Title
Modulating Protein Activity through Polymer Conjugation

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Modulating Protein Activity through Polymer Conjugation

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry

by

Caitlin Gayle Decker

2015
Protein therapeutics have become essential to the healthcare and pharmaceutical industries since the first recombinant proteins entered the clinic in the 1980s. Modification of proteins with polymers has traditionally been pursued as a means to improve protein stability and enhance pharmacokinetic properties. In addition to these benefits, polymer conjugation can also be utilized to control and modulate protein activity. The first polymer used for protein conjugation was poly(ethylene glycol) (PEG) in 1977. PEG is currently the only FDA-approved
polymer for protein conjugation and 10 PEGylated protein drugs are currently on the market. This dissertation offers three modifications to traditional PEGylation, which allow for the modulation of protein activity.

In the first example, masking and unmasking the activity of a model protein, lysozyme, was achieved by incorporating both a reducible disulfide linkage between the polymer and the protein as well as incorporating degradable cyclic ketene acetal (CKA) moieties throughout the backbone of a PEG-like polymer (Chapter 2). Specifically 5,6-benzo-2-methylene-1,3-dioxepane and poly(ethylene glycol) methyl ether methacrylate (PEGMA) were copolymerized by reversible addition-fragmentation chain transfer polymerization (RAFT) facilitated by a cysteine-reactive, pyridyl disulfide (PDS) modified chain transfer agent (CTA). Two polymers, a small ($M_n$ GPC = 10.9 kDa) and a large ($M_n$ GPC = 20.9 kDa) PDS-pPEGMA-co-BMDO, were synthesized with reasonable control (dispersities ($D$) = 1.34 and 1.71, respectively). The polymers were then conjugated to a thiol-enriched hen egg white lysozyme by disulfide exchange. Conjugation with the 10.9 kDa polymer resulted in a conjugate, which exhibited high initial activity (63%) while the larger conjugate activity was highly attenuated (20%). Lysozyme release from both polymers by reduction of the disulfide linkage and by hydrolytic cleavage, in basic media, of the polymer backbone was visualized by gel electrophoresis. Reduction of the disulfide conjugation linkage with glutathione resulted in an increase in protein activity for both conjugates.

In the next example, site-specific chemical dimerization of fibroblast growth factor 2 (FGF2) with a PEG linker, of optimized length, resulted in a FGF2 homodimer with wound healing ability at exceptionally low concentrations (Chapter 3). Homodimers of FGF2 were synthesized through site-specific conjugation to both ends of poly(ethylene glycol) (PEG).
FGF2 was conjugated to 2, 6, and 20 kDa vinyl sulfone bis-functionalized PEG, as well as to a small molecule and mono-functionalized PEG controls. The optimal linker length was determined by screening FGF2 dimer-induced proliferation of human dermal fibroblasts (HDF). The inter-cysteine distance was calculated to be approximately 70 Å, which is similar in length to a 2 kDa PEG. FGF2-PEG2k-FGF2 induced greater fibroblast proliferation than FGF2 alone, all other dimers, and all monoconjugates, at each concentration tested, with the greatest difference observed at low (0.1 ng/mL) concentration. FGF2-PEG2k-FGF2 further exhibited superior activity compared to FGF2 for both proliferation and migration in human umbilical vein endothelial cells, as well as improved angiogenesis in vitro. Efficacy in an in vivo wound healing model was assessed in diabetic mice. FGF2-PEG2k-FGF2 increased granulation tissue and blood vessel density in the wound bed compared to FGF2. The results suggest that this rationally designed construct may be useful in chronic wound healing.

Lastly, a block copolymer capable of noncovalent and releasable conjugation to histidine-6 tagged proteins, consisting of a PEG-based block and a Ni(II) nitrioltriacetic acid (NTA)-based block was synthesized (Chapter 4). The first block was synthesized via RAFT polymerization of a NTA monomer. The resulting polymer was then utilized as a macro-CTA for the polymerization of PEGMA, resulting in a pNTAMA-b-PEGMA, containing 9 NTAMA repeats and 8 PEGMA repeats, with number average molecular weight ($M_n$) (GPC) = 9.9 kDa and dispersity ($D$) = of 4.5. The high dispersity indicates a lack of control, and disproportionation was further confirmed by $^1$H-NMR. Initial studies indicated that mono-NTA-His6 interactions are not sufficient for protein conjugation, therefore extension of this work towards a multi-valent approach may prove effective in the future.
The dissertation of Caitlin Gayle Decker is approved.

Joseph A. Loo

Harold G. Monbouquette

Heather D. Maynard, Committee Chair

University of California, Los Angeles

2015
This Dissertation is Dedicated to my Parents, Glenn and Melinda Decker,

my Auntie Mommy, Rebecca Rhodes,

my brother, Aaron Pegues

and my best friend, Kathryn Margaret Trankina

“You help me exist”
# Table of Contents

Abstract of the Dissertation ........................................................................................................... ii

Table of Contents ............................................................................................................................ vi

List of Figures ................................................................................................................................... x

List of Tables ................................................................................................................................. xiii

List of Schemes ............................................................................................................................ xiv

List of Abbreviations ...................................................................................................................... xv

Acknowledgements ....................................................................................................................... xix

Vita ................................................................................................................................................ xix

**Chapter 1. General Introduction: Modulating Protein Activity Through Polymer Conjugation** ................................................................................................................................. 1

1.1 Motivation for polymer conjugation to protein therapeutics .............................................. 2

1.2 Protein-PEG conjugates with retention of activity ............................................................... 3

1.3 Protein-polymer conjugates with masked-unmasked activity ............................................. 9

1.4 Multimerized protein-polymer conjugates exhibiting enhanced activity ......................... 13

1.5 Concluding remarks on the Introduction to the Dissertation .......................................... 14

1.5. References ........................................................................................................................... 15

**Chapter 2. Degradable PEGylated Protein Conjugates Utilizing RAFT Polymerization** ... 26

2.1. Introduction .......................................................................................................................... 27

2.2. Results and Discussion ....................................................................................................... 30
2.2.1. Synthesis of PDS-p(PEGMA-co-BMDO)s 1 and 2 ........................................ 30
2.2.2. Hydrolytic Degradation of 1 and 2 ......................................................... 31
2.2.3. Conjugation of 1 and 2 to Thiolated Lysozyme ..................................... 33
2.2.4. Hydrolytic or Reductive Cleavage of 1 or 2 from Lyz-1 or Lyz-2 ........... 34
2.2.5. Activity of Lyz-1 and Lyz-2 before and after reductive treatment with glutathione ................................................................. 36
2.3. Experimental .............................................................................................. 39
  2.3.1. Materials ............................................................................................... 39
  2.3.2. Analytical Techniques .......................................................................... 39
  2.3.3. Methods ................................................................................................ 40
2.4. Conclusions .................................................................................................. 50
2.5. References .................................................................................................. 51

Chapter 3. Fibroblast Growth Factor 2 Dimer with Superagonist Activity Improves Wound Healing ............................................................................................................. 55

3.1. Introduction ................................................................................................... 56
3.2. Results and Discussion ................................................................................ 60
3.3 Experimental ................................................................................................ 76
  3.3.1. Materials ................................................................................................ 76
  3.3.2. Analytical Techniques .......................................................................... 76
  3.3.3. Methods ................................................................................................ 77
3.4. Conclusions.................................................................................................................. 93

3.5. References.................................................................................................................... 94

Chapter 4. Progress towards a Ni(II)-Nitrilotriacetic acid Functionalized Block Copolymers for the Multimerization of His6-Proteins................................................................. 99

4.1. Introduction..................................................................................................................... 102

4.2. Results and Discussion ................................................................................................. 60

4.2.1 NTA Monomer Synthesis .......................................................................................... 102

4.2.2 Block Copolymer Synthesis....................................................................................... 105

4.2.3 Synthesis of Protein-His6-Ni(II)-pNTAMA-b-pPEGMA Conjugates ..... 108

4.3 Experimental.................................................................................................................. 110

4.3.1 Abbreviations............................................................................................................. 110

4.3.2 Materials ................................................................................................................... 110

4.3.3 Instrumentation ........................................................................................................ 110

4.3.4. Methods................................................................................................................... 111

4.4. Conclusions................................................................................................................... 115

4.5. References..................................................................................................................... 116
List of Figures

Figure 2.1. PEGylated protein conjugate, released by either reduction or hydrolysis.................... 3

Figure 2.2. GPC analysis of polymer before and after incubation in 5% KOH of a) 1 for 7 days and b) 2 for 1 day.................................................................................................................. 33

Figure 2.3. SDS-PAGE gel electrophoresis, visualized by Coomassie blue staining, of Lyz-1 and Lyz-2 before and after treatment with reducing agent and/or base. ...................................................... 35

Figure 2.4. Activity of native lysozyme + one equivalent of (non attached) polymer in solution, Lyz-polymer conjugates before and after treatment with GSH, and Lyz after cleavage from Lyz-2. Lyz-1 was treated with 5 mM GSH and Lyz-2 was treated with 10 mM GSH......................... 36

Figure 2.5a. 1H-NMR (CD3CN) of PDS-p(PEGMA-co-BMDO) 1 .............................................. 42

Figure 2.5b. Kinetics for the copolymerization of PEGMA and BMDO yielding PDS-p(PEGMA-co-BMDO) 1 .............................................................................................................................. 43

Figure 2.6a. 1H-NMR (CD3CN) of PDS-p(PEGMA-co-BMDO) 2 .............................................. 44

Figure 2.6b. Kinetics for the copolymerization of PEGMA and BMDO yielding PDS-p(PEGMA-co-BMDO) 2 .............................................................................................................................. 44

Figure 2.7. Degradation kinetic analysis by GPC of PDS-p(PEGMA-co-BMDO) a)1 and b)2 .. 45

Figure 2.8. 1H-NMR (CD3CN) comparison of PDS-p(PEGMA-co-BMDO) 1 before (top) and after (bottom) hydrolysis with 5% KOH for 24 hours................................................................. 46

Figure 2.9. 1H-NMR (CD3CN) comparison of PDS-p(PEGMA-co-BMDO) 2 before (top) and after (bottom) hydrolysis with 5% KOH for 24 hours................................................................. 47
Figure 3.1. The active tetrameric complex consisting of two FGF2s (gold) two FGFRs (silver) with Cys96 in red and poly(ethylene glycol) represented in blue. .................................................. 59

Figure 3.2. Western blot of purified conjugates. ........................................................................... 63

Figure 3.3. Screening of PEG linker-length effect on HDF proliferation and migration. (a) and (b) Percent proliferation of HDFs............................................................................................................... 66

Figure 3.4. (a) Proliferation of HUVECs at various concentrations of FGF2 and FGF2-PEG2k-FGF2. .................................................................................................................................................. 68

Figure 3.5. Effect of FGF2 and FGF2-PEG2k-FGF2 at various concentrations on angiogenesis through the co-culture of HDFs and HUVECs ........................................................................................................... 70

Figure 3.6. In vivo assessment of FGF2-PEG2k-FGF2 in diabetic wounds. ................................. 72

Figure 3.7. 1H-NMR (CDCl3) of mPEG2k-VS ............................................................................. 80

Figure 3.8. 1H-NMR (CDCl3) of mPEG5k-VS ............................................................................. 81

Figure 3.9. 1H-NMR (CDCl3) of mPEG20k-VS .......................................................................... 82

Figure 3.10. 1H-NMR (CDCl3) of VS-PEG2k-VS ................................................................. 83

Figure 3.11. 1H-NMR (CDCl3) of VS-PEG6k-VS ................................................................. 84

Figure 3.12. 1H-NMR (CDCl3) of VS-PEG20k-VS ............................................................... 85

Figure 3.13. MALDI-MS of unmodified FGF2 (top) or FGF2-PEG2k-FGF2 conjugates (bottom). .................................................................................................................................................. 87

Figure 4.1. 1H-NMR (CDCl3) of NTA alcohol 1 ............................................................... 103

Figure 4.2. HSQC-inept analysis of NTA alcohol 1 (CH2, CH, or CH3 are indicated by color) .................................................................................................................................................. 103
Figure 4.3. 2D-COSY analysis of NTA alcohol 1 .......................................................... 104
Figure 4.4. 1H-NMR of NTAMA 2 ............................................................................. 105
Figure 4.5. 1H NMR of pNTAMA-b-pPEGMA 3 .......................................................... 107
Figure 4.6. GPC trace of pNTAMA-b-pPEGMA 3 ..................................................... 108
List of Tables

Table 3.1. Crosslinker length based on Flory’s radius of gyration and fully extended chain length .......................................................... 61
List of Schemes

Scheme 2.1 (a) RAFT polymerization of PEGMA and BMDO (b) Covalent modification of thiolated Lyz with polymer 1 or 2 .......................................................... 30

Scheme 2.2. Reductive or hydrolytic cleavage of polymer 1 or 2 from Lyz ......................... 34

Scheme 4.1. Synthesis of NTAMA 2 ........................................................................... 102

Scheme 4.2. Synthesis of pNTAMA-b-pPEGMA 3 via RAFT block copolymerization of NTAMA 1 and PEGMA .............................................................................. 106

Scheme 4.3. Synthesis of Protein-His6-Ni(II)-pNTAMA-b-pPEGMA conjugates ............... 108
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIBN</td>
<td>2,2-azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>BBE</td>
<td>Bovine Brain Extract</td>
</tr>
<tr>
<td>BMDO</td>
<td>5,6-benzo-2-methylene-1,3-dioxepane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlated Spectroscopy</td>
</tr>
<tr>
<td>CTA</td>
<td>Chain Transfer Agent</td>
</tr>
<tr>
<td>CRP</td>
<td>Controlled Radical Polymerization</td>
</tr>
<tr>
<td>CKA</td>
<td>Cyclic Ketene Acetal</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s Phosphate-buffered Saline</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVS</td>
<td>Divinylsulfone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial Growth Medium</td>
</tr>
<tr>
<td>EGMEA</td>
<td>Ethylene glycol methyl ether acrylate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast Growth Factor II</td>
</tr>
<tr>
<td>FGF8</td>
<td>Fibroblast Growth Factor 8</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Fibroblast Growth Factor Receptor 1</td>
</tr>
<tr>
<td>FMOC</td>
<td>Fluorenlymethoxycarbonyl</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>HDF</td>
<td>Human Dermal Fibroblasts</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence Spectroscopy</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower Critical Solution Temperature</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid Chromatography Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>Lyz</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>MADIX</td>
<td>Macromolecular Design via Interchange of Xanthates</td>
</tr>
<tr>
<td>MEEA</td>
<td>Methoxy ethoxy ethyl acrylate</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MMA</td>
<td>Methyl Methacrylate</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted Laser Desorption/ionization Time of Flight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>( M_n )</td>
<td>Number Average Molecular Weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Weight Average Molecular Weight</td>
</tr>
<tr>
<td>NTA</td>
<td>Ni(II) Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>NMP</td>
<td>Nitroxide Mediated Polymerization</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGMA</td>
<td>Poly(ethylene glycol) methyl ether methacrylate</td>
</tr>
<tr>
<td>PDS</td>
<td>Pyridyl disulfide</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible Addition-Fragmentation Chain Transfer</td>
</tr>
<tr>
<td>$R_F$</td>
<td>Flory’s Radius</td>
</tr>
<tr>
<td>RID</td>
<td>Refractive Index Detector</td>
</tr>
<tr>
<td>rROP</td>
<td>Radical Ring Opening Polymerization</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Poly(acrylamide) Gel Electrophoresis</td>
</tr>
<tr>
<td>TCEP</td>
<td>Triscarboxyethylphosphine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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</table>
Acknowledgements


I begin my Acknowledgements with those most closely associated with the work herein, followed by a reverse chronological progression towards those who have contributed historically to my academic success and personal development. Therefore, I would first like to thank my research advisor, Prof. Heather D. Maynard, for her dynamic and continued mentorship and support throughout the duration of my graduate studies as well as for her creative and inquisitive mind, for which I have to thank for all of the research presented here. Heather continually molds her mentoring style to the needs of her students, including myself, not only accounting for the diversity of the members of her lab, but also the dynamic nature of each individual’s needs as they change throughout their academic career. She has genuinely expressed and acted upon a personal investment in my success in both research and career goals, and has shown deep empathy during some of the most tumultuous times in my life. Thank you, Heather.

