely for use in this thesis.

Chapter 2 presents a peer-reviewed paper published in Chemical Communications in December 2014. It focuses on the random attachment of a polymer to lysozyme through grafting-to and grafting-from RAFT polymerization. A RAFT chain transfer agent was attached to lysozyme through EDC/NHS coupling, with the intention of grafting a polymer from the CTA. However, the attachment of the CTA caused the lysozyme to lose water solubility. This led to the development of a combined grafting-to and grafting-from RAFT polymerization. To increase water solubility, a short acrylamide oligomer was synthesized from the CTA, attached to lysozyme, and subsequently chain extended with OEOA. The enzyme activity of lysozyme was measured after each step of modification and compared to the activity of native lysozyme. The supplemental information for this publication is in the Appendix. This work was done under the guidance of Dr. Jason Berberich, Dr. Richard Page, and Dr. Dominik Konkolewicz. Saadyah Averick synthesized the RAFT CTA. Cameron McGlone performed all of the MALDI-MS with the assistance of Sameer Al-Abdul-Wahid. Sameer also ran all of the ESI-MS on the oligomer CTA. Dr. Konkolewicz, Dr. Page, Dr. Berberich, Sameer Al-Abdul-Wahid, and I collaborated to write the publication and its supplemental information.

Chapter 3 presents a peer reviewed paper published in Polymer. It focuses on applying a grafting-to RAFT polymerization method to a protease, chymotrypsin, and studying the stabilization affects associated with the addition of different polymers. Three different oligomers were synthesized from the RAFT CTA; a low MW pDMAm, a high MW pDMAm, and a pOEOA. These polymers were then grafted-to chymotrypsin using EDC/NHS coupling. The short term enzyme activity retention and the enzyme activity over a period of time was measured. The larger MW polymers caused a decrease in the short term enzyme activity when compared to native chymotrypsin. However, the larger MW polymers also shielded the chymotrypsin from autolysis when compared to the native chymotrypsin. This work was done
under the guidance of Dr. Jason Berberich, Dr. Richard Page, and Dr. Dominik Konkolewicz. The oligomers were synthesized by Samantha Sloane and coupled to chymotrypsin by SaadyahAverick, with the assistance of Shoahua Li. Cameron McGlone collected all the MALDI-MS data. Dr. Konkolewicz, SaadyahAverick, Dr. Page, Dr. Berberich, Sameer Al-Abdul-Wahid, and I collaborated to write the publication and its supplemental information.

Chapter 4 presents work done on a site-specific method of protein modification utilizing a LAP/lipoic acid ligase system combined with RAFT polymerization. The model protein for this system was green fluorescent protein that was genetically altered to include LAP sequences in different sites in the protein’s structure. Lipoic acid ligase was then used to ligate 10-azidodecanoic acid to the LAP sequence, from which an oligomer alkyne CTA was attached using copper-catalyzed alkyne-azide cycloaddition. This work was done under the guidance of Dr. Jason Berberich, Dr. Richard Page, Dr. Dominik Konkolewicz, and Dr. Joel Kaar. The plasmids containing the LAP modified GFP were constructed by Joseph Plaks at the University of Colorado-Boulder. A majority of the protein was produced and purified by Kalya Thompson. I wrote this chapter of the thesis with editing assistance from Dr. Berberich.

Chapter 5 provides conclusions on the results shown in Chapters 2, 3 and 4. It discusses the significance of these findings and suggests future work. I wrote this chapter solely for the use in this thesis.

1.8. References


CHAPTER II – The Best of Both Worlds: Active Enzymes by Grafting-To followed by Grafting-From a Protein**

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Supplemental Information published with the work is included as Appendix I.
II.1. Introduction

Protein-polymer conjugates are important and fascinating biomaterials. Tethering polymers from the protein surface or the covalent incorporation of proteins into polymeric materials can improve the stability of proteins, enhance or shift the pH/temperature optimum of enzymes, or synthesize responsive and functional materials. There are two commonly used strategies for the synthesis of protein-polymer conjugates, the “grafting-from” and the “grafting-to” methods. In grafting-from, an initiator or chain transfer agent (CTA) is attached to the protein, followed by the growth of the polymer from the protein-initiator/CTA in an aqueous medium. In the grafting-to approach, a polymer is synthesized in a separate reaction, characterized and subsequently attached to the protein using an efficient organic reaction. The advantages of grafting-from include facile purification of the protein-polymer conjugate, and in many cases a high graft density. When grafting-from, it can be a challenge to design reaction conditions that lead to well controlled polymers while maintaining protein structure. The advantages of the grafting-to approach are facile characterization of the protein and polymer before conjugation, and that the polymerization conditions do not affect protein stability. The disadvantages of grafting-to are the relatively low graft densities, especially with longer polymers, and difficulties in the purification process. Recently, reversible deactivation radical polymerization (RDRP) methods have revolutionized the field of protein-polymer conjugates. RDRP methods allow well-defined protein-polymer to be synthesized by either grafting-to or grafting-from a protein.

The three most commonly used RDRP methods are reversible addition-fragmentation chain transfer polymerization (RAFT), atom transfer radical polymerization (ATRP) and nitroxide mediated polymerization (NMP). RDRP methods, including RAFT, offer several advantages including compatibility with various functional groups, and the ability to chain extend the polymer after it is attached to the protein. Protein-polymer conjugates have been synthesized by each RDRP method however, there still remain significant challenges. The two main challenges are: ensuring that all of the native protein is conjugated with well defined polymers, and using polymerization conditions that preserve protein structure and function. This leads to the concept of reactions under “bio-relevant” conditions.
Scheme 1. The hybrid grafting-to and grafting-from method for protein-polymer conjugate synthesis. A preformed oligoCTA is attached to lysozyme, followed by chain extension by RAFT.

II.2. Results

In our preliminary work, the small molecule CTA, 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC), was attached to the protein lysozyme. However, as shown in Figure S1, even though PAETC is water soluble, the formed protein-CTA conjugate was unstable, and a significant portion of this enzyme-CTA precipitated out of solution. UV-Vis indicated that 2 PAETC molecules attached to lysozyme (Figure S2). This challenge inspired a new synthetic strategy, which combines the strengths of RAFT polymerization with the best of the grafting-to and grafting-from techniques. In this communication lysozyme was selected as a model protein due to its well-characterized structure and function, which can be used to confirm that the protein-polymer conjugate retains function and has not lead to degradation of the protein. The hybrid-grafting-to and grafting-from approach is shown in Scheme 1.
Figure 1. A) ESI-MS of the Am oligoCTA centered at approximately 7-8 units of Am. B) MALDI-MS of native lysozyme. C) MALDI mass spectrum of the lysozyme conjugated with the Am oligoCTA with average DP≈5. D) MALDI-MS of the lysozyme conjugated with the Am oligoCTA with average DP≈10. E) MALDI-MS of the lysozyme conjugated with the Am oligoCTA with average DP≈20. F) Inset of the peak MALDI-MS of the lysozyme conjugated
with the Am oligoCTA with average DP≈5, showing that the spacing of the peak is consistent with the molar mass of Am.

The combined grafting-to and grafting-from approach utilizes a short oligoCTA in the grafting-to step to ensure solubility and stability of the resultant biohybrid. The oligoCTA, in this case an oligomer of acrylamide, is less subject to the limitations of low grafting density that can affect longer polymers. Subsequently, the protein-oligomer conjugate can be extended using RAFT polymerization.

Initially a series of 3 acrylamide (Am) oligomers were synthesized by RAFT using PAETC as the CTA, since RAFT is an excellent tool for the synthesis of oligomers. Three oligomers of Am targeted degrees of polymerization (DP) of 5, 10 and 20. Figure S3 and Figure 1A show the electrospray ionization-mass spectrometry (ESI-MS) data for all three oligomers, indicating that the polymers are well defined and narrowly distributed. Table S1 confirms that the polymers were well controlled with acceptable agreement between the theoretical and experimentally determined molecular weights. Fractional precipitation of high molecular weight polymers could explain the larger measured molecular weight compared to theory.

