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Exploration of Thiol-Michael Addition Bioconjugation to Extend Polymers of a Protein-Based Hydrogel

Elaine Wallin Smith

University of Mississippi, ewsmith3@go.olemiss.edu

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EXPLORATION OF THIOL-MICHAEL ADDITION BIOCONJUGATION TO EXTEND POLYMERS OF A PROTEIN-BASED HYDROGEL

by
Elaine Wallin Smith

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2019

Approved by

____________________________
Advisor: Dr. Susan Pedigo

____________________________
Reader: Dr. Davita Watkins

____________________________
Reader: Dr. Nathan Hammer
DEDICATION

This thesis is dedicated to all of the professors who have challenged me, my family and friends who have encouraged and supported me, and the peers who have grown alongside me.
ACKNOWLEDGEMENTS

I received tremendous help from Christopher Fox, a graduate student; Dr. Susan Pedigo, my research advisor; and enjoyed my collaboration with the other students in Dr. Pedigo’s lab.

I would like to thank the Sally McDonnell Barksdale Honors College for providing me funds in the amount of $1,000 in order to purchase supplies for the expression and purification of our protein constructs.

Additionally, I thank the Department of Chemistry and Biochemistry at the University of Mississippi for granting me access to their equipment, facilities, and resources.

Finally, I thank Dr. Davita Watkins and Dr. Nathan Hammer for serving on my committee, providing important insight, and pushing my research farther.
ABSTRACT

ELAINE WALLIN SMITH: Exploration of Thiol-Michael Addition Bioconjugation to Extend Polymers of a Protein-Based Hydrogel
(Under the direction of Dr. Susan Pedigo)

Hydrogels have been explored for many biomedical applications, including targeted, in situ drug delivery to avoid the negative side effects associated with systemic delivery. In our work, we are exploiting the high affinity, calcium-dependent binding between calmodulin and its target peptides to create a biomaterial for in situ, extracellular drug delivery. Genes were engineered to make two protomers, Calmodulin Collagen-Like Protein (CCLP) and Peptide Collagen-Like Protein (PCLP), that will spontaneously self-assemble in situ due to the high Ca\(^{2+}\) concentration in the extracellular space and provide tunable, targeted drug delivery in a biocompatible hydrogel. One important factor that would dictate the potential utility and application of this hydrogel is the geometry of the protomers involved. Longer polymers have higher levels of entanglement and thus form gels with greater integrity. We used Thiol-Michael addition chemistry to bioconjugate our protomers with polyethylene glycol (PEG) crosslinkers in order to fine-tune the physical properties of the resultant hydrogel, such as elasticity and viscosity. We took advantage of the base-catalyzed Thiol-Michael addition mechanism and performed reactions between the cysteine residues of our genetically-engineered protomers and both divinyl sulfone and maleimide-based PEG crosslinking reagents. We studied the extent of bioconjugation and the effects of factors including concentration of reducing agent and
denaturant, temperature, identity of the Michael acceptor, and the ratio of crosslinker to protein. Results were assayed by gel electrophoresis. We found that maleimide-PEG reagents are far more reactive than those based on divinyl sulfone, and at a lower pH. Additionally, bioconjugation is most promoted by the presence of 1 mM TCEP (reducing agent). Temperature and urea (denaturant) do not have significant effects on the reaction. The three constructs we tested were all modifiable, but we see particular potential in our CCLP Bis-Cysteine construct because it is capable of multiple bioconjugation reactions due to its bifunctional nature. The chemistry described here can be used to fine-tune the physical properties of the hydrogels formed by our protomers for a wide array of applications.
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LIST OF ABBREVIATIONS

AU..............................................................................................................Absorbance units
CaM..............................................................................................................Calmodulin
Ca^{2+}........................................................................................................Calcium ion
CCLP............................................................................................................Calmodulin Collagen-Like Protein
CLS.................................................................................................................Collagen Linker Sequence
Collagenase.............................................................................................Matrix Metalloproteinase
Cys................................................................................................................Cysteine
DNA..........................................................................................................Deoxyribonucleic acid
EC.................................................................................................................Extracellular
Eppie.............................................................................................................Microfuge tube
E. coli.........................................................................................................Escherichia coli
g....................................................................................................................Grams
GI..................................................................................................................Gastro-intestinal
HEPES.....................................................................................................4-2-Hydroxyethyl-1-piperazineethanesulfonic acid
His-Tag.....................................................................................................Polyhistidine tag
hrs.................................................................................................................Hours
IPTG...........................................................................................................Isopropyl β-D-1-thiogalactopyranoside
Kan..............................................................................................................Kanamycin
KCl..............................................................................................................Potassium chloride
kDa..............................................................................................................Kilodaltons
L..............................................................Liter
LB..............................................................Lysogeny broth
M..............................................................Molar
mg............................................................Milligrams
mins..........................................................Minutes
mL............................................................Milliliter
MLCK.........................................................Myosin Light Chain Kinase
mM............................................................Millimolar
mm............................................................Millimeter
M13.........................................................Residue 577-602 of Myosin Light Chain Kinase
NaCl.........................................................Sodium chloride
NaN₃..........................................................Sodium azide
nM............................................................Nanomolar
NSAIDs..................................................Nonsteroidal Anti-inflammatory Drugs
PCLP.........................................................Peptide Collagen-Like Protein
pH..............................................................Potential Hydrogen
pKa..........................................................Acid dissociation constant
RNA........................................................Ribonucleic acid
RPM.........................................................Revolutions per minute
SDS-PAGE..........................Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC .............................................................. Size exclusion chromatography
Tris/HCl ........................................................ Trishydroxymethylaminomethane hydrochloride
UV ........................................................................................................................ Ultra-violet
3D ........................................................................................................................ Three-dimensional
°C ..................................................................................................................... Degrees Celsius
µg ........................................................................................................................ Micrograms
µL ........................................................................................................................ Microliters
CHAPTER 1

INTRODUCTION

Currently, significant effort is being devoted to specific targeting of pharmaceutics to affected tissues or physiological systems. One approach is in situ drug delivery in which a drug depot is embedded in specific tissues to allow delivery at the intended site rather than systemic distribution characteristic of oral or intravenous delivery. In situ drug delivery systems are advantageous because they limit pleiotropic effects and release the drug only where it is needed most. In situ drug delivery is enabled by a number of techniques including subcutaneous slow compression hypodermics which require surgery to implant, and depending on the site of the drug reservoir, surgery to refill. The work described herein is directed toward an injectable, protein-based gel that would be resident at the site where it is needed without associated mechanical devices or surgical intervention. The protein components will be covalently modified by drug such that it delivers drug in situ directly at the site where it is needed.

Hydrogels are composed of three-dimensional polymeric networks that hold large quantities of water. Many over-the-counter products are hydrogels intended for external use such as cosmetics and topical ointment and lotions.¹ The use of hydrogels for in situ drug delivery has been well established and in active use for approximately 15 years, and are traditionally composed of synthetic components such as polyethylene glycol (PEG). Protein- and carbohydrate-based hydrogels are of interest because natural components increase their biocompatibility, degradation, and excretion. Such “natural” hydrogels
have been explored for some time in a wide range of in vivo uses including seeding cell
growth in artificial tissues\(^2\) and dendrimer formation for drug delivery systems for
treatment of inflammation.\(^3\) Our project focuses on a novel hydrogel that is based on the
specific interaction between the protein calmodulin and a particular peptide that it binds
with high affinity in vivo. Since both protein components are natural proteins found in
humans, we expect our engineered proteins will be biocompatible as well. Their
degradation and excretion should occur through normal physiological mechanisms.
Further, we have designed the components of our system to provide the flexibility of
forming a protein-only hydrogel or a hybrid hydrogel composed of protein and synthetic
components. A specific feature of this calmodulin-peptide hydrogel is that calcium in the
extracellular space in vivo will promote the association of the calmodulin- and peptide-
based components into a physical hydrogel, which will serve as a drug depot. The gel can
be injected at the site of inflammation to provide a depot of drug to be released by
hydrolysis reactions in a targeted way. The following section describes the general
structure of our novel hydrogel, and introduction into inflammation and the drugs we
intend to deliver in situ.

I. Inflammation and Common Anti-inflammatory Drugs

Inflammation is a physiological reaction to injury and infection. It is a normal and
important action that engages cellular machinery to allow tissue repair and destruction of
pathogens. Because of the ubiquity of inflammatory response, we take over-the-counter
and prescription drugs to lessen the discomfort of its symptoms. The front line drugs are
nonsteroidal anti-inflammatory drugs (NSAIDs). They are inexpensive, readily available,
and well-studied. Below we will describe chronic inflammation, the disease form of the inflammatory response, and the pleiotropic effects of NSAIDs that in situ drug delivery systems are developed to avoid.

Acute inflammation is part of the body’s natural defense mechanism to address injury or infection. Pain, redness, and swelling of affected tissues are all symptoms of inflammation; mechanisms to resolve injury and infection include recruitment of neutrophils or other granulocytes to affected tissue, and then phagocytosis of damaged tissue or infectious agents. For our purposes, the two major factors that are the hallmark of inflamed tissues are the secretion of proteases and the acidification of tissue. In acute inflammatory events, acidification recruits the granulocytes, and then the secretion of proteases follows, causing the remodeling of the EC collagen network so that normal tissue can be rebuilt.

Chronic inflammation is a disease state that is due to the inability of the inflammation machinery to resolve the underlying problem. Chronic inflammation is a relevant condition to many of us since it is related to osteo- and rheumatoid arthritis, atherosclerosis, cancer, inflammatory bowel disease, endometriosis, and acute conditions such as the formation of surgical adhesions.

NSAIDs are a class of drugs primarily used for pain relief and to treat ubiquitous acute and chronic inflammation. Because of their relative safety and long history of efficacy, they are the most commonly purchased over-the-counter drug worldwide. Their general mechanism is to work as inhibitors of cyclooxygenases, critical enzymes that are necessary to synthesize prostaglandins, thromboxanes, and leukotrienes, paracrine hormones that function in specific tissues and under specific physiological
For our purposes, prostaglandins are the main concern. In the inflammatory response, they are produced by the damaged tissue and cause local swelling, acidification and secretion of collagenases, enzymes that remodel the EC collagen network. As such, NSAIDs are critical for reducing the pain and tissue remodeling effects of inflammation in order to reduce the impact of chronic conditions. NSAIDs are delivered almost exclusively orally, and are, therefore, distributed throughout the tissues systemically. Since they are absorbed in the intestinal tract, they have particularly devastating side effects on the mucosal linings in the stomach and intestine, sites where normal prostaglandin synthesis ensures the natural regeneration of the mucosal layers to prevent damage in the harsh environment of our digestive system. The destructive effects in the digestive system lining and the massive dosages required because of systemic distribution of these efficacious drugs means that inflammation and its unpleasant side effects are inadequately addressed by oral administration of the drug.