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xxiii
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“Who am I? It’s going to take a while to find myself, and where I belong”
– Caitlin, 2002

Well, I think you figured it out.
Vita

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Chapter 1

General Introduction: Modulating Protein Activity Through Polymer Conjugation
1.1 Motivation for polymer conjugation to protein therapeutics

More than 100 protein drugs are FDA approved, earning 103 billion dollars in sales in 2010\textsuperscript{1,2}. Major challenges for the development of protein therapeutics include instability during transport and storage, as well as rapid renal clearance\textsuperscript{3}. Covalent conjugation of polymers to protein therapeutics can mitigate these issues by both increasing the effective size of the protein therapeutic by enlarging the overall hydrodynamic radius and stabilizing the protein to various enzymes that would normally break it down \textit{in vivo}\textsuperscript{4}. The most commonly employed and studied polymer for therapeutic protein conjugation is poly(ethylene glycol) (PEG), the development and use of which has been extensively reviewed\textsuperscript{5-10}. Eleven PEG-based protein-conjugate therapeutics have been approved by the FDA\textsuperscript{11,12} since the first protein-PEG conjugate was reported by Abuchowski and coworkers in 1977\textsuperscript{13,14}. However, one of them, a PEGylated erythropoietin (Omontys®, peginesatide), was removed from the market due to death related to anaphylaxis. This introduction outlines the progression from the first PEGylated proteins (1.2) to second-generation PEGylated proteins, detailing the addition of degradable linkages and/or polymer backbones (1.3) as well as the adjustment of protein to PEG stoichiometry (1.4), all in the context of modulating therapeutic protein activity. Specifically, this dissertation will focus on the modulation of protein activity through non-reversible covalent, reversible covalent, and non-covalent conjugation to PEG and PEG-like polymers.
1.2 Protein-PEG conjugates with retention of activity

In order to maintain high activity of the protein therapeutic, the size, number, and shape of the polymer, as well as conjugation site(s) must be carefully assessed\textsuperscript{15,16}. In early examples of protein PEGylation, proteins were PEGylated at multiple sites (>5 conjugation sites) with low molecular weight PEGs (2-5 kDa) by nonspecific ligations to multiple amino acid types or specific ligations to lysine residues, which are abundant in most proteins\textsuperscript{8}. This was, in part, due to the limited chemical methodologies for protein conjugation at that time. However, in the early 90s, researchers found that fewer or single modifications with high molecular weight PEGs (>20 kDa) resulted in more active and less immunogenic conjugates with comparable or improved circulation times as compared to the addition of multiple, smaller PEGs. For example, Somack and coworkers modified superoxidase dismutase (SOD) at 1-4 sites with 41-72 kDa PEG, and found that the protein retained 90-100\% native activity, and exhibited less immunogenicity and longer circulation times than SOD modified at 7-16 sites with 5 kDa PEG\textsuperscript{17}. They later found that a single conjugation of a 100 kDa PEG increased circulation time in mice to 36 hours compared to 24 hours with 7 x 5 kDa PEG attachments, and that 3 x 50 kDa PEG resulted in a 5 day circulation time in dogs\textsuperscript{18}. Despite the benefits of larger PEGs, issues with clearance from the body have restricted PEG size to under approximately 30,000 kDa for therapeutic use\textsuperscript{19}. Therefore, an important focus of PEGylation research is the addition biodegradable linkages into the backbone of larger PEG analogs since degradable chains may still be eliminated from the body\textsuperscript{20}. Chapter 2 of this dissertation outlines methods to obtain high molecular weight PEG-like polymer-protein conjugates with degradable backbone linkages.
In addition to the size and number of PEGs, architecture also alters activity. Branching of PEG increases the activity or pharmacokinetic properties of the protein conjugate as compared to linear PEG\textsuperscript{21}. The first branched PEG conjugates were prepared in the late 1990s by coupling of two linear PEG chains to trichloro-s-triazine\textsuperscript{22,23} or to lysine\textsuperscript{24,25} followed by functionalization with a protein reactive group. More recently, controlled radical polymerization (CRP) has allowed for the formation of highly branched end-functionalized PEGs\textsuperscript{26,27}. Haddleton\textsuperscript{28-30}, Maynard\textsuperscript{31,32}, and Matyjaszewski\textsuperscript{33} developed the first branched PEG-protein conjugates via CRP. In the first example (2004), Haddleton utilized an N-hydroxy succinimidyl ester functionalized initiator for the atom transfer radical polymerization (ATRP) of poly(ethylene glycol) methyl ether methacrylate (PEGMA) followed by coupling of the succinimidyl ester end group to lysine side chains of lysozyme\textsuperscript{29}. The activity of this conjugate was not assessed, however. Concurrently, Maynard conjugated site-specifically to cysteine on bovine serum albumin (BSA) via a pyridyl disulfide end-modified poly(2-hydroxyethyl methacrylate)\textsuperscript{34}. Shortly thereafter another site-specific conjugation method was developed by Maynard, by polymerizing PEGMA from a streptavidin macroinitiator\textsuperscript{31}. In a separate example, Matyjaszewski and coworkers acylated an average of one, four, or eight lysines on chymotrypsin with a small molecule initiator, 2-bromoisobutyryl bromide, followed by ATRP of PEGMA\textsuperscript{33}. Although the location of modification varied (several available lysines), singly modified chymotrypsin exhibited higher activity (85\%) than multiply modified chymotrypsin (46-64\%). In addition the branched PEGMA conjugates exhibited similar activity (up to 85\%) to linear PEG conjugates (82\%). While
this early study provided a proof-of-concept for highly active branched-PEG conjugates, the effect on in vivo pharmacokinetics remained to be explored.

There are several recent examples of branched-PEG protein conjugates that exhibit both high initial activity as well as an extended circulation time and improved pharmacokinetics. One example of such highly active, long circulating branched PEG conjugates was developed by Caliceti and coworkers; PEGMA was grafted from lysine side chains (9 total modification sites) of human growth hormone (hGH) via ATRP. Despite the high level of modification, modified hGH had the same initial in vivo activity at day 1 than unmodified hGH, but exhibited higher activity on days 2-10, indicating a gradual release and reduced clearance\textsuperscript{35}. Since for many proteins, non-specific, multiple additions of branched polymers results in significantly decreased activity, Brayden and Haddleton utilized N-terminal reductive amination to compare initial activity and in vivo pharmacokinetics of branched pPEGMA-salmon calcitonin to linear PEG-salmon calcitonin\textsuperscript{36}. Both conjugates, with polymers of the same molecular weight, maintained greater than 85% native activity, with branched PEG exhibiting improved pharmacokinetics over linear PEG. Additional examples of site-specific modification leading to highly active, long circulating conjugates are provided by the Chilkoti group, who have developed highly active p(PEGMA) protein conjugates by polymerizing from the N-terminus of myoglobin\textsuperscript{37} and the C-terminus of green fluorescent protein (GFP)\textsuperscript{38,39}. Myoglobin maintained complete activity after modification and increased blood exposure by 41-fold in mice\textsuperscript{37}. GFP did not lose any fluorescence upon conjugation and exhibited improved the blood exposure 15-fold and tumor accumulation by 50-fold in mice\textsuperscript{38}. Overall, more active conjugates have been developed through the
use of site-specific conjugation techniques. These recent examples of highly active, long circulating branched PEG-protein conjugates implicate the promise of such techniques towards clinically relevant therapeutics. **Chapter 2** of this dissertation describes a branched PEG-like polymer-protein conjugate that retains 63% native activity.

Giving further support for the use of branched-PEG protein conjugates, PEG-interferon alpha 2b (Pegasys) includes a single branch-point and it is a FDA-approved PEGylated therapeutic. While the native activity is reduced to 7%, the improved pharmacokinetic properties including circulation time and stability compensate for activity loss\(^{40}\). A recent evaluation of linear and highly branched PEG-interferon alpha compared to the singly branched Pegasys revealed that highly branched PEG-interferon alpha exhibited the best *in-vivo* pharmokinetics\(^{41}\).

Site-selective conjugation is imperative to retention of native activity\(^{42}\). Conjugation can be non-specific (adding to several different amino acids) or specific (adding to one type of amino acid or residue). Polymers can be end-functionalized to react covalently (to lysine, N or C-termini, or cysteine) or non-covalently (to histidine tags or biotin)\(^{43}\). Another route to site-specific modification is through enzyme-facilitated conjugation to glutamine (glutamination)\(^{44}\) or threonine-serine (gycosylation)\(^{45}\). In addition, non-native amino acids can be incorporated by mutagenesis, allowing for even more selective, orthogonal reactions with initiators or polymers\(^{46}\). The above-mentioned examples of N (myoglobin) and C-terminal (GFP) conjugations are examples of site-specific modification, potentially explaining the high retention of activity. One prominent example of N-terminal conjugation is Neulasta®, a PEGylated version of granulocyte colony-stimulating factor (G-CSF) approved by the
FDA, which maintains high activity (>60%) and has an increased serum half life of 42 hours (from 4 hours). For more selective modification, less abundant amino acids, such as cysteine, are frequently targeted. The versatile reactivity of cysteine makes it ideal for either reversible (reaction with maleimide, pyridyl disulfide, or diselenide) or irreversible (reaction with vinyl sulfone or iodoacetamide) conjugations. For example, fibroblast growth factor 2 (FGF2), which has only two surface-exposed, free cysteines, maintained full activity when PEGylated covalently at both positions. Recently, Jevsevar and coworkers compared three different cysteine conjugation strategies for PEGylation of G-CSF: maleimide, pyridyl disulfide, and a new diselenide. Pyridyl disulfide exhibited the highest in-vivo activity. Conjugating to existing disulfide bridges in proteins can be challenging due to their role in maintaining protein structure, and disruption of these bonds is often responsible for loss of protein activity. However, conjugation to existing disulfide bridges without disruption of protein activity was achieved by Shaunak et al utilizing a bis-thiol PEG reagent with a 3-carbon linker. In order to gain even greater control of modification location, strategies for genetic modification to reduce the number of available cysteines or to incorporate cysteine in proteins lacking the amino acid entirely have been developed. In addition genetic modification allows for control over the location of polymer conjugation, in order to avoid protein active sites or receptor binding cites. Chapter 3 describes the utilization of mutagenesis to provide a single surface-exposed free cysteine distal from protein active sites and receptor binding sites.

Non-covalent interactions with protein tags, such as avidin-biotin or histidine-Ni(II)-nitrilotriacetic acid (NTA) interactions, traditionally used for chromatographic
purification of proteins\textsuperscript{52,53}, have been extended to synthesize protein-polymer conjugates. The first conjugate via an NTA-functionalized polymer was developed by Kiessling in 2004, through post-polymerization side-chain modification of poly(succimidyl methacrylate) with hydroxyethyl or NTA units\textsuperscript{54}. This polymer was then used for Ni(II) chelation and conjugation to fibroblast growth factor 8 (FGF8). The conjugate showed \textit{in-vitro} proliferative activity in engineered BaF3 cells lacking surface heparan sulfate while the unmodified protein did not, indicating that FGF8 had been multimerized by polymer conjugation. However, the activity was not as high as native protein with added heparin. These conjugates were not tested for \textit{in vivo} activity. Further development of NTA-Ni(II)-His6-protein conjugates was performed by the Szoka group who found the highest affinity (nM) for His-tagged proteins was with tri-NTA functionalized liposomes as compared to mono-NTA, however even liposome-tri-NTA-His6-protein conjugates did not improve \textit{in vivo} circulation as compared to the unmodified protein in mice\textsuperscript{55-57}. Recently, Pasut and coworkers extended this methodology to include proteins lacking His-tags by modifying a flexible 8-arm PEG with 8 NTA groups in order to maximize the number of possible interaction with dispersed histidine residues\textsuperscript{43}. In addition, Cu(II) was interchanged with Ni(II) in an attempt to avoid nickel toxicity issues for \textit{in vivo} applications. Successful conjugation was observed for hemoglobin and G-CSF, however conjugation was not successful for hGH, interferon alpha 2b, or insulin because these proteins do not contain enough surface-exposed histidine residues. G-CSF contains five histidine residues that exhibit a nM dissociation constant; however, when tested \textit{in vivo} modification did not increase the half life compared to unmodified protein. Therefore this methodology is not promising
for *in vivo* therapeutic protein delivery. Alternatively, the avidin-biotin association has a 
$K_D$ on the order of $10^{-15}$. In a recent application, Lee and coworkers developed 
radio-labeled avidin-biotin-PEG-endothelial growth factor (EGF) as a tumor imaging 
probe\textsuperscript{58}. Chapter 4 details the synthesis of NTA functionalized polymers for multimeric 
conjugation to His6-tagged proteins.

1.3 Protein-polymer conjugates with masked-unmasked activity

There are many instances in which it is beneficial to mask activity of the protein 
until delivery within a targeted cell, or to gradually release active protein over time. For 
instance neuroprotective growth factors could cause cancer in healthy cells, and so 
masked activity prior to delivery is preferable, and for insulin a gradual and slow release 
is desired to maintain stable glucose levels. One interesting masking and unmasking 
technique, developed by Hoffman and Stayton, is the conjugation of thermally responsive 
polymer(s) near a protein active site, to then allow tunable activity through temperature 
modulation\textsuperscript{59-62}. This methodology has since been extended to PEG-like polymers\textsuperscript{63}. 
Notably, Haddleton and coworkers grafted from salmon calcitonin with 
di(ethyleneglycol) methyl ether methacrylate and tri(ethyleneglycol) methyl ether 
methacrylate, resulting in conjugates exhibiting lower critical solution temperatures 
(LCSTs) of 24 °C, 37 °C, and 51 °C\textsuperscript{64}. The activity based on temperature was not 
assessed, however. Similarly, Lutz modified trypsin with oligo(ethylene glycol)-based 
branched polymers which imparted thermo-responsive activity to the protein\textsuperscript{65}. While the 
modified protein exhibited higher activity, the activity at various temperatures was not 
assessed. Nolte and coworkers created a thermo-responsive bioconjugate using enhanced
green fluorescent protein (EGFP) and ethylene glycol methyl ether acrylate (EGMEA) and methoxy ethoxy ethyl acrylate (MEEA). Aggregation was observed above 35 or 45 °C, depending on the polymer molecular weight. The intended use of these conjugates was protein purification, and therefore use as a protein drug is unlikely. Overall, thermoresponsive PEGylation may be useful to shut-down protein activity, but the applicability for masked/unmasked protein therapeutics has yet to be explored.

More extensively studied is the conjugation of very large branched PEGs (>30 kDa), through multiple conjugations, or through conjugation near an active site to mask protein activity. Unmasking is achieved through the degradation of a single cleavable linkage between the polymer and protein or multiple degradable linkages within the polymer backbone. Examples of such degradable linkages include: maleic anhydride, maleylamino peptide bonds, disulfide, hydrazone, oxime, fluorenylmethoxycarbonyl (FMOC) carbamates, azo-benzene, thioesters, bicin, benzyl carbamate, phenyl carbamate, and enzyme-degradable peptides. Each degradable linkage is designed to respond to a specific in vivo stimulus, such as reductive/low pH environment of endosomes, low pH in tumor cells, esterases present in the bloodstream, or azo-reductases present in the colon.

In the earliest degradable linkage study, Roberts and Harris observed an increase of up to 60% native activity upon hydrolysis of a maleic anhydride linked PEG-lysozyme conjugate. Since then, the only example of an FDA-approved PEGylated protein therapeutic with a degradable linkage is PEG-intron for the treatment of hepatitis C, which contains a carbamate linkage. However, alkyl-carbamate hydrolysis is slow. Therefore, substituted carbamates, which are more easily cleaved, have been developed.
Recently, Goepferich and coworkers assessed release kinetics of various substituted phenyl carbamate-linkages between PEG and lysozyme\textsuperscript{83}. Rate of complete cleavage was regulated from between 24 hours to 28 days with native activity restored to between 73 and 93%; this was achieved by altering phenyl substitutions, with electron withdrawing groups at the \textit{ortho} or \textit{para} positions accelerating hydrolysis via stabilization of the phenolate product. Schecter and coworkers have shown complete masking (<1% activity) and complete recovery of protein activity through the use of FMOC-based carbamate linkages (cleavable through β-elimination) between PEG and insulin\textsuperscript{75,77} and human growth hormone\textsuperscript{76}. Insulin conjugates exhibited an increased circulation time (30 hour half-life) and a 7-fold increase in the extent of glucose-lowering ability as compared to unmodified insulin\textsuperscript{77}. Another recently successful technique, investigated by Pluckthun and coworkers, utilized a rhinovirus 3C-protease sensitive peptide linker between a branched 40 kDa PEG and pseudomonas exotoxin A\textsuperscript{86}. The IC\textsubscript{50} was improved 80-fold in HT29 cells, and cytotoxicity decreased 25-fold in EpCAM-positive tumor cells. Full recovery of cytotoxic activity was observed after treatment with rhinovirus 3C-protease. Recently, Davis and coworkers developed a new reversible linkage based on trivalent arsenicals for conjugation of a branched PEG to the peptide salmon calcitonin, which was completely released by incubation with lipoic acid in 30 minutes. Since two cysteines within close proximity to one another are required, the extension of this chemistry to (natural) proteins may be limited\textsuperscript{88}.

