Title
Protein Stabilization and Immobilization in Three Dimensions

Permalink
https://escholarship.org/uc/item/60g7d5tf

Author
Mancini, Rock Joseph

Publication Date
2012

Peer reviewed|Thesis/dissertation
Protein Stabilization
and
Immobilization in Three Dimensions

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

By

Rock Joseph Mancini

2012
From bee venom to recombinantly produced replacement proteins, biologics have played a key role in the development of civilization. Advancement in two areas of this field is reported: three-dimensional (3-D) encapsulated hydrogel microstructures fabricated by electron-beam lithography and the stabilization of protein therapeutics to environmental insults by trehalose glycopolymer excipients.
A procedure for the fabrication of protein reactive hydrogels of orthogonal reactivity in an encapsulated configuration by electron beam (e-beam) lithography is described. Amine terminated 8-arm poly(ethylene glycol) (PEG) has been modified to produce polymers containing biotin (Biotin-PEG), and alkyne (Alkyne-PEG) end-groups. Hydroxyl terminated PEG has been used to synthesize a polymer with aminooxy functionality (AO-PEG). These polymers have in turn been employed to fabricate micro-gels by e-beam lithography, and it was determined via confocal microscopy that a nominal size exclusion effect exists for hydrogels of feature sizes ranging from 5 to 40 µm. Micro-realignment has been used to pattern Alkyne-PEG encapsulated as a core inside an outer shell of AO-PEG. Hydrogel micro-patterns with encapsulated architectures have been labeled with fluorescent dyes of complimentary reactivity and 3-D spatial orientation of the reactive end-groups has been confirmed via confocal microscopy. The proteins glucose oxidase (GOX) and horseradish peroxidase (HRP) have been modified to contain azide and carbonyl moieties along with green and blue fluorophores respectively. Upon incubation with encapsulated hydrogels it was determined that the modified GOX and HRP proteins reacted selectively with hydrogel end-groups of complimentary reactivity. Retention of enzyme activity was confirmed in this encapsulated configuration via an enzyme cascade reaction with glucose and Amplex Red substrates.

The synthesis of trehalose side chain polymers with stabilization of protein conjugates to environmental stressors is also reported. The glycomonomer 2-methacryl-4,6,4’,6’-O-dibenzylidene-α,α-trehalose was synthesized over two steps in 14% yield through dibenzylidene protection and subsequent esterification with methacryloyl chloride. A thiol reactive glycopolymers was prepared by reversible addition-fragmentation chain transfer (RAFT)
polymerization using a thiol reactive chain transfer agent (CTA). Furthermore, the glycomonomer 4,6-O-(4-vinylbenzyl)-α,α-trehalose (4,6-O-(4-vinylbenzyl)-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) was synthesized in 40% yield over two steps without the use of protecting group chemistry. Polymers containing this glycomonomer were prepared via RAFT polymerization using three different protein reactive CTAs. A range of molecular weights from 4.2 to 49.5 kDa was obtained with well-defined polydispersities for all but the highest molecular weights attempted. The polymers were conjugated to thiolated hen egg white lysozyme and purified. The glycopolymers, when covalently attached to protein or added to the formulation without attachment, significantly increased stability towards lyophilization and heat relative to wild-type protein. Up to 100% retention of activity was observed when lysozyme was stressed ten times with lyophilization and 81% activity when the protein was heated at 90 °C for 1 hour; this is in contrast to 16% and 18% retention of activity, respectively, for the wild-type protein alone. The glycopolymers were compared to equivalent concentrations of trehalose relative to glycopolymer side-chain, and the glycopolymers were found to be superior at stabilizing the protein to lyophilization and heat. When compared to poly(ethylene glycol) (PEG) the glycopolymers were superior at heat stabilization and exhibited comparable lyoprotectant effects. In addition, the protein-glycopolymer conjugates exhibited significant increases in stability when compared to adding the same concentration of un-conjugated polymer to the protein.
The dissertation of Rock Joseph Mancini is approved.

Jeffrey I. Zink
Timothy J. Deming
Andrea M. Kasko

Heather D. Maynard, Committee Chair

University of California, Los Angeles
2012
This work is dedicated to all of my friends and family who have helped me to become the human being that I am today.
# TABLE OF CONTENTS

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

Table of Contents............................................................................................................................ vii

List of Figures and Tables............................................................................................................... x

List of Abbreviations ...................................................................................................................... xiii

Acknowledgements........................................................................................................................ xvi

Vita .................................................................................................................................................... xviii

## CHAPTER 1. INTRODUCTION

1.1 Surface Immobilized Biologics ............................................................................................... 1

  1.1.1 Biosensors ......................................................................................................................... 2

  1.1.2 Oriented Bio-arrays ......................................................................................................... 2

1.2 Biological Therapeutics ............................................................................................................. 3

  1.2.1 Classes of Protein Based Treatments ............................................................................. 4

  1.2.2 Protein Conformation ..................................................................................................... 6

1.3 Protein Polymer Conjugates .................................................................................................... 7

  1.3.1 Conjugation Strategies ................................................................................................... 7

  1.3.2 Effects of Attaching Poly(ethylene glycol) to Protein Therapeutics ......................... 10

  1.3.3 Conjugation to Other Polymers .................................................................................... 11

1.4 Trehalose.................................................................................................................................. 12

## CHAPTER 2. 3-D ENCAPSULATED HYDROGEL ARCHITECTURES BY e-BEAM LITHOGRAPHY

2.1 Introduction .............................................................................................................................. 14

2.2 Results and Discussion ........................................................................................................... 15

  2.2.1 Synthesis of Protein Reactive 8-arm Poly(ethylene glycol) .......................................... 16

  2.2.2 Confocal Microscopy of Surface Immobilized Streptavidin ........................................ 17
2.2.3 Fabrication of Encapsulated Hydrogels by e-Beam Lithography ....................... 18
2.2.4 Confocal Microscopy of Encapsulated Hydrogels ............................................. 21
2.2.5 Enzyme Immobilization in Encapsulated Hydrogels ...................................... 24
2.2.6 Enzyme Cascade Reaction in Encapsulated Hydrogels .................................. 28
2.2.7 Surface Immobilized Enzyme Kinetics ............................................................ 30

2.3 Experimental ........................................................................................................ 32

CHAPTER 3. Trehalose Based Glycomonomers .............................................................. 50
3.1 Introduction ........................................................................................................... 51
3.2 Results and Discussion .......................................................................................... 53
   3.2.1 Synthesis of 2-Methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose ................ 53
   3.2.2 Characterization of 2-Methacryl-4,6,4',6'-Dibenzylidene-α,α-Trehalose ...... 58
   3.2.3 Synthesis of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose ............................... 63
   3.2.4 Characterization of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose ................. 65
   3.2.5 Conformational Analysis of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose ....... 70
3.3 Experimental ........................................................................................................ 72

CHAPTER 4. Protein Reactive Trehalose Side-Chain Glycopolymers ............................. 84
4.1 Introduction ........................................................................................................... 85
4.2 Results and Discussion ......................................................................................... 87
   4.2.1 Polymerization of 2-methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose ....... 87
   4.2.2 Polymerization of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose ....................... 89
   4.2.3 Synthesis of Novel Chain Transfer Agents ...................................................... 91
   4.2.4 Polymerization with Novel Chain Transfer Agents ....................................... 93
   4.2.5 Implications of Synthesized Glycopolymers ................................................ 97
4.3 Experimental ........................................................................................................ 98
CHAPTER 5. CONJUGATION OF Trehalose Glycopolymers to Lysozyme .............................................. 113

5.1 Introduction ................................................................................................................................. 114

5.2 Results and Discussion .............................................................................................................. 116

5.2.1 Attachment of Thiols to Hen Egg-White Lysozyme .......................................................... 116

5.2.2 Attachment of Trehalose Glycopolymers to Thiolated Lysozyme ........................................ 117

Purification of Lysozyme-Glycopolymer Conjugates ...................................................................... 118

5.3 Experimental ............................................................................................................................. 121

CHAPTER 6. STABILIZATION EFFECTS OF Trehalose Glycopolymers .............................................. 126

6.1 Introduction ................................................................................................................................. 127

6.1.1 Protein Environmental Stressors .......................................................................................... 127

6.1.2 Protein Degradation Mechanisms ....................................................................................... 128

6.1.3 Protein Drug Formulations and Stabilizing Excipients ....................................................... 129

6.2 Results and Discussion .............................................................................................................. 132

6.2.1 Lyophilization of Lysozyme and Trehalose Glycopolymers ................................................. 132

6.2.2 Heating Lysozyme and Trehalose Glycopolymers ............................................................... 134

6.2.3 Effects of Trehalose Glycopolymers Relative to Poly(ethylene glycol) ............................ 135

6.2.4 Stability Observations and Mechanistic Implications ......................................................... 137

6.2.4.1 Heat Burden .................................................................................................................. 138

6.2.4.2 Lyophilization Stress ..................................................................................................... 139

6.3 Experimental ............................................................................................................................. 141

CHAPTER 7. CONCLUSIONS ............................................................................................................ 144

7.1 Encapsulated Hydrogel Architectures by e-Beam Lithography ............................................. 145

7.2 Trehalose Based Glycopolymers ............................................................................................... 145

References ........................................................................................................................................ 147
LIST OF FIGURES AND TABLES

CHAPTER 1.

Figure 1.1: Conjugation Strategies for Attachment of Polymers to Proteins.......................... 8

CHAPTER 2.

Figure 2.1: Synthesis of Compound 2.1................................................................................ 16
Figure 2.2: Synthesis of Alkyne-PEG, Biotin-PEG, and AO-PEG ........................................ 17
Figure 2.3: Confocal Microscopy of Surface Immobilized SAv-AF488.................................. 18
Figure 2.4: Synthesis of Encapsulated Hydrogels ................................................................ 19
Figure 2.5: Confocal Microscopy of Biotin-PEG Surrounding AO-PEG................................. 22
Figure 2.6: Fluorophore Cross-Talk of Coumarin and SAv-AF488 ........................................... 23
Figure 2.7: Confocal Microscopy of AO-PEG Surrounding Alkyne-PEG................................. 24
Figure 2.8: Enzyme Cascade with Encapsulated Surface Immobilized Enzymes.................... 27
Figure 2.9: Fluorescence Microscopy of Surface Immobilized Enzymes................................. 28
Figure 2.10: Cross Reactivity of Azide-GOX-AF488 ............................................................. 28
Figure 2.11: Activity of Surface Immobilized Enzymes .......................................................... 30

CHAPTER 3.

Figure 3.1: Synthesis of compounds 3.1 and 3.2 ................................................................ 53
Figure 3.2: 1H NMR (500 MHz, D6DMSO) of Compound 3.1.............................................. 55
Figure 3.3: COSY (500 MHz, D6DMSO) of Compound 3.1................................................... 55
Figure 3.4: 1H-13C HSQC (500 MHz, D6DMSO) of Compound 3.1 ........................................ 56
Figure 3.5: Illustration of 3.1 Diastereomers ....................................................................... 56
Figure 3.6: Illustration of Compound 3.2 ........................................................................... 58
Figure 3.7: 1H NMR (500 MHz, D6DMSO) of Compound 3.2 ............................................. 60
Figure 3.8: 13C and DEPT-135 NMR (500 MHz, D6DMSO) of Compound 3.2 ................. 61
Figure 3.9: $^1$H-$^{13}$C HSQC (500 MHz, D$_6$DMSO) of Compound 3.2 ........................................ 61
Figure 3.10: COSY (500 MHz, D$_6$DMSO) of Compound 3.2 .................................................. 62
Figure 3.11: Synthesis of Compounds 3.3 and 3.4 .................................................................... 64
Figure 3.12: Illustration of Compound 3.4 ............................................................................. 65
Figure 3.13: $^1$H NMR (600 MHz, D$_6$DMSO) of Compound 3.4 ........................................... 66
Figure 3.14: COSY (500 MHz, D$_6$DMSO) of Compound 3.4 .................................................. 67
Figure 3.15: $^1$H-$^{13}$C HSQC (500 MHz, D$_6$DMSO) of Compound 3.4 ......................... 68
Figure 3.16: 2-D HSQC-TOCSY (600 MHz, D$_6$DMSO) of Compound 3.4 .................... 68
Figure 3.17: $^{13}$C NMR (500 MHz, D$_6$DMSO) of Compound 3.4 ....................................... 70
Figure 3.18: NOESY (500 MHz, D$_6$DMSO) of Compound 3.4 ............................................. 71

CHAPTER 4.