Once synthesized, the oligomers were conjugated to lysozyme using an in-situ N-Hydroxysuccinimide/ 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (NHS/EDC) coupling procedure. The effectiveness of the NHS coupling was assessed using matrix assisted laser desorption/ionization mass-spectrometry (MALDI-MS). Figure 1B presents the MALDI-MS data for native (unmodified) lysozyme. The major peak is at an m/z 14300 and there are no high molecular weight peaks. Figures 1 C, D, and E show the MALDI-MS data for lysozyme conjugated with the oligoCTAs with approximately 5, 10 and 20 Am units in the backbone. In this conjugation protocol, 60 units of carboxylic acid on the oligoCTA were used for every amine on lysozyme. Figures 1 C, D, and E indicate that as the length of the oligomer increases, the conjugation efficiency decreases dramatically, even though the concentrations of the carboxylic acid, amine group on lysozyme, etc. were the same in all cases. In particular, the short DP≈5 oligomer lead to almost complete coupling efficiency, with virtually no unmodified lysozyme. Figure 1F illustrates the saw tooth pattern in the protein polymer conjugate, with each peak being separated by approximately 70 Da, which is the molar mass of acrylamide. Careful examination of the MALDI-MS data for the DP≈5 oligomer indicate that on average two oligoCTAs were added per lysozyme on average, as described in the supporting information.
This attachment of two DP≈5 oligoCTAs per lysozyme is consistent with literature data attaching small molecule CTAs to lysozyme.\(^1\)\(^9\) When attaching poly(ethylene glycol) chains to amine residues on lysozyme through NHS coupling, lysines 33 and 97 have been found to be the most reactive.\(^2\)\(^7\) This implies that lysines 33 and 97 are expected to be the two residues to which the oligoCTA attaches. However, Figure 1 D and E indicate that when the larger oligoCTAs of DP≈10 and DP≈20 were attached to lysozyme there is also a significant peak from unmodified lysozyme. This suggests that steric hindrance caused by the longer chain reduces the conjugation efficiency of longer polymers. Furthermore, the protein-polymer conjugated peaks in Figure 1 D and E are distinctly bimodal, with the lower molecular weight peak consistent with one oligomer attached to lysozyme, and the higher molecular weight peak, consistent with two oligomers attached to lysozyme. Similar conjugation protocols were applied with the ratio of 120 oligoCTA to amine on lysozyme. Figure S4 shows the MALDI data for the protein-polymer conjugates synthesized with the ratio of 120 oligoCTA to amine in lysozyme. As seen in Figure S4, the larger ratio of oligoCTA to amine gives more complete conjugation, although the use of higher polymer concentrations is less desirable, since a larger fraction of polymer is unused in the conjugation step.

As indicated in Figure 1, protein-polymer conjugates can be formed by reacting lysozyme with oligoCTAs of acrylamide, using NHS/EDC coupling. However, it is also important to determine if the enzyme is active after the conjugation with a polymer. This can be achieved by two independent assays. The first assay examines lysis of *Micrococcus lysodeikticus* due to hydrolysis of glycosidic bonds within the cell wall. The other assay is based on the enzymatic hydrolysis of a glycosidic bonds in 4-methylumbelliferyl β-D-N,N',N"-triacetylchitotrioside \(((\text{NAG})_3\text{-MUF})\), which leads to a fluorescent product. Figure 2 gives the activities of the native enzyme compared to the conjugate with the DP≈5,10 and 20 length oligoCTA. For each length of oligomer, the activity was determined for the conjugate synthesized under a molar ratio of 60 or 120 units of oligoCTA to amine on lysozyme (denoted by 60 and 120). The key conclusion of Figure 2 is that each lysozyme-oligoCTA conjugate retains activity with the larger *Micrococcus lysodeikticus* and the smaller \(((\text{NAG})_3\text{-MUF})\) substrates. The lower activity with the *Micrococcus lysodeikticus* substrate is presumably due to steric effects, which are more significant for the larger *Micrococcus* substrate than the small \(((\text{NAG})_3\text{-MUF})\) substrate.
Figure 2. Activity of native lysozyme compared to the lysozyme-acrylamide oligoCTA conjugates. The conjugates involving oligomers of length DP=5, 10, 20 conjugated with either a 60 or 120 molar excess of polymer to lysine on lysozyme are evaluated for Micrococcus lysodeikticus and small molecule Fluorescent assay.

Given the absence of native lysozyme in the conjugate based on the DP≈5 oligoCTA of Am, this conjugate was used for chain extension, with oligo(ethylene oxide) methyl ether acrylate of average molecular weight =480 (OEOA). The polymerization was conducted at 35 °C. The target degree of polymerization was 200 units and the final monomer conversion was over 90%. This chain extension is an attractive feature of the RAFT mechanism, and central to the combined grafting-from and grafting-to method.

Figure 3A shows results from polyacrylamide gel electrophoresis (PAGE) for the native lysozyme (well 2), lysozyme-oligoCTA conjugate (well 3), and the OEOA extended conjugate (well 4). Figure 3 clearly shows an increase in molecular weight from the native lysozyme to the lysozyme-oligoCTA conjugate, and an even larger increase in molecular weight after chain extension with OEOA. The macromonomer, OEOA, leads to a particularly large increase in the molecular weight of the conjugate as expected. A lower intensity lower molecular weight band could arise from lysozyme functionalized with only one oligoCTA. It is important to note that negligible native protein remains in all protein polymer conjugates.
Figure 3. A) PAGE data for various conjugates and modified lysozyme structures. Well 1 is a series of standards, Well 2 is native lysozyme, Well 3 is the lysozyme-oligoCTA (DP=5) conjugate, and Well 4 is the lysozyme-oligoCTA conjugate chain extended with OEOA. B) Plot of relative activities for native lysozyme, DP5 lysozyme-oligoCTA and OEOA chain extended lysozyme conjugate against the fluorescent (NAG)$_3$-MUF or *M. lysodeikticus* substrates.

Finally, the activity of the chain extended conjugate was assessed using both the *Microccocus* and ((NAG)$_3$-MUF) substrates. As shown in Figure 3B, the chain extended conjugate showed negligible activity with the *Microccocus* substrate, however, the enzyme retained 25% activity with the small fluorescent ((NAG)$_3$-MUF) substrate. This indicates that steric effects are preventing lysozyme interaction with the larger *Microccocus* substrate since the enzyme retained activity with the smaller fluorescent substrate.

II.3. Conclusions

This paper outlines a new approach to the synthesis of protein-polymer conjugates, where a short hydrophilic oligoCTA was first grafted-to lysozyme followed by grafting-from the protein with a second monomer. This approach combines the advantages of grafting-from the biomolecule such as simple purification and a relatively high graft density even with moderately low concentrations of (macro)CTA or (macro)initiator during the conjugation step, with the advantage of grafting-to, such as characterization of the conjugate, improved solubility and stability of the initially formed conjugate. The activity of the lysozyme enzyme was assessed at each stage, and it was shown that the
protein retains its enzymatic activity after each stage. Since the outlined strategy results in negligible quantities of unmodified protein, and does not lead to precipitation of the CTA or initiator-protein conjugate, this is an attractive and useful strategy that combines the strengths of grafting-from and grafting-to a protein.

II.4. References

CHAPTER III – Why Synthesize Protein-Polymer Conjugates? The Stability and Activity of Chymotrypsin-Polymer BioConjugates Synthesized by RAFT**

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III.1. Introduction

The ability to precisely synthesize a macromolecule with controlled functionality and molecular architecture for a given application is an ongoing target in the field of polymer chemistry. One area where this synthetic ability is particularly important is in the preparation of bioconjugates, or materials that combine a biologically relevant molecule with a synthetic compound. Bioconjugates are an emerging class of materials that offer the benefits of activity and function in biological applications with the flexibility of chemical functionality and structure possible through synthetic chemistry. A particularly interesting group of bioconjugates are protein-polymer hybrids, wherein the conjugated polymer can provide a synthetic handle to modulate the performance of the biomaterial. The polymer attached to the protein can serve multiple roles including, stabilizing the protein-polymer conjugate, shifting the optimal pH and temperature for the enzyme, leading to responsive or “smart” biomaterials.