Pyridyl disulfide (PDS) groups allow for the facile and releasable conjugation of PEG and PEG-like polymers to cysteine residues on proteins, and PDS-PEG is commercially available. To extend this methodology to branched PEGs, developed by
CRP, Maynard and coworkers developed a PDS-functionalized ATRP initiator in 2004\textsuperscript{34} and Davis and coworkers developed a PDS-functionalized chain transfer agent (CTA) in 2007\textsuperscript{89}. Since then PDS end-functionalized, branched PEGs have been extensively used for the reversible modification of proteins\textsuperscript{90}. Chapter 2 describes the modulation of lysozyme activity through a reversible disulfide linkage to a branched PEG. As an alternative to PDS, Davis and coworkers developed a thiozolidine-2-thione based CTA, which contains a reducible disulfide linkage and reacts with lysine side-chains to form amide bonds. Reversible addition-fragmentation chain transfer (RAFT) polymerization of PEGMA with this CTA allowed for the reversible conjugation of an average of 3 pPEGMAs to lysozyme\textsuperscript{91}. Activity was masked upon conjugation (33% native activity) and unmasked upon reductive release (75% native activity).

In addition to protein release through a single degradable linkage, degradability can also be incorporated throughout the polymer backbone. Backbone-degradable polymers allow for the use of even larger polymers (for masking) because the large polymers can gradually be broken down for clearance, preventing negative effects due to polymer build-up. Several backbone-degradable PEGs have been developed\textsuperscript{92-95}, yet none have been conjugated to a protein. In addition, degradable linkages can easily be incorporated into the backbone of branched PEGs through the use of cyclic ketene acetal (CKA) comonomers for CRP\textsuperscript{96-101}. Despite their development, neither backbone degradable linear PEGs nor backbone degradable branched PEGs had yet been conjugated to a protein. Chapter 2 describes the first example of such a conjugation, specifically through RAFT copolymerization of PEGMA and a CKA followed by conjugation to lysozyme.
1.4 Multimerized protein-polymer conjugates exhibiting enhanced activity

Protein activity is often dependent on dimerization or multimerization of the protein and concurrent receptor dimerization or multimerization, and synthetic multimerization has thus been studied extensively\(^{102-115}\). Enhanced activity results from synthetic dimerization because there is an increase in the local concentration for the second ligand once the first ligand is bound to its receptor\(^{116,117}\). Small molecule ligands are often dimerized with linear PEG to block receptors in their more stable dimerized form. For instance, McAlpine and coworkers recently developed dimeric inhibitors of heat shock protein 90, which is upregulated in cancer cells\(^{118}\). Two different length PEGs were chosen based on the crystal structure of the receptor dimer complex, and both significantly increased receptor blocking as compared to the monomeric inhibitor. Despite the success of small molecule dimerization via PEG linkers, protein dimerization via PEG has not been as extensively studied. Instead, small molecule induced dimerization of protein ligands has been extensively studied, and Houk and coworkers have reviewed various linkers based on length\(^{117}\). In 2001 Seely and Richey described the synthesis of protein homodimerization via a 20 kDa bis-vinylsulfone PEG and purification by high pressure liquid chromatography (HPLC), yet no studies on protein activity were carried out\(^{119}\). A more recent example of PEG-linked protein dimerization by Imperiali and coworkers examined the effect of linker length on the activity of EGF.\(^{104}\) Superagonist activity was noted for conjugates with PEG-linker lengths close to the distance between conjugation sites, as calculated by referencing the protein: receptor
crystal structure. Chapter 3 focuses specifically on PEG-linked dimerization of FGF2, and improves on methodologies previously investigated by others\textsuperscript{120-124}, while Chapter 4 investigates a more general route to multimerization of His-tagged proteins, building on previous and ongoing work involving homodimeric and heterodimeric conjugates through telechelic CRP-based polymers\textsuperscript{125-127} and the study of dimerization through branched-PEGs and various protein-specific polymer architectures.

1.5 Concluding remarks on the Introduction to the Dissertation

The following chapters of the dissertation describe various methodologies towards highly active, activatable, or superagonist PEGylated proteins. Linear (Chapter 3) and branched (Chapters 2 and 4) PEGs are developed. Both nonspecific and site-specific conjugates are described including random lysine modification (Chapter 2), genetic modification of a protein to facilitate site-specific conjugation (Chapter 3), as well as the synthesis of a polymer capable of N-terminal His6-tag conjugation (Chapter 4). Non-reversible covalent (thioether, Chapter 3), reversible covalent (disulfide, Chapter 2), and non-covalent (Ni\textsuperscript{2+}-NTA, Chapter 4) linkages are described. Protein activity is modulated through these linkages as well as through degradable linkages within a branched-PEG backbone (Chapter 2) or through protein dimerization (Chapter 3). Successful modulation of protein activity was achieved through rational conjugate design.
1.5. References


71. Brocchini, S.; Godwin, A.; Balan, S.; Choi, J.-w.; Zloh, M.; Shaunak, S.

*Advanced Drug Delivery Reviews* **2008**, *60*, 3.


Chapter 2

Degradable PEGylated Protein Conjugates

Utilizing RAFT Polymerization†
2.1. Introduction

Covalent attachment of PEG-based polymers is known to improve the pharmacokinetics of protein therapeutics through stabilization and improved circulation time\(^1\). As a result there are many FDA-approved, PEGylated therapeutic agents on the market\(^2\). Protein conjugation to branched PEG-like polymers, such as pPEGMA, prepared by controlled radical polymerization (CRP), has also been shown to improve protein pharmacokinetics\(^3\). Despite these advantages, PEGylation has several drawbacks. Typically polymer attachment results in decreased activity of the protein\(^4\), and long-term treatment with PEGylated therapeutics can result in PEG accumulation in the liver and spleen, hypersensitivity, the development of anti-PEG IgM antibodies, and lysozomal disease syndrome\(^5\). Therefore, PEG-like polymers, containing a degradable linkage and/or degradable moieties in the backbone are important to circumvent these issues\(^6\,7\).

Degradable linkages at the site of attachment between the polymer and protein are often installed so that the protein can be released (hydrolytically, enzymatically, or reductively) from the polymer \textit{in-vivo}\(^8\). Such linkages include maleylamino peptide bonds\(^9\), carbamate\(^10\), ester\(^11\), disulfide\(^12\), hydrazone\(^13\), and oxime\(^14\) bonds. For instance, PEG-Intron\(^\text{®}\) was designed with a degradable carbamate linkage to interferon alpha-2b\(^15\). Cleavage of the polymer results in regained protein activity. Roberts and Harris reported PEGylation of lysozyme (Lyz) through a degradable ester linkage; upon hydrolysis of the ester, the activity of Lyz was regained to 60\% of the native activity\(^4\). However, in these cases the PEG backbone itself is non-degradable, and thus negative effects associated with polymer accumulation could persist. To prevent this, enzymatically or hydrolytically degradable moieties such as esters\(^16\), vinyl ethers\(^17\), acetals\(^18\), oximes, or
urethanes\textsuperscript{19}, as well as reduction sensitive disulfides\textsuperscript{20} have been installed in the backbone of PEG. However, to our knowledge, main-chain degradable PEG-like polymers have not yet been conjugated to a protein. Several backbone degradable non-PEG polymer-protein conjugates have been developed. Most of these conjugates consist of sugar-based or sugar-derived polymers such as hydroxyethyl starch\textsuperscript{21}, polysialic acid\textsuperscript{22}, dextran\textsuperscript{23} or dextrin\textsuperscript{24}. In addition, recently, ring opening polymerization has been used to synthesize a poly(ε-caprolactone) which was covalently bound to bovine serum albumin\textsuperscript{25}. However, none of these methods result in PEG-like polymers. Herein, we describe the combination of both a degradable linkage and a degradable backbone as an approach for next generation PEGylated protein therapeutics (Figure 2.1).

![Figure 2.1. PEGylated protein conjugate, released by either reduction or hydrolysis.](image)

CRP offers easy end-group functionalization, well-defined polymer molecular weights, and compatibility with a wide variety of monomers. Therefore, much attention
has been paid to the development of CRP techniques as a means to develop well-defined, PEG-like polymer-protein therapeutics\textsuperscript{26}. Coupling of radical ring-opening polymerization (rROP) of cyclic ketene acetals (CKAs) with CRP techniques including atom transfer radical polymerization (ATRP)\textsuperscript{27,28}, nitroxide mediated polymerization (NMP)\textsuperscript{29}, RAFT polymerization and macromolecular design via interchange of xanthates (MADIX)\textsuperscript{30-32} has led to polymer backbones that are degradable. These CKA polymers have been covalently conjugated to drugs\textsuperscript{33}. However to our knowledge degradable CKA polymers prepared by CRP have not been covalently attached to proteins. In the work described here, we utilized RAFT polymerization to prepare polymers that are degradable, protein-reactive, and PEG-like. Furthermore, we demonstrate the conjugation of these degradable PEG-like polymers to a protein, specifically Lyz, through a reversible disulfide linkage.
2.2. Results and Discussion

Scheme 2.1. (a) RAFT polymerization of PEGMA and BMDO (b) Covalent modification of thiolated Lyz with polymer 1 or 2.

2.2.1. Synthesis of PDS-p(PEGMA-co-BMDO)s 1 and 2

We chose to install the cysteine reactive chain-end by using a PDS-modified chain transfer agent (CTA), 3-(pyridine-2-yl disulfanyl)propyl-2-(ethylthiocarbonothioylthio) propanoate. While this CTA is not ideal for the polymerization of methacrylates, Junkers found that the addition of BMDO as a comonomer resulted in well-defined polymers with a similar CTA, and BMDO has been shown to exhibit favorable reactivity with methacrylates under typical CRP conditions. Two random copolymers, PDS-p(PEGMA-co-BMDO) (1 and 2) were synthesized by RAFT polymerization, with $M_n$ ($^1$H-NMR) of 14.8 kDa and 45.0 kDa and $M_n$ (GPC) of 10.9 kDa and 20.9 kDa, (as compared to poly(methyl methacrylate)
(PMMA) standards) with molecular weight dispersities of 1.34 and 1.71 (experimental details are in the SI). The molecular weight dispersities are broad, as observed for other CKA polymers\textsuperscript{27,30}. Yet, the resulting polymers clearly contained the resulting pyridyl disulfide end group for conjugation to free cysteines on proteins (see NMR spectra, \textbf{Figure 2.5a} and \textbf{Figure 2.6a}). The polymers had an average of 10.5\% and 9\% BMDO units per polymer chain, respectively, determined through analysis of $^1$H-NMR peak ratios. Reactivity ratios for BMDO and MMA have been reported under ATRP conditions as $r_1 = 0.53$ and $r_2 = 1.96$ by Wickel\textsuperscript{40} however Junkers assessed reactivity ratios under RAFT conditions at low conversions to avoid the affects of composition drift, and determined the ratios to be $r_1 = 0.33$ and $r_2 = 6$. These ratios indicate that our polymers are likely gradient copolymers, with more BMDO units incorporated later in the polymer chain. We also observed very low BMDO incorporation (11\% for 1 and 7\% for 2).

\subsection*{2.2.2. Hydrolytic Degradation of 1 and 2}

We next investigated the polymer degradation kinetics under acidic, basic, and physiological conditions. Stability at neutral and mildly basic conditions would be ideal for storage and delivery to the blood stream while acidic degradation would be advantageous for release upon cell uptake in the acidic endosome or for oral drug delivery. Complete degradation has been shown for similar polymers incubated in complete cell medium for one week\textsuperscript{31} In addition, p(PEGMA-co-BMDO), synthesized by ATRP, was shown to completely degrade within 24 hours after incubation with 5\% KOH, and partially degrade under acidic conditions (pH 3-5)\textsuperscript{27,28}. \textsuperscript{31}Therefore, to test the
degradation of our polymers, we applied the following conditions: 5% KOH, 0.5 M tosic acid, 100 mM carbonate / bicarbonate buffer (pH 10), MilliQ water acidified to pH 4 with HCl, and D-PBS, pH 7.4.

After incubation in 5% KOH, GPC indicated polymer degradation by a shift in molecular weights from 10.9 kDa to 4.4 kDa fragments for 1 and 20.9 to 3.0 kDa fragments for 2 with final dispersities of 1.2 and 1.4, respectively (Figure 2.2). 1H-NMR analysis of the polymers indicated that after incubation for only one day in 5% KOH complete hydrolysis was achieved as visualized by the disappearance of BMDO methylene proton signals at 5 ppm for both polymers (see supporting information Figure 2.8 and 2.9). These results confirm that both polymers can be degraded into fragments ideal for rapid clearance from the body. For 1 tosic acid and 100 mM carbonate / bicarbonate (pH 10) buffer partially degraded the polymer backbone, while D-PBS (pH 7.4) and MilliQ water acidified to pH 4.0 did not significantly degrade the polymer within one week (Figure 2.7). For the longer polymer 2, tosic acid partially degraded the polymer backbone, but 100 mM carbonate / bicarbonate (pH 10) buffer, D-PBS (pH 7.4), and MilliQ water acidified to pH 4.0 did not significantly degrade the polymer (Figure 2.7). To investigate long-term degradation, timepoints at 1 and 7 months for polymer 2 in either D-PBS or 100 mM carbonate / bicarbonate (pH 10) were assessed. The polymer was stable at pH 7.4 over the 7-month time period while at pH 10 the polymer degraded to 18.3 kDa ($D = 1.7$) after 1 month and to 8.2 kDa ($D = 1.7$) after 7 months. Therefore the polymers could be expected to remain intact at physiological pH while a very gradual degradation of the polymer could be expected under mildly basic conditions. The larger dispersities of these fragments are expected for gradient copolymers.
2.2.3. Conjugation of 1 and 2 to Thiolated Lysozyme

Lyz was chosen as a model protein for polymer conjugation. Thiols are often added to the lysine side-chains of proteins to allow for single polymer-chain conjugation. Therefore thiolated Lyz was conjugated to polymer 1 and 2 (Scheme 2.1b) resulting in Lyz-1 and Lyz-2 conjugates visualized by gel electrophoresis as smeared bands (Figure 2.3 lanes 7 and 8). Such smears are expected due to variation in polymer molecular weight, and are commonly seen with proteins conjugated to non-monodisperse polymers. Indeed, the polymers alone result in smear bands when stained with iodine (data not shown).
2.2.4 Hydrolytic or Reductive Cleavage of 1 or 2 from Lyz-1 or Lyz-2

Scheme 2.2. Reductive or hydrolytic cleavage of polymer 1 or 2 from Lyz

Enzymatic or hydrolytic degradation of the polymer chain from the conjugates should occur in-vivo. Similar polymers have been shown to degrade partially via enzymes, for example lipases, or completely in cell culture medium such as Dulbecco’s Modified Eagle Medium (DMEM). However, due to the difficulty of separating the released Lyz from other enzymes and proteins in medium, we chose to visualize the release of Lyz under basic conditions by gel electrophoresis. Since the polymer alone degraded to the greatest extent with 5% KOH, this condition was chosen to quickly visualize the hydrolytic degradation of the polymer chain from Lyz (Scheme 2.2). The purified conjugates were incubated with 5% KOH for 24 hours and complete release of
Lyz in both cases was observed (Figure 2.3 lanes 4, 5 reducing and 9, 10 nonreducing). \(^1\)H-NMR analysis of polymer 1 indicated an average of 5 BMDO units, and therefore complete cleavage of the backbone should result in 6 fragments. The expected \(M_n\) of the polymer fragments after complete hydrolysis is 10.9 kDa / 6 = 1.8 kDa. For 2, the original polymer contained 14 BMDO units / polymer chain, which would result in 15 cleavages, with \(M_n = 1.4\) kDa. Therefore, the degradation products were also analyzed by mass spec, resulting in fragments with \(m/z = 1.9\) kDa for both size conjugates indicating complete degradation. As expected some degradation of Lyz itself was observed under such harsh conditions (seen as a smear below the normal Lyz band in Figure 2.3 lanes 4, 5). However, these results serve as a proof of concept, indicating that the polymer backbone can be hydrolytically cleaved from the conjugates.