Table 4.1: Polymerization Conditions Used to Obtain Poly 1-4 ........................................... 91
Table 4.2: Polymerization Conditions Used to Obtain Poly 5-8 .......................................... 93
Figure 4.1: Synthesis of CTA 1 ............................................................................................... 87
Figure 4.2: Polymerization of 3.2 to Obtain 4.1 .................................................................. 88
Figure 4.3: Gel Permeation Chromatography Trace of 4.1 ................................................. 88
Figure 4.4: Polymerization of 3.4 to Obtain Poly 1-4 ......................................................... 90
Figure 4.5: $^1$H NMR (500 MHz, D$_6$DMSO) of Poly 2 ....................................................... 90
Figure 4.6: Gel Permeation Chromatography Trace of Poly 1-3 ......................................... 91
Figure 4.7: Synthesis of Compounds 4.2-4.4 and CTA 2,3 .................................................. 92
Figure 4.8: $^1$H NMR (500 MHz, D$_6$DMSO) of Poly 5 ......................................................... 94
Figure 4.9: Gel Permeation Chromatography Trace of Poly 5-8 ........................................ 94
Figure 4.10: UV/Vis Absorbance Spectra of Poly 5-8 .......................................................... 95
Figure 4.11: Gel Permeation Chromatography Trace of Poly 9 ............................................ 96
Figure 4.12: $^1$H NMR (600 MHz, D$_6$DMSO) of Poly 9 ................................................................. 96

**CHAPTER 5.**

Figure 5.1 Synthesis of Compound 5.1 ................................................................. 116

Figure 5.2 Addition of Free Thiols to Lysozyme to Produce LyzSH.................................. 116

Figure 5.3 Conjugation of Poly 5-8 to LyzSH .................................................................. 117

Figure 5.4 SDS-PAGE of Crude Conjugation Reactions .................................................. 118

Figure 5.5 FPLC Purification of Conjugation Reactions ................................................. 119

Figure 5.6 SDS-PAGE of Conjugates Lyz-Poly 5-8 Purified by FPLC ......................... 120

**CHAPTER 6.**

Figure 6.1 Activity of Lysozyme and Conjugates Lyz-Poly 5-8 After Lyophilization .......... 133

Figure 6.2 Activity of Lysozyme and Conjugates Lyz-Poly 5-8 After Heating ................. 135

Figure 6.3 Activity of Lysozyme stabilized by Poly 5-8 Relative to PEG (DP).................... 136

Figure 6.4 Activity of Lysozyme stabilized by Poly 5-8 Relative to PEG (M$_n$) ............... 137
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcOH</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>AIBN</td>
<td>Azobisisobutyronitrile</td>
</tr>
<tr>
<td>Alkyne-PEG</td>
<td>Alkyne Functionalized 8-arm Poly(ethylene glycol)</td>
</tr>
<tr>
<td>AO-PEG</td>
<td>Aminooxy Functionalized 8-arm Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>Azide-GOX-AF488</td>
<td>Azide Functionalized Glucose Oxidase-Alexafluor 488 Conjugate</td>
</tr>
<tr>
<td>Biotin-PEG</td>
<td>Biotin Functionalized 8-arm Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Bz</td>
<td>Benzyldene</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Deuterated Chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>Homonuclear Correlation Spectroscopy</td>
</tr>
<tr>
<td>CRP</td>
<td>Controlled Radical Polymerization</td>
</tr>
<tr>
<td>CS$_2$</td>
<td>Carbon Disulfide</td>
</tr>
<tr>
<td>CTA</td>
<td>Chain Transfer Agent</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>d</td>
<td>doublet (NMR)</td>
</tr>
<tr>
<td>D$_6$DMSO</td>
<td>Deuterated Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DBz</td>
<td>Dibenzylidene</td>
</tr>
<tr>
<td>DCC</td>
<td>$N,N'$-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Methylene Chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets (NMR)</td>
</tr>
<tr>
<td>ddd</td>
<td>doublet of doublet of doublets (NMR)</td>
</tr>
<tr>
<td>DEPT-135</td>
<td>Distortionless Enhancement by Polarization Transfer 135 (NMR)</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N'-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DMT</td>
<td>Dimethoxy Toluene</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>EA</td>
<td>Encapsulated Architecture</td>
</tr>
<tr>
<td>e-Beam</td>
<td>electron-Beam</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eq</td>
<td>Equivalents</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GOX</td>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation (Size Exclusion) Chromatography</td>
</tr>
<tr>
<td>HEMA</td>
<td>Hydroxyethyl Methacrylate</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence (NMR)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HSQC-TOCSY</td>
<td>Heteronuclear Single Quantum Coherence Total Correlation Spectroscopy (NMR)</td>
</tr>
<tr>
<td>J</td>
<td>Coupling Constant (NMR)</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>Lev-HRP-MB</td>
<td>Levulinyl Horseradish Peroxidase-Marina Blue Conjugate</td>
</tr>
<tr>
<td>Lyz-Poly</td>
<td>Lysozyme Glycopolymer Conjugate</td>
</tr>
<tr>
<td>LyzSH</td>
<td>Thiolated Lysozyme</td>
</tr>
<tr>
<td>m</td>
<td>multiplet (NMR)</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz (NMR)</td>
</tr>
<tr>
<td>M&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Number Average Molecular Weight</td>
</tr>
<tr>
<td>M&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Weight Average Molecular Weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>n-BuLi</td>
<td>n-Butyl Lithium</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Enhancement Spectroscopy</td>
</tr>
<tr>
<td>p</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate Buffer</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Poly X</td>
<td>Poly(4,6-O-(4-vinylbenzylidene)-α,α-trehalose)</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million (NMR)</td>
</tr>
<tr>
<td>p-TsOH</td>
<td>para-Toluenesulfonic Acid</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible Addition-Fragmentation Chain-Transfer</td>
</tr>
<tr>
<td>s</td>
<td>singlet (NMR)</td>
</tr>
<tr>
<td>SAM</td>
<td>Self Assembled Monolayer</td>
</tr>
<tr>
<td>SATP</td>
<td>N-Hydroxysuccinimidyl-S-Acetythiopropionate</td>
</tr>
<tr>
<td>Sav-AF488</td>
<td>Streptavidin-Alexafluor 488 Conjugate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Poly(acrylamide) Gel Electrophoresis</td>
</tr>
<tr>
<td>t</td>
<td>triplet (NMR)</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethyl Amine</td>
</tr>
<tr>
<td>Tg</td>
<td>Glass Transition Temperature</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>U</td>
<td>Units of Lysozyme Activity</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Diphosphate</td>
</tr>
<tr>
<td>XRD</td>
<td>X-Ray Diffraction</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction Coefficient (UV/Vis)</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First I would like to thank my friend at the University of Pittsburgh, Prof. George Bandik, for challenging me to attend graduate school and for his guidance throughout my time here at UCLA. I would also like to thank my advisor Prof. Heather Maynard for allowing me to be an integral part of her research group, and for allowing me to pursue projects of interest to me. I am indebted to Prof. Yong Chen, for his SEM equipped to perform e-beam lithography (Chapter 2), and Juneyoung Lee for his assistance with the trehalose glycopolymer project (Chapters 3-6). Jane Strouse assisted in preparing the HSQC-TOCSY pulse sequence (Chapter 3). Robin Higgins assisted in synthesizing chain transfer agent precursors (Chapter 4). Kathy Nguyen was instrumental in preparing thiolated lysozyme and in FPLC purification (Chapter 5).

Portions of chapters 3-6 are reproduced from Mancini, R. J.; Lee, J. Y.; Maynard, H. D. “Trehalose Glycopolymers for Stabilization of Protein Conjugates to Environmental Stressors” J. Am. Chem. Soc., 2012, 134, 20, 8474-8479. Lastly, I would also like to acknowledge the NIH and NSF for project funding as well as for the Materials Creation Training Program (MCTP) Fellowship.
VITA

Education

2003 Hampton High School
Allison Park, Pennsylvania

2007 University of Pittsburgh
B.S., Chemistry with Honors

2012 University of California, Los Angeles
Ph.D., Chemistry

Research Experience

2005 NSF: Research Experience for Undergraduates Fellow
Washington State University
Prof. Alexander D. Q. Li, Ph.D.

2006 Intern
Valspar Global Technical Center
Pittsburgh, PA

2006-2007 Undergraduate Research Assistant
University of Pittsburgh
Prof. Toby M. Chapman, Ph.D.

2007-2012 Graduate Research Assistant
University of California, Los Angeles
Prof. Heather D. Maynard, Ph.D.

2008 Intern
DuPont Fluoroproducts
Wilmington, DE

Awards

2005 NSF: Research Experience for Undergraduates Fellowship

2006 Valspar Award

2008 NSF IGERT: Materials Creation Training Program Fellowship
Publications


Presentations

(7) “Multi-Component 3-D Protein Arrays by Electron Beam Lithography” Oral presentation at the 243rd ACS meeting, San Diego, March 26, 2012.

(6) “Multi-Component 3-D Protein Arrays by Electron Beam Lithography” Poster presentation at the Seaborg Symposium, UCLA, November 5, 2011.

(5) “Multi-Component 3-D Protein Arrays by Electron Beam Lithography” Poster presentation at the Materials Creation Training Program IGERT Conference, UC, Santa Barbara, October 21, 2011.

(4) “Multi-Component 3-D Hydrogel Architectures Created by Electron Beam Lithography” Oral presentation at the Zing Polymer Conference, Cancun, Mexico, November 19, 2010.

(3) “Sustainable Fluoroproduct Formulations” Oral presentation at the Materials Creation Training Program IGERT Conference, UCLA, November 6, 2009.


(1) “Patterns of Aminooxy Groups for Protein Conjugation” Poster presentation at the Materials Creation Training Program IGERT Conference, UCLA, November 14, 2008.
CHAPTER 1

INTRODUCTION
SURFACE IMMobilIZED BIOLOGICS

Biosensors

Improved efficiency of biomarker discovery has resulted in a demand for high-throughput protein screening methods to identify disease at early onset, as well as to monitor the progression of treatments through biosignature detection. Surface immobilization at this scale allows for detection of multiple biomarkers on the same surface, resulting in a more resolved biosignature and a more robust protein array. As such, methods for fabrication of reactive surface arrays for site-specific conjugation to molecules of biological interest have been sought.

Oriented Bio-arrays

The ability to pattern bioactive arrays of proteins at the micron or nano-scale has been comprehensively examined. Several serial and parallel techniques to produce oriented bio-arrays are currently known. Parallel techniques have the advantage of increased speed for high throughput processing often with multiplexed capabilities. In contrast, higher resolution is traditionally achieved with serial techniques. Parallel approaches to surface immobilized bioactive patterns include self assembled monolayer (SAM) formation, micro-contact printing or stamping as well as photo-lithography. Resolution among these processes is comparable and typically in the nano-scale regime. Serial techniques include electron-beam lithography and dip-pen atomic force microscopy (AFM)/lithography. Resolution for e-beam lithography is theoretically limited by the wavelength of an electron, which is on the
order of 10 pm for typical acceleration voltages; single molecule resolution may be obtained in the case of AFM.18

Several methods of chemical immobilization have been utilized to attach biomolecules to surfaces. One method involves nonspecific adsorption onto a surface; however, this often results in major conformational changes.19-21 Alternatively, biomolecules may be covalently linked to the surface. Early routes included carbodiimide coupling of the free amine groups of the protein to a surface bearing terminal carboxylic acid moieties or reversible coupling with surface carbonyl groups via imine chemistry.22, 23 These methods however, are not site-selective and often result in reduction of protein bioactivity.24

To achieve site-specific coupling, free cysteines have been targeted. However, while effective, free cysteines are rare in natural proteins and additional steps of adding a surface reactive amino acid through techniques such as mutagenesis may be required.25, 26 Alternatively, Huisgen cycloaddition or “click” chemistry has been used by modifying the biomolecule to contain an azide or alkyne moiety with an alkane thiol of complementary reactivity patterned on the surface.27,28 Phosphonate protein interactions29 as well as Diels–Alder reactions30 have also been exploited, and this methodology has been extended to control cellular environments.31 Another approach involves reaction of an aminooxy moiety with a protein containing a ketone or aldehyde to form oxime bonds.32-34

SAMs are a viable option for the fabrication of bioactive arrays required for high-throughput screening. This is because SAMs present well-defined surfaces for protein immobilization. These patterning methods are easily transferred to optical sensing techniques
such as localized surface plasmon resonance\textsuperscript{35} for subsequent investigation of biomolecules at biologically relevant concentrations.\textsuperscript{36}

Serial techniques have been used to create the highest resolution arrays. This high resolution has the advantage of maximal utilization of sensor space and often facilitates multiplexed detection capabilities. Surface cross-linked hydrogels fabricated by photolithography\textsuperscript{37-40} or e-beam lithography\textsuperscript{41} have been employed for biomolecule immobilization,\textsuperscript{42} and most immobilization techniques employed in these areas are analogous to the conjugation chemistries used to produce protein-polymer conjugates in solution. As such we sought to determine the reactive heterogeneity of encapsulated hydrogel architectures fabricated by e-beam lithography with subsequent protein immobilization in this encapsulated configuration (Chapter 2).

\textbf{BIOLOGICAL THERAPEUTICS}

\textit{Classes of Biological Therapeutics}

Protein based drugs possess several inherent advantages over small molecule therapies as biological therapeutics display a complex set of functions that are highly specific, relatively non-immunogenic, and in the case of replacement proteins, otherwise endogenous.\textsuperscript{43} Since the first use of insulin,\textsuperscript{44} the field of biological therapeutics has advanced significantly to include a wide range of drugs and delivery methods. In humans these treatments may be classified according to biologic type\textsuperscript{43} including carbohydrates,\textsuperscript{45} gene therapy,\textsuperscript{46,47} proteins,\textsuperscript{48-53} targeting anti-bodies,\textsuperscript{54} and even whole cells.\textsuperscript{55}
Protein based treatments may be further divided into the sub-categories: replacement, pathway augmentation, interference, delivery vector, vaccine, and diagnostic proteins. Replacement proteins are therapeutics administered to replace deficiencies or mutations. This class includes: hormones such as insulin,\textsuperscript{56-67} insulin-like growth factor,\textsuperscript{68, 69} and other growth hormones;\textsuperscript{70-73} haemostasis regulators including clotting factors,\textsuperscript{74-78} antithrombin,\textsuperscript{79, 80} and protein C;\textsuperscript{81} as well as enzymes\textsuperscript{49, 51, 53, 82-93} or immunoglobulins.\textsuperscript{94} Proteins administered to augment existing pathways encompass: the immune-regulators such as interferon-\textalpha,\textsuperscript{95-112} interferon-\textbeta,\textsuperscript{113-117} interferon-\textgamma,\textsuperscript{56, 118-120} and interleukins,\textsuperscript{121-123} growth-regulators including bone morphogenic proteins,\textsuperscript{124} gonadotropin releasing hormone,\textsuperscript{125, 126} and other growth factors;\textsuperscript{127-130} as well as proteins used to treat various endocrine pathway disorders.\textsuperscript{131-137} Other classes of protein therapeutics include protein based vaccines,\textsuperscript{138-143} and proteins administered for diagnostic\textsuperscript{144-155} or imaging purposes.\textsuperscript{156-164}