The synthesis of bioconjugates, including protein-polymer conjugates, typically involves one of two strategies, the “grafting-to” and the “grafting-from” approaches. In grafting-to, a polymer is first synthesized, and subsequently attached to the protein, or other biomolecule, using an efficient organic reaction. In contrast, the grafting-from approach first attaches a small molecule initiator or chain transfer agent (CTA) to the protein, or biomolecule of interest, and then directly grows the polymer from the protein in an aqueous solution. The advantages of grafting-from include simple purification, and in many cases a higher grafting-density. However, the difficulties with grafting-from include potential loss of protein stability upon attaching the initiator or CTA, and choosing reaction conditions that preserve protein stability while giving well controlled polymers. In contrast, grafting-to offers the advantages of simple synthesis and characterization of the polymer and protein before conjugation, and that the polymerization conditions do not affect protein stability. The disadvantages of grafting-to include difficulty achieving high graft density, particularly with high molecular weight polymers, and difficulty purifying the polymer from the conjugate after synthesis. A representation of the grafting-from and grafting-to strategies is given in Scheme 1.
Scheme 1. Top shows a grafting-from strategy for synthesizing a protein-polymer bioconjugate, and bottom shows a grafting-to strategy for bioconjugate synthesis.

Reversible deactivation radical polymerization (RDRP) methods have revolutionized the fields of polymer chemistry and material science.\textsuperscript{15} Nitroxide mediated polymerization (NMP),\textsuperscript{16} atom transfer radical polymerization (ATRP),\textsuperscript{17,18} and reversible addition-fragmentation chain transfer polymerization (RAFT),\textsuperscript{19} are the three most commonly used RDRP methods. Each of these three RDRP methods has been used to create well controlled protein-polymer conjugates.\textsuperscript{10,20-29} RDRP methods are particularly well suited to protein-polymer conjugate synthesis since both the grafting-from and grafting-to methods can be used to create well defined biohybrids.\textsuperscript{1} This manuscript focuses on RAFT polymerization, as a tool to synthesize well defined protein polymer conjugates. RAFT is well suited to the synthesis of bioconjugates,\textsuperscript{30,31} including protein polymer conjugates,\textsuperscript{8,12,23,25,32,33} since it creates living polymers from a wide variety of functional groups, and offers excellent control over short chains.\textsuperscript{34,35}

This paper uses $\alpha$-chymotrypsin as the enzyme to be conjugated with synthetic polymers made by RAFT. Chymotrypsin is a protease, an enzyme that digests other proteins, including other $\alpha$-chymotrypsin molecules (autolysis), by catalyzing peptide bond hydrolysis.\textsuperscript{36,37} Due to promiscuous activities, conjugation with synthetic polymers can dramatically improve the stability and useful lifetime of proteases such as trypsin and chymotrypsin.\textsuperscript{7,38-40} Although chymotrypsin polymer bioconjugates have been synthesized by ATRP,\textsuperscript{7,20} to the best of our
knowledge there are no examples of chymotrypsin-polymer conjugates with the polymer synthesized by RAFT.

**III.2. Results and Discussion**

In this paper RAFT polymerization was used to synthesize polymers containing a single carboxylic acid group, from the R group of the CTA. RAFT was used to synthesize the polymers of N,N-dimethylacrylamide (DMAm) and oligo(ethylene oxide)methyl ether acrylate (OEOA) of average molecular weight = 480. Polymers with number average molecular weight below ~5000 were chosen since the short chain facilitates grafting-to processes. Subsequently, each polymer was conjugated to free amine groups on chymotrypsin to create amide bonds through an *in-situ*-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling strategy. Subsequently the activity and stability of these bioconjugates was determined and compared to that of the unmodified chymotrypsin. A summary of this approach is given in Scheme 2.
Scheme 2. Synthesis of pDMAm and pOEOA chains containing a single carboxylic acid by RAFT polymerization, followed by the subsequent conjugation of the oligomers to the protein through EDC/NHS coupling.
III.2.1. Synthesis and Characterization of Polymers made by RAFT.

In this approach, three polymers were synthesized, and subsequently attached to chymotrypsin. 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC) was used as the chain transfer agent. RAFT polymerization was used to create the poly(DMAm) (pDMAm) and the poly(OEOA) (pOEOA) based chains, using AIBN (0.2 mole equivalents to CTA) as the initiator, at 63 °C, with methanol being the solvent. The temperature of 63 °C was chosen to be below methanol’s boiling point of 64.65 °C, and at 63 °C AIBN has a half life of approximately 12.4 h. The three polymers synthesized are labeled pDMAm-low MW for a polymer with a target of 10 repeat units of DMAm giving a targeted molecular weight of ~1200, pDMAm-high MW for a polymer with a target of 48 repeat units of DMAm giving a targeted molecular weight of ~5000, and pOEOA for a polymer with a target of 10 repeat units of OEOA giving a targeted molecular weight of ~5000. In all cases the monomer conversion after 24h of reaction time was over 95%, the limit of NMR measurement.

As indicated in Figure 1, the pDMAm-low MW oligomer was very well controlled, with a narrow molecular weight distribution, centered around 9 repeat units of DMAm, as measured by electrospray ionization mass-spectrometry (ESI-MS). This is in good agreement with the targeted degree of polymerization of 10 units. For the pDMAm-high MW, matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), was used to characterize the polymer. As shown in Figure S1(a), the pDMAm-high MW polymer gave a well-defined peak centered at a molecular weight of 4500. This corresponds to an average degree of polymerization of approximately 43 units, in acceptable agreement with the targeted degree of polymerization of 48 units.Similarly, MALDI-MS for the pOEOAoligoCTA, shown in Figure S1(b), identified a molecular weight distribution centered around 10 repeat units of OEOA.

Each of the MS data sets were fitted with Gaussian functions to determine the number averaged molecular weight (M_n), corresponding number averaged degree of polymerization (DP_n), weight averaged molecular weight (M_w), and the dispersity (M_w/M_n). These values are given in Table 1 and the raw MS data and fitted Gaussian functions are shown in Figure S2.
III.2.2. Conjugation of the OligoCTA to Chymotrypsin

Once synthesized and characterized, all polymers were conjugated to chymotrypsin to produce well-defined protein polymer conjugates. Polymer conjugation may afford hybrids that avoid autodigestion of chymotrypsin. The DMAm and the OEOA based polymers were conjugated to free amine residues on chymotrypsin using an in-situ EDC/NHS coupling strategy. This is possible since the pDMAm and pOEOA based polymers contain a single carboxylic acid group from the R group of the CTA, which is a useful reactive handle for EDC/NHS coupling. A 30 to 60-fold excess of polymer to amine groups (both terminal amine and lysine residues) was used.

Successful grafting of the pDMAm-low MW oligomer to the protein is shown in Figure 2. Figure 2 A shows the MALDI-MS data for the unmodified chymotrypsin, which has a single sharp peak at 25600 m/z, which agrees well with the molecular weight of chymotrypsin. There are no other major or broad peaks in the native chymotrypsin sample. Figure 2B gives the MALDI-MS data for the chymotrypsin-pDMAm-low MW conjugate. As seen in Figure 2B,
there is complete modification of the native chymotrypsin by the pDMAm oligomer, as indicated by a shift of the MALDI-MS spectrum to a peak centered at approximately 30000 m/z. This corresponds to an average of 4 pDMAm oligomers attached per chymotrypsin molecule. MALDI-MS is used for the analysis, since it directly shows the molecular weight of the singly charged complex. Although sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) could be used to characterize these complexes, we have not done so here as SDS-PAGE measures apparent molecular weights which can be skewed by differential interactions of protein-conjugated polymers with the polyacrylamide gel matrix relative to those interactions for protein-based molecular weight standards lacking conjugated polymers. Figure S3 gives the aqueous size exclusion chromatography data for native chymotrypsin, and the chymotrypsin-pDMAm-low MW conjugate. Although the SEC data in Figure S3 indicate a broader distribution than that obtained from the MALDI-MS, the SEC data and the MALDI-MS data both indicate efficient conjugation of the pDMAm-low MW oligomer to the protein, with the MALDI-MS indicating negligible unmodified protein. Similarly Figure S4a indicates by SEC the coelution of the pDMAm-low MW based oligomer containing the RAFT end group at 309 nm, and the protein at 280 nm. This suggests conjugation of the polymer and the protein, and negligible free polymer since the unconjugated would elute at lower molecular weight.