![Figure 2.3. SDS-PAGE gel electrophoresis, visualized by Coomassie blue staining, of Lyz-1 and Lyz-2 before and after treatment with reducing agent and/or base (lane 1: protein marker; lane 2: Lyz-1 reducing conditions; lane 3: Lyz-2 reducing conditions; lane 4: Lyz-1 KOH treated, reducing conditions; lane 5: Lyz-2 KOH treated, reducing conditions)](image-url)
conditions; lane 6: Lyz; lane 7: Lyz-1; lane 8: Lyz-2; lane 9: Lyz-1 KOH treated; lane 10: Lyz-2 KOH treated).

2.2.5 Activity of Lyz-1 and Lyz-2 before and after reductive treatment with glutathione

In the body, the polymer chain may either hydrolytically degrade off of the conjugates, or reductively cleave prior to hydrolytic degradation. To visualize the reductive cleavage of the polymer chain from Lyz, the conjugates were incubated with 0.65 M DTT at 95 °C for 6 minutes (Scheme 2.2). Complete reduction of the disulfide bond and release of Lyz was observed (Figure 2.3, lanes 2 and 3).

![Bar chart](image)

**Figure 2.4.** Activity of native lysozyme + one equivalent of (non attached) polymer in solution, Lyz-polymer conjugates before and after treatment with GSH, and Lyz after
cleavage from Lyz-2. Lyz-1 was treated with 5 mM GSH and Lyz-2 was treated with 10 mM GSH.

More physiologically relevant reducing conditions (5 or 10 mM GSH) were used to analyze the activity of Lyz released from the polymers (Figure 2.4). Original activity of Lyz-1 was 63 +/- 3 % of native Lyz, which is fairly high. For comparison, Roberts and Harris observed 60% activity only after cleavage of the polymer^4. This high activity is likely due to the smaller size of the polymer as well as the addition of a single polymer chain per Lyz. After treatment with 5 mM GSH for 6 hours at 4 °C, the activity of Lyz-1 increased to 77 +/- 2% indicating incomplete release of Lyz within that time period. The activity of conjugate Lyz-2 was 19.6 +/- 1.9%. This lower activity was likely due to the large size of the polymer. However, after treatment with 5 mM GSH at 4 °C for 6 hours, the activity did not significantly increase and incomplete cleavage of the polymer chain from Lyz as visualized by gel electrophoresis and FPLC (data not shown). The large size of polymer 2 may shield Lyz from the reducing agents. After treatment with 10 mM GSH at room temperature for 4 days, the activity increased to 39.4 +/- 2.0%. To ensure that the released Lyz was active, we purified the reduced conjugate Lyz-2 by FPLC, and separated the released Lyz from the uncleaved conjugates. This released Lyz exhibited 100.7 +/- 3.7% native activity, indicating that the released Lyz is completely active. It should be noted that adding 1 or 2 did not result in decreased activity demonstrating that the observed decreases were a result of covalent conjugation.

Overall, these results indicate that the initial activity of a protein therapeutic can be adjusted by varying the length of the polymer chain, and that the protein activity
increases under reducing conditions that are similar to those found in the endosome or lysosome. There are some cases when high initial activity of a protein-polymer conjugate would be desired. Polymer 1 is a good choice in this case. However, there are other cases when it is desirable to mask the activity of the protein until a triggered release in order to avoid off-target effects. In this case, polymer 2 is a good choice because the activity is reduced considerably, yet 100% active protein is produced upon cleavage of the polymer.

It is the degradability of the polymer main chain that is the significant advantage of these polymers for biomedical use. Small polymers (less than 30 kDa for PEG) are eliminated from the body\(^5\). Yet current data on the observation of PEG aggregates indicates that this is a significant challenge, particularly for replacement therapies where the protein conjugate would be administered over a long period of time. For instance, PEGs as small as 20 kDa have been found to cause vacuolization with repeated administration or with high dosage\(^5\). Furthermore, it is known that larger polymers result in better pharmacokinetics. Yet, polymers that are too large cannot be used because they are not cleared from the body. With the approach reported herein, it is expected that the polymer will be released from the protein and subsequently degraded, avoiding polymer accumulation for any size polymer and allowing secretion of even larger polymers. Thus, by having a dually degradable polymer (through backbone hydrolysis and reductive cleavage) as described herein, such conjugates should exhibit the advantage of enhanced pharmacokinetics, with enhanced activities and reduced accumulation in the body.
2.3. Experimental

2.3.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. The chain transfer agent (CTA), 3-(pyridine-2-yldisulfanyl)propyl-2-(ethylthiocarbonothioylthio) propanoate was synthesized as previously described\textsuperscript{34}. 2,2-azobis(2-methylpropionitrile) (AIBN) was recrystallized from acetone.

2.3.2 Analytical Techniques

NMR spectra were obtained on an Avance 500 MHz DRX spectrometer. Proton NMR spectra were acquired with a relaxation delay time of 2 seconds for small molecules and 10 seconds for all polymers. MALDI-TOF mass spectrometry was performed on an Applied Biosystems Voyager-DE STR and operated in linear mode with an external calibration. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 µm mixed D columns. LiBr (0.1 M) in dimethylformamide (DMF) at 40 °C was used as an eluent (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) (PMMA) standards from Polymer Laboratories. SDS-PAGE was performed using Bio-Rad Any kD Mini-PROTEAN-TGX gels. SDS-PAGE protein standards were obtained from Bio-Rad (Precision Plus Protein Pre-stained Standards). For SDS-PAGE analysis, approximately 5 µg of protein was loaded into each lane. Samples were incubated with 0.65 M DTT in laemmli buffer (20 µL total) at 95 °C for 6 minutes for reducing lanes. Fast protein liquid
chromatography (FPLC) was performed on a Bio-Rad BioLogic DuoFlow chromatography system equipped with a GE Healthcare Life Sciences Superdex 75 10/300 column. For Lyz and Lyz conjugates, D-PBS (pH 7.4) at 4 °C was used as the solvent (flow rate: 0.5 mL/min). Protein and conjugate concentrations were determined using the Thermo Scientific Pierce Coomassie Plus (Bradford) Protein Assay. Lyz activity was determined using the EnzChek® Lysozyme Assay Kit (E-22013) from Molecular Probes.

2.3.3. Methods

Synthesis of 5,6-benzo-2-methylene-1,3-dioxepane (BMDO)

BMDO was synthesized following literature procedures, taking extra precaution to prevent hydrolysis of the product. All glassware was oven-dried overnight. 20 mL of tert-butanol was distilled into a round bottom flask. 5,6-benzo-2-(bromomethyl)-1,3-dioxepane (4.5 g, 18.6 mmol) and potassium tert-butoxide (2.1 g, 18.6 mmol) were then added to the flask with stirring, under argon. The solution was brought to reflux and then heated at 100 °C for 12 hours, with a water-cooled condenser. The solvent was removed by rotatory evaporation and immediately placed back under inert atmosphere. 25 mL ether, dried by passing through an activated alumina column, was added to the crude mixture, which was then immediately filtered over celite to remove any remaining solids, into an oven-dried collection flask. Ether was then removed from the filtrate via rotatory evaporation. The crude product (a yellow oil) was then distilled under vacuum. The product was collected at 200 atm, 80 °C as a hard, white solid. Some fluffy white
crystals were collected at 70 °C, but contained impurities, so a second vacuum distillation was performed. Yield: 1.87 g (62%). δ ¹H-NMR 500 MHz (CDCl₃): 7.41-6.95 (m, 4H), 5.07 (s, 4H), 3.73 (s, 2H). δ ¹³C-NMR 500 MHz (CDCl₃): 164.27, 135.85, 127.46, 126.21, 72.10, 69.54.

**Synthesis of PDS-p(PEGMA-co-BMDO) (1)**

Reversible addition-fragmentation chain transfer (RAFT) polymerization was employed to copolymerize poly(ethylene glycol) methyl ether methacrylate (PEGMA) and 5,6-benzo-2-methylene-1,3-dioxepane (BMDO). An initial feed ratio of 0.5:1:50:50 for AIBN:CTA:PEGMA:BMDO was used. AIBN (2.0 mg, 12.3 μmol), the CTA (9.7 mg, 24.7 μmol), PEGMA (0.35 mL, 1.23 mmol), BMDO (200.0 mg, 1.23 mmol), and 2.0 mL of dry DMF were placed into a 100 mL schlenk tube and subjected to five freeze-pump-thaw cycles before immersion in an oil bath at 70 °C. Aliquots were taken for time points and diluted in DMF and CD₃CN for analysis by GPC and ¹H-NMR, respectively. Percent conversion was calculated by comparing the sum of the integrations of vinylic protons from PEGMA (6.18-5.96 ppm) in the ¹H-NMR spectrum to the integration of regions where both PEGMA and the growing polymer chain overlap (4.46-3.99 ppm). BMDO conversion was not calculated due to the close proximity of the monomer peaks and those of the growing polymer chain. The polymerization was stopped at 71% PEGMA conversion after 4.62 hours by exposing the reaction mixture to atmosphere, and cooling with liquid nitrogen. The polymer was purified by extensive dialysis (Spectra/Por® Regenerated Cellulose Dialysis Membrane, MWCO 6-8000) against MeOH followed by 1:1 MeOH : MilliQ water, and then MilliQ water alone before
lyophilization to remove water. The final molecular weight was determined by comparing the integrations for the aryl BMDO units (subtracting one proton for the PDS end-group) from 7.67-6.89 ppm and the PEGMA side-chain protons from 4.49-3.17 ppm to the PDS end-group proton at 8.44 ppm. Using this analysis, the final polymer was found to contain 5.3 BMDO units and 45.3 PEGMA units, resulting in an $M_n$ of 14.8 kDa, and a BMDO content of 10.5%. $\delta$ $^1$H-NMR 500 MHz (CD$_3$CN): 8.41 ppm (1H, PDS end-group NCH), 7.75 ppm (2H, PDS end-group NCCHCH and NCHCH), 7.57-6.90 ppm (22 H, PDS end-group NCCHCH and BMDO aryl CH), 5.26-4.87 ppm (10 H, backbone BMDO ester COOCH$_2$CCCH$_2$), 4.68 ppm (2H, Z end-group after BMDO unit, CCH$_2$S), 4.39-3.17 ppm (PEGMA side-chains), 3.00-0.27 ppm (polymer backbone). $M_n = 10.9$ kDa by GPC, $D = 1.34$ by GPC.

Figure 2.5a. $^1$H-NMR (CD$_3$CN) of PDS-p(PEGMA-co-BMDO) 1
**Figure 2.5b.** Kinetics for the copolymerization of PEGMA and BMDO yielding PDS-p(PEGMA-co-BMDO) 1

**Synthesis of PDS-p(PEGMA-co-BMDO) (2)**

An initial feed ratio of 0.5:1:200:200 for AIBN:CTA:PEGMA:BMDO was used. AIBN (1.0 mg, 6.2 µmol), the CTA (4.9 mg, 12.3 µmol), PEGMA (0.70 mL, 2.47 mmol), BMDO (400.0 mg, 2.47 mmol), and 4.3 mL of dry DMF were placed in a 100 mL schlenk tube and subjected to five freeze-pump-thaw cycles before immersion in an oil bath at 70 °C. Aliquots were taken for time points and diluted in DMF or CD$_3$CN for analysis by GPC and $^1$H-NMR, respectively. Percent conversion was calculated as described previously. The polymerization was stopped at 59% PEGMA conversion after 4.75 hours, by exposing the reaction mixture to atmosphere, and cooling with liquid nitrogen. The final molecular weight, by $^1$H-NMR, was determined as described above. The polymer chain was found to contain 141.1 PEGMA units and 13.9 BMDO units, with a final $M_n$ of 45.0 kDa, and BMDO content of 9%. $\delta$ $^1$H-NMR 500 MHz (CD$_3$CN): 8.44 ppm (1H, PDS end-group NCH), 7.79 ppm (2H, PDS end-group NCCHCH and NCHCH), 7.67-6.89 ppm (57 H, PDS end-group NCCCHCH and BMDO aryl CH), 5.31-4.93 ppm (26 H, backbone BMDO ester COOCH$_2$CCCH$_2$), 4.73 ppm (2H, Z end-group
after BMDO unit, CCH₂S), 4.49-3.17 ppm (PEGMA side-chains), 3.04-0.29 ppm (polymer backbone). $M_n$ (GPC) = 20.9 kDa, $D$ (GPC) = 1.71.

Figure 2.6a. $^1$H-NMR (CD₃CN) of PDS-p(PEGMA-co-BMDO) 2

Figure 2.6b. Kinetics for the copolymerization of PEGMA and BMDO yielding PDS-p(PEGMA-co-BMDO) 2

Hydrolytic Degradation of 1 and 2
7.8 mg of either polymer (0.7 μmol for 1 and 0.4 μmol for 2) was weighed into each of five 1.5 mL eppendorf tubes. 1 mL of either: 5% KOH, 0.5 M tosic acid in MilliQ water, MilliQ water acidified to pH 4 with HCl, D-PBS (pH 7.4), or 100 mM Carbonate / Bicarbonate (pH 10) was added to a tube and the sample placed on a rotating plate at 4 °C. Degradation was analyzed over one week, with timepoints taken at 1, 3, and 7 days. For 1 in D-PBS, day 4 was analyzed instead of day 3. Timepoints at 1 and 7 months for polymer 2 were also taken for samples diluted in D-PBS and 100 mM Carbonate / Bicarbonate (pH 10) to assess long-term stability under mild conditions. 250 μL of each sample was lyophilized, dissolved in DMF, 0.1 M LiBr, filtered through a 0.2 μ filter, and analyzed by GPC.

![Figure 2.7](image)

**Figure 2.7.** Degradation kinetic analysis by GPC of PDS-p(PEGMA-co-BMDO) a) 1 and b) 2

**1H-NMR Analysis of Hydrolytic Degradation of 1 and 2**

20 mg of either polymer (1.83 μmol for 1 and 0.96 μmol for 2) was dissolved in 500 μL of 5% KOH in a 1.5 mL eppendorf tube, placed on a rotary plate at room temperature,
and allowed to incubate for 24 hours. The solution was then lyophilized to dryness and the resulting gel dissolved in CD$_3$CN for $^1$H-NMR analysis.

Figure 2.8. $^1$H-NMR (CD$_3$CN) comparison of PDS-p(PEGMA-co-BMDO) 1 before (top) and after (bottom) hydrolysis with 5% KOH for 24 hours
Figure 2.9. **1**H-NMR (CD$_3$CN) comparison of PDS-p(PEGMA-co-BMDO) 2 before (top) and after (bottom) hydrolysis with 5% KOH for 24 hours

**Typical Conjugation of Thiolated Lysozyme 1 or 2 (Lyz-1, Lyz-2)**

Lyz from hen egg-white was thiolated as previously described$^{37,38}$, and an average of 0.7 thiols / protein (verified by Ellman’s assay) were installed. 2.83 mg (0.20 μmol) of thiolated Lyz (stored on TCEP resin at 4 °C), dissolved in 500 μL of D-PBS was placed in a LoBind eppendorf tube. 10 equivalents (based on end-group determined molecular weight by 1H-NMR) of either polymer was then dissolved in 1 mL of D-PBS and added to the eppendorf tube. For the 10.9 kDa polymer (14.8 kDa by 1H-NMR), 29.2 mg (1.98
μmol) was added. The solution was then placed on a rotating plate at room temperature for 4 hours, followed by concentration by ultracentrifugation (10 kDa MWCO Centriprep®, Millipore). This solution was then purified by FPLC. Unmodified Lyz eluted around 35 minutes, while Lyz-1 and Lyz-2 eluted between 20-31 minutes. Conjugates were characterized by SDS-PAGE, the concentration was determined by Bradford assay, and the activity was analyzed using the EnzChek® Lysozyme Assay Kit.