There are several obstacles associated with the use of biological therapies in humans. These challenges include \textit{in vivo} complications such as immunogenicity,\textsuperscript{165} pathway mutation\textsuperscript{166} or modulation,\textsuperscript{167} drug resistance\textsuperscript{168} and delivery process of the therapeutic.\textsuperscript{169} While delivery modes commonly attempted include oral,\textsuperscript{170} buccal,\textsuperscript{171} ocular,\textsuperscript{172} intranasal,\textsuperscript{173} pulmonary,\textsuperscript{174} and transdermal\textsuperscript{175} methods, injections (intramuscular, intravenous, or subcutaneous) remain the most common method for administration inherently limiting patient compliance.\textsuperscript{176} In addition, proteins or peptides have a pharmacokinetic profile that is markedly differentiated from typical small molecule drugs.\textsuperscript{177} External factors such as storage conditions,\textsuperscript{178} batch variability,\textsuperscript{179} and non-specific adsorption\textsuperscript{180-184} also affect the practical utilization of protein based agents.
Protein conformation

One major driver in the rigorous storage and administration directives required of protein based drugs is the conformational structure that must be maintained\textsuperscript{185} as subtle changes in sequence or folding can have great effects on activity.\textsuperscript{186} Characterization of proteins includes complete elucidation of primary, secondary, tertiary, and quaternary structure. Techniques to deduce the primary sequence of proteins involve Edman degradation\textsuperscript{187, 188} and more recently, mass spectrometry.\textsuperscript{189, 190} Specifically, tandem MS techniques coupled with collision-activated dissociation,\textsuperscript{191-193} electron-capture dissociation,\textsuperscript{192, 194-196} or electron-transfer dissociation\textsuperscript{197-201} can be employed to unambiguously assign amino acid sequences. Protein secondary structure involves interactions of the primary chain to produce various conformations including alpha helices and \(\beta\)-sheets. These secondary interactions may be estimated using various spectroscopic techniques such as circular dichroism,\textsuperscript{202-210} infrared spectroscopy,\textsuperscript{211} NMR,\textsuperscript{212-226} or via chemical means.\textsuperscript{227} Tertiary structure is the 3-D atomic structure of the protein as a whole entity and is responsible for the folding/unfolding and active/denatured state of a protein. This higher order structure may be probed by NMR,\textsuperscript{228-232} dual-polarization interferometry,\textsuperscript{233-235} or assigned by X-ray scattering.\textsuperscript{236-239} Protein quaternary structure includes interactions of folded substructures of proteins that combine to form higher order complexes. These interactions may be partially discerned by size exclusion chromatography,\textsuperscript{240, 241} analytical ultracentrifugation,\textsuperscript{242} or static light scattering.\textsuperscript{243, 244} For many proteins, structure may be unambiguously assigned by x-ray crystallography. Lastly, it should be noted that several examples are known whereby proteins exist in a disordered native state but regain folding, order and activity shortly before utilization.\textsuperscript{245-247}
Proteins are known to denature or lose activity through a variety of mechanisms including unfolding, agglomeration, and precipitation; however, even completely denatured proteins exist with some ordered structure, and cases of activity retention in the denatured state are known. In fact, proteins do not typically unfold to a homogenous references state but rather enter a heterogeneous number of partially folded states collectively termed the denatured state. Attempts to mitigate this loss of structure have been performed through several methods including formulation techniques and use of polymer excipients of various morphologies.

PROTEIN POLYMER CONJUGATES

Conjugation Strategies

The field of protein-polymer conjugates has been thoroughly reviewed both for conjugation strategies as well as current commercial PEGylated drug formulations. For controlled radical polymerization (CRP) techniques, two methodologies towards protein-polymer therapeutics are commonly employed. Each starts with a small molecule that both exhibits reactivity with proteins and participates in polymerization as an initiator or chain transfer agent (CTA). In the first methodology, the protein reactive initiator or CTA is first attached to a protein before subsequent polymerization. The second strategy involves polymerization with the protein reactive initiator or CTA to obtain a protein reactive polymer, which can be attached to the protein of interest in a grafting-to approach.

Several types of conjugation strategies exist to covalently attach initiators, CTAs, or polymers to proteins (Figure 1.1). The most commonly employed methods are similar to
chemistry used for surface protein immobilization and target free amines or cysteines on proteins. Methods to target amines include functionalization with chlorotriazines,\textsuperscript{255} thioimidoesters,\textsuperscript{256} and aldehydes.\textsuperscript{257} In the case of reductive amination, selectivity may be obtained by adjusting pH such that imine formation occurs exclusively at the $N$-terminus before reduction with sodium cyanoborohydride.\textsuperscript{258,259} Free cysteines are rare in proteins, therefore targeting thiol groups is inherently site-selective. Chemistry employed to conjugate to free thiols in proteins includes vinyl sulfones,\textsuperscript{260-262} maleimides,\textsuperscript{263-265} and activated disulfides such as methoxy carbonyl disulfide\textsuperscript{266} or pyridyl disulfide.\textsuperscript{267} In the case of vinyl sulfone irreversible Michael addition of thiols or alcohols occurs to couple hydroxyl functionalized polymer with thiol functionalized protein. Maleimides may be similarly added post-polymerization or polymerized in protected form as the furan Diels-Alder adduct.

**Figure 1.1:** Various conjugation strategies employed to covalently attach biomolecules to polymers. From top to bottom other chemistry employed involves oxime bond formation, azide-alkyne cycloaddition, ligand-protein binding as in biotin-avidin interactions, and conjugation to cofactors (heme with horseradish peroxidase shown)
Other conjugation strategies also exist whereby the biomolecule of interest is modified to contain a polymer reactive group. Examples compatible with the previously mentioned strategies include modification with N-succinimidyld-S-acetythiopropionate, or Traut’s reagent to install free thiols via reaction with primary amines. Conversely, free amines may be added at sites of free sulfhydryl moieties by reaction with N-(iodoethyl)trifluoroacetamide followed by deprotection under basic conditions. Techniques to install non-natural functionality typically target free amines or sulfhydryl groups for conversion or coupling to the moiety of interest. Another reaction involves N-terminal oxidation with pyridoxial-5-phosphate (PLP) to produce an aldehyde or ketone functionality that is reactive towards aminooxy groups installed on polymer chains. PLP is the active form of vitamin B₆ and is employed as a cofactor in transamination reactions. Similarly, when the cofactor is mixed with proteins of interest, Schiff base formation occurs followed by tautomerization to the corresponding glyoxal imine. This is then followed by hydrolysis to give a protein modified to contain an aldehyde or ketone at the N-terminus. This particular reaction sequence has been thoroughly screened for reactivity. Several types of aminooxy terminated polymers have been conjugated to free ketone or aldehyde moieties on proteins including hydrogels formed from bis-functionalized proteins. Other common conjugation techniques include alkyne-azide “click” chemistry as the high yield of this reaction presents an attractive approach to bio-conjugates. In addition, the copper catalyst employed to produce a polymer by ATRP may also be used for subsequent reaction with alkyne labeled proteins to form protein-polymer conjugates. Proteins have also been combined with avidin for later reaction of the fusion protein with biotin polymers. Lastly, protein cofactors may be linked to polymers and then coordinated to the
corresponding protein as this method has the advantage of preserving the endogenous amino acid sequence in the protein of interest.\textsuperscript{278-280}

**Effects of Attaching Poly(ethylene glycol) to Protein Therapeutics**

Diminutive modifications in protein structure or sequence can result in magnified effects with regards to protein activity and shelf-life.\textsuperscript{281} For therapeutic use, protein activity must be maintained at strictly constant levels such that the active dose at the time of administration is well-established. More robust protein formulations may be obtained through lyophilization of the therapeutic mixture,\textsuperscript{282} however, this process inherently imparts the environmental insults of freezing and desiccation on the protein formulation at the time of freeze-drying.\textsuperscript{283, 284} One typical excipient employed to shield protein formulations from environmental stress is poly(ethylene glycol) (PEG).\textsuperscript{285} There are several mechanistic aspects with regards to how PEG stabilizes proteins to environmental stressors, however, it is thought that the method through which stabilization is conferred is largely dependent on changes in solution glass transition temperature (Tg) as well as interactions of the polymer with surrounding hydration sphere of the complex.\textsuperscript{286}

Covalent attachment of PEG or other polymers to proteins to form protein-polymer conjugates imparts several changes to the therapeutic. In addition to increasing the stability of protein therapeutics, conjugation of polymers to protein based drugs also modulates pharmacokinetic properties.\textsuperscript{287-290} Typically, circulation half-lives are increased,\textsuperscript{291} enzymatic degradation and immunogenic effects are diminished\textsuperscript{292} and biodistribution is modulated.\textsuperscript{293} For example, the PEGylated anti-VEGF drug Pegaptanib has a circulation half life of 131 hours
relative to 1.4 hours for the non PEGylated vector. In addition, PEGylation also allows the use of peptide fragments such as antibody epitopes that would otherwise be rapidly excreted due to their small size. Receptor binding, cellular uptake, and endosomal escape are also strongly inhibited, although these factors may be controlled. Concentration dependent effects of polymer relative to protein are enhanced due to the increase in local concentration, and increasing the size of the macromolecule has additional pharmacodynamic benefits including the enhanced permeability retention (EPR) effect as has been observed in oncological therapies with >20 kDa PEG. Bio-distribution and metabolism of PEG conjugates typically involves accumulation in cancer cells and liver before hepatocyte degradation and renal clearance with minimal toxicity. Furthermore, with the invention of hetero α-ω telechelic polymer constructs with protein reactive end-groups, attachment of therapeutic drugs or proteins to targeting moieties is also possible. Although in its infancy, this strategy has been used to link cytotoxic agents such as calicheamicins, auristatins, and maytansinoids to cancer targeting antibodies. Lastly, attachment of polymers to proteins has been employed in areas beyond therapeutic use including molecular switches and anti-freeze protein materials.

**Conjugation to Other Polymers**

Selection of the class of polymer used in bio-conjugation reactions is critical as covalent attachment can have the beneficial effect of contributing the materials properties of the polymer to the protein, or the deleterious consequence of amplifying known inhibiting effects of certain polymers. Various choices of polymer exist including styrene,
carbohydrates, PEG, and poly hydroxy ethyl methacrylate (HEMA) as well as “smart” side-chain polymers such as PEG (methyl)acrylates (PEG[MA]), and N-isopropyl acrylamide (NIPAM). Recently, zwitterionic conjugates have been reported leading to a new class of biomaterials, and these particular bio-conjugates have shown increased bioactivity relative to PEG counterparts.

**Trehalose**

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) significantly stabilizes proteins to environmental stresses. The sugar protects biomolecules during desiccation, freezing, heating, and to oxidation, it has also been shown to prevent aggregation. Trehalose is found in bacteria, fungi, and plants. Whole animals such as insects are protected by this unique disaccharide. For example, tardigrades (water bears) can survive desiccation for close to five years and can be cooled to -196 °C without adverse effect on survival. These remarkable cryptobiotic effects are attributed, at least in part, to the significant increases in trehalose concentration in the animal body. For some organisms, trehalose can be up to 15% of the dry weight after exposure to an environmental stress such as desiccation. Although the mechanism of trehalose protection is not proven, it has been hypothesized that the α,α linkage is required for protection from dessication, and computational results demonstrate that rotation is indeed restricted for the α,α linked anomer compared to neotrehalose. The exact means by which trehalose protects biomolecules, cells, and organisms is much debated. Possible mechanisms include vitrification (the formation of a trehalose based glass around proteins that restrict
mobility), water replacement (with trehalose hydrogen bonding to biomolecules), and water entrapment (the formation of a strong water layer between the biomolecule and sugars that maintain hydration). Likely, the properties are a result of several of these factors as each one is not mutually exclusive. Regardless of the exact mechanism, the protective properties of the non-reducing sugar trehalose make it an attractive target as an excipient to stabilize proteins. As such we sought to attach a polymerizable group to trehalose (Chapter 3), polymerize via controlled radical polymerization to produce a glycopolymer with a protein reactive end-group (Chapter 4), attach the glycopolymer to a protein of interest to obtain a trehalose glycopolymer-protein conjugate (Chapter 5), and measure stabilizing effects relative to other common excipients such as trehalose or PEG (Chapter 6).
CHAPTER 2

3-D ENCAPSULATED HYDROGEL ARCHITECTURES BY e-BEAM LITHOGRAPHY
INTRODUCTION

Several procedures have been developed that employ the fabrication of protein reactive poly(ethylene glycol) (PEG) hydrogel arrays via electron beam (e-beam) lithography induced cross-linking. However, unambiguous assignment of the 3-D spatial orientation of immobilized species has yet to be addressed for encapsulated hydrogel constructs fabricated by e-beam lithography. Many approaches exist to generate micro or nano arrays of multiple proteins patterned within nano-scale proximity including bias-assisted atomic force microscopy, self-assembled monolayers, and e-beam lithography. Of these techniques all have been used to generate protein arrays immobilized through covalent reactions with PEG, and architectures of multiple proteins in layered configurations have been fabricated by e-beam lithography. However, the general interaction at the interface of these layers is as of yet unknown and unambiguous 3-D characterization of immobilized species in this configuration has not been reported. While a diverse selection of immobilization strategies exist for biomolecules, some of the most common used for PEG hydrogel surface arrays include avidin-biotin affinity, oxime bond formation, and 1,3 Huisgen cyclo-addition. Therefore, it would be useful to understand polymer distribution and permeability throughout PEG hydrogels particularly in constructs with multiple instances of these protein conjugation strategies positioned in close proximity. As such we sought to establish encapsulated hydrogel architectures whereby a shell hydrogel forms a contiguous border in 3 dimensions around an inner core hydrogel of orthogonal reactivity.
RESULTS AND DISCUSSION

Synthesis of Protein Reactive 8-arm Poly(ethylene glycol)

Polymers of orthogonal reactivity were prepared based on poly(ethylene glycol) PEG. Best yields for modification to obtain alkyne end-groups (Alkyne-PEG) were achieved by first activating pentynoic acid as the NHS ester via carbodiimide coupling under basic conditions (Figure 2.1) before treatment with 8-arm amine terminated poly(ethylene glycol). Next, 8-arm amine terminated PEG was modified to contain biotin (Biotin-PEG) through carbodiimide mediated coupling, and hydroxyl terminated poly(ethylene glycol) (PEG-OH) was subjected to Mitsunobu conditions to produce a PEG with aminooxy end-groups (AO-PEG) upon deprotection with hydrazine (Figure 2.2).