Figure 2. A. MALDI-MS data for native chymotrypsin. B. MALDI-MS data for the chymotrypsin-pDMAm conjugate, indicating a clear shift towards higher molecular weight.
A similar EDC/NHS conjugation protocol was used to attach the pDMAM-high MW and the pOEOA polymers to chymotrypsin. Figure S5 shows the MALDI-MS data for the chymotrypsin-pDMAm-high MW conjugate. The chymotrypsin-pDMAm-high MW conjugate had significantly poorer signal to noise than chymotrypsin-pDMAm-low MW conjugate, however, no native protein and the peaks corresponding to 1, 2 and 3 attachments could clearly be seen in the MALDI-MS data. Unlike the pDMAm based conjugates, ionization efforts with a library of MALDI-MS matrices proved to be a challenge for the pOEOA based conjugate and failed to produce enough ions to obtain any discernable signal. However, as indicated in Figure 3 aqueous SEC clearly indicated successful conjugation between chymotrypsin and the pOEOA oligomers. There was a significant shift of the molecular weight distribution to higher molecular weight, after chymotrypsin was conjugated with the pOEOA based oligomers. Additionally the SEC data indicate only a small amount of native chymotrypsin, indicating conjugation between the polymer and the protein. Similarly Figure S4b indicates by SEC the coelution of the pOEOA based CTA at 309 nm, and the protein at 280 nm. This indicates conjugation of the polymer and the protein and negligible free polymer since the unconjugated would elute at lower molecular weight.
Figure 3. Aqueous SEC molecular weight distributions for native chymotrypsin and chymotrypsin conjugated with the pOEOA oligomer.

III.2.3. Enzymatic Activity and Stability of the Protein-Polymer Conjugates

Finally, the enzymatic activity of the chymotrypsin-polymer conjugates was assessed, and the stability was determined by measuring the activity as a function of time. Since chymotrypsin is a protease, the enzymatic activity of chymotrypsin can decrease over time, due to proteolytic digestion of one chymotrypsin by a second chymotrypsin molecule. However, conjugating a polymer to chymotrypsin offers the potential to stabilize the enzyme by decreasing the rate of proteolytic degradation while maintaining enzymatic activity against smaller substrates. To determine the activity and stability of chymotrypsin and the chymotrypsin-polymer conjugates, a colorimetric N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA) hydrolysis assay was used. The activity of the native chymotrypsin was compared to the chymotrypsin-pOEOA and the chymotrypsin-pDMAm-low MW and pDMAm-high MW conjugates in Figure 4A. As indicated in Figure 4A, the pOEOA based conjugate had essentially the same activity as the native chymotrypsin, while the pDMAm-low MW based conjugate had approximately 2/3 the activity of the native chymotrypsin, and the pDMAm-high MW based
conjugate had approximately 1/3 the activity of the native chymotrypsin all measured against the Suc-AAPF-pNA substrate. The decrease in activity for the pDMAm based bioconjugate is most likely due to the functional group potentially interfering with the active site, steric effects, or a less efficient stabilization of chymotrypsin against enzymatic degradation. To discriminate between these effects, the normalized activity of each conjugate was measured as a function of time, as displayed in Figure 4B and 4C.

Figure 4B displays the activity of native chymotrypsin, and both the pDMAm based conjugates measured at several time points over a 2.5 h period. The native enzyme lost almost 90% of its activity over the 2.5 h measurement. This is likely due to the digestion of one chymotrypsin molecule by a second chymotrypsin. Similarly the chymotrypsin-pDMAM-low MW conjugate exhibited a near identical loss of enzymatic activity over time. In contrast, the chymotrypsin-pDMAM-high MW conjugate showed improved stability over time. The pDMAM-high MW conjugate lost 50% of its original activity as a function of time, compared to a loss of 80-90% activity for the native protein and the pDMAM-low MW based conjugate. This indicates that higher molecular weight polymers allow for better stabilization of the protein against digestion by a protease.

Finally, Figure 4C compares the enzymatic activity of the native enzyme, the pDMAm-high MW conjugate and the pOEOA conjugate as a function of time. As shown in Figure 4C the pOEOA conjugate is significantly better stabilized against autolytic digestion than the pDMAm-high MW conjugate. In both cases the average molecular weight of the attached polymer is approximately 5000, and the difference in stability suggests that the OEOA based polymer is better at stabilizing the enzyme than the DMAm based polymer.
Figure 4. A. Activity of native, pOEOA-conjugated and pDMAm-conjugated chymotrypsin for hydrolyzing the N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate. B. Stability of the native, pDMAm-low MW conjugated and pDMAm-high MW conjugated chymotrypsin as a function of time, measured from the activity as a function of time for hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Data points represent the average of three independent trials ± standard deviation (dashed lines). C. Stability of the native, pOEOA-conjugated and pDMAm-high MW
conjugated chymotrypsin as a function of time, measured from the activity as a function of time
for hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. Data points represent the average
of three independent trials ± standard deviation (dashed lines).

**III.3. Conclusions**

RAFT polymerization was used as a tool to synthesize well-defined protein-polymer
conjugates. Two monomers, N,N-dimethylacrylamide (DMAm) and oligo(ethylene oxide)methyl
ether acrylate (OEOA) of average molecular weight = 480 were used to create narrowly
distributed polymers containing one carboxylic acid group per chain. The carboxylic acid group
was used as a reactive handle, to conjugate the polymer to amine groups on chymotrypsin using
EDC/NHS coupling. Well-defined conjugates were synthesized, with negligible unmodified
protein. Finally, the activity of chymotrypsin was determined using Suc-AAPF-pNA. The results
of these activity assays indicate that the pOEOA conjugate has essentially the same activity as
the native enzyme, while a third of the activity is lost in the low molecular weight pDMAm
conjugate, and two thirds of the activity were lost in the high molecular weight pDMAm
conjugate. Additionally, the pOEOA conjugate is stable and shows negligible loss of activity
over time. In contrast, the high molecular weight DMAm conjugate displayed a relatively low
loss of stability over time, while the native enzyme and the pDMAm low molecular weight
conjugate show significant losses of activity over time, with a half-life of approximately 20 min.
These results indicate that in certain cases, such as the pOEOA based conjugate, the presence of
the polymer can significantly improve the stability of the enzyme without compromising the
enzymatic activity.

**III.4. References**

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CHAPTER IV – Lipoic Acid Ligase-Mediated Chain Transfer Agent Attachment for Site-Specific RAFT Polymerization from Proteins

Rebecca Falatach
IV.1. Introduction

Recent interest in the protein-polymer materials has led to the development of new approaches for their synthesis [1][2][3]. Preliminary work has demonstrated that polymer attachment can affect enzyme activity [4], pH and temperature stability [5][6][7], resistance to proteolytic degradation [8], and increased circulation time by changing the type of polymer and sites of attachment [9]. There are two key aspects to consider when designing these systems; the method of attaching the polymers to the proteins and the strategy for creating the polymer. The most popular methods for attaching a polymer to a protein is by EDC/NHS coupling targeting the terminal amine and lysine residues and maleimide chemistry targeting cysteine residues. These methods have been widely used for creating protein-polymer systems, however they can create heterogeneous populations due to variations in number and sites of attachments, depending on the prevalence and reactivity of the targeted amino acid [10]. The site of attachment can affect enzyme activity and protein folding if the targeted amino acid is close to the active site or if it is a site that is critical to protein folding or protein stability [4][8][11][12].

For this reason, many site specific methods of attaching polymers to proteins have been in development [13][14]. One method of site specifically attaching polymers or small molecules to a protein is by the subtraction or addition of natural amino acids such as lysine and cysteine residues in desired locations [15]. Then EDC/NHS coupling or maleimide chemistry can be utilized. However, adding or subtracting one of these natural amino acids in a new location can cause misfolding or a change in solubility of the protein. Another method developed involves the addition of non-canonical amino acids to the protein’s backbone [16][17][18]. These non-canonical amino acids can be synthesized to include almost any functional group, typically one that is not naturally found in the protein’s structure. This allows any subsequent reaction to be site specific since it will only react with the unique functional group added in a precise location [9]. However, adding an unnatural amino acid can cause perturbations in the protein’s structure and function, as well as low expression levels of the modified protein, especially those with multiple insertion sites [11][19]. Also, the azide on the unnatural amino acid can become reduced to an amine if exposed to light for a long period of time, removing its ability to react with the alkyne [20]. This makes protein production and purification slightly more difficult.