Clearly, inflammation is an important issue to be addressed, and NSAIDs would be more potent and less harmful if they were delivered in a targeted way. We envision our hydrogel will be injected at the site of inflammation. For example, it could be placed in the abdominal cavity to prevent the adhesions that form due to inflammation in conditions such as endometriosis, or after surgery. Other applications could include pain and inflammation relief due to constrictions or compressions of spinal nerves or cartilage damage in synovial joints. Further, targeted, in situ drug delivery systems are particularly important when the drug in question is toxic in general circulation, or has low solubility in the blood. These factors motivate our interest in developing the calmodulin-peptide hydrogel under study here.
Our plan is to modify the hydroxyl and/or amino groups on the protomers in the hydrogel with an NSAID via hydrolysable ester linkages to create a responsive, targeted drug delivery system. The responsive nature of this hydrogel is based on a variation in pH; these ester linkages are increasingly hydrolysable at lower pHs. Some sources have speculated that inflammation causes a decrease in tissue pH, which in turn makes the ester linkages more subject to hydrolysis, meaning they are cleaved at a faster rate and more drug is released to combat the increase in inflammation. Therefore, this hydrogel system is responsive to bodily conditions, providing targeted and tunable treatment of inflammation. The affixing of the drug analogue will be done via a propargylation reaction followed by a Huisgen 1,3-dipolar cycloaddition. The resulting modification will be evaluated through mass spectrometry.

II. Description of the Calmodulin-Peptide Hydrogel

The motivation for using the calmodulin-peptide interaction as the basis for the assembly of the hydrogel is threefold. First, as mentioned above, the protein components are present in human physiology, and so we would expect that they would not elicit an immune response, as this would be counter-productive to the effect of the drug. Using a “natural” hydrogel, such as this one, reduces the risk of adverse reaction because it is broken down through naturally occurring enzymes, MMP-1, -8, and -13. Second, calmodulin and peptide interact with very high affinity and with high specificity at 1 mM calcium levels, the normal extracellular concentration in vivo. As such, the environment in which the hydrogel is injected will have the necessary calcium to stabilize its structure and allow for spontaneous self-assembly in situ. Finally, protein-based drug delivery
systems provide precise genetic control over the chemistry of the constructs. For example, we altered the genetic sequence of each construct to control factors like the rate of hydrolysis and the reactivity of amino acid residues in bioconjugation reactions.

In this section, first I will describe the salient features of calmodulin and the peptide. Second, I will describe how these components are situated into an engineered protein context that contains design elements necessary for the assembly of the hydrogel, and how they assemble into a hydrogel. Finally, I will show that hydrogel formation is a reality.

A. Calmodulin and Peptide

Calmodulin is a very important intracellular regulatory protein that has been extensively studied as recorded in the literature. A quick keyword search of PubMed shows 4900 articles on calmodulin in the last 5 years. Its protein chemistry and physiological roles are well studied, providing a deep literature base for its use in protein engineering. This protein was a clear choice for this biomaterial because it reversibly denatures and has high affinity binding with its peptide binding partners. It is soluble from a dried powder. Its function is unperturbed by treatment in boiling water. This remarkable resiliency simplifies purification and, we hypothesize, will allow it to retain function after repeated exchanges between aqueous and organic solvents. There is ample literature that would direct mutations to modify calcium binding affinity and peptide binding affinity, possibilities that may be important in the future to broaden the properties of the hydrogel to suit a diverse range of purposes. Our hydrogel uses the protein sequence for wild type vertebrate calmodulin.
A ribbon drawing of the crystal structure of calmodulin is depicted in Figure 1.1 below. There are 148 amino acids in calmodulin, divided into two distinct globular domains separated by a central “helix” segment. The pairs of calcium binding sites in each domain are in the EF-hand family (helix-loop-helix). The “loop” contains the amino acids responsible for calcium binding (yellow) regions. At the back of each pair of sites is a short region of β-sheet. The $K_d$ for calcium binding to calmodulin is ~1 µM in the C-terminal sites and ~10 µM in the N-terminal sites, well below the 1 mM level found in the EC-space in vivo.\(^{19}\)

The peptide component of our hydrogel is M13, a 26 amino acid peptide derived from skeletal muscle myosin light chain kinase. Its sequence is very basic (KRRWKKNFIAVSAANRFKKISSGAL; 5 lysines (K) and 2 arginines (R), and no acidic residues) with hydrophobic components tryptophan (W, position 4) and a FIAVSA segment that is strikingly extended and hydrophobic compared to the rest of the sequence. It binds calmodulin in the presence of Ca\(^{2+}\) with an affinity < 10 nM and a 1:1 stoichiometric ratio.\(^{25}\) This high affinity in the presence of calcium is useful because the

\(\text{Figure 1.1. Ribbon and cartoon drawings of calmodulin. A) Ribbon drawing of calmodulin.}^{23}\) The N-domain (Sites 1 and 2; blue) and the C-domain (Sites 3 and 4; red) are separated by the central helix. The middle of the central helix will lose its helical character when it is in solution or in equilibrium with binding peptides. The calcium binding sites are in yellow. B) Schematic that illustrates the exposure of hydrophobic surface area at the base of each domain (orange crescents) when calcium binds. C) Ribbon drawing of the crystal structure of calmodulin binding the M13 peptide (green helix).\(^{24}\) D) Schematic representation of calmodulin binding M13 peptide (green cylinder). (Pedigo Lab Archive)
Ca\textsuperscript{2+} intrinsically present in the extracellular space will be sufficient to promote hydrogel formation.

**B. Engineered Calmodulin and Peptide Protomers**

The general design of calmodulin and peptide protomers are shown in Table 1.1 below. CCLP stands for Calmodulin Collagen-Like Protein and PCLP is Peptide Collagen-Like Protein. The protomers have repeating units consisting of either calmodulin (CaM) or M13, and the collagen-like sequences. Preceding the first repeat in each construct is a short segment with a His-Tag sequence (His\textsubscript{6}) and a thrombin cleavage site. These two features at the N-terminus of the protomers allow us to purify the protomers with His-Tag Chromatography, and then to cleave off the N-terminal segment after His-Tag Chromatography is complete. A C-terminal cysteine residue is useful as a site of modification, or for crosslinking of protomers to create dimeric linear protomers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLP2</td>
<td>X B X B SH</td>
</tr>
<tr>
<td>PCLP2</td>
<td>X B X B SH</td>
</tr>
<tr>
<td>CCLP3</td>
<td>X B X B SH</td>
</tr>
<tr>
<td>PCLP3</td>
<td>X B X B SH</td>
</tr>
<tr>
<td>CCLP Bis-Cys</td>
<td>HS X B X B SH</td>
</tr>
</tbody>
</table>
In the Table 1.1 above, the yellow represents a His\textsubscript{6} sequence used in HisTag purification. The blue X is CaM, whereas the green X is M13. The blue B contains three collagen-like cleavable sequences, whereas the green B has only one collagen-like cleavable sequence. The SH represents the terminal cysteine residue.

Each construct is also engineered to contain collagen-like sequences (CLS) that are the sites of hydrolysis by collagenases, proteases that are specific for collagen. The collagen-like sequence is GPQG/\text{I}WGQ. The slash indicates the cleavage site. Optimizing these cleavage events is of active interest in the lab. My colleagues, Hunter Berry and Christopher Fox, are currently experimenting on the factors affecting the rate of hydrolysis of the protomers and the hydrogel by collagen-specific proteases.

CCLP2 and PCLP2 are so named because they have 2 repeating units, each consisting of their respective protein, CaM or M13, and their collagen-like sequences. We redesigned the gene to have 3 repeats instead of two, producing CCLP3 and PCLP3. Finally, we made an alternate design of CCLP3 with a cysteine residue on both the C and N termini, named CCLP Bis-Cys. The structures of each of these constructs is depicted above in Table 1.1, and the detailed properties are below, in Table 1.2.
<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular Weight (g/mol)</th>
<th>Extinction Coefficient (M⁻¹ cm⁻¹)</th>
<th>Relative solubility in water</th>
<th># of Active Sites</th>
<th>Residues</th>
<th>Isoelectric point</th>
<th>Estimated net charge at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLP2</td>
<td>46,216</td>
<td>46,940</td>
<td>good</td>
<td>2</td>
<td>423</td>
<td>pH 4.01</td>
<td>-44.1</td>
</tr>
<tr>
<td>CCLP3</td>
<td>58,675</td>
<td>32,430</td>
<td>requires salt</td>
<td>3</td>
<td>530</td>
<td>pH 3.94</td>
<td>-67</td>
</tr>
<tr>
<td>CCLP Bis-Cys</td>
<td>58,707</td>
<td>32,430</td>
<td>good</td>
<td>3</td>
<td>530</td>
<td>pH 3.94</td>
<td>-67.1</td>
</tr>
<tr>
<td>PCLP2</td>
<td>18,470</td>
<td>53,200</td>
<td>poor (denaturant required)</td>
<td>2</td>
<td>177</td>
<td>pH 11.54</td>
<td>19.6</td>
</tr>
<tr>
<td>PCLP3</td>
<td>19,307</td>
<td>35,420</td>
<td>poor (denaturant required)</td>
<td>3</td>
<td>179</td>
<td>pH 11.8</td>
<td>22.6</td>
</tr>
</tbody>
</table>

When engineering these new constructs, several interesting features have been added. The CCLP Bis-Cys has two cysteines. The second thiol increases the likelihood of linear polymerization of the construct. To optimize the reactivity of the sulfhydryl on the terminal cysteine, we included an arginine residue next to the terminal cysteine(s) of CCLP3 and CCLP Bis-Cys. A basic residue in this position has been shown to increase its reactivity in Thiol-Michael addition reactions, which will be discussed in part III of this chapter.²⁶

The mixture of the CCLP and PCLP protomers produces a gel. Figure 1.2 below shows the schematic of the interactions between the PCLP and CCLP protomers to form a noncovalent, interlinked gel matrix. Our initial protomers contained only two repeats, so the interaction options were few. As mentioned above, we have several iterations of
these constructs with different numbers of CLS sequences, calmodulin and M13 inserts, and cysteines. The purpose behind the experiments reported in this thesis is to change the properties of the gel by covalently linking the protomers together to increase the total number of repeats on each chain. The studies reported here are establishing the chemical protocols for these linkage reactions.