Figure 2.1: Synthesis of NHS-activated ester of pentynoic acid via carbodiimide mediated coupling. Activation under basic conditions was found to be essential for subsequent amidation with amine terminated 8-arm poly(ethylene glycol).
Figure 2.2: Synthesis of alkyne (Alkyne-PEG), biotin (Biotin-PEG), and aminooxy (AO-PEG) functionalized 8-arm poly(ethylene glycol). Alkyne-PEG and Biotin-PEG were synthesized via amidation of amine terminated 8-arm PEG with the corresponding carboxylic acid. AO-PEG was synthesized in two steps via Mitsunobu reaction of N-hydroxy phthalimide with 8-arm PEG followed by deprotection with hydrazine.

Confocal Microscopy of Surface Immobilized Streptavidin

Once polymers of varied reactivity were obtained, the first parameter investigated was protein distribution throughout PEG-hydrogel micro-patterns with varied feature sizes. Hydrogel arrays of Biotin-PEG were patterned by e-beam lithography (110 µC/cm²) to contain feature sizes ranging from 5 to 40 µm. The patterns were subsequently incubated with a green fluorescent streptavidin (SAv-AF488) for 3 hours at room temperature, and washed by immersion in D-PBS, pH 7.4 for 5 minutes 3 times. Confocal microscopy was performed using a 100x oil objective (full width at half max 640 nm) taking 400 nm sections in both z and y directions, thereby observing fluorescence of the micro-gel in three dimensions (Figure 2.3).
The protein was found to be homogenously dispersed throughout the microstructure for a feature size of 5 µm. For larger feature sizes, a decrease in fluorescence indicative of a slight size-exclusion effect was observed in the z-dimension for the center of the hydrogel feature relative to the edge. For the largest feature sizes increased fluorescence was found around the border of the hydrogel which may be attributed to the increase in height due to backscattered electrons as has been previously reported.\textsuperscript{41}

**Figure 2.3:** a) Fluorescent image of SAv-AF488 conjugate immobilized on Biotin-PEG hydrogels of feature sizes varying from 40m (top), 20m (middle), and 5m (bottom). b) Fluorescence intensity plot of the red line in the x-y dimensions for each feature size. c) Fluorescence intensity plot of the profiled through the z dimension corresponding to d) the edge (purple) or bulk (green) of each feature.

**Fabrication of Encapsulated Hydrogels by e-Beam Lithography**

Encapsulated architectures of PEG hydrogels are of biological significance as encapsulated constructs have been fabricated to contain enzymes\textsuperscript{411} and even whole cells.\textsuperscript{412} Photolithographic techniques have been employed to produce multi-enzyme constructs\textsuperscript{413} as
Encapsulation increases the ratio of signal to noise and also increases viability of unique cell types such as islets of Langerhans. Therefore, e-beam fabrication of hydrogel architectures that contain 2 different polymers of orthogonal reactivity patterned in an encapsulated configuration was pursued. The procedure utilized micro-realignment methodology requiring 3 lithographic steps (Figure 2.4). The morphology of the polymer networks along with 3-D localization of species conjugated to the polymer reactive end-groups was investigated among polymers containing 3 different end-groups. Proteins, enzymes, and nano-particles were also immobilized to demonstrate retention of bioactivity and to further investigate the permeability of the polymer networks.

Figure 2.4: Synthesis of an encapsulated PEG hydrogel by e-beam lithography. a) A methanolic solution of AO-PEG is spin coated onto silicon substrate before b) crosslinking by exposure to e-beam and c) removal of un-reacted polymer by developing in Milli-Q water. The process is then repeated first d) with a polymer of orthogonal reactivity (Alkyne-PEG) followed by capping with e) the original polymer. Confocal microscopy was performed to obtain a maximum transparency projection in the f) xz-y and g) xy-z dimensions of the Alkyne-PEG core stained with Alexa-488 Azide (green) patterned inside the contiguous shell of AO-PEG stained with maleimide functionalized coumarin (blue) illustrating the reactive heterogeneity in EA-2.
Two different encapsulation strategies were employed: encapsulation of AO-PEG inside Biotin-PEG and encapsulation of Alkyne-PEG inside AO-PEG. The AO-PEG and Biotin-PEG have a large difference in optimal reactive doses (80 µC/cm² and 120 µC/cm² respectively); therefore, to prevent overexposure in subsequent lithographic steps, 50% of the optimal dose for each polymer was used. Encapsulated structures were fabricated over three patterning steps each consisting of spin coating a 1% methanolic solution of polymer (4000 rpm, 60 s), microalignment, e-beam lithography, and development by immersion in Milli-Q water. Thereby, a 40 µm box of Biotin-PEG (60 µC/cm²) was patterned followed by a 20 µm box of AO-PEG (40 µC/cm²) with a final 40 µm box of Biotin-PEG (60 µC/cm²) patterned on top of the two preceding features to complete the encapsulated architecture (EA-1).

The second encapsulation strategy consisted of AO-PEG patterned as a shell around an inner core of Alkyne-PEG. This pair of polymers was found to be most compatible with the multiple exposure steps in the fabrication process. This was likely due to the relatively low optimal reactive e-beam doses (80 and 30 µC/cm² respectively) for each polymer. As before, to prevent overexposure in subsequent lithography steps, 50% of the optimal dose for each polymer was used. Again, encapsulated structures were fabricated over three patterning steps each consisting of spin coating a 1% methanolic solution of polymer (4000 rpm, 60 s), microalignment, e-beam induced crosslinking, and development by immersion in Milli-Q water. Thereby, a 40 µm box of AO-PEG (40 µC/cm²) was patterned followed by a 20 µm box of Alkyne-PEG (15 µC/cm²) with a third 40 µm box of AO-PEG (40 µC/cm²) patterned on top of the two preceding features completing the encapsulated architecture (EA-2).
**Confocal Microscopy of Encapsulated Hydrogels**

To investigate the distribution of polymer reactive end-groups in the EA-1 milieu, fluorescent dyes were attached to the polymer end-groups retained in the hydrogel. Thus, EA-1 was first exposed to streptavidin-Alexafluor-488 (SAv-AF488) conjugate for 3 hours at 4°C followed by washing by immersion in Milli-Q water. The surfaces were then incubated with maleimide-coumarin (0.1 mg/mL in 10% DMSO$_{(aq)}$) for 1 hour at room temperature in the dark and washed in similar fashion thereby covalently attaching a blue coumarin dye to the aminooxy end-groups. Fluorescence of the hydrogel micro-patterns was observed and the images were sectioned by confocal microscopy using a 100x oil objective to determine the reactive heterogeneity of the polymer architecture (Figure 2.5). Profiling the z-dimension through the center of reacted EA-1, the maximum intensity of the green channel was found to span the entire signal observed for the blue channel. It was thought that the green signal observed in the core of the hydrogel architecture was not a result of Biotin-PEG, but rather that this signal originated from the coumarin fluorophore. To test this, the microscope was focused halfway through EA-1, and a fluorescence image was obtained in the x-y dimension only. In the first case, two laser lines were used. One laser line at 400 nm is strongly absorbed by the coumarin dye, and to a lesser extent Alexafluor 488. The second line at 488 nm is outside the absorption spectrum of coumarin and is therefore only absorbed by the Alexafluor 488. As expected, when both lasers are used to illuminate the sample, green fluorescence is clearly observed to envelop the blue fluorescence signal and also to occur throughout the center of EA-1. When only the 488 nm laser line is employed however, the green signal in the center of EA-1 disappears thereby revealing the unambiguous location of maleimide-coumarin and SAv-
488 dyes (Figure 2.6). This result indicates that the blue maleimide-coumarin signal is encapsulated within the green SAv-488 fluorescence signal, and this may be correlated to polymer end-group to conclude that the AO-PEG is encapsulated as a core within an outer shell of Biotin-PEG.

Figure 2.5: Reactive heterogeneity of the polymer architecture is observed by a) first patterning AO-PEG on top of Biotin-PEG before b) completing the encapsulated architecture with a second layer of Biotin-PEG. Subsequent staining with Streptavidin-Alexafluor 488 conjugate (green) followed by maleimide-coumarin (blue) allowed for c) fluorescence imaging by confocal microscopy (blue maleimide-coumarin has been digitally replaced with yellow coloring for clarity) with d) profiles through the center of the microstructure in the z dimension (blue circle) confirming encapsulation as the maximum intensity of the green channel (top) completely surrounds the maximum intensity of the blue channel (bottom).
Figure 2.6: Fluorescence profile in the x-y dimension approximately halfway through the microstructure in the z dimension. Pronounced signals in both the a) green and b) blue channels when the substrate is simultaneously illuminated with laser wavelengths at 400 (coumarin absorbance) and 490 nm (Alexafluor 488 absorbance) are observed. c) Fluorescence in the core is no longer observed when the sample is only illuminated with the 490 nm laser line.

To investigate the distribution of polymer reactive end-groups in EA-2, fluorescent dyes were again covalently attached to the alkyne or aminooxy end-groups. In this case, EA-2 was first exposed to Alexafluor-488 azide® according to manufacturer instructions followed by washing with Milli-Q water. Next, EA-2 was incubated with maleimide-coumarin (0.1 mg/mL in 10% DMSO_{aq}) for 1 hour at room temperature in the dark and washed in similar fashion. The
fluorescent images were sectioned by confocal microscopy using a 100x oil objective to determine the reactive heterogeneity of the polymer architecture (Figure 2.7, d-i). Via this method it was observed that the Alkyne-PEG hydrogel (green) was contained as a core inside a contiguous shell of AO-PEG thereby confirming the encapsulated architecture (Figure 2.7, e,f).

Figure 2.7: Fabrication of an encapsulated micro-pattern achieved by a) patterning a 40µm box consisting of AO-PEG hydrogel, development and realignment to pattern b) a 20µm box of alkyne-PEG on top of the center of the existing microstructure. c) Subsequent capping with an additional layer of AO-PEG patterned as a 40µm box to complete encapsulation. Microstructures were stained with maleimide-coumarin (blue) and Alexa-488 Azide (green) before measurement of the fluorescence profile of the d) xy-through z maximum fluorescence projection with e) blue and f) green channels. Confocal slices were obtained in the z direction with g) z-profiles in the center and on the border of the microstructure. h) The blue channel fluorescence was found to surround a contiguous border of the i) green channel fluorescence.

Enzyme Immobilization in Encapsulated Hydrogels

Once retention of polymer end-group reactivity in EA-2 was established, enzyme immobilization was attempted in this encapsulated configuration. Horseradish peroxidase (HRP) and glucose oxidase (GOX) were chosen as these enzymes have been used to effect enzyme cascade reactions when contained in polymersomes. Thus, HRP and GOX were
functionalized with levulinyl or azido moieties, respectively, for fabrication of GOX immobilized as a core encapsulated by a HRP shell (Figure 2.8).

Azide modification was chosen for GOX as several azides are known inhibitors of HRP. Therefore, GOX was modified to contain free azide moieties through the reported procedure with imidazole-1-sulfonyl azide hydrochloride, utilizing 3 equivalents of the diazo transfer reagent. Upon purification by centriprep ultra-centrifugation (MWCO 3 kDa), the protein conjugate was assayed for azide moieties by reaction with Alexafluor 594 Click It® alkyne. Degree of labeling determined by UV absorbance at 280 nm (protein) and 590 nm (dye) was found to be 1.4 azides per GOX protein. Likewise, HRP was modified to contain levulinyl moieties via incubation with N-hydroxy succinimidyl levulinate (3 eq, Dulbecco’s Phosphate Buffered Saline [DPBS], 3h, rt) and purified by centriprep (MWCO 3 kDa). The conjugate was assayed for free ketones by reaction with Alexafluor 488® hydrazide under reducing conditions with NaBH₃CN. Degree of labeling determined by UV absorbance at 280 nm (protein) and 490 nm (dye) was found to be 1.00 levulinyl moiety per HRP protein.

Fluorophore selection for the immobilized conjugates was found to be non-trivial as well. Specifically, GOX contains a flavin adenine dinucleotide (FAD) redox cofactor with strong absorption in the blue region. These findings necessitate that for a blue/green pair of fluorophores, GOX be labeled green with HRP labeled as blue. Furthermore, when a fluorophore contained in the core contains a fluorophore with emission in the absorption range of the shell fluorophore, cross-talk and Förster Resonance Energy Transfer (FRET) are observed as with EA-1. Therefore, azide modified GOX and levulinyl modified HRP were made fluorescent by incubation with TFP-Alexa 488® and Marina Blue® NHS ester to produce the
fluorescent protein conjugates Azide-GOX-AF488 and Lev-HRP-MB, respectively. Protein conjugates were purified and concentrated by centriprep (MWCO 3 kDa) to a final concentration of 0.1 mg/mL in DPBS, pH 7.4. Degree of labeling studies were conducted by UV absorbance and it was found that Azide-GOX-AF488 contained 0.7 fluorophores per protein (280 nm protein, 490 nm dye) with 0.68 fluorophores per protein observed for Lev-HRP-MB (280 nm protein, 350 nm dye).