**Typical Reduction of 3 and 4 with Dithiothreitol**

1 to 5 μg of Lyz-1 or Lyz-2 were diluted in 20 μL of lamelli buffer with 0.65 M DTT. The samples were incubated at 95 °C for 6 minutes before loading into a gel lane for SDS-PAGE analysis.

**Typical Hydrolytic Cleavage of 3 and 4 with 5% KOH**

Lyz-polymer conjugates (about 58 μg of Lyz-1 or Lyz-2) were diluted in 200 μL of degassed MilliQ water, 5% KOH (final concentration 0.29 mg/mL) and allowed to incubate on a rotating plate at 4 °C for 24 hours. The solution was then neutralized by ultracentrifugation (10 kDa MWCO Centriprep®, Millipore) with D-PBS for four ten-minute cycles at 12 rpm to a final volume of 40 μL, to be used for SDS-PAGE analysis.

**Reduction of Lyz-1 and Lyz-2 with Glutathione**

Lyz-polymer conjugates (0.71 mg/mL for Lyz-1 and 0.51 mg/mL for Lyz-2) were incubated with 5 mM glutathione (GSH) on a rotating plate at 4 °C for 6 hours. The polymer was not released under these conditions for Lyz-2, and therefore treatment with
10 mM GSH on a rotating plate at room temperature over 4 days was analyzed. FPLC purification of Lyz-2 after reduction separated unmodified Lyz (33-39 minutes) from uncleaved conjugates (20-31 minutes). Concentrations were determined by Bradford assay and activity analyzed using the EnzChek® Lysozyme Assay Kit.
2.4. Conclusions

In this report we described the synthesis of two protein-reactive, PEGylated, backbone degradable polymers by RAFT polymerization and the conjugation of these polymers to Lyz. RAFT copolymerization of cyclic ketene acetal (CKA) monomer 5,6-benzo-2-methylene-1,3-dioxepane (BMDO) with PEGMA yielded two polymers with number-average molecular weight ($M_n$) (GPC) = 10.9 and 20.9 kDa and molecular weight dispersities ($D$) = 1.34 and 1.71, respectively. Hydrolytic degradation of both polymers from Lyz with base as well as the reductive cleavage of the polymer chains from Lyz with DTT was observed. The smaller 10.9 kDa polymer-Lyz conjugate retained 63% activity and the larger 20.9 kDa polymer-Lyz conjugate retained only 19.6% native activity, which regained 100% activity when slowly released by reduction with GSH. These results indicate that such degradable polymers should be explored in the field of polymer-protein therapeutics both with PEG-like and tailored polymers, and these studies are underway.
2.5. References

†Portions of Chapter 2 have been published as: Decker, C. G.; Maynard, H. D. *Eur. Poly. J.* 2015, *65*, 305.


Chapter 3

Fibroblast Growth Factor 2 Dimer with Superagonist Activity Improves Wound Healing
3.1 Introduction

Healing in chronic wounds including venous, arterial, diabetic\(^1\), and pressure\(^2\) ulcers is impaired due to decreased growth factor production, keratinocyte and fibroblast proliferation and migration, granulation tissue formation, and angiogenesis. Treatment of chronic wounds costs over 9.5 billion dollars annually, worldwide.\(^3\) Diabetes alone is projected to affect 439 million adults (ages 20-79) by 2030 globally, and 15% of diabetic patients develop chronic foot ulcers\(^4\). Viable treatment of chronic wounds represents a significant challenge to the medical community.

Fibroblast growth factor 2 (FGF2), a growth factor whose expression is impaired in both diabetic and pressure ulcers,\(^1,2\) moderates cell proliferation, differentiation and migration of multiple cell types. FGF2 is critical in wound healing, angiogenesis, bone regeneration, neuroregeneration, and can even result in scarless healing\(^5,6\). While FGF2 alone appears to be a good candidate for the treatment of chronic wounds, and is approved in Japan (Fiblast or Trafermin) for skin ulcers, US and European clinical trials (Phase II for treatment of peripheral arterial\(^7\) and coronary\(^8\) disease and Phase III for neuropathic diabetic foot ulcers\(^9\), respectively) have shown minimal effectiveness. Previous clinical trials have also shown minimal improvement, likely due to the low level of receptor expression in chronic wounds and increased inflammation with high dosage\(^10\). One concern with growth factor treatment in general is an increased risk of cancer, especially with high dosages, and an increased observance of malignancies was found in some clinical trials\(^11\). A highly effective growth factor treatment with low dosage would mitigate these challenges currently experienced at the clinical level. We have developed
an FGF2 with superagonist activity that improves the quality of wound healing in vivo at exceptionally low doses through intentional modification of FGF2.

FGF2 activity is dependent on the formation of a tetrameric complex, consisting of two FGF2 proteins and two FGF receptors (FGFR1)\textsuperscript{12,13}. Many proteins, like FGF2, exist or self-assemble into homodimers or multimers in their native or active state and these structures are often required for protein activity.\textsuperscript{14,15} Synthetic routes to protein dimerization are pursued in the scientific community as a means to study protein interactions and to create superagonist protein therapeutics\textsuperscript{16-19}. Preorganization of dimeric ligands is known to increase the effective local concentrations, thereby facilitating activation of receptors\textsuperscript{20,21}. Nonspecific chemical crosslinking of FGF2 lysine side-chains through reaction with short (11.4 Å)\textsuperscript{22-24} tethers such as bis(sulfosuccinimidyl) suberate has been performed as a means to study the interaction of FGF2 with heparin oligomers, mimics of membrane-bound heparan-sulfates which are known to facilitate receptor binding and dimerization. Towards the same end FGF2 has also been oligomerized through biotin-streptavidin binding\textsuperscript{25}. In addition, the oxidative disulfide dimerization of FGF2 has led to enhanced biological activity at extremely low concentrations compared to FGF2 alone\textsuperscript{26}. While small-molecule chemical dimerization or disulfide dimerization of FGF2 prior to delivery has previously resulted in enhanced activity as a result of increased local concentration and preorganization, we hypothesized that much more significant activity could be obtained by 1) conjugating site-selectively to residues spatially separated from both the heparin-binding domain and receptor binding sites and 2) probing the ideal length of the dimerizer.
Site-specific conjugation is imperative in the development of protein homodimers in order to avoid the formation of protein multimers or complex protein-polymer networks. In addition, polymer conjugation at or near an active site, or the addition of multiple polymers to a protein therapeutic can shut down protein activity. Therefore targeting a single reactive site is ideal to maintain protein activity. Cysteine is an ideal target for site-specific protein modification due to its low abundance and nucleophilicity. FGF2 contains two surface-exposed free cysteines (Cys-78 and Cys-96). The mutation of either cysteine, as shown by Lappi et al., is not detrimental to protein activity and Kang et al observed a retention of activity after PEGylation at both surface-exposed cysteines with a 5 kDa PEG. Therefore we chose to install the genetic modification cysteine to serine at amino acid 78 (C78S), resulting in an FGF2 containing a single surface-exposed cysteine, Cys96 (shown in red, Figure 3.1).

In addition to site-selective dimerization, the length of the tether is also imperative to protein activity. Linker length is essential to receptor activation for similar growth factors. Based on the crystal structure of the tetrameric FGF2:FGFR1 complex, we hypothesized that a flexible linker with a length close to the inter-cysteine distance of 70 Å would induce the greatest activity. Poly(ethylene glycol) (PEG) was chosen as the linker based on the ease of modification and previous use as a probe for ideal dimerization lengths. PEG is known to improve pharmacokinetics through stabilization and improved circulation time, and many FDA-approved, PEGylated therapeutic agents are on the market. Here, we describe the development of PEG-linked FGF2 dimers as superagonists in wound healing.
**Figure 3.1.** The active tetrameric complex consisting of two FGF2s (gold) two FGFRs (silver) with Cys96 in red and poly(ethylene glycol) represented in blue. Modified from PDB 1CVS using Chimera software.
3.2 Results and Discussion

Conjugate Synthesis

FGF2 was genetically engineered (C78S) to contain a single surface-exposed free cysteine, Cys96, to facilitate site-specific polymer conjugation and therefore stoichiometric homodimerization. This mutation was made on a pET29c(+)hFGF-2 plasmid provided by the Helmholtz Centre for Infection Research, Braunschweig, Germany. Employing *E. coli* host BL21(DE3), the mutant was expressed and purified through a cationic membrane adsorber, a heparin column, and an anionic membrane adsorber as previously described. The endotoxin level after purification was ≤ 0.002 EU/µg. Activity of the mutant was confirmed in an *in vitro* proliferation assay in human dermal fibroblasts (HDFs) and no significant difference was observed as compared to unmodified FGF2.

Pure and active mutant protein (hereby called simply “FGF2”) was obtained as visualized by Western Blot ([Figure 3.2](#), lane 2, procedure described in Methods section) with a molecular weight of 17.2 kDa measured by MALDI mass spectrometry ([Figure 3.13](#)). The mutation was further verified by trypsin-digestion followed by liquid chromatography tandem mass spectrometry, and the expected peptide (GVVSIKGVSANR) showing the C78S mutation was found.

PEG linker lengths were chosen based on the inter-cysteine distance (Cys96 of one FGF2 to Cys96 in the second FGF2) in the 2:2 FGF2:FGFR1 tetrameric complex ([Figure 3.1](#)), which is approximately 70 Å. Based on prior work, we hypothesized that in the active tetrameric complex the PEG would be stretched slightly from its normal
random coil configuration. As a guide we considered Flory’s Radius ($R_F = aN^{0.6}$) where $a =$ monomer unit length and $N =$ # monomers or degree of polymerization, to estimate the random coil length of various PEGs. We also used the equation for a fully-extended polymer chain of length $L = aN$ to estimate the maximum possible length. We hypothesized that the best candidate would be a PEG with an $R_F < 70 \text{ Å}$ and an $L > 70 \text{ Å}$ (both 2 and 6 kDa PEG fit this description, Table 1). For comparison we also chose a small molecule linker, divinyl sulfone (DVS), where both $R_F$ and $L << 70 \text{ Å}$ and a large 20 kDa PEG control where both $R_F$ and $L >> 70 \text{ Å}$ (Table 3.1).

Table 3.1. Crosslinker length based on Flory’s radius of gyration and fully extended chain length.

<table>
<thead>
<tr>
<th>Crosslinker</th>
<th>MW*</th>
<th>$a^{+}$</th>
<th>N</th>
<th>$R_F^{+}$</th>
<th>$L^{+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVS</td>
<td>118</td>
<td>&lt;10</td>
<td>1</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VS-PEG2k-VS</td>
<td>2000</td>
<td>3.5</td>
<td>45</td>
<td>35</td>
<td>160</td>
</tr>
<tr>
<td>VS-PEG6k-VS</td>
<td>6000</td>
<td>3.5</td>
<td>136</td>
<td>67</td>
<td>480</td>
</tr>
<tr>
<td>VS-PEG20k-VS</td>
<td>20000</td>
<td>3.5</td>
<td>454</td>
<td>138</td>
<td>1600</td>
</tr>
</tbody>
</table>

*Molecular weight (MW) in g/mol

† in Å

The Michael addition of cysteine residues to vinyl sulfone (VS) is known to occur preferentially over conjugation to lysine or N-terminal amines at pH 7-9\textsuperscript{37} and FGF2 is stable between pH 5 and 9\textsuperscript{38}. Therefore PEGs (2, 6, and 20 kDa) were modified at both ends with VS according to literature procedures\textsuperscript{39}. Since PEGylated proteins often exhibit enhanced activity due to increased stabilization or conversely can decrease activity due to steric hinderance, monoconjugated controls were developed. Towards that
end, poly(ethylene glycol methyl ether) (mPEGs) with molecular weights of 2, 5, and 20 kDa were modified on one end with VS.

In order to synthesize the conjugates, FGF2 was incubated with 0.5 eq. of polymer (2, 6, or 20 kDa VS-PEG-VS, 2, 5, or 20 kDa mPEG-VS) or small molecule crosslinker DVS in 100 mM Tris buffer, pH 9 at room temperature for 12 hours. A range of buffer types, pHs, polymer equivalents, times, and addition of organic solvents were screened, and in all cases the maximum yield of the desired conjugate (monoconjugate or homodimer) was obtained using the conditions described below. Although excess polymer is typically used to maximize monoconjugate yield, excess polymer resulted in multiple polymer additions, which were difficult to remove. Kang et al. also observed up to four polymer additions for monoconjugations and hypothesized that with increased time the two internal cysteines are also able to react\(^{29}\). This was avoided by using 0.5 equivalents of polymer incubated with the protein at 73 \(\mu\)M concentration in 100 mM Tris, pH 9, 5 mM EDTA for 12 hours. Conjugates were purified by fast protein liquid chromatography (FPLC). Pure monoconjugates and homodimers were obtained for each length PEG as visualized by western blot (Figure 3.2). For FGF2-PEG20k-FGF2 (Figure 3.2, lane 9) the homodimer appears as a smear, which is often observed with larger polymer-conjugates.
Figure 3.2. Western blot of purified conjugates. Lane 1: Protein Ladder; Lane 2: FGF2; Lane 3: FGF2-VS-FGF2; Lane 4: mPEG2k-FGF2; Lane 5: FGF2-PEG2k-FGF2; Lane 6: mPEG5k-FGF2; Lane 7: FGF2-PEG6k-FGF2; Lane 8: mPEG20k-FGF2; Lane 9: FGF2-PEG20k-FGF2.

Conjugate Screening in Human Dermal Fibroblasts

Efficacy of FGF2 in wound healing is often first assessed in vitro by fibroblast and endothelial cell proliferation, migration, vasculogenesis, and angiogenesis. Human dermal fibroblast (HDF) proliferation was chosen to screen and compare the activity of all PEG-linked dimers, monoPEGylated controls, the small molecule control, and FGF2 alone. HDFs were serum and growth factor starved, followed by treatment with samples for 3 days.

FGF2-PEG2k-FGF2 induced significantly greater proliferation in HDFs than all other linker lengths, all monoPEGylated controls, and free FGF2 at every concentration tested (Figure 3.3 a). FGF2-PEG2k-FGF2 induced the most marked difference in proliferation at very low (0.1 ng/mL) concentrations where the dimer induced 169% compared to blank media (set to 100%) while FGF2 alone and monoconjugate was only
118% and 131% respectively. FGF2-PEG2k-FGF2 induced the highest proliferation overall, 181% compared to blank media, at 1 ng/mL while FGF2 alone induced only 144% proliferation at 1 ng/mL. The increase in proliferation compared to blank plateaued at 1 ng/mL for FGF2-PEG2k-FGF2 but even with this plateau, the increased proliferation is greater than all other conjugates and FGF2 alone. The small molecule linked homodimer, FGF2-VS-FGF2, does induce significantly greater growth than FGF2 at low concentrations (0.1 and 1 ng/mL). However this increase was small and only 9 and 7% greater than FGF2 at the two concentrations, respectively, likely due to the steric restrictions of such a short linker. The fact that there was increased activity indicates that there was some flexibility in the 2:2 FGF2:FGFR tetrameric complex as compared to what is observed in the crystal structure. While FGF2-PEG6k-FGF2 did induce greater proliferation than FGF2 at low concentrations (0.1 and 1 ng/mL), it did not significantly increase proliferation beyond that of its monoPEGylated control (mPEG5k-FGF2) indicating that the increase in activity might be due solely to the added stability induced by PEG. Again, the enhanced activity was only slightly increased (14 and 13%, respectively) compared to FGF2 at the two lower concentrations. For FGF2-PEG20k-FGF2, a decrease in activity as compared to FGF2 was observed. FGF2-PEG20k-FGF2 induced 13% and 18% less proliferation than FGF2 at 1 and 10 ng/mL, respectively. While we expected the longer linker to be less effective than the shorter linkers (less pre-organization, increased entropy as seen by Krueger et al.\textsuperscript{16}) decreased activity as compared to FGF2 was not expected. We hypothesized that this might be due to steric hindrance towards receptor docking due to the bulk of 20 kDa PEG. Taken together, these results supported our hypothesis that the ideal linker length was: $R_F (35 \text{ Å}) <$ inter-
cysteine distance (70 Å) and L (160 Å) > the inter-cysteine distance. We then tested a greater number of concentrations (0.01, 0.05, 0.1, 1, 2, 5, 10 ng/mL) of FGF2 and FGF2-PEG2k-FGF2 on HDF proliferation, to assess the lowest effective concentration (Figure 3.3, b). Even at 0.01 ng/mL FGF2-PEG2k-FGF2 induced a 139% increase in HDF proliferation as compared to 105% for FGF2. Thus, FGF2-PEG2k-FGF2 exhibited superagonist activity, even at extremely low concentrations, in the proliferation of HDFs, making it a good target as a superagonist in wound healing.