Another approach used for site specific modification of proteins is through enzyme-mediated ligation reactions [21]. There are many different enzyme ligation systems that can be
modified to site specifically attach a wide variety of functional groups to a protein. Ting et al. developed a site specific enzyme-mediated method of attaching an azide molecule to a protein, through the modification of lipoic acid ligase. In this approach, lipoic acid ligase has been modified to accept an alkyl azide acid as a substrate, which it will attach to the lysine residue contained in the 13 amino acid sequence called the LAP (Lipoic Acid Ligase Acceptor Peptide) sequence. From the azide acid, azide-alkyne cycloaddition reactions can be performed to attach a polymer or small molecule with an alkyne end group. The advantage to this method is nearly unaffected protein expression levels, even with multiple sites in one protein, demonstrated site selectivity, and the high efficiency of the following click reaction [19][22].

The next aspect to consider with protein-polymer systems is the method of polymer synthesis. The most commonly used method is a grafting-to method which involves growing a polymer in solution and subsequently attaching it to a protein, such as PEGylation [11]. Issues with this strategy include steric hindrances associated with attaching a large molecular weight polymer and purification issues associated with separating polymers of similar molecular weight to the protein-polymer conjugate. To overcome these issues, grafting-from methods have been developed to attach an initiator molecule to a protein, then grow a polymer directly from the protein-initiator conjugate (macroinitiator) [3][23]. The benefit to using a grafting-from method is the simpler purification since only small monomers need to separated, as well as the ability to more efficiently create large protein-polymer conjugates due to the reduction in steric hindrance associated with trying to conjugate to high molecular weight polymers [6][24]. A possible issue with the grafting-from approach is the decrease in water solubility of the protein-initiator molecule associated with the attachment of a somewhat hydrophobic initiator. This can be overcome by using an initiator molecule with a short, water soluble oligomer attached [4]. The most common polymerization method used for grafting-from is controlled radical polymerization (CRP) techniques such as atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT) polymerization. RAFT polymerization is a promising approach because it can be done in protein friendly conditions [24], does not require the use of a metal catalyst, and is compatible with a range of different monomers [25].

The utility of the lipoic acid ligase-mediated approach of site specific attachment combined with grafting-from RAFT polymerization to create well defined protein-polymer conjugates was demonstrated using green fluorescent protein (GFP) as the model protein. The
LAP sequence was inserted into 3 different sites on three different GFP mutants to show the effect of attaching a polymer in different locations on the same protein. Lipoic acid ligase was then used to attach 10-azidodecanoic acid to these mutants, from which an oligomer RAFT chain transfer agent (CTA) containing an alkyne end group was clicked to the azide using a copper-catalyzed azide-alkyne cycloaddition reaction. From this GFP-oligomer CTA conjugate, different sizes and types of polymers were grown using grafting-from RAFT polymerization.

Scheme 1. Scheme of ligation, click and chain extension of LAP containing GFP mutants.
IV.2. Materials and Methods

IV.2.1. Materials

Tryptone, yeast extract, sodium chloride, and ampicillin used for making LB media were purchased from Fisher Scientific. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology. Adenosine 5'-triphosphate (ATP), copper sulfate, Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), aminoguanadinehydrochloride, (+)-sodium L-ascorbate, propargyl alcohol, 4-(dimethylamino)pyridine (DMAP), 2,2'-azobis(2-methylpropionitrile) (AIBN), and 10-bromodecanoic acid were obtained from Sigma Aldrich. VA-044 was obtained from Wako chemicals. Imidazole, sodium azide, and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were purchased from Fisher Scientific. All materials were used without further purification. Dialysis in each step was performed using Fisher brand regenerated cellulose bags with a molecular cutoff weight of 12-14 kDa.

IV.2.2. Protein Expression and Purification

All GFP constructs were expressed in BL21 DE3 cells [19]. Initially, 10 mL overnight cultures were used to inoculate 500 mL of LB media and grown at 37°C and 200 rpm. Once the cells reached an OD of between 0.4-0.6, they were induced with 1 mM IPTG. After 3 hours of induction, the cells were centrifuged at 5000 rpm for 15 minutes. The media was poured off and the cell pellet was resuspended in 50mM Tris (pH 8), 250 mMNaCl, 5 mM imidazole, with the addition of 1 mg/ml lysozyme and subsequently kept in a -80°C freezer until purification. The cells were then thawed at room temperature and lysed by sonicating at 30% duty for 8 minutes on and 10 minutes off. After 2 cycles, the cells were then centrifuged at 13,000 rpm for 15 minutes. The supernatant was filtered through a 0.45 µm syringe filter and loaded onto a 5 mL GE HisTrap chromatography cartridge. Protein containing N-terminal polyhistadine tag was eluted in lysis buffer containing 500 mM imidazole and subsequently dialyzed into 20 mM sodium phosphate pH 7.
IV.2.3. Synthesis of 10-Azidodecanoic Acid

To synthesize the 10-azidodecanoic acid, 1 g (3.98 mmol) of bromodecanoic acid was added to 10 ml of N,N-dimethylformamide (DMF). To this, 0.5 g (7.69 mmol) of sodium azide was added and allowed to stir at room temperature overnight. The reaction was monitored by thin layer chromatography (TLC) using 1:2 hexanes:ethyl acetate. The solvent was then removed under vacuum, then 15ml of 1M HCl was added. This was then washed 3 times with 15ml of ethyl acetate. The organic layer was dried over sodium sulfate and the solvent was removed under vacuum. The 10-azidodecanoic acid was then separated by a silica gel column using a solvent gradient of hexane and ethyl acetate. The identity of the acid was confirmed by infrared spectroscopy (IR) and nuclear magnetic resonance (NMR). \[^1\text{H NMR (CDCl}_3\text{): 3.23ppm (t, 2H), 2.32ppm (t, 2H), 1.57ppm (m, 5H), 1.31ppm (m, 9H). IR: 1706.32 cm}^{-1}\text{ and 2093.28 cm}^{-1}\].

IV.2.4. Synthesis of Macro-RAFT Chain Transfer Agent

The alkyne CTA (prop-2-ynyl propanoate)yl ethyl trithiocarbonate (PYPETC)) [26] was synthesized from 2-(((ethylthio)-carbonothioyl)thio) propanoic acid (PAETC) [4]. First PAETC (0.909g, 4.34 mmoles) was added to 50 mL of dichloromethane. To that propargyl alcohol (1.21g, 21.58 mmoles), EDC (1.65g, 1063 mmoles), and DMAP (0.5521g, 4.52 mmoles) were added and allowed to stir for 2 hours on ice, then overnight at room temperature. This crude product was first washed 4 times with 70 mL of 0.2M HCl. It was then washed once with 70 mL of DI water, 3 times with 50 mL of 0.5M sodium carbonate, and once more with 70 mL of DI water. The resulting product was dried under vacuum and the purity was determined using proton NMR.

From the purified alkyne CTA, acrylamide oligomers of 5, 10 and 20 monomer units were synthesized using RAFT polymerization. This is done by adding alkyne CTA (0.104g, 0.4mmoles), acrylamide (DP5 0.15g, DP10 0.3g and DP20 0.6g), and AIBN (6.9mg, 0.0422mmoles) to 80/20 methanol/water (DP5 0.6mL, DP10 0.93mL, and DP20 1.6mL). The reaction was stirred overnight at 65°C. Monomer conversion was determined to be >90% by \[^1\text{H NMR}.\] The oligomer alkyne CTA was the purified by precipitation into THF and hexanes. Entire reaction mixture was added dropwise to 50 mL THF with 20 mL hexanes. The resulting solid was gravity filtered and dried under vacuum. The average molecular weight and molecular weight distribution were determined using ESI-MS.
IV.2.5. Ligation of 10-Azidodecanoic Acid

The ligation of 10-azidodecanoic acid to LAP-containing GFP constructs was performed by incubating 0.1 μM LplA, 10 μM GFP, 600 μM 10-azidodecanoic acid, 2 mM ATP, and 2 mM MgCl₂ in 25 mM sodium phosphate at pH 7.2 at 30°C for 4 hours (Joe’s paper and Yao’s paper). The LplA was expressed in BL21 DE3 cells and purified protein was dialyzed in 20mM Tris, 0.01% β-mercaptoethanol, and 10% glycerol at pH 7.5. Purified LplA was then kept at -80°C until needed. These reactions were then dialyzed at 4°C overnight using Fisherbrand regenerated cellulose dialysis bags with a molecular cutoff weight of 12-14 kDa in 50 mM sodium phosphate buffer at pH 7.