Activity was confirmed through an enzyme cascade reaction between HRP and GOX. When hydrogen peroxide is present, HRP is able to modify numerous substrates in solution including 2-hydroxy-1-naphthaldehyde salicyloylhydrazone, phenylene diamines (for colorimetric analysis), and tetramethylbenzidenes (common for analysis of activity by e-beam microscopy). Fluorescent substrates including Amplex Red® may be used to quantify enzymatic activity, and may also be used to measure hydrogen peroxide concentration. Additionally, GOX is known to liberate hydrogen peroxide as a byproduct in the conversion of glucose to glucanoic acid. This implies that glucose and GOX coupled with a fluorescent HRP substrate and HRP may be used to probe activity of the enzyme pair as glucose oxidation by GOX provides one of the substrates (hydrogen peroxide) required for HRP activity. Therefore, activity retention was confirmed for both proteins by effecting an enzyme cascade reaction with Amplex Red® (Figure 2.8).
Figure 2.8: Enzyme cascade between Azide-GOX-AF488 and Lev-HRP-MB immobilized in an encapsulated configuration.  

a) Glucose is oxidized to produce hydrogen peroxide and gluconolactone before subsequent hydrolysis to glucanoic acid.  

b) Lev-HRP-MB mediated conversion of Amplex Red® to the red fluorescent molecule resorufin via liberated hydrogen peroxide substrate completes the cascade reaction.

Multi-component hydrogels were fabricated as before to contain an Alkyne-PEG hydrogel core encapsulated within an AO-PEG shell (EA-2). The microstructure was then incubated with Lev-HRP-MB (40 µL, 0.1 mg/mL) for 24 h at 4°C. The surfaces were washed 3 times with DPBS before exposure to Azide-GOX-AF488 (20 µL, 0.1 mg/mL, DPBS pH 7.4), CuSO₄ (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) in DPBS for 24 hours at 4°C. The surfaces were washed again 5 times with reaction buffer and the rinsing solutions from each wash cycle were assayed for enzyme activity with no activity detectable after 3 washes. The immobilized enzymes were then viewed by fluorescence microscopy (Figure 2.9). Furthermore, chemical immobilization and cross reactivity was probed by fabrication of a wafer to contain Alkyne-PEG patterned next to AO-PEG. This wafer was incubated as before with Azide-GOX-AF488 (20 µL, 0.1 mg/mL, DPBS pH 7.4), CuSO₄ (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) in DPBS for 24 hours at 4°C. The surface was washed 5 times with reaction buffer and viewed by fluorescence microscopy. As expected a large fluorescence signal was present within
the Alkyne-PEG hydrogel with only minimal fluorescence detected from the AO-PEG hydrogel (Figure 2.10).

**Figure 2.9:** a) A 40 µm box consisting of an AO-PEG hydrogel is patterned, developed and realigned to pattern b) a 20 µm box of Alkyne-PEG on top of the existing microstructure before subsequent capping with c) an additional layer of AO-PEG patterned as a 40µm box. Fluorescence microscopy of patterns subsequently treated with Azide-GOX-AF 488 and Lev-HRP-MB highlighting d) multi-channel, e) green-channel, and f) blue-channel fluorescence.

**Figure 2.10:** Cross reactivity study for Azide-GOX-AF488. After incubation of the entire wafer with Azide-GOG-AF488, CuSO₄, and sodium ascorbate with subsequent washing, a) a large increase in fluorescence is observed for the enzyme immobilized on Alkyne-PEG relative to b) AO-PEG (hydrogel is patterned within the white dashed box).

**Enzyme Cascade Reaction in Encapsulated Hydrogels**

Next, the patterned surface was incubated with glucose (10 mM) and Amplex Red (50 µM) substrates at 4°C and fluorescence of resorufin was measured after 2h. Parallel control experiments were also conducted. Surfaces with no patterns or patterns of unmodified PEG-
OH were incubated with both enzymes under identical conditions. These surfaces were then washed in identical fashion to EA-2 and exposed to a solution that included substrates for both enzymes (glucose, 10 mM, hydrogen peroxide, 300 ppm, and Amplex Red, 50 µM). In a separate experiment, Azide-GOX-AF488 was immobilized alone on a surface of Alkyne-PEG. As such Alkyne-PEG hydrogel arrays were fabricated by e-beam lithography and Azide-GOX-AF488 was immobilized on the patterns using conditions identical to immobilization of the enzyme in EA-2. The surface immobilized Azide-GOX-AF488 was then incubated with a solution of glucose substrate along with Lev-HRP-MB (1 µg/mL) and corresponding HRP substrate, Amplex Red (50 µM). To quantify the maximum surface enzyme activity possible for HRP, Lev-HRP-MB was immobilized alone on a surface of AO-PEG. This was achieved by fabricating patterns of AO-PEG and incubating with Lev-HRP-MB in identical fashion to EA-2. The surface was then exposed to both substrates for HRP (Amplex Red, 50 µM, and hydrogen peroxide, 300 ppm). Evolution of resorufin in all samples was measured after 2 h and the experiments were compared relative to Lev-HRP-MB activity when immobilized alone. Furthermore, an additional enzyme immobilized EA surface was mounted on a trans-well membrane and exposed to glucose (10 mM) and Amplex Red (50 µM) to visualize resorufin emanating from the microstructure. (Figure 2.11).
Figure 2.11: a) Time-lapse pictures of resorufin emanating from enzymes immobilized on a multi-component hydrogel with encapsulated architecture. b) The substrate mounted on a trans-well membrane with resorufin (orange) evolved from the micro-pattern. c) Surface activity of positive and negative control surfaces along with enzyme activity when immobilized in the encapsulated architecture.

**Surface Immobilized Enzyme Kinetics**

Although by visual inspection protein was clearly observed to adsorb onto the surface of the bare silicon substrate, lack of enzymatic activity in this experiment indicated that non-specific adsorption to the surface inactivates both enzymes. Additionally, the wash solutions from the bare silicon surface were assayed for activity, and no activity was present after 3 wash cycles (out of 5 performed) indicating that no active enzymes remained in the final wash solution. This implies that all enzyme activity in subsequent experiments is a result of enzymes present in the PEG hydrogels. For PEG-OH hydrogels a minimal level of enzyme activity was observed. This was likely due to enzymes that diffused into the PEG-OH hydrogel array, but were not chemically immobilized. A similar effect likely occurs for the other hydrogel arrays as well. When Lev-HRP-MB was immobilized alone on the surface it exhibited slightly lower
activity than Azide-GOX-AF488 immobilized alone. This was likely because in the case of Azide-GOX-AF488, free Lev-HRP-MB was added to solution to complete the enzyme cascade. This enzyme was free to diffuse into the PEG hydrogel (as shown in the PEG-OH example) and could have increased reaction kinetics due to the local Lev-HRP-MB concentration. Lastly, the encapsulated surface EA-2 with immobilized enzymes Azide-GOX-AF488 and Lev-HRP-MB displayed comparable activity to Lev-HRP-MB immobilized alone. This implied that conversion of Amplex Red to resorufin was the rate limiting step. The result is consistent and expected as the hydrogen peroxide substrate was free to diffuse throughout EA-2. Overall, activity of the two enzyme process in EA-2 was similar to activity of either enzyme immobilized alone. It was thereby demonstrated that enzyme encapsulation via this technique could prove useful in biosensors, or binary heterogeneous reaction catalysis.
EXPERIMENTAL

Materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. Poly(ethylene glycol) and 8-arm amine terminated poly(ethylene glycol) were purchased from JenKem Inc. Amplex Red, Alexa Fluor 488 and Maleimide-Coumarin were purchased from Invitrogen. All other reagents including horseradish peroxidase and glucose oxidase were purchased from Sigma-Aldrich.

Analytical Techniques

NMR spectra were recorded on a Bruker Avance 500 or 600 MHz spectrometer. UV-Visible spectroscopy was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments). Mass spectra were acquired using a Thermo Finnigan LCQ Deca Ion Trap MS. Infrared spectra were obtained with a Perkin-Elmer Spectrum One instrument equipped with a universal ATR accessory. Wafers were patterned with a JEOL 5910 scanning electron beam microscope. For multi-component PEGs, gold features were fabricated on the Si chips prior to PEG spin-coating and writing. Pattern files were created in Design CAD 2000 and written with a JC Nabity lithography system (Nanometer Pattern Generation System, Ver. 9.0). A Leica confocal SP2 1P microscope with fluorescence correlation spectroscopy was used for all confocal sectioning images. A Zeiss fluorescence microscope was used to observe a 2-D picture of immobilized enzymes (Figure 2.9), cross-reactivity control (Figure 2.10) and evolution of resorufin through use of a transwell membrane (Figure 2.11).

32
**2,5-Dioxopyrrolidin-1-yl pent-4-ynoate (2.1)**

To a flame dried round bottom flask containing 100 mL of freshly distilled methylene chloride (DCM) was added N-hydroxysuccinimide (NHS, 1.0 g, 8.7 mmol, 1 eq), pentynoic acid (0.94 g, 9.6 mmol, 1.1 eq), triethylamine (1.4 g, 14 mmol, 1.6 eq), and 4-dimethylaminopyridine (DMAP) (0.11 g, 0.87 mmol, 0.1 eq). The resulting solution was stirred for 30 min before addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 1.8 g, 9.6 mmol, 1.1 eq). The reaction was stirred for 4 h at 22 °C, and the solvent was removed *in vacuo* to give a crude product which was subjected to column chromatography (100% DCM, *R* f 0.4). This product was further purified by recrystallization from 1 : 4 ethyl acetate : hexanes to obtain the title compound as a white solid (0.69 g, 40% yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 2.90–2.84 (m, 5H, COCH$_2$ + NHS), 2.62 (dt, 2H, $J$ = 7.5, 2.6 Hz, CH$_2$CH$_2$C), 2.05 (t, 1H, $J$ = 2.6 Hz, CCH); $^{13}$C (400 MHz; CDCl$_3$) $\delta$ (ppm): 169.1, 167.2, 81.0, 70.2, 30.4, 25.7, 14.2; DEPT-135 $^{13}$C (400 MHz; CDCl$_3$) $\delta$ (ppm): 69.9(-), 30.18(+), 25.5(+), 14.0(+) ppm. FTIR $\nu_{\text{max}}$/cm$^{-1}$: 3304, 2948, 2258, 1815, 1787, 1734, 1430, 1415, 1437, 1203, 1085, 1067, 993, 906.
$^1$H NMR (400 MHz, CDCl$_3$)
The compound 2.2 was synthesized similarly to a known procedure.\cite{320} To a flame dried round bottom flask containing 100 mL of freshly distilled DCM was added levulinic acid (1.0 g, 8.6 mmol, 1 eq), NHS, (1.1 g, 9.5 mmol, 1.1 eq), TEA (1.7 g, 17 mmol, 2 eq), and DMAP (0.11 g, 0.86 mmol, 0.1 eq). The resulting solution was cooled to 0°C and stirred for 30 min before addition of DCC (1.8 g, 9.5 mmol, 1.1 eq). The reaction was warmed to room temperature and stirred for an additional 6 h. The crude reaction mixture was added to ethyl acetate previously cooled to -20°C. The white precipitate was filtered off and ethyl acetate removed \textit{in vacuo}. The reaction product was further purified by silica gel column chromatography (2.5% MeOH in HCCl$_3$) to obtain the purified product in 52% yield. Small aliquots (~10 mg) of the title compound were further purified by sublimation (0.02 Torr, 60°C) prior to use. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 2.81 (m, 4H), 2.77 (m, 4H), 2.14 (m, 3H). $^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$: 205.1, 169.2, 168.2, 37.5, 29.6, 25.6, 25.0 ppm.
8-Arm amine-terminated PEG was added (20 mg/mL) to a solution of 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (5 mg/mL) in DCM and allowed to react for 1 h at 23 °C. The solvent was removed in vacuo. The crude product was then re-dissolved in Milli-Q water and purified by centrifugal filtration (3,000 MWCO) followed by lyophilization to isolate the pure product. $^1$H NMR (500 MHz, D$_6$DMSO) δ: 3.69–3.49 (m, PEG peaks), 2.78 (CCH end group 1H). Conversion = 93% by comparison of the integrations of the alkyne end-group with the CH$_2$CH$_2$ peak of the PEG backbone.
To a flame dried round bottom flask was added 8-arm amine-PEG (10.9 kDa, 100 mg, 0.01 mmol, 1eq), biotin (130 mg, 0.55 mg, 60 eq), EDC (120 mg, 0.64 mmol, 70 eq), anhydrous TEA (60 mg, 0.59 mmol, 65 eq), and anhydrous DMF (10 mL) and the reaction was cooled to 0°C before addition of DMAP (1 mg). The reaction was allowed to warm to room temperature and was stirred for an additional 24 hours. The crude reaction mixture was first purified by ultra-centrifugation (3 kDa MWCO against Milli-Q water) before precipitation 3 times in diethyl ether.
The white precipitate was dissolved in water and lyophilized before analysis by NMR. $^1$H NMR (500 MHz, CDCl₃) δ: 6.46-640 (m, 2H), 4.35-4.16 (m, 2H), 3.91-3.43 (m, PEG signals 56H), 3.26-2.54 (m, 7H), 2.22-2.19 (m), 1.74-1.32 (m, overlapping with water peak). The percent substitution was estimated from the $^1$H NMR spectrum, by comparing the integration of the biotin peaks between 3.3-3.1 ppm to the methylene group of the 8-arm amine-terminated PEG at 3.91-3.43 ppm. This provided a substitution of 79% of the chain ends.