To further assess the viability of FGF2-PEG2k-FGF2, HDF migration was assessed in a scratch assay (Figure 3.3, c). A scratch was made through a confluent layer of growth factor starved cells followed by the application of sample and incubation for 18 hours. Incubation time was restricted to 18 hours to ensure that differences were not due to proliferation. FGF2-PEG2k-FGF2 induced a slight, but significantly greater migration at the lowest concentration tested (0.05 ng/mL) resulting in 131% increased wound closure compared to 117% for FGF2 alone. While both FGF2 and FGF2-PEG2k-FGF2 induced significantly greater migration as compared to blank at all higher concentrations, there was no significant difference between the two. These results indicate that the FGF2 dimer does not greatly enhance migration of HDFs and that proliferation is a stronger effect.
Figure 3.3. Screening of PEG linker-length effect on HDF proliferation and migration. (a) and (b) Percent proliferation of HDFs. All samples are normalized to blank media per treatment set (n=6). Error bars indicate s.e.m. Statistical analysis was carried out using a student’s t-test. # = statistically greater than FGF2 control at that concentration, p < 0.05. * or ** = statistically greater than both FGF2 and mPEG-FGF2, p < 0.05 and p < 0.001, respectively. (c) Migration scratch assay of HDFs treated with FGF2 or FGF2-PEG2k-FGF2 for 18 hours. Each treatment was repeated 5-7 times. All samples are normalized to a blank media per treatment set. Error bars indicate s.e.m. Statistical analysis was carried out using a student’s t-test. * = p < 0.05. The distance across the scratch was measure in imageJ in pixels and the % increased wound closure was calculated as 100 – distance at T / distance at T₀ *100.

Conjugate Screening in Human Umbilical Vein Endothelial Cells (HUVECs)

Next the in vitro proliferation and migration of human human umbilical vein endothelial cells (HUVECs) was assessed with FGF2-PEG2k-FGF2 and compared to free FGF2 (Figure 3.4). Since the most drastic improvements in activity occurred at lower concentrations in HDFs, we focused our test on low concentrations for HUVEC proliferation. FGF2-PEG2k-FGF2 significantly increased proliferation compared to FGF2 at low concentrations and exhibited similar activity at 1 ng/mL (Figure 3.4 a). Specifically, FGF2-PEG2k-FGF2 increased proliferation as compared to FGF2 by 28%, 21% and 21% at 0.01, 0.05 and 0.1 ng/mL, respectively. Compared to blank media, FGF2-PEG2k-FGF2 increased proliferation most effectively at 0.1 ng/mL, to 148% compared to blank media. HUVEC migration was investigated over a range of
concentrations in a scratch-assay using the same protocol as described for HDFs (Figure 3.4 b and c). FGF2-PEG2k-FGF2 significantly increased migration into the wound compared to FGF2 at 0.01, 0.05, 0.1, and 1 ng/mL. The greatest increase in migration was observed at 0.1 ng/mL. Wound closure was increased by 163% compared to blank media for FGF2-PEG2k-FGF2 while FGF2 increased wound closure by only 108%. These results further indicated that FGF2-PEG2k-FGF2 was a good candidate for improved wound healing at low concentrations.

Figure 3.4. (a) Proliferation of HUVECs at various concentrations of FGF2 and FGF2-PEG2k-FGF2. Each treatment was repeated 5-6 times and the entire study repeated
twice. All samples are normalized to blank media per treatment set. Error bars indicate s.e.m. Statistical analysis was carried out using a student’s t-test. # = statistically greater than FGF2 control at that concentration, p < 0.05. * = statistically greater than FGF2, p < 0.05. (b) Migration scratch assay of HUVECs treated with FGF2 or FGF2-PEG2k-FGF2 for 18 hours. Each treatment was repeated 4-7 times and the entire study repeated twice (n=9-12 total). All samples are normalized to a blank media per treatment set. Error bars indicate s.e.m. Statistical analysis was carried out using a student’s t-test. * = p < 0.05, *** = p < 0.001. The distance across the scratch was measured using ImageJ in pixels and the % increased wound closure was calculated as 100 – distance at T / distance at T₀ *100. (c) Representative images of HUVECs (5x magnification) at 18 hours and 0 hours after scratching and treatment with 0.1 ng/mL of each sample.

**FGF2-PEG2k-FGF2 Increases Angiogenesis in a Co-culture of HDFs and HUVECs**

In addition to increased proliferation in HDFs and HUVECs, as well as increased migration in HUVECs, FGF2-PEG2k-FGF2 also improved angiogenesis in a co-culture of HDFs and HUVECs. A standard co-culture assay of angiogenesis was utilized\(^{44,45}\). HDFs were first allowed to grow to confluency before HUVECs were added with the respective experimental sample. Sample solutions were refreshed at days 3 and 6. After 10 days the cells were fixed and tubules stained for CD31. FGF2-PEG2k-FGF2 induced a significant increase in the number of nodes, tubules, and total length of tubules compared to FGF2 at low concentrations (0.1 and 0.5 ng/mL) and performed as well as FGF2 at higher concentrations (1, 5, and 10 ng/mL) (Figure 3.5). Specifically, at 0.1 ng/mL, cells treated with FGF2-PEG2k-FGF2 increased the average number nodes by 97,
tubules by 186, and increased the total length of tubules to 9 times that of FGF2. At 0.5 ng/mL, FGF2-PEG2k-FGF2 increased the average number of nodes by 120, tubules by 229, and total length of tubules was increased to 4 times that of FGF2. Thus at these low concentrations, FGF2-PEG2k-FGF2 improved all tested aspects of angiogenesis compared to free FGF2.

Figure 3.5. Effect of FGF2 and FGF2-PEG2k-FGF2 at various concentrations on angiogenesis through the co-culture of HDFs and HUVECs (n=3). (a) representative images of select sample sets, tubules stained (b) comparison of the number of nodes per condition / sample set (c) comparison of the number of tubules per condition / sample set.
(d) the average total tubule length per image (pixels) per condition / sample set. Error bars indicate s.e.m. Statistical analysis was carried out using a student’s t-test. * = p < 0.05.

**FGF2-PEG2k-FGF2 Increases Granulation Tissue and Blood Vessel Density in Wounded, Diabetic TallyHo Mice**

Since FGF2-PEG2k-FGF2 significantly enhanced *in vitro* proliferation, migration, and angiogenesis, the efficacy of FGF2-PEG2k-FGF2 in wound healing was investigated *in vivo*. TallyHo/JngJ mice, which develop a type II diabetic phenotype, were wounded (8 mm punch biopsy) and then treated daily for 5 days with 0.02 μg of FGF2, FGF2-PEG2k-FGF2 or D-PBS control. The wound closure was measured at days 0, 1, 4, 7, 10, and 14. There was no significant difference in wound closure among the cohorts of mice (*Figure 3.6 a,b*). In contrast, the granulation tissue area and the blood vessel density in the wound bed were significantly greater in the FGF2-PEG2k-FGF2 treat mice than either FGF2 treated mice (p<0.05) or D-PBS control (p<0.01) (*Fig, 6c,d,e*). These results indicate that the low concentration of FGF2-PEG2k-FGF2 was highly effective in promoting granulation tissue and angiogenesis in the wound bed.
**Figure 3.6.** *In vivo* assessment of FGF2-PEG2k-FGF2 in diabetic wounds. Sterile wounds (8 mm punch biopsy) were performed on TallyHo/JngJ diabetic mice and the wounds were covered (Tegaderm) and treated daily for 5 days with 0.02 µg of FGF2, FGF2-PEG2k-FGF2 or D-PBS control. (A) Representative photographs of the skin wounds in treated diabetic wounds. (B) Mean wound area ± s.e.m. (C) Representative histology (H&E stained) at low magnification (left panels) and high magnification of the boxed dotted area (right panels) with black arrows indicating wound edges and white arrows indicating blood vessels. Quantitative analysis of granulation tissue area (D) and blood vessel density (E). *p<0.05, **p<0.01 FGF2-PEG2k-FGF2 versus FGF2 or D-PBS (n=5-10/group).

Chronic wounds represent a widespread and increasing socioeconomic problem. Depressed levels of growth factors and their receptors, including FGF2, are a main factor in non-healing wounds. While FGF2 is approved for use in diabetic ulcers in Japan, recent clinical trials in Europe have shown only marginal improvements for neuropathic diabetic foot ulcers\textsuperscript{7,8,11}. In addition, overexpression of FGF2 and its receptors is linked to cancer\textsuperscript{46}. The development of a more active FGF2, effective at lower doses, therefore, is necessary for clinical applicability.

Currently, advanced wound care therapies for non-healing ulcers include the use of collagen, growth factors, bioengineered skin, gene and stem cell therapy, silver products, ozone oxygen therapy, and negative pressure wound therapy\textsuperscript{47}. However, even the most promising results, including bioengineered skin, platelet derived growth factor (PDGF), and negative pressure treatment, have only a low level of evidence for improved
wound healing. Currently, PDGF is the only FDA-approved growth factor treatment for wound healing, marketed as Regranex®. However, Regranex® does increase the likelihood of malignancies and mortality due to malignancies. Due to the low efficacy and risks of currently available treatments, we sought to develop a more effective growth factor treatment, requiring low dosages. FGF2-PEG2k-FGF2 represents a promising candidate for non-healing wound treatment.

The goal of this work was to design and test the efficacy of a potentially improved chronic wound-healing therapeutic. Utilizing available knowledge of FGF2 activity and its crystal structure with its receptor, we were able to rationally design a superactive protein for wound healing. Mutation of the protein was undertaken in order to obtain a single reactive cysteine for modification, separate from residues required for receptor binding and protein activity. Homodimerization of the protein was achieved through Michael addition to bis-reactive linear PEG tethers, varying the length based on both the spatial restrictions informed by the active protein-receptor crystal structure, as well as taking in to account PEG flexibility and stretch. Although 2 kDa PEG was the best linker-length determined in this work, the ideal linker length could be determined empirically at lengths consecutively closer to the inter-cysteine distance. The simplicity of synthesis and purification as well as the effectiveness of the current model represents a viable candidate for clinical studies.

While increased rate of closure was not observed in vivo, this is likely due to the extremely low concentrations tested. For instance, Orgill and coworkers tested wound closure in C57BL/KsJ db+/db+ mice, and found no increase in wound closure when 1.5 cm² wounds were treated with 10 µg of PDGF each day for five days, but did see an
increase in granulation tissue\textsuperscript{50}. Hubbell and coworkers tested genetically modified superagonists of vascular endothelial growth factor (VEGF), PDGF, and bone morphogenic protein-2 at low concentrations (200 ng/wound) and found that the modified growth factors increased granulation tissue formation compared to no treatment while unmodified growth factors induced no increase at this concentration\textsuperscript{51}. Therefore increased granulation tissue area is a good indicator of superagonist activity at low concentrations \textit{in vivo}.

FGF2-PEG2k-FGF2 showed enhanced proliferation, migration, and angiogenesis \textit{in vitro} as well as enhanced granulation tissue and blood vessel density in diabetic mice \textit{in vivo}, all at exceptionally low effective concentrations as compared to unmodified FGF2. In addition, although wound healing and cancer risk should be assessed in higher order mammals such as a rabbit ear model and primates prior to human trials, we expect that FGF2-PEG2k-FGF2 will not significantly increase changes of malignancies due to the low effective concentration and therefore may be safe for use in non-chronic wounds to prevent scarring and keloids.
3.3 Experimental

3.3.1 Materials
All chemicals and reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. Enzyme-linked immunosorbent assay (ELISA) Development DuoSet kit was purchased from R&D Systems. Normal Human Dermal Fibroblasts (HDFs), HUVECs, and cell media were purchased from ATCC.

3.3.2 Analytical Techniques
NMR spectra were obtained on an Avance 500 MHz DRX spectrometer. Proton NMR spectra were acquired with a relaxation delay time of 10 seconds. MALDI-MS was performed on an Applied Biosystems Voyager DE-STR system and operated in linear mode with external calibration. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 µm mixed D columns. LiBr (0.1 M) in dimethylformamide (DMF) at 40 °C was used as an eluent (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse poly(methyl methacrylate) (PMMA) standards from Polymer Laboratories. Fast protein liquid chromatography (FPLC) was performed on a Bio-Rad BioLogic DuoFlow chromatography system equipped with a GE Healthcare Life Sciences Superdex 75 10/300 column. Protein concentration was determined using the Human FGF basic Duo ELISA kit from R&D Systems. Cell images were taken in bright field with a Zeiss Axioskope equipped with an AxioCam MRm at 5x magnification.
3.3.3 Methods.

Mutagenesis

A pET29c(+)hFGF-2 plasmid from the Helmholtz Centre for Infection Research, Braunschweig, Germany, as used by Chen et. al., was graciously provided. Plasmid sequence determination, plasmid mutagenesis, and protein expression were all performed at the UCLA MBI PETC (University of California, Los Angeles, Molecular Biology Institute, Protein Expression Technology Center).

Mutations were introduced into the plasmid by PCR-amplification with Pfu Turbo polymerase (Agilent, Santa Clara, CA) using the Quikchange protocol (Agilent, Santa Clara, CA) and mutagenic primers (Valuegene, San Diego, CA) incorporating the specific mutations (C78S). PCR products were treated with DpnI enzyme (New England Biolabs, Ipswich, MA) to digest the parental plasmid template, transformed into competent E. coli DH5alpha cells (New England Biolabs, Ipswich, MA) and plated on LB plates containing kanamycin. Putative positive plasmids were sequenced (South Plainfield, Piscataway, NJ) to confirm that the proper mutation had been introduced.

Based on the DNA sequence of the plasmid, the amino acid sequence of the mutant (with cysteines in bold and the mutated amino acid in red) is:

MAAGSITTLP ALPEDGGSGA FPPGHFKDPK RLYCKNGGFF LRIHPDGRVD GVERKSDPHI KLQLAEEERG VVSIGVSAN RYLAMKEDGR LLASKCVTDE CFFFERLESN NYNTYRSRKY TSWYVALKRT GQYKLGSKTG PGQKAILFLP MSAKS
Protein Expression and Purification:

Protein expression in E. coli BL21(DE3) was performed as previously described using a BioEngineering NLF22 fermentor on a scale of 12 liters. After lysis, the supernatant was purified with a sartobind S75 absorber (cationic), a heparin column (5 mL) and a sartobind Q75 absorber (anionic) to remove endotoxin. The protein was eluted from the heparin column using a salt gradient and fractions which eluted at 2 and 2.5 M NaCl were collected and concentrated for further analysis and use. The final yield of protein was 0.47 mg purified protein per 1 gram of cell pellet. The final endotoxin level was determined using the ToxinSensor Chromatogenic LAL Endotoxin Assay Kit from Genscript to be ≤ 0.002 EU/µg. According to European Pharmacopoeia, < 5 EU/kg * hr is accepted for clinical application. The purified mutant protein contained 3 cysteine residues, verified by Ellman’s assay.

After protein expression and purification, the mutant protein FGF2(C78S) was digested with trypsin followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Applied Biosystems Q-STAR Elite Quadrupole-TOF Hybrid LC-MS System at the UCLA Molecular Instrumentation Center (MIC). The peptide (sequence in previous section) was identified with 78% sequence coverage and a Mascot sequence search score of 54, which is considered significant.

Modification of poly(ethylene glycol) and poly(ethylene glycol methyl ether) with vinyl sulfone
The end-group alcohol(s) of PEG (2, 6, and 20 kDa) or mPEG (2, 5, and 20 kDa) were modified with VS, according to literature procedures, resulting in bis (VS-PEG-VS) and mono (mPEG-VS) reactive PEGs, respectively. As an example, the synthesis of 2 kDa VS-PEG-VS is described below.