**Figure 1.2.** Hydrogel formation. (A) Schematic representing the association of CCLP and PCLP proteins to form a hydrogel in the presence of Ca\textsuperscript{2+}. (B) A photographic image of a gel formed from CCLP3 and PCLP3 (10% CCLP3-PCLP3; 10 mM Ca\textsuperscript{2+}).

III. Our Experiment

The primary objective of this project is to establish the framework for the development of an injectable, protein-based hydrogel that self-assembles *in situ* and provides targeted drug delivery of NSAIDs. The component protomers interact to form the hydrogel through purely non-covalent interactions. We primarily focused on the PCLP3 and CCLP3 protomers because they each have three interaction sites for gel formation, and they each have a single cysteine. The immediate goal of the experiments in this thesis is to create bioconjugated protein dimers by the use of bifunctional reagents including divinyl sulfone – polyethylene glycol (VS-PEG-VS) and maleimide – polyethylene glycol (Mal-PEG-Mal). With a Thiol-Michael addition between the
protomers and PEG reagents, we are able to double the protomer length and therefore double the number of reactive sites, theoretically affecting the physical properties of the resultant hydrogel, like elasticity and viscosity. Thus, we would expect that the use of PEG spacers between the protomers would affect the physical properties of the resultant hydrogel; judicious use of crosslinking agents can allow tailoring of gels to specific uses. The physical properties of gels dictate their potential applications. Hydrogels that can endure high levels of stress could be placed in a joint, whereas gels with relatively weak structural integrity could be used in soft tissues. These same physical properties are dictated by the length of the gel’s constituent protomers; longer polymers have higher levels of entanglement, which increases the gel’s integrity.

My focus was developing protocols for modifying our protomers through Thiol-Michael addition bioconjugation reactions, and determining which conditions best promote bioconjugation. To this end, we studied specific factors such as the identity of the Michael acceptor, reaction temperature, presence of reducing agent, solution conditions (pH and presence of denaturant), and the ratio of bifunctional PEG crosslinker to our protomers. While the terminal cysteine residues enable the formation of disulfide-linked dimers between our constructs, we prefer the bioconjugated dimers because they are “permanent” and not subject to reduction, so we maintain reducing conditions in order to lessen the formation of the disulfide protein dimer. Thiol-Michael addition reactions will be described in the following section.
A. Thiol-Michael Addition

“Click” reactions have been characterized as “high yielding, wide in scope,” rapid, specific, environmentally friendly, and efficient. They are also selective, straightforward to perform, create only easily removable byproducts, and occur under mild conditions. Thiol-Michael addition reactions fall under this broader “click” category. Michael addition reactions involve creating new chemical bonds between an “enolate-type nucleophile” reacting with an electron-deficient \( \alpha,\beta \)-unsaturated carbonyl. This reaction is robust, but also is associated with an enhanced level of control over the chemistry while avoiding formation of “significant side products.” Thus, we used Thiol-Michael addition click chemistry to crosslink our protomers.

The Thiol-Michael addition reaction is particularly useful because of the “inherent electron density of the S atom” which ensures its reactivity under mild conditions. Figure 1.3 below shows two mechanisms for the formation of the thiolate (Michael donor), and then the subsequent reaction with the Michael acceptor. The thiolate serves as a nucleophile (and Michael donor), attacking the electrophilic carbon-carbon double bond of the Michael acceptor. Cysteine is the only amino acid with a thiol group, thus affording selectivity over the position of the reaction site in biochemical reactions. In our case, we focused on two different Michael acceptors, vinyl sulfone and maleimide.
Figure 1.3. Two mechanisms for thiolate formation and the Thiol-Michael addition reaction. The reaction can proceed under either a base- or nucleophile-catalyzed mechanism. (A) Base-catalyzed thiolate formation. (B) Nucleophile-catalyzed thiolate formation. (C) Thiol-Michael addition mechanism. (Figure adapted from Nair, et al.\textsuperscript{27})

In both the base- and nucleophile- catalyzed mechanisms, the thiol of the terminal cysteine residue is deprotonated to become a thiolate anion. The thiolate then serves as a nucleophile, attacking the electrophilic carbon-carbon double bond of the Michael acceptor (vinyl sulfone or maleimide). This reaction forms a carbon-sulfur bond to form our Michael product, the modified protomer.

The thiolate anion produced is unstable and highly reactive, resulting in efficient reaction kinetics.\textsuperscript{27} The kinetics and yield of the Thiol-Michael addition reaction depend on the strength and concentration of the catalyst, the pK\textsubscript{a} of the thiol, the nature of the...
electron-withdrawing group on the Michael acceptor, steric hindrance, and the solvent polarity and pH. At physiological pH, thiols react at least 1 order of magnitude faster than amines, so thiolates are the predominant nucleophile and this reaction proceeds unimpeded by competition from other nucleophiles.

In our case, the Michael donor is the thiol of the protomer’s terminal cysteine residue. As demonstrated in Figure 1.4, the \( \text{pK}_a \) of this thiol is slightly above 8. Therefore, basic conditions seem intuitive in order to promote deprotonation of the cysteine thiol and form the nucleophilic thiolate, however the best reaction pH depends on the identity of the Michael acceptor, as detailed below. The thiolate is the strongest nucleophile present in the reaction, and it initiates the addition of the anion across the Michael acceptor’s electrophilic carbon-carbon double bond. Thiolates are typically considered the reactive species in this reaction, so the reaction rate is strongly dependent on the reaction pH and the thiol \( \text{pK}_a \). Generally, the reaction rate increases with pH because basic conditions result in the formation of more thiolate, the reactive species.

![Figure 1.4. \( \text{pK}_a \)s of various thiols. (Figure adapted from Nair, et al.)](image-url)
Michael acceptors have electron deficient carbon-carbon double bonds that often contain an electron withdrawing group. A variety of Michael acceptors and their relative reactivities are depicted in Figure 1.5 below. We initially experimented with various Michael acceptors, but we ultimately focused on vinyl sulfones and maleimides because of their promising chemistry.

![Diagram of Michael acceptors](image)

**Figure 1.5.** Relative reactivity of various Michael acceptors.
(Figure adapted from Nair, et al.27)

Due to the breadth of Michael acceptors explored in this work, I have compiled the structures of these reagents. Table 1.3 introduces the various chemicals discussed and/or utilized in our Michael addition reactions.
Table 1.3. Michael acceptors and polyethylene glycol reagents.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Structure</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divinyl sulfone (DVS)</td>
<td><img src="image" alt="Structure of Divinyl sulfone" /></td>
<td>118.15 g/mol</td>
</tr>
<tr>
<td>Methyl-vinyl sulfone (MVS)</td>
<td><img src="image" alt="Structure of Methyl-vinyl sulfone" /></td>
<td>106.14 g/mol</td>
</tr>
<tr>
<td>VS-PEG-VS</td>
<td><img src="image" alt="Structure of VS-PEG-VS" /></td>
<td>~3500 g/mol</td>
</tr>
<tr>
<td>Mal-PEG-Mal</td>
<td><img src="image" alt="Structure of Mal-PEG-Mal" /></td>
<td>~3500 g/mol</td>
</tr>
</tbody>
</table>

Vinyl sulfones are useful because they are more stable in aqueous and alkaline solutions as compared to maleimides. They react with thiols to form stable thioester linkages, reacting especially quickly in basic pH. The thioester sulfone bond formed by the Thiol-Michael addition with a vinyl sulfone is not easily hydrolysable, making it very useful for this type of application.

Maleimides are considered the most reactive Michael acceptor. The Maleimide carbon-carbon double bond is so reactive because of the associated ring strain and the cis-conformation of its carbonyl groups. They are more reactive at lower pHs, between 6.5-
7.5, which is remarkable because the Michael-addition mechanism involves the deprotonation of a cysteine thiol, which has a $pK_a \sim 8$, as seen in Figure 1.4. Maleimide is able to undergo this reaction without either catalyst if in a polar enough solvent. Maleimides undergo Thiol-Michael addition reactions to form succinimide bonds. This type of bond is more subject to hydrolysis than the vinyl sulfone thioester bond, but it is still widely used for this type of application because of its rapid kinetics and selectivity.

While these Michael acceptors can be reacted with our protomers by themselves, we used PEG (polyethylene glycol) to further lengthen the protomers and make the modification more visible via gel electrophoresis and mass spectrometry. Bioconjugation with PEG, or PEGylation, is a commonly used approach in the modification of peptides because of PEG’s hydrophilicity and low susceptibility to degradation by human enzymes. Therefore, PEGylation can “dramatically extend the stability and improve the pharmacology of biomolecule(s).” Due to PEG’s hydrophilicity, it can also increase the solubility of its binding partners, which we hope to use to increase the solubility of our PCLP construct. PEG can be toxic, however this usually only occurs with higher molecular weight PEG and at high doses.

The bifunctionality of these Michael acceptors is what makes them so useful to us; the reactive groups on either side mean that they can react with two separate protomers, which would serve to connect two protomers and thereby double the length, doubling the number of active sites and theoretically increasing the structural integrity of the resultant hydrogel.
Through bioconjugation with these compounds, various modified protomers are expected as products. Table 1.4 below shows some of the possible results of these Thiol-Michael addition reactions.

**Table 1.4. Schematic of possible bioconjugation products.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>MW g/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unreacted protomer (“X”)</td>
<td></td>
<td>Protein MW</td>
</tr>
<tr>
<td>Mono-modified protomer (“X-PEG”)</td>
<td></td>
<td>Protein MW +3500</td>
</tr>
<tr>
<td>Bioconjugated dimer (“X-PEG-X”)</td>
<td></td>
<td>2*(protein MW) + 3500</td>
</tr>
<tr>
<td>CCLP Bis-Cys with 2 PEGs</td>
<td></td>
<td>~65,700</td>
</tr>
<tr>
<td>CCLP Bis-Cys biocojugated dimer</td>
<td></td>
<td>~124,400</td>
</tr>
<tr>
<td>CCLP Bis-Cys biocojugated dimer with extra PEG</td>
<td></td>
<td>~127,900</td>
</tr>
<tr>
<td>Bioconjugated trimer</td>
<td></td>
<td>~186,600</td>
</tr>
</tbody>
</table>

The purple and blue boxes represent a protomer (CCLP, PCLP, or CCLP Bis-Cys). The yellow circle represents either VS or Mal, and the green star represents PEG. The first three (with the blue boxes) are possible with CCLP3, PCLP3, and CCLP Bis-Cys. The purple ones that follow only apply to CCLP Bis-Cys because it has two cysteines available to react with the PEG reagents. The last figure, “bioconjugated trimer,” illustrates that this bifunctionality can link three or more protomers, whereas the other constructs are limited to two.