$^1$H NMR (500 MHz, CDCl₃)
Aminooxy-Poly(ethylene glycol) (AO-PEG)

Hydroxyl-terminated 8-arm PEG (2 g, 0.1 mmol), N-hydroxyphthalimide (157 mg, 0.96 mmol) and triphenylphosphine (PPh3, 252 mg, 0.96 mmol) were dissolved in dry DCM (15 mL). S5 Diisopropyl azodicarboxylate (DIAD, 173 μL, 0.88 mmol) was then added drop wise and the mixture was stirred at 23 °C under Argon for 18 h. To precipitate the polymer an excess of cold diethyl ether was added. The solids were collected by filtration and dried under vacuum. The product was re-subjected to the same procedure to afford the N-hydroxyphthalimide-8-arm PEG.\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\): 7.84-7.83 (m, 2H, C\(_6\)H\(_4\)), 7.76-7.74 (m, 2H, C\(_6\)H\(_4\)), 4.37 (t, 2H, CH\(_2\)ON), 3.87-3.49 (m, PEG signals). The N-hydroxyphthalimide-8-arm PEG (1.75 g, 0.083 mmol) was refluxed with hydrazine hydrate (500 μL, 16.5 mmol) in CH\(_2\)Cl\(_2\) (10 mL) for 30 min immediately prior to use. The crude reaction product was filtered to remove the white solid bi-product, and aminooxy-8-arm PEG (AO-PEG) was isolated after removal of the solvent and drying under vacuum. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\): 3.83-3.48 (m, PEG signals). Percent substitution was calculated from the N-hydroxyphthalimide-8-arm PEG by comparison of the integrations of the N-hydroxyphthalimide end group proton peaks at 7.85 ppm with the integrations of the PEG proton peaks at 3-4.5 ppm assuming the molecular weight of the PEG as 20,000. This gave a substitution of 94%.
$^1$H NMR (500 MHz, CDCl$_3$)
**Imidazole-1-sulfonyl Azide Hydrochloride (2.3)**

Synthesis of imidazole-1-sulfonyl chloride proceeded in 62% yield following a literature procedure. The product was isolated as colorless needles.\(^1\)\(^H\) NMR (500 MHz, D\(_2\)O) \(\delta\): 9.34 (1H), 7.99 (1H), 7.57 (1H). \(^1^3\)C NMR (500 MHz, D\(_2\)O) \(\delta\): 137.6, 123.1, 120.0 FTIR \(\nu_{\max}/\text{cm}^{-1}\): 3104, 3059, 2499, 2425, 2169,1913, 1582, 1508, 1425, 1298, 1229, 1189, 1159, 1137, 1103, 1068, 982, 967.

**Levulinyl Horseradish Peroxidase Marina Blue Conjugate (Lev-HRP-MB)**

Horseradish Peroxidase (1 mg/mL, DPBS pH 7.4) was combined with NHS levulinate (3 eq, as a 0.1 mg/mL solution in DMSO) and the reaction was stored at 4°C for 24 h. The crude reaction mixture was purified by centrifprep ultracentrifugation (MWCO 3 kDa) to a final volume of 1 mL in 100 mM PB, pH 8.0. Degree of labeling was determined by reaction with Alexafluor 488 hydrazide. A 100\(\mu\)L aliquot of the purified conjugate was combined with Alexafluor 488 hydrazide (40\(\mu\)L, 1mg/mL in DMF), and the reaction was stored for 24 h at 4°C. The crude reaction mixture was purified by centrifprep ultra-centrifugation (MWCO 3kDa) to a final volume of 100 \(\mu\)L. Degree of labeling was determined to be 1.00 by comparing the protein absorbance at 280 nm to the fluorophore absorbance at 490 nm with a correlation factor of 0.11 for the fluorophore. Next 40 \(\mu\)L of Marina Blue NHS ester (5 mg/mL in DMSO) was added to the remaining purified conjugate solution and the reaction mixture was stored at room temperature for 3 hours before a second addition of 40 \(\mu\)L of Marina Blue NHS ester (5 mg/mL
in DMSO). The mixture was held at 4°C for 24 hours and then purified by centriprep ultra-filtration (3 kDa MWCO) to a final volume of 0.5 mL. Activity retention was confirmed by reaction with Amplex Red (50 µM) and 100 ppm hydrogen peroxide. Fluorophore degree of labeling was determined to be 0.68 based on the relative absorbance of the conjugate at 350 nm relative to absorbance of the native protein at 280 nm.

![HRP calibration curve](image)

Azido Glucose Oxidase Alexafluor 488 Conjugate (Azide-GOX-AF488)

Glucose Oxidase was prepared at a concentration of 1mg/mL in DPBS, pH 7.4. The enzyme was modified to contain free azide moieties similar to the reported procedures with imidazole-1-sulfonyl azide hydrochloride\(^{421, 422}\) except in this case only 3 equivalents of the diazo transfer reagent were added. The reaction mixture was purified by centriprep ultracentrifugation (MWCO 3kDa) to a final volume of 0.5 mL and the modified protein was assayed for azide moieties. An aliquot of the modified GOX (100µL) was incubated with Alexafluor 594 Click It® alkyne (20µL, 1mg/mL, DMSO). Degree of labeling was determined to be 1.4 azides per...
protein by comparing the relative UV absorbance of the protein at 280 nm to that of the dye at 590 nm with a correlation factor of 0.56 for the dye. Next, modified protein was incubated with TFP-Alexafluor 488 ester (40 µL, 1mg/mL in DMF) at 4°C for 24 hours. The crude reaction was then purified by centriprep ultra-centrifugation to a final volume of 0.5 mL. Degree of labeling studies were conducted by UV absorbance and it was found that Azide-GOX-AF488 contained 0.7 fluorophores per protein (280 nm protein, 490 nm dye) with a correlation factor of 0.11.

![GOX Calibration Curve](image)

\[ y = 1.0651x \]
\[ R^2 = 1.0000 \]

**Fabrication of AO-PEG Encapsulated within Biotin-PEG (EA-1)**

Biotin-PEG (10µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer with gold alignment marks (4000 rpm, 1 min). **CAUTION: PIRANHA SOLUTION REACTS VIOLENTLY WITH ORGANIC MATERIALS.** The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 80µC/cm² area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. The wafer was subsequently spin coated (4000 rpm, 1 min)
with a solution of **AO-PEG** (10 µL, 1wt% in MeOH). The surface wafer was then patterned by e-beam lithography (23 nm spot size, 30 kV accelerating voltage, 40 µC/cm² area dose) as an array of 20 µm boxes patterned in the center of the **Biotin-PEG**. The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. Next, the wafer was spin coated (4000 rpm, 1 min) with a second layer of **Biotin-PEG** (10 µL, 1wt% in MeOH). The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 80 µC/cm² area dose), and developed by immersion in Milli-Ω water for 30 seconds. The patterns were observed using an inverted bright-field microscope to confirm fabrication of the encapsulated hydrogel (EA-1).

**Fabrication of Alkyne-PEG Encapsulated within AO-PEG (EA-2)**

**AO-PEG** (10 µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer with gold alignment marks (4000 rpm, 1 min). **CAUTION: PIRANHA SOLUTION REACTS VIOLENTLY WITH ORGANIC MATERIALS.** The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 40 µC/cm² area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. The wafer was subsequently spin coated (4000 rpm, 1 min) with a solution of **Alkyne-PEG** (10 µL, 1wt% in MeOH). The surface wafer was then patterned by e-beam lithography (23 nm spot size, 30 kV accelerating voltage, 20 µC/cm² area dose) as an array of 20 µm boxes patterned in the center of the **AO-PEG**. The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope.
bright-field microscope. Next, the wafer was spin coated (4000 rpm, 1 min) with a second layer of AO-PEG (10 μL, 1wt% in MeOH). The wafer was then patterned by e-beam lithography as an array of 40 μm boxes (23 nm spot size, 30 kV accelerating voltage, 40 μC/cm² area dose), and developed by immersion in Milli-Q water for 30 seconds. The patterns were observed using an inverted bright-field microscope to confirm fabrication of the encapsulated hydrogel (EA-2).

***Fluorophore Immobilization: AO-PEG Encapsulated within Biotin-PEG***

The encapsulated hydrogel architecture EA-1 was incubated with Streptavidin Alexafluor 488 conjugate (0.1 mg/mL, 100 mM PB, pH 7.0) for 1 hour at room temperature before washing by immersion in Milli-Q water for 30 s. Next, the pattern was incubated with the aminooxy reactive dye, coumarin functionalized maleimide (0.1mg/mL in 10% DMSO) for 1 hour at room temperature before washing by immersion in 10% DMSO followed by Milli-Q water for 1 30 s each. The patterned hydrogel arrays were observed by confocal microscopy. The surface was illuminated using laser lines at 400 and 488 nm, and images were sectioned in the z dimension using a 100x oil objective advancing 620 nm with each section. Next, the focus of the microscope was shifted to approximately halfway through the microstructure. The 400 nm laser line was switched off and only the 488 nm line was used to illuminate a cross-section of the hydrogel architecture.

***Fluorophore Immobilization: Alkyne-PEG Encapsulated within AO-PEG***

The encapsulated hydrogel architecture EA-2 was incubated with Alexafluor-488 azide® (20 μL, 1mg/mL in DMSO), CuSO₄ (10μL, 1mg/mL), and sodium ascorbate (10μL, 5mg/mL)
followed by washing by immersion first in 10% DMSO then in Milli-Q water for 30 s each. Next, **EA-2** was incubated with maleimide-coumarin (0.1 mg/mL in 10% DMSO\(_{aq}\)) for 1 hour at room temperature in the dark and washed in similar fashion. The patterned hydrogel arrays were observed by confocal microscopy using a 100x oil objective. Images were sectioned in the xy through z dimensions advancing 620 nm with each section. Images were also sectioned in the xz through y dimension using similar sectioning parameters.

**Fabrication and Enzyme Immobilization on Control Surfaces**

**Cross-Reactive Surface**

**AO-PEG** (10 µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer with gold alignment marks (4000 rpm, 1 min). **CAUTION: PIRANHA SOLUTION REACTS VIOLENTLY WITH ORGANIC MATERIALS.** The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 40 µC/cm\(^2\) area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. The wafer was subsequently spin coated (4000 rpm, 1 min) with a solution of **Alkyne-PEG** (10 µL, 1wt% in MeOH). The surface wafer was then patterned by e-beam lithography (23 nm spot size, 30 kV accelerating voltage, 20 µC/cm\(^2\) area dose) as an array of 40 µm boxes patterned next to the existing **AO-PEG** features. The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. Next, **Azide-GOX-AF488** (20 µL in DPBS), CuSO\(_4\) (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) were exposed to the surface for 24 hours
before washing by immersion in 5 mL aliquots of 10% DMSO in DPBS 5 times for 30 s each. The wafer was then characterized by standard fluorescence microscopy.

**HRP Immobilized Surface**

*AO-PEG* (10 µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer (4000 rpm, 1 min). *Caution: Piranha solution reacts violently with organic materials.* The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 40 µC/cm² area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. Next, *Lev-HRP-MB* (40 µL in DPBS), was incubated with the surface for 24 hours before washing by immersion in 5 mL aliquots of DPBS 5 times. Activity was assayed by adding the enzyme immobilized surface to a solution of hydrogen peroxide (300 ppm), and Amplex Red (50 µM) in DPBS, pH 7.4 and measuring the absorbance of resorufin at 560 nm after 2 h at 4°C.

**GOX Immobilized Surface**

*Alkyne-PEG* (10 µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer (4000 rpm, 1 min). *Caution: Piranha solution reacts violently with organic materials.* The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 20 µC/cm² area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. Next, *Azide-GOX-AF488* (20 µL in DPBS), CuSO₄ (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) were exposed to the surface for 24 hours before washing by
immersion in 5 mL aliquots of 10% DMSO in DPBS 5 times for 30 s each. Activity was assayed by adding the enzyme immobilized surface to a solution of HRP (1 µg), glucose (10 mM), and Amplex Red (50 µM) in DPBS, pH 7.4 and measuring the absorbance of resorufin at 560 nm after 2 h at 4°C.

**PEG-OH Control Surface**

8-Arm poly(ethylene glycol) (10.9 kDa, 10 µL, 1wt% in MeOH) was spin coated onto a piranha cleaned silicon wafer (4000 rpm, 1 min). *CAUTION: PIRANHA SOLUTION REACTS VIOLENTLY WITH ORGANIC MATERIALS.* The wafer was then patterned by e-beam lithography as an array of 40 µm boxes (23 nm spot size, 30 kV accelerating voltage, 60 µC/cm² area dose). The wafer was developed by immersion in Milli-Ω water for 30 seconds, and patterns were confirmed using an inverted bright-field microscope. The wafer was first exposed to **Lev-HRP-MB** (40 µL in DPBS), for 24 hours before washing by immersion in 5 mL aliquots of DPBS, pH 7.4, 5 times for 30 s each. Next, **Azide-GOX-AF488** (20 µL in DPBS), CuSO₄ (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) were exposed to the surface for 24 hours before washing by immersion in 5 mL aliquots of 10% DMSO in DPBS 3 times for 30 s each. Activity was assayed by adding the enzyme immobilized surface to a solution of HRP (1 µg), glucose (10 mM), and Amplex Red (50 µM) in DPBS, pH 7.4 and measuring the absorbance of resorufin at 560 nm after 2 h at 4°C.

**Alignment Wafer Control Surface**

A silicon wafer with gold alignment marks was cleaned by immersion in piranha solution. *CAUTION: PIRANHA SOLUTION REACTS VIOLENTLY WITH ORGANIC MATERIALS.* The wafer was subsequently
incubated with **Azide-GOX-AF488** (20 µL in DPBS, pH 7.4), and **Lev-HRP-MB** (20 µL in DPBS, pH 7.4) at room temperature for 3 hours. The surface was washed by immersion in 5 mL aliquots of DPBS, pH 7.4 5 times. Enzyme removal from the surface was probed by adding 10 mM glucose, 100 ppm hydrogen peroxide and 50 µM Amplex red to each wash mixture. No activity was observed in the 3rd wash solution aliquot. Remaining activity on the surface was assayed by adding the bare alignment wafer surface to a solution of glucose (10 mM), hydrogen peroxide (300 ppm), and Amplex Red (50 µM) in DPBS, pH 7.4 and measuring the absorbance of resorufin at 560 nm after 2 h at 4°C.

**Enzyme Immobilization in Encapsulated Hydrogels**

The encapsulated architecture **EA-2** was exposed to **Lev-HRP-MB** (40 µL, DPBS, pH 7.4, 3 h) at room temperature followed by immersion in 5 mL aliquots of DPBS, pH 7.4 5 times. Next, the surface was incubated with a solution of **Azide-GOX-AF488** (20 µL, DPBS, pH 7.4), CuSO₄ (10 µL, 1 mg/mL), and sodium ascorbate (10 µL, 5 mg/mL) for 3 hours at room temperature. The surface was washed by immersion in 10% DMSO in DPBS, pH 7.4 3 times. Activity was assayed by adding the enzyme immobilized surface to a solution of glucose (10 mM), and Amplex Red (50 µM) in DPBS, pH 7.4 and measuring the absorbance of resorufin at 560 nm after 2 h at 4°C.
CHAPTER 3

TREHALOSE BASED GLYCOMONOMERS
INTRODUCTION

Trehalose polymers may confer protective properties to biologics that are similar to the parent disaccharide. Therefore we sought to modify trehalose to contain a polymerizable group that could be further employed in controlled radical polymerization (CRP) by either atom transfer radical polymerization (ATRP) or reversible addition-fragmentation chain transfer (RAFT) polymerization.