Briefly, sodium hydride (12.5 mmol, 25 eq.) was weighed into an oven-dried round-bottom flask under inert atmosphere. The flask was cooled in an ice-bath, and 100 mL of dry dichloromethane (DCM) was added. PEG, 2 kDa, (0.5 mmol, 1 eq.), previously freeze-dried from benzene to remove water, was dissolved in 50 mL dry DCM. The PEG-alkoxide was then added dropwise to the cooled slurry of sodium hydride and DCM, to prevent gelation of the PEG. It was imperative that the solution was dilute (3 mM for PEG in this case) to prevent gelation, and this was especially important for smaller PEGs (2 and 6 kDa). After hydrogen evolution (30 minutes stirring), divinyl sulfone (30 mmol, 60 eq.) was added quickly. The solution was allowed to come to room temperature and stir for 3 days under argon. The solution was then neutralized with acetic acid, concentrated, and purified by precipitation into cold ether five times.

$^1$H-NMR (CDCl$_3$) was used to approximate the percentage end-group modification by comparing the integration of the new vinylic protons (6.0, 6.3, and 6.7 ppm, 1H) to the main-chain methylene protons (3.4-3.9 ppm) (Figures 3.7-12). For VS-PEG2k-VS, 2000 Da / 44 Da per (-CH$_2$CH$_2$O-) repeat = 45.45 repeats x 4 H / repeat = 181.8 H’s in the backbone at 3.4-3.9 ppm (Figure 3.10). The integration for the vinylic H at 6.3 ppm is set to 1. For monofunctionalized mPEG-VSs, percent functionalization was calculated by:
Integration from 3.4-3.9 ppm (expected / observed) * 100

For bisfunctionalized VS-PEG-VS, percent functionalization was calculated by:

Integration from 3.4-3.9 ppm (expected / (observed * 2)) * 100

% Functionalization per polymer by $^1$H-NMR:
mPEG2k-VS: $181.8/181.46\times 100 = 100\%$ functionalization
mPEG5k-VS: $454.5/536.81\times 100 = 85\%$ functionalization
mPEG20k-VS: $1818.2/3088.95\times 100 = 59\%$ functionalization
VS-PEG2k-VS: $181.8/(94.43\times 2)\times 100 = 96\%$ bis-functionalization
VS-PEG6k-VS: $545.5/(330.01\times 2)\times 100 = 83\%$ bis-functionalization
VS-PEG20k-VS: $1818.2/(1071.67\times 2)\times 100 = 85\%$ bis-functionalization

Figure 3.7. $^1$H-NMR (CDCl$_3$) of mPEG2k-VS
Figure 3.8. $^1$H-NMR (CDCl$_3$) of mPEG5k-VS
Figure 3.9. $^1$H-NMR (CDCl$_3$) of mPEG20k-VS
Figure 3.10. $^1$H-NMR (CDCl$_3$) of VS-PEG2k-VS
Figure 3.11. $^1$H-NMR (CDCl$_3$) of VS-PEG6k-VS
Conjugation of FGF2 to mPEG-VS and VS-PEG-VS

Each polymer, mPEG (2, 5, or 20 kDa) or VS-PEG-VS (2, 6, or 20 kDa) was dissolved in 100 mM Tris, pH 9, 5 mM EDTA. 0.5 equivalents of each polymer was added into a separate tube containing 1.3 mg (77 nmol) FGF2 in an eppendorf lobind tube, so that the final concentration of protein was 73 μM.

The solutions were placed on a rotating plate at room temperature for 12 hours. The solutions were then concentrated / solvent exchanged into D-PBS + 10 mM DTT using Centriprep MWCO 10,000 for 2, 5, and 6 kDa PEG conjugates, and using MWCO 30,000 for 20 kDa PEG conjugates, at 6 x 10 min cycles. 16.1 rcf (13.2 rpm). Ultracentrifugation resulted in the removal of unreacted FGF2 from mPEG20k-FGF2 and
removal of all unreacted FGF2 and monoconjugate from FGF2-PEG20k-FGF2. All other conjugates required purification by fast protein liquid chromatography (FPLC).

Each vial was concentrated to a final volume of 200 μL, and 100 μL was loaded onto a size exclusion column (Superdex 75 10/300 GL) at 0.4 mL/min in D-PBS, 10 mM DTT, pH 7.4. Fractions were collected every 0.25 mL, for a total of 15 mLs. After concentration by ultracentrifugation, SDS-PAGE gel electrophoresis was run to assess which fractions contained the respective desired product. Fractions containing the desired conjugate were then combined, concentrated by ultracentrifugation, and buffer exchanged into D-PBS (–Mg²⁺ -Ca²⁺) for use in cell or animal studies. The fractions in which each product was found is listed below:
mPEG2k-FGF2 (13.5-14 mLs)
FGF2-PEG2k-FGF2 (11-11.25 mLs)
mPEG5k-FGF2 (11.5-12 mLs)
FGF2-PEG6k-FGF2 (10-10.5 mLs)
FGF2-PEG20k-FGF2 (9-9.75 mLs)
**Figure 3.13.** MALDI-MS of unmodified FGF2 (top) or FGF2-PEG2k-FGF2 conjugates (bottom). Top: m/z = 17.2 kDa (FGF2), 34.5 kDa (naturally occurring disulfide dimer). Bottom: m/z = 17.2 kDa (FGF2), 18.2 kDa (FGF2-PEG2k some cleavage), 19.3 (FGF2-PEG2k), 36.5 kDa (FGF2-PEG2k-FGF2), 38.4 kDa (PEG2k-FGF2-PEG2k with one additional nonspecific polymer addition).

**Western Blot**

All reagents for western blot were purchased from Bio-Rad except as noted. 4-20 ng of FGF2 or conjugate was diluted in Laemmeli buffer, 0.65 M DTT (Sigma) and heated to 95 °C for 6 minutes before loading onto a mini-ProTEAN TGX any-Kd gel. Precision Plus Protein™ WesternC™ Standards (6 µL) were loaded into the first lane of the gel.
The gel was run in Tris/Glycine/SDS buffer at 200 V for 28 minutes, then transferred onto nitrocellulose in Tris/Glycine buffer at 100 V for 2 hours. The nitrocellulose membrane was blocked overnight with Tris-buffered Saline Tween 20 (TBST) + 5% fat-free dry milk, 1% BSA (Sigma) (blocking buffer). The membrane was then incubated with the primary antibody (rabbit-antiFGF2, “FGF Antibody (Center)”, Abgent) diluted 1:100 in blocking buffer for 16 hours at 4 °C on a rotating plate. The membrane was then washed with TBST 3 x 5 minutes, and incubated with secondary antibody (goat anti-rabbit IgG-HRP conjugate) diluted 1:1000 in blocking buffer as well as the secondary antibody for the protein standards (Strep-Tactin HRP conjugate) diluted 1:10,000 at room temperature on a rotating plate for 30 minutes. The membrane was then washed with TBST 3 x 5 minutes, and incubated with Clarity™ Western ECL Substrate for 5 minutes. Chemiluminescence was imaged on an Alpha Innotec FluorChem® FC2 Imaging System at the UCLA-DoE Biochemistry Instrumentation Core Facility.

**HDF Proliferation Assay**

HDF cells (passage 4 - ATCC) were suspended in UltraCULTURE™ (Lonza) serum-free medium supplemented with 2 mM L-Glutamine and 100 μg/ml penicillin and 100 μg/ml streptomycin and plated at 2000 cells/well in a 96-well plate. The cells were allowed to adhere for 16 hours at 37 °C, 5% CO₂. After 16 hours the medium was removed by aspiration replaced with 100 μL of unmodified FGF2, mPEG-FGF2, or FGF2-PEG-FGF2, diluted in UltraCULTURE™ medium (original concentration determined by ELISA). The cells were incubated with the samples for 72 hours at 37 °C, 5% CO₂, after which cell proliferation was assessed using a CellTiter-Blue® assay. All experimental
groups were normalized to the control group, which received only blank medium. Each group was done with six replicates.

**HUVEC Proliferation Assay**

HUVEC cells (passage 5 - ATCC) were suspended in EGM\textsuperscript{TM} (-BBE) medium supplemented with 100 ug/ml penicillin and 100 μg/ml streptomycin and plated at 1000 cells/well in a 96-well plate. The cells were allowed to adhere for 16 hours at 37 °C, 5% CO\textsubscript{2}. After 16 hours the medium was removed by aspiration replaced with 100 μL of unmodified FGF2, mPEG-FGF2, or FGF2-PEG-FGF2, diluted in EGM\textsuperscript{TM} (-BBE) medium (original concentration determined by ELISA). The cells were incubated with the samples for 72 hours at 37 °C, 5% CO\textsubscript{2}, after which cell proliferation was assessed using a CellTiter-Blue\textsuperscript{®} assay. All experimental groups were normalized to the control group, which received only blank medium. Each group was done with six replicates.

**HDF Migration Assay**

Two horizontal lines were drawn on the back of each well of a 0.2% gelatin-coated 24-well plate. HDFs from ATCC (P4) were seeded in fibroblast growth medium with full supplements at 70,000 cells/well (400 μL/well) and allowed to incubate for 24 hours at 37 °C, 5% CO\textsubscript{2}. The media was then removed, and the wells rinsed with warm D-PBS 2x, followed by the addition of starvation media, DMEM + 2% FBS and the cells incubated for 24 hours. A vertical scratch was then made using a P200 pipette tip. The wells were then rinsed 2x with warm D-PBS, and then the diluted samples of FGF2 or FGF2-PEG2k-FGF2 (in DMEM + 2% FBS) were added into 4 wells per concentration.
(n\textsubscript{max}=8) as well as 4 wells containing blank media. The cells were then incubated for 18 hours. Bright field images were taken just above and just below the drawn line, and analyzed using the ImageJ, by measuring the distance (pixels) of the cell-free area at T=0 and T=18. The study was blinded before image analysis.

**HUVEC Migration Assay**

Two horizontal lines were drawn on the back of each well of a 0.2% gelatin-coated 24-well plate. HUVECs (P5) were seeded in complete Epidermal Growth Media (EGM), at 60,000 cells/well (400 μL/well) and allowed to incubate for 24 hours at 37 °C, 5% CO\textsubscript{2}. The media was then removed, and the wells rinsed with warm D-PBS 2x, followed by the addition of starvation media, DMEM + 2% FBS and the cells incubated for 24 hours. A vertical scratch was then made using a P200 pipette tip. The wells were then rinsed 2x with warm D-PBS, and then samples diluted in DMEM + 2% FBS, were added at each concentration into 4 separate wells (n\textsubscript{max} = 8), as well as 4 wells of blank media. The cells were then incubated for 18 hours. Pictures were taken at 5X magnification at T=0 and T=18 hours. Bright field images were taken just above and just below the drawn line, and analyzed using the ImageJ, by measuring the distance (pixels) of the cell-free area at T=0 and T=18. One of the two full repeats was blinded before image analysis.

**In vitro Angiogenesis**

Methods for angiogenesis co-culture and tubule staining were adapted from literature procedures\textsuperscript{44,45}. Normal human dermal fibroblasts (HDFs) (P3) were plated at 12,500 cells/well in a 48-well plate, in endothelial growth medium (EGM) + full supplement and
incubated at 37°C for 72 hours or until cells reached confluency. The cells were then starved for 18 hours with EGM (-BBE) (-EGF). Human umbilical vein endothelial cells (HUVECs) P4 were trypsonized and resuspended in EGM (-BBE) (-EGF). Starvation media was aspirated and HUVECs were plated on top of the HDF monolayer at 10,000 cells/well in EGM (-BBE) (-EGF) followed by sample (either FGF2 or FGF2-PEG2k-FGF2) diluted in EGM (-BBE) (-EGF). Final concentrations of FGF2 and FGF2-PEG2k-FGF2 were 10 ng/mL, 5 ng/mL, 1 ng/mL, 0.5 ng/mL, and 0.1 ng/mL. After 72 and 144 hours the sample solutions were refreshed with samples EGM (-BBE) (-EGF) at the appropriate concentrations.

10 days after HUVEC and the first sample addition, the medium was removed and each well fixed with 70% EtOH (at -20°C) for 30 minutes. After fixing, the wells were rinsed with 0.5 mL of 1% BSA in D-PBS three times. Next, endogenous alkaline phosphatase was removed by incubation with 0.3% H₂O₂ in MeOH at room temperature for 15 minutes. The H₂O₂ solution was removed once the solution was cloudy. The wells were washed 3 times with 1% BSA. The wells were then incubated with primary antibody (mouse anti-human PECAM1/CD31, R&D Systems) at 1 ug/mL in 1% BSA for 60 minutes at 37°C. The wells were then rinsed 3 times with 1% BSA. The wells were then incubated with secondary antibody (goat anti-mouse IgG – alkaline phosphatase, Sigma Aldrich) at 3 ug/mL in 1% BSA for 60 minutes at 37°C and then wells were rinsed 3x5 minutes with milliQ water. Next, the wells were incubated with BCIP/NBT solution (one tablet dissolved in 10 mL milliQ water, filtered) at room temperature for 6-15 minutes. After the tubules were visually stained the BCIP/NBT (Life Technologies) was removed and the wells were washed 3 times with milliQ water and allowed to dry. Plates were
stored for up to 60 days at -80°C. To assess extent of angiogenesis 5 bright field images were captured per well at 5x magnification. Number and length of tubules as well as number of nodes were calculated manually using NIH ImageJ Software while blinded. The values for each of 5 images per well were summed and then the sums were averaged across 3 wells. The sample identities remained blinded until after results were calculated.

**In vivo Assessment of Wound Healing in Diabetic Mice**

Eight to nine-week-old male TallyHo/JngJ mice (Jackson Laboratories, Bar Harbor, ME) were used as a model of type II diabetes. Briefly, 1 day prior to wounding, dorsal hair was shaved and depilated and mice were housed individually. Full-thickness wounds were created on the dorsum of mice (8 mm punch biopsy; Acuderm, Inc., Lauderdale, FL). The wound bed was covered with Tegaderm™ (3M, St. Paul, MN) and treated with 20 µl of FGF2 or FGF2-PEG2k-FGF2 (0.02 µg total) or D-PBS control (-Mg²⁺, -Ca²⁺) by injecting through the covering into the wound bed daily for 5 days. The covering was removed on day 7. Digital photographs taken on day 0, 1, 2, 3, 4, 7, 10, and 14 and wound area was measured using ImageJ software. The wound beds were excised en bloc with the surrounding soft tissue and fixed with 10% formalin solution and histologic analysis was performed on day 14. The granulation tissue area and blood vessel density were measured as previously described⁵².
3.4. Conclusions

Site-specific chemical dimerization of fibroblast growth factor 2 (FGF2) with intentional adjustment of linker length resulted in a FGF2 homodimer with wound healing ability at exceptionally low therapeutic concentrations. Homodimers of FGF2 were synthesized through site-specific linkages to both ends of poly(ethylene glycol) (PEG). The ideal linker length was determined by screening dimer-induced proliferation of human dermal fibroblasts (HDFs). The ideal length was found to be that closest to the inter-cysteine distance, 70 Å, corresponding to 2 kDa PEG. FGF2-PEG2k-FGF2 induced greater human dermal fibroblast proliferation than FGF2 alone, all other dimers, and all monoconjugates, at each concentration tested. The greatest difference in HDF cell proliferation was observed at a low concentration (0.1 ng/mL). FGF2-PEG2k-FGF2 further exhibited superior activity compared to FGF2 for both proliferation and migration in human umbilical vein endothelial cells, as well as improved angiogenesis in an in vitro co-culture of fibroblasts and endothelial cells. In an in vivo a wound healing model in diabetic mice, FGF2-PEG2k-FGF2 induced increased granulation tissue and blood vessel density in the wound bed. The results indicate that this rationally designed construct may be useful in chronic wound healing.
3.5 References


Chapter 4

Progress towards a Ni(II)-Nitrilotriacetic acid Functionalized Block Copolymers for the Multimerization of His6-Proteins
4.1. Introduction

Many proteins are tagged with histidine to facilitate purification\(^1\). Typically purification is accomplished on an affinity column through chelation to transition metals, such as Ni(II), Cu(II), Co(II) or Fe(III) associated with a nitrilotriacetic acid (NTA) resin.\(^2\) This methodology can be extended to develop conjugates of His6-tagged proteins with NTA-modified peptides, surfaces, drugs, imaging agents, DNA, nanoparticles, liposomes, or polymers\(^3-9\). Polymer conjugation to therapeutic proteins is known to increase stability and \textit{in vivo} circulation time, thereby improving the pharmacokinetics of such proteins\(^10\). In addition, many proteins are activated by dimerization or multimerization; therefore synthetic multimerization of proteins can result in enhanced activity\(^11,12\). Polymers modified with multiple NTA functionalities can be conjugated to His6-tagged proteins, combining the benefits of polymer conjugation with those of protein multimerization. In addition, the NTA-metal-His6-Protein interaction is reversible, allowing for protein release from the polymer over time.