IV.2.6. Copper-Catalyzed Attachment of Oligomer Chain Transfer Agent

The macro-CTA was attached to the 10-azidodecanoic acid through a copper-catalyzed azide-alkyne cycloaddition [19]. Initially, stock solutions of aminoguanadine hydrochloride (100 mM), sodium ascorbate (100 mM), macro-CTA (20 mM), CuSO₄ (20 mM), and THPTA (50 mM) were prepared in 50 mM sodium phosphate at pH7. A mixture of CuSO₄ and THPTA was prepared by combining 1 part CuSO₄ to 2 parts THPTA prior to addition to the reaction. Then for a 1 mL reaction volume, 865 μL of 10 μM ligated GFP was incubated with 15 μL of the CuSO₄ and THPTA mixture, 50 μL of aminoguanidine hydrochloride, 20 μL of the macro-CTA, and lastly 50 μL of sodium ascorbate for 4 hours at 37°C. This reaction was then dialyzed in 20 mM Sodium Phosphate at pH 7 and concentrated to 10 mg/ml using an ultrafiltration stirred cell dialysis unit with a 10 kDa molecular cutoff weight cellulose membrane.

IV.2.7. Grafting-from RAFT Polymerization

Grafting-from RAFT polymerization was performed by adding VA-044 (1.03mg, 1.6 μmol) and monomer (for DP50 acrylamide: 2.27mg, 31.9μmoles) to 1 mL of 15mg/ml macroCTA-GFP in 20 mM sodium phosphate at pH 7 in a 10 mL schlenk flask. This solution was degassed with nitrogen for 15 minutes and heated to 40°C for 6 hours. ¹H NMR was used to determine the monomer conversion.
IV.3. Results and Discussion

IV.3.1. Synthesis of Oligomer Alkyne CTA

First the alkyne CTA (PYPETC) was synthesized according to Scheme 2. The crude product was extracted and dried under vacuum, yielding a yellow oil.

Scheme 2. Scheme for synthesis of the alkyne CTA.

The purity of the product was determined using proton NMR. $^1$H-NMR (300MHz, CDCl$_3$) δ ppm, 2.509 (1H, t, H-C≡C), 4.749 (2H, d, C≡C-CH$_2$-O), 4.835 (1H, q, C(O)-C(S)H-CH$_3$), 1.630 (3H, d, C(S)H-CH$_3$), 3.353 (2H, q, S-CH$_2$-CH$_3$), 1.353 (3H, t, S-CH$_2$-CH$_3$).
Figure 1. The NMR of the purified alkyne CTA in chloroform-d.

Any peaks not associated with the product are a result of the NMR solvent, DCM, and water. No starting reactants were seen in the NMR spectrum suggesting that the alkyne CTA was successfully purified. Once purity was confirmed, the alkyne CTA was used to synthesize DP5, DP10, and DP20 acrylamide oligomers using RAFT polymerization. Electrospray ionization mass spectrometry (ESI-MS) was performed on each sample, shown in Figure 2.
Figure 2. ESI MS of DP5, DP10 and DP20 Oligomer Alkyne CTA.
Each ESI-MS spectrum was fit to a Gaussian distribution to determine the average degree of polymerization and the dispersity of the oligomers. The calculated averages and dispersity of all three oligomers are shown in Table 1.

**Table 1. Summary of ESI data of Oligomer Alkyne CTA.**

<table>
<thead>
<tr>
<th>Average DP</th>
<th>( \frac{M_w}{M_n} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP 5 Alkyne CTA</td>
<td>4.5</td>
</tr>
<tr>
<td>DP 10 Alkyne CTA</td>
<td>9.6</td>
</tr>
<tr>
<td>DP 20 Alkyne CTA</td>
<td>21.9</td>
</tr>
</tbody>
</table>

Each of the average degree of polymerizations are similar to the expected value and the dispersities are all less than 1.2, which displays very good control over the acrylamide polymer synthesized.

**IV.3.2. Site-Specific Attachment of Oligomer Alkyne CTA**

A large batch of 172 GFP, a mutant with a single LAP insertion between E172 and D173, was purified. Then 10-azidodecanoic acid was ligated to the 172 GFP using purified LplA, to which all three oligomers were clicked. MALDI was used to confirm that the ligation (shown in Figure 3) and click reactions (MALDI not shown here) were successful in attaching a single oligomer alkyne CTA.
Figure 3. MALDI of native and ligated single LAP insertion GFP.

This MALDI-MS shows a shift of approximately 195 Da, which corresponds to the molecular weight of the 10-azidodecanoic acid minus a water that is lost in the reaction. An SDS PAGE was also performed to confirm the attachment of the DP20 alkyne CTA, shown in Figure 4.

Figure 4. SDS PAGE of 172 GFP at each step of modification. Lane 1: Protein standard, Lane 2: Native 172 GFP, Lane 3: Ligated 172 GFP, Lane 4: DP20 clicked 172 GFP.
Both methods confirmed that one oligomer alkyne CTA was attached in each case. The shift in molecular weight on the SDS PAGE and the smeared appearance of the DP20 clicked GFP lane suggests that an oligomer has been attached since the oligomer MW has a distribution. The band appearing at the higher molecular weight corresponds to a dimer, possibly due to the aggregation of the clicked 172 GFP after concentration to 15 mg/ml.

A fluorescence spectrum of native, ligated, and clicked 172 GFP. The samples were excited at 450nm and the emission spectrum was collected from 480 to 600nm using a Biotek Synergy H1 micro-plate reader. As seen in Figure 5, the three spectrums follow a similar shape suggesting that the structure of the 172 GFP was not perturbed by the attachment of the 10-azidodecanoic acid and DP20 alkyne CTA.

![Fluorescence emission spectrum of native, ligated and clicked 172 GFP.](image)

**Figure 5.** Fluorescence emission spectrum of native, ligated and clicked 172 GFP.
IV.3.3. Chain Extension of Oligomer Alkyne CTA with Acrylamide

A grafting-from RAFT polymerization was performed from the DP20 alkyne CTA on the 172 GFP. As a result of concentrating the clicked GFP and subsequently heating it at 40°C, a portion of the protein became insoluble in the pH 7 phosphate buffer. The insolubility could be due to either heating the protein for a long period of time or because the pH of the buffer is close to the pI of GFP. The chain extension of DP50 and DP100 acrylamide had a monomer conversion of less than 80% and the DP200 did not proceed at all.

To help improve the solubility of the clicked 172 GFP, it was buffer exchanged with a 20mM Tris buffer at pH 8 with 500 mM imidazole. The polymerization reaction was run again with the same conditions except the reaction time was shortened to 6 hours. The solubility of the protein was improved, however the polymerization did not proceed. This could be due to the increase in pH. Increasing the pH from 7 to 8 could have resulted in the VA-044 initiator becoming deprotonated, preventing it from initiating the polymerization reaction at 40°C.

From here, a DP200 alkyne CTA was synthesized and clicked to all three single LAP insertion mutants, N GFP, 157 GFP, and a new batch of 172 GFP, using the method outlined above. According to SDS PAGE (not shown here), the click reaction did not proceed efficiently and left a large portion of all the constructs unmodified. This is most likely due to the steric hindrances associated with attaching a large polymer to the protein. The click reaction was run at a polymer to protein molar ratio of 10 to 1. This ratio could be increased to ensure the modification of all the protein in solution.

IV.4. References


CHAPTER V – Conclusions and Future Work

Rebecca Falatach
**IV.1. Conclusions**

The goal of this research was to produce protein-polymer conjugates using random (uncontrolled) modification and site-specific techniques. The random method of attaching a RAFT chain transfer agent and subsequently grafting a polymer from it was demonstrated using lysozyme and chymotrypsin. With lysozyme the disadvantage of decreased water solubility of the CTA modified conjugate was found when using the grafting-from approach. To address this issue, a short, water soluble oligomer of acrylamide was synthesized and subsequently attached to lysozyme in an added grafting-to step. This grafting-to step did decrease the efficiency of the EDC/NHS coupling reaction but not significantly since the oligomer was relatively small at a MW of about 600 Da, including the CTA. This did however increase the water solubility of the conjugated lysozyme. Once a soluble lysozyme conjugate was synthesized, the grafting-from step was successfully demonstrated by chain extension with OEOA. Our results showed the catalytic activity of lysozyme decreased with each step of modification. It was observed that the reduction in activity decreased more rapidly with a larger substrate, suggesting that the decrease in catalytic activity was due to steric hindrance of the active site. This led to a side study which revealed that one of the lysine residues modified by the EDC/NHS reaction was adjacent to an active site residue.