The Bis-Cys construct is particularly intriguing because the cysteine residues on either side mean that it could theoretically form a hydrogel by itself. (This is useful
because of its simplicity and PCLP’s solubility issues.) Because of the cysteine residues on either end of the construct, and the two Michael acceptors on VS-PEG-VS and Mal-PEG-Mal, the reaction could theoretically continue indefinitely because no matter which of the two is terminal, the reaction could continue to propagate. Methyl vinyl sulfone (MVS) could be used as an end-cap to terminate this chain polymerization reaction.

Figure 1.6 below depicts the possibility for chain polymerization between CCLP Bis-Cys and VS-PEG-VS.

**Figure 1.6.** Chain polymerization with CCLP Bis-Cys and Michael acceptor. This figure illustrates the potential for chain polymerization between bifunctional CCLP Bis-Cys (blue helical structure) and either DVS or bifunctional Maleimide. The last step shows how this reaction could be terminated: through the addition of a unifunctional sulfone like Methyl-vinyl sulfone (MVS). Figure by Christopher Fox.

These modifications will be visualized through gel electrophoresis. This is another reason for the utilization of PEG: modification is more easily monitored when 3500 g/mol is added than it would be for vinyl sulfone or maleimide alone (~100 g/mol).
Chapter 2 of this thesis details the reaction conditions that enabled us to explore these chemistries.

To explore these promising reactions further, we designed a series of experiments that examined the impacts of the following variables on the reaction’s efficacy and the physical properties of the hydrogel: temperature, identity of Michael acceptor, and the presence or absence of TCEP, a reducing agent, and urea, a denaturant.

The reactivity of the thiol, in our case cysteine, is instrumental in dictating the reaction rate and product specificity. A “linear correlation” has been found between the concentration of thiolate and the rate of the reaction, confirming the thiolate’s role as the reactive species. Researchers also emphasize the connection between the concentration of thiolate and the rate of the reaction, stating that the thiolate concentration has the largest effect on the reaction rate. The concentration of thiolate depends on its pKₐ, which can be manipulated by altering its electrostatic environment. Positively charged residues nearby the cysteine have been found to decrease the thiol’s pKₐ and thus increase the rate of the Thiol-Michael addition reaction. For each positive charge, the pKₐ is decreased approximately 0.2 units. Negatively charged residues showed the opposite trend. This pKₐ shift promotes deprotonation of the thiol, and is often induced by “electrostatic interactions with neighboring inonizable amino acids and/or polar residues.” They found that the number of positive charges affects the reaction rate more than the charge’s position. This positive charge is thought to influence the transition energy and thus kinetics of the reaction.

Using this information, we modified the gene design of CCLP to include an arginine residue near the terminal cysteine(s) on CCLP3 and CCLP Bis-Cys. By
modifying our gene, we are partaking in rational peptide design to improve the kinetics and selectivity of our reaction. Therefore, we can also design an experiment comparing the reactivities of CCLP3 and CCLP2, with and without an arginine residue next to the cysteine, to compare the effects of this difference in thiol pKₐ.

My focus was on exploring factors that influence the extent of bioconjugation. Manipulating the reaction time and ratio of the various components would provide a range of products of varying lengths, viewable by gel electrophoresis and mass spectrometry. Once the conditions that best promote the formation of the bioconjugated dimer are uncovered, we will create a hydrogel with these bioconjugated protomers and use microrheological techniques to examine how its physical properties differ from a hydrogel composed of un-modified protomers. Ultimately, these varying lengths due to various extents of modification would produce hydrogels with differing physical properties, and thus, potentially different applications.
CHAPTER 2
MATERIALS AND METHODS

I. Gene Design

In order to create the hydrogel, we first had to design genes to synthesize CCLP and PCLP. We designed a gene with calmodulin or M13 peptide, respectively, collagen-like sequences for hydrolysis, and histidine residues to allow for purification via His-Tag Chromatography as described in the Introduction and Table 1.1 above. Recombinant plasmids were synthesized by Genscript. Genes were sequenced to confirm they were correct. Via transformation, we created both maintenance (DH5α) and expression (BL21(DE3)) cell lines, and froze them as glycerol-containing cell stocks. The plasmid has a kanamycin-resistance gene to select for bacteria that have been transformed.

II. Expression and Purification

Sterile LB Agar plates were prepared with 30 µg/mL kanamycin, then inoculated with CCLP or PCLP BL21(DE3) by streaking of the frozen cell stocks, and were grown overnight. An overnight liquid culture was then prepared by picking a single colony from the plate and inoculating a 50 mL liquid LB culture with kanamycin added. A 5 mL volume of this culture was used to inoculate a 1 L liquid culture with 50 mL 1 M potassium phosphate at pH 7.4 with kanamycin added. After growing cells to midlog stage at 37°C, expression of the protein was induced via the addition of 2 mL of 0.4 M IPTG. CCLP continues at 37°C, while PCLP is induced at room temperature. The
bacterial cells are allowed to grow post-induction for 5 hours (CCLP) or 18 hours (PCLP), at which time the cells are harvested by centrifugation. The supernatant is then decanted, then cells are resuspended in 20 mM HEPES, 100 mM KCl, pH 7.4, and then frozen overnight. The cells are then thawed and sonicated in order to lyse them, then the contents are centrifuged. After centrifugation, the supernatant and the pellet are separated. CCLP constructs are soluble and are in the supernatant fraction. PCLP constructs are not soluble and are in the pellet fraction. At this point, their purification procedures differ and will be described sequentially.

CCLP, a soluble protein, is present in the supernatant after this step. Calcium is added to the supernatant, and then it is heated to 80°C for 10 minutes, cooled, and centrifuged. This step causes most bacterial proteins to precipitate and to pellet. In later preparations, a protease inhibitor cocktail was added. CCLP is then dialyzed into a high calcium, low salt buffer (Buffer A) for Hydrophobic Interaction Chromatography for purification in the next step.

After the expression step, PCLP is present in the pellet. To remove bacterial membrane components from the pellet, we use a series of Triton X-100 washes to further purify the PCLP, which remains in the pellet. The pellet is then dialyzed in His-Tag Binding Buffer with urea added to solubilize the protein for purification in the next step. For PCLP, we use His-Tag Chromatography because its low solubility means that it will not allow for Hydrophobic Interaction Chromatography. For CCLP, we use Hydrophobic Interaction Chromatography because we need a reducing agent present which is incompatible with the His-Tag column. Samples are taken at each step and assayed using
a 17% polyacrylamide gel (SDS-PAGE) to visualize the purification process of each protein.

We confirm the presence of our purified protein in specific fractions via UV-Vis spectroscopy, then prepare the CCLP-containing samples for lyophilization by dialyzing them with water. The lyophilization process turns the purified CCLP into a solid, which is a more stable form for storage. To prepare for lyophilization while also keeping CCLP soluble, we use ammonium bicarbonate (NH₄HCO₃) in our buffers. During lyophilization, the ammonium bicarbonate evaporates, and therefore does not affect the mass of the solid protein. It served as a completely volatile salt and also as a pH buffer, enabling us to get pure, solid CCLP.

PCLP is dialyzed into a buffer containing 6 M urea, and then aliquoted and frozen.

**III. Maintaining Reducing Conditions**

Because the Thiol-Michael addition involves the terminal cysteine, it is important that our protein’s sulfhydryl groups stay reduced so they are available to react. We maintained anoxic conditions during our experiments. We bubbled in argon to our reaction buffers in order to exclude O₂ and maintain anoxic conditions. Throughout the preparation of the protein, the proteins are kept in solution with 1 mM dithiothreitol (DTT), a reducing agent. While we ran an experiment examining the impact of another reducing agent, Tris(2-carboxyethyl)phosphine (TCEP), on the Thiol-Michael addition reactions, the default reaction condition was 1 mM TCEP in order to increase the
proportion of reactive sulfhydryls. More information about the reducing agents is available in Appendix D.

**IV. Thiol-Michael Addition Reactions**

We pulled from literature and utilized trial and error to settle on an effective protocol for performing the Thiol-Michael addition reactions. The active species is a thiolate, so the cysteine thiol must be deprotonated to a thiolate through either a base- or nucleophile-catalyzed mechanism. For the vinyl sulfone reactions, we used a 0.3 M triethanolamine (TEOA) buffer to promote the base-catalyzed mechanism. Maleimide is so reactive that it does not need basic conditions when the reaction is done in a polar solvent (water)\(^2\). While our actual attempted experiments utilize the base-catalyzed thiolate formation, we also explored the nucleophile-catalyzed mechanism, using PPY (4-pyrrolidinopyridine) as the nucleophile. We found no significant difference in the results of bioconjugation between the two mechanisms (see Appendix B). The contents and pH of the four buffers utilized in these reactions are detailed in Table 2.1 below.

**Table 2.1. Contents of the various buffers used in the Thiol-Michael addition reactions.**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Concentration TEOA/KH(_2)PO(_4)</th>
<th>Concentration NaCl</th>
<th>Concentration NaN(_3) (w/v)</th>
<th>Concentration urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.5</td>
<td>0.3 M TEOA</td>
<td>100 mM</td>
<td>0.05%</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>8.5</td>
<td>0.3 M TEOA</td>
<td>100 mM</td>
<td>0.05%</td>
<td>6 M</td>
</tr>
<tr>
<td>3</td>
<td>6.5</td>
<td>0.3 M KH(_2)PO(_4)</td>
<td>100 mM</td>
<td>0.05%</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>6.5</td>
<td>0.3 M KH(_2)PO(_4)</td>
<td>100 mM</td>
<td>0.05%</td>
<td>6 M</td>
</tr>
</tbody>
</table>
The high pH buffers, 1 and 2, were used in the VS-PEG-VS reactions because vinyl sulfones are more reactive at basic pHs. TEOA serves as both a buffer and a base in buffer 1 and 2. Buffers 3 and 4, at pH 6.5, were used for the Mal-PEG-Mal reactions because maleimides are more reactive at lower pHs. KH₂PO₄ is used as a buffer. Experimenting with the PCLP construct necessitates the use of buffers with 6 M urea, and we also used these buffers to explore the effect of a denaturant on the extent of bioconjugation. Sodium azide was used to prevent microbial growth.