Trehalose was first synthesized in 1954 by Lemieux. Although trehalose derivatives are ubiquitous across the literature, most modifications are symmetrical, including dideoxy, halogenated derivatives, and thiodisaccharides. Furthermore, Johnson’s protocol for 6,6’ acylated trehalose facilitates access to a wide-range of symmetrical analogs functionalized at these positions. As with other disaccharides, cyclization derivatives are also known for example as 6,6’ deoxy isocyanato adducts.

Unsymmetrical modifications are less common and often stem from Lee’s intermediate whereby the 4-position is left as a free hydroxyl group in the hepta-acetate adduct. This was the first unsymmetrical product reported to be obtained in a 1 pot procedure exploiting the differences in reactivity of the hydroxyl positions with 6,6’>2,2’>3,3’>4,4’. Additionally synthesis of trehalose glycolipids especially unsymmetrical trehalose cord factors is known. This class of chemicals has been correlated to tuberculosis and the bacterium has been shown to incorporate a number of synthetic analogs into the mycobacterial envelope. Further unsymmetrical derivatives include modifications to the glucopyranosidic ring structure to yield allo, manno, or xylopyranosides as well as trehalose halogenated selectively at the 6 position following benzylidene protection. Benzyldiene
protection at the 4,6 or 4,6 and 4′,6′ positions has been employed to afford the mono and
dibenzylidene acetals in 40 and >90% yield respectively.\textsuperscript{503,504} This particular modification is
the basis of our recently reported synthetic strategy to produce a trehalose glycomonomer.\textsuperscript{505}
RESULTS AND DISCUSSION

Synthesis of 2-Methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose

In our synthetic efforts towards a trehalose glycomonomer, we envisioned that trehalose could first undergo benzylidene protection at the 4,6/4',6' positions to afford dibenzylidene trehalose. This could be followed by reaction with any number of hydroxyl reactive monomers such as methacryloyl chloride to link the disaccharide to a polymerizable group via an ester bond. This specific example would yield 2-methacryl-4,6-4',6'-O-dibenzylidene-α,α-trehalose as the protected glycomonomer (Figure 3.1).

![Synthesis of 2-Methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose](image)

**Figure 3.1**: Synthesis of the known protected carbohydrate 4,6,4',6'-O-dibenzylidene-α,α-trehalose (3.1) followed by regioselective esterification at the 2-hydroxyl position with methacryloyl chloride to obtain 2-methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose (3.2)

Therefore, 4,6,4',6'-O-dibenzylidene-α,α-trehalose (3.1) was synthesized. Trehalose was made anhydrous according to known procedures as this was found to greatly increase the yield of benzylidination. Addition of dimethoxy toluene (DMT) in small portions over 40
minutes was also found to be critical for acceptable reaction yields, especially with larger batches. To date the reaction has been scaled to produce over 100 g of the dibenzylidene product in comparable yields. The resulting product was easily purified by removal of reaction solvent under vacuum followed by recrystallization once in benzene and once in a 30 % solution of ethanol. The $^1$H NMR spectra (Figure 3.2) of the purified reaction product was characterized according to standard spectroscopic techniques including NMR experiments typically employed in carbohydrate chemistry.\textsuperscript{507} For the dibenzylidene protected disaccharide, double quantum filtered phase sensitive correlation spectroscopy (COSY) (Figure 3.3) was first used in tandem with $^1$H-$^{13}$C heteronuclear single quantum coherence spectroscopy (HSQC) (Figure 3.4) to assign initial couplings of the $^1$H spectra. This strategy allowed two starting points for proton assignment. In the HSQC spectra the diasteriotopic protons may be unambiguously observed as two distinct $^1$H signals that correlate to the same $^{13}$C signal; in the COSY spectra the 1,1’ protons known to occur near 5.0 ppm were well resolved with crosspeak signals that could be mapped back to the remainder of the pyranosyl ring. Identifying these two key elements allowed clean elucidation of most $^1$H peaks in the parent structure. These resonances could then be mapped back onto the HSQC spectra to assign all $^{13}$C signals. As expected, diastereomers are not observed in the formation of the two new stereo centers indicating strong preference for the acetal proton to adopt the axial position thereby holding the phenyl groups equatorial to the protected carbohydrate (Figure 3.5)
Figure 3.2: $^1$H NMR (500 MHz, D$_6$DMSO) of the known compound 4,6,4',6'-O-dibenzylidene-α,α-trehalose.

Figure 3.3: Double quantum filtered phase sensitive correlation spectroscopy (COSY, 500 MHz, D$_6$DMSO) of the dibenzylidene protected carbohydrate 3.1.
Figure 3.4: $^1$H-$^{13}$C Heteronuclear single quantum coherence spectroscopy (HSQC, 500 MHz, D$_6$DMSO) of 3.1 highlighting the diasteriotopic ring system (red circle).

Figure 3.5: Only one diastereomer of benzylidene protected trehalose is observed with both phenyl groups held in equatorial positions.

The protected carbohydrate 3.1 was found to be highly hygroscopic. This interfered with statistical esterification at the 2 position as excess dibenzylidene trehalose was required to effect mono-substitution. Therefore, dibenzylidene trehalose was made anhydrous by
processing through a dean stark trap (50% toluene in benzene) immediately prior to use. Initial functionalization studies screened relatively mild methods to substitute dibenzylidene trehalose in an attempt to maximize selectivity for the 2 position. It has been hypothesized that this selectivity is due to steric effects. Therefore, to further maximize regioselectivity, addition of large functional groups was attempted including trityl, dimethoxy trityl, and p-toluene sulfonyl chlorides. In the latter case 2-hydroxyl displacement with the p-toluene sulfonyl adduct of HEMA was also attempted along with carbodiimide mediated coupling of methacrylic acid. Further elaboration of 3.1 was not observed in any of the attempted reactions. This led to attempts at functionalization with smaller acid halides.

Reaction with methacryloyl chloride was found to proceed slowly over 48 hours with optimal yields obtained when the reaction mixture was cooled to -78°C prior to drop-wise addition of the acid chloride over 3 hours. The product 2-methacryl-4,6,4’,6’-O-dibenzylidene-α,α-trehalose (3.2) was significantly hydrophobic and could be purified from the bulk reaction mixture by standard silica gel column chromatography (Hexanes : EtOAc 70:30). However, the molecule was isolated as a mixture of mono-substituted products functionalized at the 2 or 3 position in a ratio of 13:1 based on integrations of proton signals at the 1,1’ positions.
Characterization of 2-Methacryl-4,6,4',6'-O-Dibenzylidene-α,α-Trehalose

Figure 3.6: Illustration of protected glycomonomer complete with 28 inequivalent sets of protons, 2 diasteriotopic pairs of protons, 2 new stereo centers (relative to trehalose) and 6 different ring systems (A-F).

Once purified 3.2 was obtained, structural characterization commenced utilizing a similar suite of NMR techniques as with 3.1. In this case, assignment was non-trivial as the protected glycomonomer featured 28 inequivalent sets of protons, 2 pairs of diasteriotopic protons, 2 new stereo-centers (relative to trehalose) and 6 ring systems (Figure 3.6). Based on the initial $^1$H spectra (Figure 3.7), ring elaboration could be confirmed by the appearance of a doublet of doublets at 4.75 ppm with integration equal to vinyl peaks at 6.26 and 5.8 ppm respectively. It could also be confirmed that mono-substitution had occurred via integrations relative to the benzylidene signals from rings A and F near 7.5 ppm. The acetal proton signal was observed to evolve into two different signals at 5.68 and 5.58 ppm indicating the presence of the two inequivalent acetal ring systems B and E. The 1,1' signals at 5.27 and 5.01 ppm respectively demonstrated a similar occurrence in the formation of the two inequivalent
pyranoside ring systems C and D. The remaining hydroxyl protons could be assigned based on chemical shift with 3, 2’ and 3’ hydroxyls occurring at 5.67, 5.48, and 5.35 ppm respectively. Doublet splitting due to pyranoside ring protons also supported these assignments. Further structural explication involved 2-D NMR techniques. DEPT-135, $^{13}$C NMR (Figure 3.8) and HSQC (Figure 3.9) were used to first solve the diasteriotopic protons coupled in ring systems B and E. From these assignments, COSY (Figure 3.10) could be employed to assign adjacent protons in the bridged ring systems C and D, however, resolution was not great enough to continue this assignment cascade throughout the entire molecule. Therefore, assignment by COSY was attempted starting from 3 key points, namely, the protons at the 1, 1’ and 2 positions. Via this method, $H_i$ was observed to couple neatly to $H_k$ at the substituted 2 position thereby distinguishing it from $H_j$ at the 1’ position. The 2’ position was then assigned via cross-peak with $H_j$ to give $H_s$ as the 2’ pyranosidic ring proton with subsequent coupling to $H_g$ confirming assignment of the corresponding 2’ hydroxyl proton. Proceeding around the ring, $H_s$ was confirmed to couple to $H_p$ by alignment of the cross-peaks of attached hydroxyl $H_h$; hydroxyl $H_g$ crosspeak with $H_s$ was also observed before loss of resolution for the remaining protons belonging to ring systems B and C.
Figure 3.7: Standard $^1$H NMR of protected glycomonomer 3.2 complete with signal assignment.
Figure 3.8: $^{13}$C (top) and phase sensitive DEPT-135 (bottom) spectra of protected glycomonomer 3.2. Vinyl carbons (15) and carbons with diasteriotopic protons (25 and 26) are highlighted in red.

Figure 3.9: $^1$H-$^{13}$C HSQC NMR of 3.2 at a) full scale and b) enlarged to illustrate coupling within C and D ring protons complete with labeled key cross-peaks.
Similarly, ring D was assigned based on H_K cross-peaks beginning with correlation to H_I at the 1 position of 3.2. H_K coupling was also employed to elucidate H_M with subsequent correlation observed for H_E as the attached 3-hydroxyl proton. Associations for this proton could be carried forward to further assign H_R however the COSY signal was again unresolved in this region preventing further elucidation by this route. Therefore, assignment of the bridging protons for the B/C and D/E ring systems were given tentative assignments according to the dibenzylidene starting material to give H_R and H_T at the 4 and 4’ positions respectively. This revealed coupling between H_R and H_N in the COSY spectra, and additional cross-peaks between H_T and H_Q completed unambiguous assignment of all protons in the glycomonomer. All carbon signals were either fully resolved in the $^{13}$C and DEPT-135 experiments or correlated to resolved proton signals via HSQC. This allowed complete assignment of the $^{13}$C spectra to be accomplished through simply mapping the known proton assignments onto the HSQC spectra to complete full characterization of all H and C nuclei in the protected glycomonomer 3.2.
Once protected glycomonomer was confirmed by NMR, attempts were made towards deprotection. Typical cleavage of benzylidene acetals occurs under acidic hydrogenolysis conditions, however in this case reductive conditions were found to be prohibitive due to the highly sensitive methacrylate functionality. Acid hydrolysis was attempted with sulfuric or acetic acid as well as boronic or Lewis acids; however, the ester functionality exhibited preferential reactivity in all cases. With these results it was thought that the heightened reactivity of the methacrylate ester was due to the unsaturation. Efforts were shifted to other methods of trehalose functionalization, as well as direct polymerization of 3.2 in protected form followed by deprotection of the polymer.

**Synthesis of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose**

As 4,6,4′,6′-O-dibenzylidene trehalose could be prepared in high (>90%) yield, we envisioned that reaction with 4-vinylbenzaldehyde diethyl acetal (3.3) would exhibit similar regio-selectivity to install a polymerizable group in one step without the use of protecting group chemistry. Furthermore, mono-substitution could be effected under conditions modeled after the synthesis of the monobenzylidene acetal. Terephthaldehyde monodiethyl acetal was employed to form 4-vinylbenzaldehyde diethyl acetal via Wittig reaction in 97% yield. This product was used in a tran-acetal reaction with trehalose to achieve mono-substitution exclusively at the 4,6 position. As mentioned previously, reactions with trehalose necessitating anhydrous conditions along with statistical monosubstitution of unmodified trehalose are generally difficult to achieve. This is partially due to the dramatic decrease in solubility of anhydrous trehalose in organic solvents relative to the dihydrate form. It was therefore
expected that the reaction product would be significantly more soluble in the reaction solvent than the trehalose starting material. In fact, upon addition of functionalized acetal to an acidic mixture of trehalose and DMF, a significant amount of the carbohydrate was observed to dissolve in the solvent indicating that reaction was proceeding similar to protection with DMT. Excess trehalose could be removed from the crude reaction mixture by extraction with ethyl acetate, however, the greatest yields were obtained by direct HPLC purification of the crude reaction mixture to afford the product 4,6-O-(4-vinylbenzylidene)-α,α-trehalose (3.4). Although a significant amount of bis-functionalized byproduct was formed, production of the desired product 2 occurred in 41% yield after HPLC purification; no other isomers were observed indicating that preference for the 4,6 position was retained for 4-vinylbenzaldehyde diethyl acetal (Figure 3.11).

**Figure 3.11:** Synthesis of known 4-vinylbenzaldehyde diethyl acetal (3.3) with subsequent trans-acetal reaction found to be regioselective for the 4,6 hydroxyl positions on trehalose to form the glycomonomer 4,6-O-(4-vinylbenzylidene)-α,α-trehalose (3.4).
Figure 3.12: Glycomonomer 4,6-O-(4-vinylbenzylidene)-α,α-trehalose (3.4) complete with 4 ring systems.