This decrease in enzyme activity with random modification yielded a need for an effective site-specific method of modification. The LAP/lipoic acid ligase system was demonstrated using green fluorescent protein (GFP). Three GFP constructs were created each with one LAP sequence engineered at different locations on the protein surface. Lipoic acid ligase was then used to site-specifically attach 10-azidodecanoic acid, from which an oligomer alkyne CTA could be attached through copper-catalyzed azide-alkyne cycloaddition. Although the site-specific modification of GFP was successful, it was found that GFP was insoluble at the high concentrations that are necessary for efficient chain extension by RAFT.

**IV.2. Future Work**

Future work is necessary to effectively synthesize site-specific protein polymer conjugates using RAFT. Optimization of conditions is needed to improve the efficiency of chain extension from the oligomer alkyne CTA attached to GFP. One approach would involve identifying buffer conditions that would improve GFP solubility allowing polymerizations to be
performed at a higher protein concentration. Another approach would be to find an alternative method to better initiate the RAFT polymerization at the lower protein concentrations. Also, extending these approaches to multiple LAP containing GFP constructs would demonstrate the flexibility of this approach for site-specific modification. Once these site specific protein-polymer conjugates are successfully produced, a study of stability against thermal, chemical, and/or protease degradation of each of the modified constructs would be of interest.

In addition, alternative methods for protein-polymer conjugation should be explored to find conditions that are compatible with a wider variety of proteins. Although the conditions used in this study were relatively protein-friendly, they still included reactions that are run at 30-40°C for anywhere between 6 and 24 hours. Many of the model proteins used for research in this area remain stable under these conditions; however, many proteins of therapeutic and industrial interest are more thermo-sensitive. This challenge currently limits the utility of the grafting-from approach to more thermally stable proteins.
APPENDIX-Supplemental Information for CHAPTER II

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A.1. **Experimental**

**Materials.** Hen Egg White Lysozyme was purchased from MP Biomedicals. Acrylamide and hydrochloric acid were obtained from Fisher Scientific. Micrococcus lysodeikticus, 4-methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside, azobisisobutyronitrile (AIBN), ethanethiol, carbon disulfide, 2-bromopropionic acid, and oligo(ethylene oxide) methyl ether acrylate (OEOA) were purchased from Sigma Aldrich. Deuterated DMSO was obtained from Acros Organics. N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Thermo Scientific. Sodium hydroxide was obtained from Ricca chemicals, and sodium carbonate was obtained from Tedia. 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.

**1H Nuclear Magnetic Resonance.** $^1$H NMR (300 MHz) was recorded on Bruker 300 MHz NMR spectrometer equipped with a broadband inverse probe. A standard pulse-acquire sequence was used with a 30° tip angle ($\pi/2$).

**Matrix Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS).** MALDI-TOF spectra were collected on a Bruker Autoflex III MALDI-TOF mass spectrometer (Billerica, MA). Mass spectra were calibrated using Bruker Protein Calibration Standard I (spanning 5.7 kD to 16.9 kD) as an external standard. Generally, 0.5 µL of sample (0.5 mg/ml) was mixed with 0.5 µL of saturated sinapinic acid (Fluka, WI) solution (0.1 % TFA, 40% acetonitrile) directly on the target plate and allowed to dry at room temperature. Samples were analysed in the positive ion linear mode to detect [M + H]$^+$ ions.

**Electrospray Ionization-Mass Spectrometry (ESI-MS).** Direct injection ESI-MS data was collected on a Bruker Esquire-LC mass spectrometer (Billerica, MA) operating in negative ion mode. Samples were injected at a rate of 7 µL / minute, with a nebulization gas pressure of 7 psi and a heated nitrogen drying gas flow rate of 4 L / minute at a temperature of 300 °C. The capillary voltage was set to 4000 V, and ions were allowed to accumulate in the trap for 10
ms. The nominal target mass was set to 750 m/z. 40 scans, spanning 50 – 2200 m/z, were collected in order to obtain sufficient signal-to-noise.

**Synthesis of 2-(((ethylthio)carbonothioyl)thio)propanoic acid (PAETC).**

Sodium hydroxide (20.5g, 0.51 mol) was dissolved in 200 mL of water. Ethanethiol (42 mL, 36.1 g, 0.58mol) was added dropwise, and allowed to react for 20 min, after which point carbon disulfide (40 mL, 61.6g, 0.81 mol) was added dropwise over approximately 10 min. The reaction mixture was stirred at room temperature for 40 min, affording a dark orange solution. The aqueous solution was washed with 4 x 100 mL of dichloromethane, and the solvent was removed to afford an orange solid of sodium ethyltrithiocarbonate. To a clean round bottom flask sodium ethyltrithiocarbonate (48g, 0.30 mol) was added. To this flask acetone (150 mL) was added followed by the dropwise addition of 2-bromopropionic acid (43 g, 0.30 mol). The reaction mixture was allowed to stir overnight giving a yellow solution. The solid of sodium bromide was removed by vacuum filtration, and the solvent removed under reduced pressure. The product was redisolved in acetone and a white product was recrystallized from the yellow solution. The acetone was removed under reduced pressure, and finally the product was purified by dissolving the product in diethyl ether (150 mL), and washed with saturated sodium carbonate (150 mL). The ether layer was discarded and the aqueous layer was washed with 4 x 100 mL of diethyl ether. A final layer (100 mL) of ether was added, and the aqueous layer was acidified with concentrated hydrochloric acid. The ether layer was washed once with water (50 mL) and the ether layer was collected. The solvent was removed under reduced pressure to afford 34 g (53% isolated yield) 2-(((ethylthio)carbonothioyl)thio)propanoic acid or (propionylacid)ylethyltrithiocarbonate (PAETC). The purity was confirmed by NMR and was found to be greater than 97%. $^1$H-NMR(300 MHz, CDCl$_3$) δ ppm1.63 (3H, d, $J = 7.4$ Hz, CH$_3$CH(S)COOH) 4.87 (1H, q, $J = 7.4$ Hz, CH$_3$CH(S)COOH), 3.38 (2H, q, $J = 7.4$ Hz, CH$_3$CH$_2$S), 1.36 (3H, t, $J = 7.4$ Hz, CH$_3$CH$_2$S).

**Conjugation of PAETC to Lysozyme.** For NHS/EDC coupling reactions, lysozyme, CTA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysulfosuccinimide (sulfo-NHS) were combined in 0.1M phosphate buffer at pH 7.4 in a flask and allowed to react at room temperature for 2hrs before being quenched by glycine. Lysozyme was always added at a final
concentration of 1mg/ml, sulfo-NHS was always kept at a final concentration 5mM, and EDC was added at a molar ratio of 1 EDC to 1 CTA. A sample reaction is as follows: Lysozyme (5mg, 0.35μmol), CTA (25.73mg, 0.122mmol), EDC (23.46mg, 0.122mmol), and NHS (5.429mg, 25μmol) were added to the reaction vial using stock solutions all prepared in the reaction buffer. The molar ratio of CTA to amines on the lysozyme (6 lysine residues plus 1 terminal amine) was varied to determine optimal conditions. Higher ratios of CTA to amine resulted in higher modification as determined by MALDI, although the solubility was lower. This conjugated protein was washed with 20mM MES buffer at pH 6 using an Amicon stirred ultrafiltration cell with a 10kDa MWCO membrane to remove excess CTA, EDC, and NHS until the absorbance at 309nm of the solution inside the cell held constant.

UV-Vis Spectroscopy. A SpectronicGenesys 5 spectrophotometer was used to collect UV-Vis spectrums. UV-Vis spectroscopy was used to determine the approximate molar ratio of CTA attached to lysozyme. A calibration curve was developed to determine that the extinction coefficient of the PAETC at 309nm is 11700 M⁻¹cm⁻¹. Once the conjugated lysozyme was washed using the stirred ultrafiltration cell the absorbance spectrum was recorded and the concentration of the protein determined by the peak at 280nm was compared to the concentration of the CTA determined by the peak at 309nm to get an approximate ratio of CTA to lysozyme.