The Michael-addition reactions were between the cysteine thiol of our protomers and the Michael acceptors already bioconjugated to PEG (VS-PEG-VS and Mal-PEG-Mal). We investigated three of our constructs: PCLP3, CCLP3, and CCLP Bis-Cys. Each coupling reaction was done in a small vial. After weighing out the solid protein, we solubilized it in the appropriate, degassed buffer, then added TCEP, then the Michael acceptor. They were left stirring for 1 day, with samples taken at the following time points: 0 hours, 0.5 hours, 1 hour, 2 hours, and 24 hours. At each of the time points, 10 µL samples were taken and then quenched with 10 µL of 2x SDS-PAGE reducing loading buffer. In order to more clearly visualize the results on the gel, samples were diluted to a final CCLP3 concentration of 25 µM before loading 2.5 µL per lane.

Each reaction series, including the variables tested, is detailed in the Table 2.2 below. “+” means the component was present in the reaction, “-” means it was absent, “c” means it was kept constant in each reaction of the series, and “*” means it was the independent variable of the reaction. Details of each experimental setup can be found in Appendix C.
Table 2.2. Matrix depicting Thiol-Michael reaction conditions.

<table>
<thead>
<tr>
<th>Reaction Component</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I_a</th>
<th>I_b</th>
<th>J</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS-PEG-VS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>diMal-PEG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Buffer</td>
<td>1</td>
<td>1</td>
<td>1,2</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3,4</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TCEP</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temp</td>
<td>c</td>
<td>*</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>*</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>Urea</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ratio of reactants</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>*</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>*</td>
<td>c</td>
<td>c</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

We utilized SDS-PAGE for the qualitative comparison of the band density at the distances corresponding to the different outcomes of bioconjugation. By using electrophoresis, we were able to visualize the relative band density and make conclusions regarding the impact of each variable, and suggest which conditions best promote bioconjugation.
CHAPTER 3

RESULTS

I. Expression and Purification of Constructs

Genes for proteins were transcribed and recombinant proteins were expressed from cultures of transformed *E. coli* cell lines. Figure 3.1 shows SDS-PAGE analysis of the fractions from the expression of CCLP constructs. The Molecular Weight (MW) standard (left lane) allows estimation of the MW of each expressed construct based on its migration in the gel. Table 3.1 compares the MW based on the gene sequence (Actual) to the estimated MW (Apparent) for each of the constructs.

![Figure 3.1. Expression of CCLP constructs.](image)

The CCLP constructs were clearly overexpressed as witnessed by the prominent band in lanes 1 in Figures 3.1 A-C. In Figures 3.1 A, B and C, lane 2 shows the
supernatant after centrifugation of the material in lane 1. The fact that the expressed protein appears in the supernatant illustrates that the protein is soluble in an aqueous HEPES/KCl buffer.

A similar gel illustrating the expression of PCLP2 is shown in Figure 3.2. In contrast to the CCLP constructs, the PCLP construct does not appear in the supernatant after centrifugation of the sonicated expression culture. This indicates that PCLP2 (and PCLP3, data not shown) is not soluble in the aqueous HEPES/KCl buffer. The PCLP constructs required 6 M urea to bring the expressed protein into solution (lane 4, Figure 3.3). Table 3.1 below contains the actual and apparent molecular weights of each of the five protein constructs.

![Figure 3.2. Expression of PCLP2. SDS-PAGE analysis expression (lane 1) and supernatant (lane 2) of PCLP2 are shown.](image)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Actual MW</th>
<th>Apparent MW</th>
<th>Soluble ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCLP2</td>
<td>46,216 g/mol</td>
<td>46,340 g/mol</td>
<td>Yes</td>
</tr>
<tr>
<td>CCLP3</td>
<td>58,675 g/mol</td>
<td>53,950 g/mol</td>
<td>Yes</td>
</tr>
<tr>
<td>CCLP3-Bis-Cys</td>
<td>58,707 g/mol</td>
<td>52,970 g/mol</td>
<td>Yes</td>
</tr>
<tr>
<td>PCLP2</td>
<td>18,470 g/mol</td>
<td>18,020 g/mol</td>
<td>No</td>
</tr>
<tr>
<td>PCLP3</td>
<td>19,307 g/mol</td>
<td>18,408 g/mol</td>
<td>No</td>
</tr>
</tbody>
</table>
Proteins were purified using chromatographic methods as noted in Chapter 2, and SDS-PAGE analysis of the resultant purified proteins are shown in Figure 3.3. Purification of these constructs from the bacterial cultures was successful with >95% purity of the working stocks. CCLP2 (lane 1), CCLP3 (lane 2), CCLP3 Bis-Cys (lane 3) were purified using Hydrophobic Interaction Chromatography. PCLP3 (lane 4) was purified using His-Tag Chromatography using buffers containing 6 M urea.

**Figure 3.3.** SDS-PAGE analysis of purified proteins.

**II. Thiol-Michael Addition**

We set out to determine the factors affecting the bioconjugation of both VS-PEG-VS and Mal-PEG-Mal to our protomers, focusing on the following factors: concentration of the reducing agent TCEP, the reaction temperature, the presence or absence of the denaturant urea, and the ratio of PEG:Protein. The independent variables and reaction conditions are summarized in Table 2.2 in the Methods section, and Appendix C. The
results are grouped by the conclusions we drew from them: the variable has no apparent effect, the variable has a positive effect, the variable has mixed effects.

**A. Independent Variables with no Apparent Effect on Bioconjugation**

1. **Urea**

Because steric hindrance of the thiol has been shown to be a major factor in Thiol-Michael addition, we wanted to see if our cysteines were sterically hindered at all by testing the effect of a denaturant (urea) on the reaction. The impact of urea on the extent of bioconjugation between CCLP3 and VS-PEG-VS (part A) and between CCLP3 and Mal-PEG-Mal (part B) is shown in Figure 3.4. A general inspection of these two figures indicates that the presence of urea during bioconjugation of CCLP3 to either of the two Michael acceptors was inconsequential in the results of the study. Lane 2 of (A) and (B) contains unmodified protomer; the large band ~55 kDa corresponds to unmodified CCLP3 (values listed in Tables 1.2 and 1.4). The subsequent lanes show multiple bands, indicating that a reaction occurred that produced a material with a higher MW than unmodified CCLP3. These higher MW bands represent the bioconjugated products; their creation was the goal of our experiments. The band directly above the one at ~55 kDa represents the addition of VS-PEG-VS (in the case of 3.4 (A)) or Mal-PEG-Mal (in the case of 3.4 (B)). This “mono-modified protomer” is represented by “X-PEG” to the right of the gels. “X-PEG-X” represents two CCLP3s bioconjugated through two separate Thiol-Michael additions to form CCLP-VS-PEG-VS-CCLP (in the case of (A)), or CCLP-Mal-PEG-Mal-CCLP (in the case of (B)). We refer to these constructs as the “bioconjugated dimer” as it is two CCLPs connected via bioconjugation. The X-PEG-X
band is ~120 kDa, which corresponds to the mass of 2*CCLP3 (~117 kDa) plus the mass of VS-PEG-VS or Mal-PEG-Mal (both ~3500 Da).

In comparing lanes 3-6 (A) and lanes 7-10 (A), one can see that the proportions of each band do not differ significantly between the two conditions, 0 M urea and 6 M urea. In fact, 6 M urea may even hinder the reaction, as shown in (B). The reactions with 6 M urea added (lanes 6-8) have more unmodified protomer (“X” band) than the 0 M reaction (lanes 3-5). The 0 M reaction seems to have a larger proportion of the mono-modified (“X-PEG”) band. Given the poor quality of this gel, we argue here that there is not a large effect from the presence of urea on the fraction of protomers that are bioconjugated.
Urea has no substantial effect on Thiol-Michael addition bioconjugation. (A) Effect of urea on bioconjugation of CCLP3 and VS-PEG-VS [Study C]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition without urea, lanes 7-10 correspond to Michael addition in 6 M urea. (B) The impact of urea and on bioconjugation between CCLP3 and Mal-PEG-Mal [Study G]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-5 correspond to Michael addition without urea and with a 2:1 PEG:CCLP3 ratio; lanes 6-8 correspond to Michael addition in 6 M urea.

2. Temperature

Temperature was the next variable that was tested. In Figure 3.5 (A) and (B), gels show the analysis of samples taken at 4 time points for each of three temperatures. The
general trend is that increasing the temperature does not result in an increased proportion of mono-modified protomer or bioconjugated dimer. However, for the reactions with vinyl sulfone as the Michael acceptor (A), there is less protein on the gel overall at 37°C. The band corresponding to unmodified CCLP3 certainly decreased between 22°C and 37°C, but this is not indicative of increased bioconjugation because we do not see a corresponding increase in the X-PEG or X-PEG-X bands. It could be that there are very large MW species formed that do not enter the gel, or that the protein is being degraded by endogenous proteases.