Kiessling has used Ni(II)-NTA as a non-covalent linker in a protein-polymer conjugate by modifying a poly(succinimidyl methacrylate) with a Ni(II)-NTA complex post-polymerization.\(^13\) This random copolymer of NTA was used to multimerize fibroblast growth factor 8 (FGF8), which must be dimerized with its receptor to activate cells. Proliferative activity was monitored in a cell line lacking surface heparan sulfate, which normally facilitates dimerization of FGF8. The conjugate was able to elicit proliferation without the addition of exogenous heparin, indicating that multimerization had occurred. In addition, the activity was concentration dependent, with 1:10
polymer:FGF8 resulting in the highest activity. This indicated that the polymer could facilitate chelation of up to 10 his-tagged FGF8s.

While these results are promising, others have found that the chelation is not stable \textit{in vivo}. Szoka and coworkers developed a tri-NTA liposome conjugate with nM affinity for His6-tagged proteins\textsuperscript{14,15}. However, when tested in mice, there was no improvement in the circulation time for the conjugates as compared to unmodified protein.\textsuperscript{16} The authors hypothesize that this is due to competition with plasma proteins or removal of nickel by natural chelators in the blood stream. Similarly, Pasut and coworkers found that a flexible 8-arm PEG, when chelated via Cu(II) to 8 NTAs, dissociated from granulocyte stimulating factor (G-CSF) \textit{in vivo}\textsuperscript{17}. While this G-CSF contained five histidine residues throughout its structure, it was not His6-tagged. Improving on these methods, Paik and coworkers, who previously developed mono-NTA end-functionalized polystyrenes, have now developed tri-NTA end-functionalized polystyrenes for the multimeric aggregation of green fluorescent protein\textsuperscript{18-21}. However, these conjugates have not been tested \textit{in vitro} or \textit{in vivo}.

Here we describe the synthesis of a NTA monomer for the synthesis of His6-protein reactive polymers that does not require post-polymerization modification. Furthermore we describe the synthesis of poly(nitrilotriacetic acid methacrylate)-\textit{block}-poly(polyethylene glycol methyl ether methacrylate) (pNTAMA-\textit{b}-pPEGMA) towards multimeric conjugation of His6-tagged proteins.
4.2. Results and Discussion

4.2.1 NTA Monomer Synthesis

Scheme 4.1. Synthesis of NTAMA 2

A histidine-reactive NTA monomer was synthesized in two steps (Scheme 4.1). The double N-alkylation of serine tert-butyl ester with tert-butyl bromoacetate resulted in NTA alcohol 1, obtained as a white solid after lyophilization, in 94% yield. It is interesting to note the effect of the diastereotopicity of protons D and D’ on the alcohol proton C as observed in $^1$H-NMR analysis (Figure 4.1) with proton C appearing as a doublet of doublets at 4.3 ppm. This assignment is confirmed by HSQC-inept and 2D-COSY (Figures 4.2 and 4.3).
Figure 4.1. $^1$H-NMR (CDCl$_3$) of NTA alcohol 1
Figure 4.2. HSQC-inept analysis of NTA alcohol 1 (CH$_2$, CH, or CH$_3$ are indicated by color)

![HSQC-inept analysis of NTA alcohol 1](image)

Figure 4.3. 2D-COSY analysis of NTA alcohol 1

Esterification of 1 with methacryloyl chloride produced the yellow, viscous NTA monomer 2 in 78% yield. Monomer 2 was stored at -20 °C to prevent auto-polymerization, which was observed upon storage at higher temperatures. Both diastereotopic protons D and D’ are visible in the $^1$H spectra (Figure 4.4). Proton E is observed as a distinct triplet, whereas for 1 the signal overlapped with signals from B and D.
4.2.2 Block Copolymer Synthesis

Scheme 4.2. Synthesis of pNTAMA-b-pPEGMA 3 via RAFT block copolymerization of NTAMA 1 and PEGMA
Reversible addition-fragmentation chain transfer (RAFT) polymerization of NTAMA was mediated by the chain transfer agent (CTA) S-thiobenzoyl-2-thiopropionate and initiated by AIBN (Scheme 4.2). After 6 hours at 70 °C, the polymerization was stopped at 70% conversion. The next block was synthesized by adding additional AIBN and the second monomer, PEGMA, with pNTAMA as the macro-CTA. The polymerization was stopped after 6 hours at 70 °C, resulting in pNTAMA-b-PEGMA, which was characterized by ¹H-NMR analysis (Figure 4.5). Comparison of the integration from 4.92-4.20, corresponding to NTAMA -CO₂CH₂CH- protons, or the integration from 3.50-3.19, corresponding to PEGMA side-chain –OCH₃, to the integration at 1.05 ppm for the CTA end-group -O₂CCHCH₃- protons gave a NTAMA:PEGMA ratio of 9:8 and a molecular weight of 6.7 kDa.
Figure 4.5. $^1$H NMR of pNTAMA-\textit{b}-pPEGMA 3

The lack of peaks in the aromatic region, specifically around 8.1 ppm, indicates that there has been a loss of the dithioester end-group. This could be due to the high amount of AIBN used. In addition, the small peaks at 5.3 and 6.3 ppm indicate some disproportionation, indicating that the polymerization was not well controlled. In addition, the molecular weight is bimodal, as visualized by gel permeation chromatography (GPC) with maxima at $M_n = 3.9$ kDa and 48.5 kDa (Figure 4.6). The overall number average molecular weight is 9.9 kDa with a dispersity of 4.5. The lack of control is likely due to the mismatch between CTA and monomer reactivity. This polymerization should be performed again with a more appropriate CTA and less AIBN.
4.2.3 Synthesis of Protein-His6-Ni(II)-pNTAMA-b-pPEGMA Conjugates

Scheme 4.3. Synthesis of Protein-His6-Ni(II)-pNTAMA-b-pPEGMA conjugates

Future work on this project involves the deprotection of t-butyl protecting groups with trifluoroacetic acid, metal chelation, and conjugation to His6-tagged proteins. This should result in dimeric and multimeric protein-polymer conjugates. The conjugates can then be characterized by fast protein liquid chromatography (FPLC) and native gel electrophoresis.

It will also be important to control the number of proteins which conjugate to the synthesized block copolymer as well as their spacing. Meier recently evaluated the multimerization of His6-tagged yellow fluorescent protein or collagenase through Cu(II)
mediated chelation to poly(N-isopropylacrylamide-co-tris-nitritriacetic acid acrylamide) and found that the distance between NTA units must be at least the width of one protein\textsuperscript{22}. Therefore, the block copolymer synthesized in this chapter may limit the number of proteins in the conjugate due to steric restrictions.

Concurrently with this work, conjugations between His-tagged calmodulin as well as major vault protein and polymers functionalized at one end with mono-NTA were unsuccessful. These results, in addition to previously described results in the literature, indicated that mono-NTA-His\textsubscript{6} interactions are not sufficient for protein conjugation or multimerization, and tri-NTA units are necessary. However, direct polymerization of a tri-NTA monomer would involve multi-step synthesis of the monomer resulting in a bulky monomer, which would likely be difficult to polymerize due to steric hindrance. Since the aim of this project was to develop a facile polymerization method for NTA block-copolymers and consequent protein multimerization, further work in this area was discontinued.
4.3. Experimental

4.3.1 Abbreviations
AIBN: 2,2’-azobis(2-methylpropionitrile); BHT: 3,5-di-tert-butyl-4-hydroxytoluene; CTA: chain transfer agent; D_2O: deuterated water; DCM: dichloromethane; DMF: N,N-dimethylformamide; EtOAc: ethyl acetate; GPC: gel permeation chromatography; MALDI-TOF MS: matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MeOH: methanol; MWCO: molecular weight cut off; NTA: nitrilotriacetic acid; PEGMA: poly(ethylene glycol) methacrylate; PMMA: poly(methyl methacrylate); pPEGMA: poly(poly(ethylene glycol) methacrylate).

4.3.2 Materials
Chemicals were purchased from Sigma-Aldrich, Fisher Scientific, Acros, Chem-Impex Int’l Inc. and used as received unless otherwise specified. AIBN was recrystallized from acetone. DCM was distilled over CaH_2 and stored under argon.

4.3.3. Instrumentation
NMR spectra were obtained on a Bruker Avance (DRX or ARX) 500 MHz spectrometer. ¹H-NMR spectra were acquired with a relaxation delay of 2 sec for small molecules, and a relaxation delay of 30 sec for all polymers. MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager-DE STR and operated in linear mode with an external calibration. GPC was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and
two Polymer Laboratories PLgel 5 µm mixed D columns. LiBr (0.1 M) in DMF at 40 °C was used as an eluent (flow rate: 0.80 mL/min). Calibration was performed using near-monodisperse PMMA standards from Polymer Laboratories.

4.3.4. Methods

Synthesis of NTA alcohol (tert-butyl-2-di(tert-butyloxycarbonylmethyl)amino-2-hydroxypropanoate) (1)

Following the procedure of Meunier et al., serine tert-butyl ester hydrochloride (600 mg, 3.04 mmol) was suspended in 35 mL acetonitrile. Upon dropwise addition of 6 equivalents of Hünig’s base (N,N-diisopropylethylamine) (3.173 mL, 18.22 mmol), the solution became clear. Four equivalents of tert-butyl bromoacetate (1.793 mL, 12.14 mmol) was added, and the solution was refluxed at 100 °C for 24 hours under inert atmosphere. The solution was then cooled to room temperature, purified by column chromatography (8:2 hexanes:EtOAc, Rf = 0.4), and dried on high vacuum overnight to yield 1.12 g of a white solid (94% yield). ¹H NMR (500 MHz, CDCl₃) δ: 4.33-4.27 (dd, J = 11.00, 1.52 Hz, 1H), 3.80-3.71 (td, J = 11.00, 3.73 Hz, 1H), 3.56-3.42 (m, 7H), 1.47-1.43 (O-C-(CH₃)₃, 27H). ¹³C NMR (500 MHz, CDCl₃) δ: 171.62, 170.52, 82.02, 81.67, 68.50, 60.44, 55.23, 28.45, 28.34. FT-IR(cm⁻¹): 3460, 2981, 2971, 2933, 2824, 1745, 1725, 1457, 1426, 1393, 1368, 1303, 1268, 1248, 1225, 1214, 1140, 1077, 1058, 1019, 988, 967, 941, 873, 848, 827, 801, 759, 741, 654. MALDI-TOF MS expected (found): [MNa⁺]: 412.23 (412.25); [MH⁺]: 390.24 (390.28).
Synthesis of NTAMA ((di-tert-butyl 2,2'-(1-(tert-butoxy)-3-(methacryloxy)-1-oxopropan-2-yl)azanediyl)diacetate) (2)

1 (1.12 g, 2.87 mmol) was dissolved in 10 mL DCM. Under inert atmosphere, five equivalents of Hünig’s base (2.50 mL, 14.4 mmol) were added slowly. The solution was allowed to stir for 10 minutes and then cooled to 0 °C with an ice-bath. Two equivalents of freshly distilled methacryloyl chloride (0.556 mL, 5.75 mmol) were added dropwise over 15 minutes. The solution was allowed to come to room temperature and stirred for 12 hours. The reaction mixture was then washed three times with saturated NaHCO₃, once with brine, and dried over MgSO₄. The product was purified by column chromatography (8:2 hexanes:EtOAc, Rf = 0.6) and dried under high vacuum overnight to yield 1.02 g of a yellow viscous oil (78% yield). ¹H NMR (500 MHz, CDCl₃) δ: 6.14-6.01 (s, 1H), 5.61-5.48 (s, 1H), 4.58-4.48 (dd, J = 11.1, 5.47 Hz, 1H), 4.45-4.34 (dd, J = 11.2, 6.27 Hz, 1H), 3.82-3.70 (t, J = 5.83 Hz, 1H), 3.68-3.48 (q, J = 20.08, 4H), 1.95-1.88 (s, 3H), 1.47-1.40 (O-C-(CH₃)₃, 27H). ¹³C NMR (500 MHz, CDCl₃) δ: 170.79, 169.76, 166.78, 136.10, 128.47, 126.03, 81.81, 80.97, 64.40, 64.02, 54.70, 28.31, 28.24, 28.22, 18.38. FT-IR(cm⁻¹): 2978, 2932, 1722, 1638, 1479, 1456, 1393, 1367, 1319, 1295, 1248, 1221, 1136, 1032, 988, 940, 846, 813, 751, 680. MALDI-TOF MS expected (found): [M_{Na⁺}]: 480.26 (480.32); [M_{Na⁺H⁺}]: 481.27 (481.31).

Synthesis of pNTAMA-b-pPEGMA (poly(nitrilotriacetic acid methacrylate)-block-poly(polyethylene glycol methyl ether methacrylate)) (3)
NTAMA 2 (700 mg, 1.53 mmol) was dissolved in dry benzene and pushed through a neutral alumina plug to remove BHT. The solution was then transferred to a dry Schlenk tube, charged with AIBN (4.2 mg, 30 μmol), the CTA, S-thiobenzyol-2-thiopropionate, (13.0 mg, 51.5 mmol), and the volume adjusted to 5 mL. The Schlenk tube was then sealed and subjected to five freeze-pump-thaw cycles to remove oxygen, refilled with Ar and placed in an oil bath at 70 °C. Timepoints were assessed by ¹H-NMR to determine percent conversion, which was calculated by comparing the integration at 5.3 ppm, corresponding to the monomer vinyl proton (C=C-H), to the integration from 2.7-2.1 ppm, corresponding to the growing polymer backbone methylene. The polymerization was stopped after 6 hours at 70% conversion by cooling the solution and exposing atmosphere. PEGMA (459 mg, 1.53 mmol) and additional AIBN (4.2 mg, 30 μmol) were added to the Schlenk tube in 2 mL dry benzene and the same freeze-pump-thaw and Ar refill was performed. The Schlenk was placed at 70 °C in an oil bath for 6 hours. The polymerization was stopped by cooling and exposing to atmosphere, and the block copolymer was purified by dialysis in 1 L of 1:1 MeOH:H₂O in 3500 MWCO dialysis tubing. The solution was exchanged after 24 hours, exchanged once more into H₂O after an additional 24 hours, and the product dried by lyophilization. NTAMA:PEGMA = 9:8 and $M_n = 6.7$ kDa (¹H-NMR). $M_n = 9.9$ kDa and $Đ = 4.5$ (GPC). ¹H-NMR (500 MHz, CDCl₃) δ: 4.92-4.20 (18H, -CO₂CH₂CH₂-), 4.20-3.50 (156H, -CO₂CH-, N-CH₂-, CO₂CH₂CH₃, and PEGMA side-chain –O-CH₂CH₂-), 3.50-3.19 (24H, PEGMA side-chain –OCH₃), 2.72-2.18 (22H, end-group CH₃CH₂O₂CCH- and PEGMA and NTAMA backbone –OCCH₂-), 2.11-1.34 (105H, end-group CH₃CH₂O₂-, PEGMA and NTAMA backbone –CH₃, and NTA side-chain –CO₂-C-(CH₃)₃), 1.05 (3H, s, end-group -
O$_2$CCH$_3$-) ppm. FT-IR (cm$^{-1}$): 2875, 1725, 1452, 1392, 1367, 1350, 1247, 1138, 1104, 1037, 1028, 987, 947, 847, 747.
4.4. Conclusions

A NTA monomer, NTAMA, was synthesized in two steps in 73% yield. RAFT polymerization of this monomer resulted in a macro-CTA, pNTAMA. RAFT polymerization of PEGMA mediated by this macro-CTA resulted in a block copolymer, pNTAMA-\(b\)-PEGMA with number average molecular weight \(M_n\) (GPC) = 9.9 kDa and dispersity \(D\) = of 4.5. The polymer contains a block of 9 NTAMA units and a block of 8 PEGMA units as assessed by \(^1\text{H}-\text{NMR. Disproportionation was visualized by} \(^1\text{H}-\text{NMR and GPC, indicating that polymerization was not controlled. Since parallel studies indicated that mono-NTA-His6 interactions are not sufficient for protein conjugation, further work in this area was not pursued.}}\)
4.5. References