Lysozyme Activity Assays. Two different sized substrates were used to determine the activity of the protein at each stage of modification. The large substrate was Micrococcus lysodeikticus, a common bacteria used to test the activity of lysozyme. The reaction between lysozyme and this bacteria’s cell wall can be monitored by the change in absorbance at 450nm over time. The reaction was done in a 66mM KH₂PO₄ buffer at pH 6.2. This assay was done using a BioTekSynergy HT plate reader and used the kinetic feature to measure the absorbance every 15 seconds over a 4 minute time period. The slope of the line produced from graphing absorbance vs time represented the rate of reaction and when compared to the rate of reaction of a native lysozyme control the percent activity retention could be determined. The smaller substrate used was 4-methylumbelliferyl β-D-N,N’,N”-triacetylchitotrioside ((NAG)₃-MUF), which when hydrolysed by the lysozyme produces a fluorescent product. This reaction was monitored using the fluorescence function on the same plate reader using an excitation/emission setting of
360/460. This reaction took place in a 50mM Acetate buffer at a pH of 5.5, but the fluorescent product fluoresces at a higher pH so the reaction was stopped using glycine buffer at a pH of 10.8 before measuring the fluorescence. This assay also requires time point measurements but the reaction takes place over an hour, so time points were taken at time 0, 30 minutes and 60 minutes. In between measurements, the plate was kept at 37°C and in a dark place to reduce exposure of the substrate to light.

**RAFT polymerization of Acrylamide with CTA.** To synthesize the oligoCTA, RAFT polymerization was used to grow a short oligomer of acrylamide from the CTA in a 50:50 mixture of methanol and water. Dimethylformamide (DMF) was added at 4% of the volume strictly for use in conversion determination using $^1$H NMR. Molar ratio for CTA to AIBN was 1 to 0.1. The molar ratio of CTA to Am was adjusted based on desired length of oligomer (i.e. DP=5 had a molar ratio of CTA to Am of 1 to 5). The reaction conditions used to produce the DP=5 oligoCTA used in the OEOA chain extension was as follows: CTA (1.99g, 9.2mmol), acrylamide (3.389g, 47.6mmol), AIBN (0.156g, 0.95mmol) and 0.5ml of DMF were added to a round bottom flask with 12.6ml of the methanol/water mixture. This reaction was purged with nitrogen for 5 minutes to remove oxygen, then placed in an oil bath at 65°C for 21 hrs. The conversion was determined using $^1$H NMR. These oligoCTAs were precipitated by adding the reaction mixture drop-wise to tetrahydrofuran (THF) that was cooled in an ice bath. The precipitated oligoCTA was vacuum filtered and rinsed into a pre-weighed round bottom flask. The solvent was then removed under reduced pressure. ESI-MS was used to determine the average molecular weight of the oligoCTA and the distribution of oligomer lengths and $^1$H NMR in d-DMSO was used to determine the monomer conversion.

**Conjugation of oligoCTA to lysozyme.** The conjugation of the oligoCTA to lysozyme was done using the same NHS/EDC coupling method as previously mentioned. However, the efficiency greatly decreased with an increase in the oligomer chain length so the molar ratio of oligoCTA to amine was significantly increased to overcome this. The conditions used to conjugate the DP=5 oligoCTA to lysozyme for use in the chain extension reaction are as follows: Lysozyme (100mg, 7μmol), oligoCTA (3.3g, 5.87mmol), EDC (1.126g, 5.87mmol), and NHS (57.5mg, 0.5mmol) were added to the reaction flask at a final volume of 100ml of the 0.1M
phosphate buffer at pH 7.4. These conjugates were purified by dialysis using an Amicon stirred ultrafiltration cell dialysis unit and were washed with 20mM MES buffer at pH 6 until the absorbance at 309nm of the solution inside the cell held constant.

**RAFT polymerization of OEOA from oligoCTA-Lysozyme conjugate.** OEOA, oligoCTA-Lysozyme conjugate, and VA-044 were combined in a 5 ml round bottom flask. The conjugated lysozyme, in the 20mM MES buffer at pH6, was concentrated to 10 mg/ml. OEOA (45.25μl, 49.318mg, 102.75μmol) and VA-044 (0.831mg, 2.569μmol) were added to 400μl of the concentrated oligoCTA-lysozyme conjugate. This solution was degased for 5 minutes with nitrogen and sealed to remove all oxygen. It was then heated in an oil bath set at 35°C for 21 hours. $^1$H NMR was used to determine that the monomer conversion went to over 90%. The electrophoresis gel shown in Figure 3 shows that very little conjugated lysozyme was left unmodified.

**SDS-PAGE for Native, oligoCTA- and OEOA-Lysozyme conjugates.** Samples containing 7.8 μg each of native, oligoCTA- and OEOA-Lysozyme conjugate samples were mixed with 4x LDS loading buffer containing 50mM DTT. Samples were incubated at 70°C for 10 minutes prior to loading on a TGX 4-20% gradient polyacrylamide gel. Protein bands stained with GelCode Blue protein stain were visualized by fluorescence at 700nm using a LI-COR Odyssey Fc imager.

**A1.2. Additional Figures and Characterization Data**

**Characterization of the CTA-lysozyme conjugate.**
Figure S1. Protein-CTA conjugate precipitated out of solution due to high modification and therefore a loss of solubility. Conjugation performed under the conditions: Lysozyme (5mg, 0.35μmol), CTA (25.73mg, 0.122mmol), EDC (23.46mg, 0.122mmol), and NHS (5.429mg, 25μmol), with Lysozyme at 1 mg/mL.

The ratio of the peak at 309 nm to the peak at 280 nm was used to approximate the average number of CTA attachments on the lysozyme. These ratios were then compared to the MALDI-MS spectrums. Figure S2 shows the absorbance spectrum of native lysozyme compared to the CTA-lysozyme conjugate shown in Figure S1. The reduced peak at 280nm shows that the precipitate in Figure S1 is protein. The shoulder at 309nm is due to the CTA from which the ratio of CTA to lysozyme was determined to be about 2, which represents an average of 2 CTA attachments per lysozyme. This method, however, could not be used for the oligoCTAs because the broad peak at 309nm obscures the protein peak at 280nm making it difficult to determine an accurate protein concentration.
**Figure S2.** Absorbance spectrum of lysozyme-CTA conjugate shown in Figure S1 compared to native lysozyme.

**Characterization of the oligomers that are grafted to lysozyme.**

Figure S3 shows the ESI-MS results for oligoCTAs of chain lengths DP=10 and DP=20 the DP=5 oligomer in Figure 1A. Table S1 contains theoretical and experimentally determined molecular weights for these three oligoCTAs.
Figure S3. ESI-MS data for A the DP = 10 oligoCTA and B the DP = 20 oligoCTA.

Table S1. Estimated average degree of polymerization and dispersity for the DP = 5, 10 and 20 oligoCTA, as determined by fitting a Gaussian distribution to the ESI-MS data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Degree of Polymerization (from ESI-MS)$^a$</th>
<th>Estimated Dispersity (from ESI-MS)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP 5 Am oligoCTA</td>
<td>7.9</td>
<td>1.07</td>
</tr>
<tr>
<td>DP 10 Am oligoCTA</td>
<td>11.4</td>
<td>1.07</td>
</tr>
<tr>
<td>DP 20 Am oligoCTA</td>
<td>18.7</td>
<td>1.08</td>
</tr>
</tbody>
</table>

$^a$ Estimated as the mean of a Gaussian distribution fitted to the ESI-MS data.

$^b$ Estimated as $1+\mu^2/\sigma^2$, where $\mu$ is the mean and $\sigma$ is the standard deviation of a Gaussian distribution fitted to the ESI-MS data.

Additional Characterization of Protein Polymer Conjugates.
The MALDI-MS of each of the oligoCTA-lysozyme conjugates that were produced using a ratio of 120 oligoCTA per amine are shown in Figure S4

![MALDI-MS graphs](image)

**Figure S4.** MALDI-MS data for lysozyme conjugated with either the DP=5, 10 or 20 oligomer with a ratio of macroCTA to amine on lysozyme of 120:1 using insitu NHS/EDC coupling.

To determine the average number of oligoCTAs per lysozyme, the theoretical molecular weight of an oligoCTA-lysozyme conjugate with one, two, and three attachments was determined and then compared to the MALDI-MS spectrum. For example, a lysozyme with one attachment would have an approximate theoretical MW of 14,300Da (lysozyme) + 210Da (CTA) + 7.9*71Da (acrylamide with DP=5 from ESI) = 15071Da. This is repeated for two, three, or even four attachments then peaks at those approximate molecular weights are marked. To determine an approximate average number of attachments, the relative intensities at each of those peaks are used to find a weighted average.