Another thing of note is that in the vinyl sulfone reaction (Figure 3.5 (A)), time dependence is exhibited, especially at lower temperatures. At 8°C, T_{30} (lane 3 of (A)), the modified protein bands are far smaller than they are after the reaction goes overnight (T_{ov}, lane 6). Interestingly, this time dependence is only significant in at the lower temperature, and only for VS-PEG-VS. In Figure 3.5 (B), the maleimide reaction, the proportions of each band largely stay the same between 30 mins and 24 hours. In (B) there is also no significant difference between the relative proportion of the bands as a function of temperature.
Figure 3.5. Temperature has no clear effect on Thiol-Michael addition bioconjugation. (A) depicts the effect of temperature on the VS-PEG-VS and CCLP3 bioconjugation reaction [Study B]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition at 8°C, lanes 7-10 correspond to Michael addition at 22°C; lanes 11-14 correspond to Michael addition at 37°C. (B) shows the varying extents of bioconjugation between Mal-PEG-Mal and CCLP3 as affected by temperature [Study F]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition at 8°C, lanes 7-10 correspond to Michael addition at 22°C; lanes 11-14 correspond to Michael addition at 37°C.
B. Variables with Distinct Positive Effects on Bioconjugation

1. TCEP

Figure 3.6 depicts the impact of reducing agent TCEP (tris(2-carboxyethyl) phosphine) on Thiol-Michael addition between VS-PEG-VS and CCLP3 (part A) and Mal-PEG-Mal and CCLP3 (part B). In both Figures 3.6 (A) and (B), the band around 55 kDa represents CCLP3 (MW 58,675) and the band above it represents the mono-modified protomer (“X-PEG” (~62,000)). The band below 130 kDa represents the bioconjugated dimer (“X-PEG-X” (~120,000)). Over half of the CCLP3 was modified, but there is still a substantial amount of unmodified protomer, and the bioconjugated dimer band is small. Compare the starred lanes (5 and 13) of (A). Based on visual inspection, they have a similar total band density, but note that lane 13 (1 mM TCEP) has higher MW bands corresponding to “X-PEG” and “X-PEG-X” than does lane 5 (no TCEP). This indicates that 1 mM TCEP promotes bioconjugation, presumably by increasing the availability of reduced sulphydryl groups. In Figure 3.6 (B), the effects of TCEP are not as positive as seen for the vinyl sulfone acceptor, but it is clear that TCEP has no negative impact on bioconjugation with maleimide. When you examine lanes 3-6 of (B) and compare them to lanes 11-14 of (B), the results look very similar, but the 1 mM TCEP seems to have slightly more mono-modified protomer (X-PEG). Notice the higher MW bands (~ 250 kDa) in the gel in Figure 3.6 A. We do not have an explanation for them. They are higher MW than could be formed based on predicted chemistry.
Figure 3.6. 1 mM TCEP promotes bioconjugation.
(A) Impact of TCEP on the bioconjugation of CCLP3 and VS-PEG-VS [Study A]. An initial sample was not taken for this reaction. Lane 1 contains ladder; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 2-5 correspond to Michael addition in the absence of TCEP; lanes 6-9 correspond to Michael addition in 0.1 mM TCEP; lanes 10-13 correspond to Michael addition in 1 mM TCEP. (B) shows the impact of TCEP concentration on the bioconjugation between CCLP3 and Mal-PEG-Mal [Study E]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition in the absence of TCEP; lanes 7-10 correspond to Michael addition with 0.1 mM TCEP; lanes 11-14 correspond to Michael addition with 1 mM TCEP.

2. Identity of Michael Acceptor

Different Michael acceptors have different reactivities and are better suited to different applications, so we wanted to see if maleimide and divinyl sulfone produced distinct results or if either seemed better suited to our reaction. By comparing Figure 3.7 (A) and (B), we see that the reaction with VS-PEG-VS (A) is less reactive than with Mal-PEG-Mal (B); Maleimide as the Michael acceptor (B) yields a much denser band
corresponding to the mono-modified protomer (“X-PEG”) and the bioconjugated dimer (“X-PEG-X”). All other variables were constant. When comparing (C) and (D), it is clear that the VS-PEG-VS reaction (C) has a greater proportion of unmodified protomer (“X” band), and the Mal-PEG-Mal reaction (D) has a greater proportion of both mono-modified protomer (“X-PEG”) and the bioconjugated dimer (“X-PEG-X”). Because every other variable in the reaction was constant, this difference in bioconjugation suggests that maleimide is a more reactive Michael acceptor than divinyl sulfone.

Figure 3.7. Maleimide is a more reactive Michael acceptor than divinyl sulfone. (A) shows the results of bioconjugation between CCLP3 and VS-PEG-VS [Study A]. Lane 1 contains ladder; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 2-5 correspond to Michael addition in the absence of TCEP; lanes 6-9 correspond to Michael addition in 0.1 mM TCEP; lanes 10-13 correspond to Michael addition in 1 mM TCEP. (B) shows the reaction between CCLP3 and Mal-PEG-Mal [Study E]. The samples were taken in the same way as (A). (C) shows the effect of temperature on the reaction between CCLP3 and VS-PEG-VS [Study B]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition at 8°C; lanes 7-10 correspond to Michael addition at 22°C; lanes 11-14 correspond to Michael addition at 37°C. (D) shows temperature’s effect on the bioconjugation of CCLP3 and Mal-PEG-Mal [Study F]. Time points were taken in the same manner as (C).
C. Independent Variables with Mixed Effects on Bioconjugation

1. PEG:Protein Ratio

Figures 3.8 and 3.9 indicate opposite trends in terms of the effect of PEG:Protein ratio on the bioconjugation of CCLP3 and PCLP3. For CCLP3 (Figure 3.8), a higher proportion of PEG promotes bioconjugation. For PCLP3 (Figure 3.9), a higher proportion of protein promotes bioconjugation.

Figure 3.8 below depicts three gels in which a higher PEG:Protein ratio seems to have a positive effect on bioconjugation. (A) and (B) depict the same reaction conditions, CCLP3 and VS-PEG-VS, but at different ratios of PEG:CCLP3. There is more unmodified protomer (“X” band) in the 1:2 and 1:1 (A) than the 2:1 (B). This corresponds with the greater proportion of mono-modified protomer (“X-PEG”) and bioconjugated dimer (“X-PEG-X”) caused by the 2:1 PEG:CCLP3 ratio (B). The effect is more drastic on the formation of the mono-modified protomer, but there is also more bioconjugated dimer formed. In (C), the same effect is shown for CCLP3 with maleimide as the Michael acceptor. Comparing lanes 5 and 8 (starred), the $T_{120}$ time points of the 2:1 and 1:2 ratios, respectively, we see that the higher amount of PEG in lane 5 results in larger bands of X-PEG and X-PEG-X, with a corresponding decrease in the amount of unmodified protomer (band “X”). In lane 8, with twice the CCLP3 as PEG, more CCLP3 is left unreacted (band “X”), and a smaller portion is actually bioconjugated.
Figure 3.8. Bioconjugation with PEG and CCLP3 is promoted by a higher PEG:CCLP3 ratio. (A) depicts the effect on bioconjugation of manipulating the ratio of VS-PEG-VS:CCLP3 [Study D]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition with a 1:1 VS-PEG-VS:CCLP3 ratio; lanes 7-10 corresponds to the 1:2 VS-PEG-VS:CCLP3 ratio. (B) depicts the same reaction conditions as (A) but at a 2:1 PEG:CCLP3 ratio [Study C]. (C) shows the reaction between CCLP3 and Mal-PEG-Mal at the three different ratios, as labeled above the gel [Study H].

Figure 3.9 below shows the PCLP3 bioconjugation results that demonstrate the benefit of a 1:2 PEG:Protein ratio, contrary to the results in Figure 3.8. In both figures, the dark band between 25 kDa and 15 kDa represents unmodified PCLP3 (MW = 19,307 g/mol). There is a very faint band slightly above 25 kDa which likely corresponds to PCLP3 bioconjugated with one PEG. Compared to our other constructs, this was not an appreciable modification whatsoever. However, in Figure (B), there is a significant band ~55 kDa corresponding to the bioconjugated dimer, PCLP-PEG-PCLP (MW ~44,000).
The only PCLP3 sample to be appreciably modified was the 1:2 PEG:PCLP3 ratio of (B), the maleimide reaction. Further, the bioconjugated dimer (“X-PEG-X”) is present seemingly without any mono-modified protomer (“X-PEG”). This is another result unique to PCLP3; in all the other reactions, we saw more mono-modified protomer than bioconjugated dimer. Additionally, we see that PCLP3 is generally less subject to bioconjugation than CCLP3, but can still undergo it. Figure 3.9 is another example of maleimide’s higher reactivity as the VS-PEG-VS reaction seems ineffective, but the Mal-PEG-Mal reaction is able to produce a significant modification.

Figure 3.9. Bioconjugation with PEG and PCLP3 is promoted by a lower PEG:PCLP3 ratio.

(A) shows the bioconjugation of PCLP3 with VS-PEG-VS and the effect of altering the PEG:PCLP3 ratio [Study J]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to a 2:1 PEG:PCLP3 ratio; lanes 7-10 correspond to Michael addition with a 1:1 PEG:PCLP3 ratio; lanes 11-14 correspond to a 1:2 PEG:PCLP3 ratio. (B) shows the bioconjugation of PCLP3 with Mal-PEG-Mal and the effect of altering the PEG:PCLP3 ratio [Study K]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to a 2:1 PEG:PCLP3 ratio; lanes 7-10 correspond to Michael addition with a 1:1 PEG:PCLP3 ratio; lanes 11-14 correspond to a 1:2 PEG:PCLP3 ratio.
III. CCLP Bis-Cys

We applied the results from the CCLP3 bioconjugation experiments to modification of the CCLP Bis-Cys construct in order to provide the best possible conditions for bioconjugation. In Figure 3.10, the band above 55 kDa (“X”) represents CCLP Bis-Cys (MW=58,707 Da). The band above that represents the addition of one PEG (“X-PEG” (~62,000 Da)), and the band above that illustrates the reactivity of both cysteines of CCLP Bis-Cys: two PEGs were added, forming “PEG-X-PEG” (MW ~66,000 Da). The band above 100 kDa corresponds with the bioconjugated dimer (“X-PEG-X” (MW~120,000 Da)). The band below 130 kDa is likely two distinct bands that migrated together, representing the addition of 1 or 2 more PEGs (“PEG-X-PEG-X-(PEG)” (MW~123.5 kDa or ~127 kDa)). Most significantly, the band above 180 kDa (“(PEG)-X-PEG-X-PEG-X-(PEG)”) shows that it is possible to bioconjugate 3 CCLPs. It represents CCLP-PEG-CCLP-PEG-CCLP (~184,000 Da). The bands above that represent the addition of additional PEGs onto the terminal cysteines of CCLP Bis-Cys, PEG-CCLP-PEG-CCLP-PEG-CCLP-PEG-CCLP (MW ~187.5 kDa) or PEG-CCLP-PEG-CCLP-PEG-CCLP-PEG-CCLP-PEG (~191 kDa). Any other compound with a molecular weight similar to these or above these would have all combined to form those top bands, so we cannot rule out the possibility of a 4- or 5- CCLP bioconjugation product. Interestingly, there is not an apparent difference between the two Michael acceptors. We do see a slight time dependency, as the To samples have a higher proportion of the modified protomers and less unmodified CCLP Bis-Cys when compared to the T30 samples.
**Figure 3.10. Bioconjugation of CCLP Bis-Cys.**

Bioconjugation of CCLP Bis-Cys with VS-PEG-VS and Mal-PEG-Mal [Study I]. Lane 1 contains ladder; lane 2 contains an initial sample of protein; samples were taken at 30 mins, 60 mins, 120 mins, and approximately 24 hours; lanes 3-6 correspond to Michael addition with VS-PEG-VS as the Michael acceptor, lanes 7-10 correspond to Michael addition with Mal-PEG-Mal.
CHAPTER 4
DISCUSSION

As we examined the results from these gels, we qualitatively compared the proportion of modified vs. unmodified protomer and determined which variables led to the highest proportion of bioconjugation. The T₁ lanes contain unmodified protomer at the same concentration as protein is present in all of the other lanes, so we can compare other lanes with the T₁ lane to how much protein has been modified and how much remains unmodified. The Discussion will follow the same organization as the Results: part I will discuss the variables that had no significant effect, part II will discuss the variables that positively impacted bioconjugation, part III will discuss the variable effects of manipulating the PEG:Protein ratio, and part IV will discuss the optimization of these factors and the promising results of bioconjugation with CCLP Bis-Cys.

I. Independent Variables with no Apparent Effect on Bioconjugation

A. Urea

The kinetics and yield of the base-catalyzed Thiol-Michael addition mechanism depend on the steric accessibility of the thiol, so it is important to address the possibility that the thiol may be sterically hindered. We suspected that including urea as a denaturant might increase the efficacy of the reaction by decreasing any secondary or tertiary structural elements that would decrease the reactivity of the cysteine thiol.
However, the results from Figure 3.4 show that urea does not have a significant effect. We do see that the proportion of bioconjugated CCLP3 increases over time.

Figure 3.4, part (B) shows that 6 M urea can even decrease the amount of bioconjugation. The unmodified CCLP3 bands in 6 M urea have a higher relative density than the ones for 0 M urea. This could be due to an impact on CCLP3 solubility. As a denaturant, urea may affect the solubility of CCLP3 by increasing the exposure of hydrophobic residues to solution, which may have affected the reaction in unintended ways.

Because urea does not have a noticeable effect on the bioconjugation reactions, we concluded that the cysteine sulfhydryl groups were already sufficiently exposed and reactive; no denaturant was needed to expose the thiol, so urea had no substantial effect on the reaction. The cysteine residues are genetically engineered to be terminal, so it makes sense that they would not be very sterically hindered.

**B. Temperature**

Increased temperature is generally favored in order to increase the rate of a reaction. The increased temperature increases the kinetic energy of the molecules and brings them into contact with each other more often, theoretically contributing to an increased proportion of bioconjugated protein. Higher temperatures increase the rate of reactions by lowering the transition state energy barrier relative to the energies of the reactants and products, but our results did not show a significant effect. We anticipated that a higher temperature would increase the kinetics and yield of the reaction, but Figure 3.5 shows that temperature did not have a significant effect on our bioconjugation
reactions. Part (A) suggests a slight trend favoring 37°C because there is far less unmodified CCLP3, but we do not see the proportions of the higher MW bands increasing, and there is an overall decrease in the amount of protein in the samples. As such, it is not a strong argument that the temperature has a large impact on the amount of modified protein formed. Perhaps an unintended reaction occurred that produced concatenated products that are high MW such that they do not appear on the gel. Proteolysis is unlikely because we do not see fragments below ~55 kDa that would correspond to small pieces of a cleaved protein. Additionally, we do not think it was due to protein precipitation, as pellet particulates would likely be soluble in SDS and thus appear in the gel.

In part (B), the temperature effect is even less significant. This is likely due to maleimide being more reactive under every condition, so the difference is not apparent; the increased reactivity means that the Mal-PEG-Mal reactions do not rely as much on other factors such as temperature or TCEP in order to successfully undergo bioconjugation.

The effect of temperature on reaction rate is most apparent at 8°C where there is a clear time dependence of the bioconjugation reaction.

II. Independent Variables that Positively Affect Bioconjugation

A. TCEP

Reducing agent is important in this reaction because it increases the proportion of reactive sulfhydryl groups available to participate in the Michael addition reaction. The reaction rate depends on the concentration of the thiolate\textsuperscript{27}, so if the thiolate is unable to
be formed (i.e. the cysteine thiol is taking part in a disulfide linkage), the reaction rate and yield will decrease.

Our results are in accordance with this prediction. We see a clear increase in the modified CCLP3 with 1 mM TCEP. The presence of a reducing agent keeps the cysteine reduced to a sulfhydryl rather than allowing oxidation to form a disulfide linkage. The disulfide linkage does connect two protomers, but it is less stable than the dimer created by bioconjugation with PEG. We cannot observe this dimer on the gels in this document, because the SDS-PAGE loading buffer contains a reducing agent. Earlier studies in our lab showed that <10% of the protein appeared as disulfide-linked dimer, even after exposure to oxidizing reaction conditions (data not shown).

We found that a concentration of 1 mM TCEP was most effective in promoting formation of bioconjugated CCLP3-PEG, and the bioconjugated dimer. The low concentration of TCEP, 0.1 mM, has decent proportions of modified protein, but not in the same quantity as the higher 1 mM concentration. One can see that the absence of TCEP produced the least amount of bioconjugated dimer.

The results from Figure 3.6 part (B) show that it appears that TCEP does not have as large of an effect on the Mal-PEG-Mal reaction as it does with VS-PEG-VS. This could be because we better maintained reducing conditions while we were performing the experiment so TCEP had less of a role to play, or because maleimide is more reactive and the conditions matter less. The T<sub>i</sub> sample of (B) (CCLP3 without TCEP) has no appreciable band around ~120 kDa, indicating that our anoxic conditions are generally sufficient to suppress the formation of the CCLP-CCLP disulfide dimer.
B. Identity of Michael Acceptor

From Figure 3.7, we can see that the Mal-PEG-Mal reactions have a dramatically increased proportion of modified CCLP3 as compared to the VS-PEG-VS reactions. Both the mono-modified protomer (“X-PEG”) band at ~62,000 Da and the bioconjugated dimer (“X-PEG-X”) band at ~120 kDa are much larger than observed for any of the vinyl sulfone reactions. A majority of the CCLP3 is bioconjugated, while only a small portion remains unmodified, illustrating Mal-PEG-Mal’s reactivity. In (B) we can clearly see that bioconjugation with Mal-PEG-Mal results in increased modification and a far higher proportion of modified CCLP3 than VS-PEG-VS. This increased reactivity is due to the high reactivity of maleimide’s carbon-carbon double bond. It is very reactive and electrophilic due to the bond angle distortion and ring strain, in addition to the cis conformation of its carbonyl groups. Maleimide’s higher reactivity has been noted in other studies and is affirmed by our results. The maleimide reactions were interesting because it is more reactive despite not having basic or nucleophilic conditions. Instead, the thiolate is thought to form through interactions with highly polar solvents.

III. Independent Variables with Mixed Effects on Bioconjugation

A. PEG:Protein Ratio

In Figure 3.8, it is clear that bioconjugation with CCLP3 is more successful at a higher PEG:CCLP3 ratio. Comparing the results from (B) with the results from (A), which is admittedly difficult because the gel type was not standardized and different amount of samples were loaded, you can see that the 2:1 PEG:CCLP3 ratio (B) has a higher proportion of bioconjugation. The 1:1 and 1:2 ratio samples of (A) have far over
half of the CCLP3 unmodified, as shown by the big band ~55 kDa. Comparatively, the
2:1 ratio lanes (B) have over half of the CCLP3 modified. These results demonstrate that
the 2:1 PEG:CCLP3 ratio best promotes formation of the bioconjugated PEG-CCLP
construct.

This is contrary to our prediction, because PEG is bifunctional, so one would
expect that you would need twice the CCLP3 for every PEG, and the expected ratio
would thus be 1:2 PEG:CCLP3. We believe these results indicate the importance of a
high concentration of PEG in order to promote the bioconjugation to the mono-modified
protomer, CCLP3-PEG. The bioconjugated dimer cannot be formed without sufficient
CCLP3-PEG, so we do not see an increase in the bioconjugated dimer band either, even
with the 1:2 PEG:CCLP3 ratio. The reaction rate depends on both the concentration of
the thiolate and concentration of Michael acceptor, so increasing the amount of Michael
acceptor (Mal-PEG-Mal and VS-PEG-VS) available to react increases the likelihood that
a thiolate will perform a nucleophilic attack on it and form the bioconjugated product.
This also suggests that we have a sufficient concentration of thiolate, as increasing the
[PEG] increases the extent of bioconjugation.

When you examine the lanes 5 and 8 in particular on (C), there is clearly more
unmodified CCLP3 with the 1:2 ratio. Again, this indicates that an excess of PEG is best
for promoting the bioconjugation between CCLP3 and our PEG Michael acceptors. It
appears that as you increase the ratio in favor of PEG, you see a shift from majority
unmodified to a majority modified. The effect on the proportion of the bioconjugated
dimer is less significant.
Contrary to the apparent positive effect that a higher PEG:Protein ratio has on bioconjugation with CCLP3, the results from PCLP3 were the opposite, as seen in Figure 3.9. Figure 3.9 shows that PCLP is also subject to bioconjugation with PEG, although it happens at a much more appreciable quantity with Mal-PEG-Mal than VS-PEG-VS. In both figures, there is a faint band corresponding to the mono-modified protomer. The only significant bioconjugation band in the PCLP reactions is at the 1:2 PEG:PCLP3 ratio, and only for the Mal-PEG-Mal reaction (B). There, there is a significant band ~55 kDa corresponding to the bioconjugated dimer, PCLP-PEG-PCLP (MW ~44,000). We are unsure why the ratio’s effect would be opposite in CCLP and PCLP. The base-catalyzed reaction is affected by the size of the Michael donor, and PCLP is less than half the size of CCLP and lacks conformation, so it is intuitive that it reacts more rapidly and efficiently at lower levels of PEG. This 1:2 ratio is what anticipated would be most effective because PCLP3 has one reactive site compared to two on Mal-PEG-Mal and VS-PEG-VS, so it makes sense that having double the protein and evening out the ratio of reactive groups would be beneficial for the bioconjugation.

IV. Bioconjugation Results for CCLP Bis-Cys

Figure 3.10 illustrates the vast potential that bioconjugation with CCLP Bis-Cys has. There are bands corresponding to a bioconjugated trimer, something unachievable with the other constructs. Additionally, there is the possibility for further modification to a bioconjugated tetramer or pentamer. Since there is not much resolution at the higher molecular weights, we would like to run another Bis-Cys reaction on a different MW
ladder or use MALDI to more precisely determine the extent and limits of bioconjugation between PEG and CCLP Bis-Cys.

Clearly, the addition of 1 or 2 PEGs to a single CCLP happens most often, evidenced by the density of those bands ~55 kDa. Di-modification occurs to an appreciable extent as well, whereas the bands representing the trimer are less dense, but still substantial. This gel is an important proof-of-concept, showing that both cysteines are indeed reactive, and this construct has the potential to create a hydrogel by itself, as it can continue to lengthen indefinitely. While the CCLP3 reactions had a good proportion of mono-modified protomer, the CCLP3 Bis-Cys construct has a more even distribution of modified protein in many intermediate forms. This shows that CCLP3 Bis-Cys has the most potential to form a hydrogel with fine-tunable properties; modification results in a variety of construct lengths. Based on these results, the CCLP3 Bis-Cys construct seems the most promising for this type of bioconjugation reaction. We are unsure if the reason for this better reactivity is due simply to the increased number of thiols on the protomer, or if there is another underlying reason.

The difference between Mal-PEG-Mal and VS-PEG-VS is not as apparent here; both conditions are able to produce the triple-CCLP construct to an appreciable extent. Perhaps this is because we used all the reaction conditions we had found to be favorable throughout the CCLP3 studies and thus overcame any barrier VS-PEG-VS had to reacting at the same level.

Our results show bioconjugation of the three constructs tested. A complimentary method such as MALDI or HPLC is needed to quantify the data and to more accurately assess the results.
CHAPTER 5
CONCLUSION

The overall goal of this experiment was to explore Thiol-Michael addition reactions to manipulate the extent of bioconjugation between our protomers and PEG crosslinkers. By investigating factors that could affect the reaction, we can better understand how to promote the formation of longer polymers, and use this information to create hydrogels that have different sets of physical properties and that can be used in different applications. When polymers are lengthened via bioconjugation, they have higher degrees of entanglement which would theoretically produce a hydrogel with greater structural integrity.

Generally, the results show that Thiol-Michael addition is a feasible way of manipulating the length and geometries of the protomers and potentially fine-tuning the physical properties of the hydrogel they create. In summary, 1 mM TCEP has universally positive effects on bioconjugation by keeping the cysteine thiols reduced, and thus reactive. Urea and temperature have no significant effect on the extent of bioconjugation, but the urea studies show that our cysteine thiols are not sterically hindered. Maleimide is certainly a more reactive Michael acceptor than vinyl sulfone, so that, along with its properties and lower preferred pH, should be taken into account when choosing a Michael acceptor reagent in future studies.

The ratio of PEG:Protein had a variable effect on the reaction. For CCLP3 constructs, an excess of PEG promoted bioconjugation. For PCLP3, the trend was
reversed. This ratio manipulation will be an interesting strategy for promoting the formation of bioconjugated dimer in future studies.

Additionally, we saw that PCLP also has the potential to undergo this bioconjugation. This is positive because a hydrogel with both PCLP and CCLP bioconjugated dimers could have an even greater difference in physical properties. This modification has the added benefit of increasing the solubility of PCLP because of PEG’s hydrophilicity, making PCLP easier to work with in aqueous solution.

All of the constructs are modifiable, but CCLP Bis-Cys is the most promising for the future. Our results showed that both cysteines are reactive with both Mal-PEG-Mal and VS-PEG-VS, enabling the bioconjugation of multiple CCLPs through this reaction. CCLP Bis-Cys has the most potential out of all of our constructs because it has more opportunities for bioconjugation and chain polymerization. Our results suggest that we could create a hydrogel entirely constructed with bioconjugated CCLP Bis-Cys.

Throughout all of the conditions explored, we were unable to fully covert the unmodified protomer to bioconjugated protein. Though it varied based on the conditions, we were never able to convert more than ~75% of it to bioconjugated protein. Future reactions should explore other ways to push the formation of bioconjugated protein dimer so we can further impact the physical properties of the hydrogel.

The studies explored here imply that our protomers are easily functionalized with PEG crosslinkers and are impacted by variables such as temperature, reducing agent, and the ratio of PEG:Protein. By manipulating these variables, we hypothesize that we will be able to use the different extents of bioconjugation to fine-tune the physical properties of the hydrogel, increasing its utility in a variety of applications.
The next step is to actually form hydrogels with these bioconjugated protomers and do microrheology studies to determine the tangible effect of these modifications. We would examine how the extent of bioconjugation impacts physical hydrogel properties such as elasticity and viscosity.


APPENDICES
APPENDIX A – Gene design

Figure A.1. Plasmid.
(Novagen)
APPENDIX B – Nucleophile- versus base-catalyzed reactions

<table>
<thead>
<tr>
<th>T&lt;sub&gt;i&lt;/sub&gt;</th>
<th>Mal</th>
<th>Mal</th>
<th>DVS</th>
<th>DVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEOA</td>
<td>PPY</td>
<td>TEOA</td>
<td>PPY</td>
<td></td>
</tr>
</tbody>
</table>

Figure A.2. Modification of PCLP3 with Mal-PEG-Mal and VS-PEG-VS with both nucleophile- and base-catalyzed mechanisms. The left lane has the molecular weight standard. The next lane has PCLP3, then PCLP modified with Mal-PEG-Mal via TEOA (base), then PCLP modified with Mal-PEG-Mal via PPY (nucleophile), then PCLP modified with VS-PEG-VS via TEOA (base), then PCLP modified with VS-PEG-VS via PPY (nucleophile).

From this gel, we determined that there was no appreciable difference between using PPY and TEOA, so we chose to proceed with the base-catalyzed mechanism. Additionally, it further shows that Mal-PEG-Mal is more reactive than VS-PEG-VS.
APPENDIX C – Michael-addition reaction protocols

Table A.1. Study A - Effect of TCEP on bioconjugation of CCLP3 and VS-PEG-VS.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. VS-PEG-VS</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22 °C</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22 °C</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22 °C</td>
</tr>
</tbody>
</table>

Table A.2. Study B - Effect of temp. on bioconjugation of CCLP3 and VS-PEG-VS.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. VS-PEG-VS</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>8°C</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>37°C</td>
</tr>
</tbody>
</table>

Table A.3. Study C - Effect of urea on bioconjugation of CCLP3 and VS-PEG-VS.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. VS-PEG-VS</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (0 M Urea)</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>2</td>
<td>2 (6 M Urea)</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
</tbody>
</table>

Table A.4. Study D - Effect of VS-PEG-VS:CCLP3 ratio on bioconjugation.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. VS-PEG-VS</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1:1)</td>
<td>1</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>2 (1:2)</td>
<td>1</td>
<td>1 mM</td>
<td>0.25 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
</tbody>
</table>

Table A.5. Study E - Effect of TCEP on bioconjugation of CCLP3 and Mal-PEG-Mal.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. Mal-PEG-Mal</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
</tbody>
</table>

Table A.6. Study F - Effect of temp. on bioconjugation of CCLP3 and Mal-PEG-Mal.

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. Mal-PEG-Mal</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>8°C</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>37°C</td>
</tr>
</tbody>
</table>
**Table A.7. Study G - Effect of urea on bioconjugation of CCLP3 and Mal-PEG-Mal.**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. Mal-PEG-Mal</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 (0 M urea)</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>2</td>
<td>4 (6 M urea)</td>
<td>1 mM</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
</tbody>
</table>

**Table A.8. Study H - Effect of Mal-PEG-Mal:CCLP3 ratio on bioconjugation.**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. Mal-PEG-Mal</th>
<th>Conc. CCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1:1)</td>
<td>3</td>
<td>1 mM</td>
<td>0.5 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
<tr>
<td>2 (1:2)</td>
<td>3</td>
<td>1 mM</td>
<td>0.25 mM</td>
<td>0.5 mM</td>
<td>22°C</td>
</tr>
</tbody>
</table>

**Table A.9. Study I – Bioconjugation of CCLP Bis-Cys with VS-PEG-VS and Mal-PEG-Mal.**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. PEG</th>
<th>Conc. CCLP Bis-Cys</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1 mM</td>
<td>1 mM VS-PEG-VS</td>
<td>0.5 mM</td>
<td>37°C</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1 mM</td>
<td>1 mM Mal-PEG-Mal</td>
<td>0.5 mM</td>
<td>37°C</td>
</tr>
</tbody>
</table>

**Table A.10. Study J – PCLP3 Bioconjugation with VS-PEG-VS.**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. VS-PEG-VS</th>
<th>Conc. PCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2:1)</td>
<td>2</td>
<td>1 mM</td>
<td>0.2 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
<tr>
<td>2 (1:1)</td>
<td>2</td>
<td>1 mM</td>
<td>0.1 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
<tr>
<td>3 (1:2)</td>
<td>2</td>
<td>1 mM</td>
<td>0.05 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
</tbody>
</table>

**Table A.11. Study K – PCLP3 Bioconjugation with Mal-PEG-Mal.**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>Buffer</th>
<th>Conc. TCEP</th>
<th>Conc. Mal-PEG-Mal</th>
<th>Conc. PCLP3</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (2:1)</td>
<td>4</td>
<td>1 mM</td>
<td>0.2 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
<tr>
<td>2 (1:1)</td>
<td>4</td>
<td>1 mM</td>
<td>0.1 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
<tr>
<td>3 (1:2)</td>
<td>4</td>
<td>1 mM</td>
<td>0.05 mM</td>
<td>0.1 mM</td>
<td>37°C</td>
</tr>
</tbody>
</table>
### Table A.12. Structure, function, and molecular weight of additional chemicals.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Function</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine (TEOA)</td>
<td><img src="image" alt="Triethanolamine" /></td>
<td>Base</td>
<td>149.18 g/mol</td>
</tr>
<tr>
<td>Tris(2-carboxyethyl) phosphine (TCEP)</td>
<td><img src="image" alt="Tris(2-carboxyethyl) phosphine" /></td>
<td>Reducing agent</td>
<td>286.65 g/mol</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td><img src="image" alt="Dithiothreitol" /></td>
<td>Reducing agent</td>
<td>154.24 g/mol</td>
</tr>
</tbody>
</table>