Characterization of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose

Similar to the previous glycomonomer synthesis, characterization of 3.4 by NMR involved elucidation of 4 ring systems, 1 pair of diasteriotopic protons in the acetal ring, and 26 inequivalent proton signals (Figure 3.12). In addition to $^1$H NMR (Figure 3.13), $^{13}$C NMR, COSY (Figure 3.14), and HSQC-INEPT-135 (Figure 3.15), heteronuclear single quantum coherence total correlation spectroscopy (HSQC-TOCSY) experiments were performed to verify both the selectivity of the reaction as well as the integrity of the product ring systems (Figure 3.16). Furthermore, NOESY was undertaken to demonstrate retention of the unique confirmation of the native disaccharide whereby the two glucose subunits are held in a rigid clamshell shape about the α,α-1,1-glycosidic linkage (Figure 3.17). This result is important as it has proven to be a critical component in generating the unique physiochemical and protective properties of the carbohydrate.
Assignment of the $^1$H nuclei in the glycomonomer commenced with the standard $^1$H spectra, and it was found that most signals could be successfully resolved by increasing NMR field strength from 500 MHz to 600 MHz. Initial assignments were first attributed to the 4-vinylbenzylidene peaks at 7.517-7.451, 6.798-6.751, 5.901-5.872, and 5.329-5.310 ppm. Additionally the acetal proton could be observed as before at 5.590 ppm with only one diastereomer observed for the acetal ring system. Further resolved peaks included protons at the 1,1' positions (4.990 and 4.921 ppm respectively), and the primary 6’ hydroxyl proton observed as a triplet at 4.452 ppm. This provided a firm starting point for further delineation by 2-D NMR techniques.
Figure 3.14: a) COSY of 4,6-(4-vinylbenzylidene)-α,α-trehalose 3.4 with b) enlarged view of H-H coupling throughout the pyranosyl ring systems

Further characterization of the glycomonomer by 2-D NMR began from 4 key areas: the diasteriotopic protons in the acetal ring system confirmed by HSQC-INEPT-135, the 1 and 1’ protons, and also the 6’ hydroxyl proton. Observation of cross-peaks with the 6’-primary hydroxyl signal indicated 6’ methoxy protons H\text{I} and H\text{V}. Next, starting from the 1’ proton H\text{K}, cross-peak signals for the 2’ pyranosyl ring proton H\text{Y} and corresponding hydroxyl proton H\text{I} could be obtained. Starting from H\text{J}, cross-peaks corresponding to the 2 pyranosyl ring proton H\text{W} could be observed with correlation to the attached 2-hydroxyl position H\text{H}. With H\text{W} assigned, H\text{Q} was able to be confirmed based on COSY cross-peaks with subsequent elucidation of H\text{K} in a similar manner. The correlation of H\text{Q} to hydroxyl proton H\text{G} further supported this assignment. Lastly, diasteriotopic protons H\text{P} and H\text{O} were observed to couple with H\text{P} thereby completing assignment of H nuclei in ring systems A, B, and C.
Figure 3.15: $^1$H-$^{13}$C HSQC-INEPT 135 experiment of 3.4 at a) full scale and b) enlarged pyranosyl region. Cross-peaks phased negative are shown in red.

Figure 3.16: 2-D HSQC-TOCSY experiment illustrating two resolved glycoside ring systems as $^{13}$C resolution was translated into the $^1$H dimension. Cross peaks observed by HSQC-INEPT 135 are mapped as blue and red circles.
Further 2-D characterization was required for ring system D, and moving to HSQC-INEPT-135 confirmed assignment of the two sets of diasteriotopic protons, H_U/H_V and H_O/H_S. The most up-field proton signal was H_2 with corresponding $^{13}$C cross-peak by HSQC-INEPT 135 near 71 ppm. As this signal was well resolved in the HSQC spectra, a HSQC-TOCSY coupled experiment was used to translate the $^{13}$C resolution into the $^1$H spin systems. From this experiment it could be concluded that H_Z, H_R, and H_T were all part of a spin system that includes previously assigned protons in ring D, and Cross-peaks were observed between H_2 and H_R and H_T by COSY. Both of these facts indicated that H_2 was the 4’ pyranosidic ring proton coupled to the 4’ hydroxyl proton H_L. Of the two remaining ring D protons, only one correlates to a hydroxyl proton. Therefore, H_T must be the proton at the 3’ position with adjacent H_M hydroxyl group. This leaves H_R as the final 5’ ring D proton to complete assignment of all H nuclei in the glycomonomer. Assignment of the $^{13}$C spectra was performed by mapping the known $^1$H assignments onto the HSQC-INEPT 135 spectra. In this case carbons containing diasteriotopic protons are confirmed and most signals are well resolved (Figure 3.17).
Conformational Analysis of 4,6-O-\{4-vinylbenzylidene\}-α,α-trehalose

Further study was performed to investigate the shape of the glycomonomer. Thereby, NOESY was employed to observe through-space transient NOE coupling (Figure 3.18). Interglycoside ring system transient NOEs were observed and well-resolved cross-peaks were obtained for the interaction of free hydroxyl protons between ring systems C and D. This combined with the weak interaction between 1’ proton H₉ and H₁₆ at the 5 position on ring C support the hypothesis that the “clam shell” shape is retained in the functionalized glycomonomer.
Figure 3.18: NOESY experiment performed on the glycomonomer illustrating retention of the “clam shell” configuration of trehalose. Inter-glycoside ring system transient NOEs from H_6 (Blue), H_N (Red), and H_O (Green) are highlighted.
EXPERIMENTAL

Materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. Trehalose was purchased from The Endowment for Medical Research (Houston, TX) and rendered anhydrous prior to use via known procedures.  

Analytical Techniques

NMR spectra were recorded on a Bruker Avance 500 or 600 MHz spectrometer. UV-Visible spectroscopy was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments). Mass spectra were acquired using a Thermo Finnigan LCQ Deca Ion Trap MS. Infrared spectra were obtained with a Perkin-Elmer Spectrum One instrument equipped with a universal ATR accessory. Preparatory reverse phase HPLC was carried out on a Shimadzu HPLC system equipped with a UV detector using a Luna 5 µm C18 100A column (preparatory: 5 µm, 250 × 21.2 mm) with monitoring at λ = 254 and 220 nm.
This product was synthesized similar to the published route. Briefly, to a flame dried flask was added anhydrous trehalose (5 g, 14.61 mmol, 1 eq) and anhydrous DMF (75 mL), and the trehalose suspension was brought to 100°C. Next, p-TsOH (0.11 g, 0.58 mmol, 0.04 eq) was added and the mixture was placed under argon protection. Dimethoxytoluene (4.9 g, 32 mmol, 2.2 eq) was added in 2 parts over 20 minute increments. As the reaction proceeded trehalose could be observed to dissolve in the DMF solution indicating formation of the protected disaccharide. The reaction was stirred for an addition 20 min at 100°C and, the majority of the DMF was removed in vacuo to produce crude product as a viscous oil. The crude product was added to cold benzene to form white crystals, and the white solid was washed with saturated sodium bicarbonate. The white product was further recrystallized from benzene followed by recrystallization from a 30% solution of ethanol to obtain the product as white needlelike crystals (90% yield).
$^1$H NMR (500 MHz D$_6$DMSO)

$^1$H-$^{13}$C HSQC (500 MHz D$_6$DMSO)
2-methacyrloyl-4,6,4’6’-O-dibenzylidene-α,α-trehalose (3.2)

2-Methacyrloyl-4,6,4′,6’-O-dibenzylidene-α,α-trehalose (3.2) was synthesized by reaction of the protected sugar 3.1 with methacryloyl chloride. 4,6,4′6’-dibenzylidene-α,α-trehalose was made anhydrous via Dean-Stark trap with benzene, and was crystallized immediately prior to reaction. The anhydrous 4,6,4′6’-dibenzylidene-α,α-trehalose (1 g, 1.93 mmol, 1 eq) was dissolved in DCM (20 mL) and cooled to -78°C under argon protection in a flame dried round bottom flask. Imidazole (13 mg, 0.19 mmol, 0.10 eq) was added to anhydrous TEA (0.59 g, 5.79 mmol, 3 eq), and the solution was added to the bulk reaction mixture. Next, freshly distilled methacryloyl chloride (0.30 g, 2.9 mmol, 1.5 eq) was diluted in anhydrous DCM (5 mL), and the solution was added drop-wise over 3 h. The reaction was allowed to warm to room temperature over an additional 3 h before stirring for an additional 48 h. Pyridine salts were filtered form the crude reaction mixture before purification by silica gel column chromatography (30% EtOAc in Hexanes, Rf = 0.3). The product was obtained as a white solid after removal of solvents in vacuo 31 % yield, and could optionally be purified by preparatory HPLC chromatography (30 to 90% MeOH in water linear gradient, 30 min, 10 mL/min) to obtain the product after 13 min. MS (ESI-MS) calc for C₃₀H₃₄O₁₂Na⁺: 609.19 observed: 609.13. IR: δ = 3335, 2925,2356, 1727, 1452, 1377, 1148, 1073, 982, 759, 699 cm⁻¹.
$^1$H NMR (500 MHz $\text{D}_6\text{DMSO}$)

$^{13}$C NMR (500 MHz $\text{D}_6\text{DMSO}$)
The bis-functionalized glycomonomer 2,2’-dimethacryloyl-4,6,4’,6’-O-dibenzylidene-\(\alpha\),\(\alpha\)-trehalose was obtained as a byproduct from reaction of 4,6,4’,6’-O-dibenzylidene-\(\alpha\),\(\alpha\)-trehalose with methacryloyl chloride. The byproduct eluted from the column after 23 minutes upon HPLC purification (30 to 90% methanol in water, linear gradient, 30 min, 10 mL/min). Solvent was removed \textit{in vacuo} and the product was obtained as a white solid in 25% yield.

\(\textsuperscript{1}H\) NMR (500 MHz D\textsubscript{6}DMSO)
4-vinylbenzaldehyde diethyl acetal (3.3)

4-vinylbenzaldehyde diethyl acetal was synthesized following a literature procedure.\textsuperscript{513} Methyltriphenylphosphonium bromide (5.4 g, 14.5 mmol) and 45 mL of anhydrous tetrahydrofuran (THF) were added to a flame-dried 250 mL round bottom flask. The reaction mixture was cooled to -78 °C and stirred for 20 min. n-Butyl lithium (n-BuLi) (1.6 M in Hexanes, 9.15 mL, 14.7 mmol) was added drop wise over 20 min. The reaction was stirred for 30 min at -78 °C, warmed to 25 °C and stirred for an additional 10 min. The orange-red colored reaction solution was then cooled to -78 °C. Terephthaldehyde monodiethyl acetal (2.5 g, 12.0 mmol) dissolved in 7.5 mL of THF was added drop wise over 1 h. After stirring at -78 °C for 30 min, the reaction flask was warmed to 0 °C and stirred for 3 h. Then the reaction was warmed to 25 °C and stirred for an additional 1 h. The reaction was quenched by adding 10 mL saturated NaHCO\textsubscript{3} solution. The organic layer was collected after adding 50 mL of H\textsubscript{2}O, and the aqueous layer was extracted with 20 mL of ether three times. The combined organic layers were dried over MgSO\textsubscript{4}. After purification by silica gel column chromatography (Hex : EtOAc = 20 : 1), 1 (2.40 g, 97% yield) was obtained as a liquid. \textsuperscript{1}H NMR (500 MHz, in CDCl\textsubscript{3}) \(\delta\): 7.55-7.45 (m, 2H), 7.45-7.36 (m, 2H), 6.78-6.68 (m, 1H), 5.81-5.74 (d, J = 17 Hz, 1H), 5.57-5.51 (s, 1H), 5.29-5.22 (d, J = 11 Hz, 1H), 3.70-3.50 (m, 4H), 1.40-1.10 (t, J = 7 Hz, 6H). \textsuperscript{13}C NMR (500 MHz in CDCl\textsubscript{3}) \(\delta\): 138.80, 137.43, 136.59, 126.84, 125.93, 113.77, 101.00, 60.65, 15.10.
$^1$H NMR (500 MHz D$_6$DMSO)
To a flame dried round bottom flask $\alpha,\alpha$-trehalose (4.15 g, 12.1 mmol, 5.00 eq) and 150 mL DMF were added, and the resulting mixture was warmed to 100 °C. Next, 3.3 (0.500 g, 2.42 mmol, 1.00 eq) and $p$-toluenesulfonic acid ($p$-TsOH, 184 mg, 0.97 mmol, 0.4 eq) were added, and the solution was stirred for 3 h at 100 °C. Solvent was removed from the resulting crude reaction product in vacuo to give a white solid that was further purified by HPLC (60 to 90% methanol in water linear gradient, 30 min, 10 mL/min). After removal of solvent the resulting product 2 (456 mg, 0.99 mmol, 41% yield) was obtained as a white solid. $^1$H NMR (600 MHz, D$_6$DMSO) $\delta$: 7.510 (d, 2H, 8.4Hz), 7.458 (d, 2H, 8.4Hz), 6.775 (dd, 1H, 10.8, 17.4Hz), 5.887 (d, 1H, 17.4Hz), 5.590 (s, 1H), 5.320, (d, 1H, 11.4Hz), 5.259 (d, 1H, 4.8Hz), 5.033-5.016 (m, 2H), 4.990 (d, 1H, 17.4Hz), 4.921 (d, 1H, 3.6Hz), 4.868-4.853 (m, 2H), 4.452 (dd, 1H, 6, 6Hz), 4.137 (dd, 1H, 6, 6Hz), 4.036 (ddd, 1H, 4.8, 9.6Hz), 3.795 (ddd, 1H, 4.8, 5.4, 9.3Hz), 3.746 (ddd, 1H, 2.1, 4.5, 9.9Hz), 3.691 (dd, 1H, 10.2, 10.2Hz), 3.644-3.595 (m, 2H), 3.530 (ddd, 1H, 5.4, 5.7, 11.7Hz), 3.454-3.402 (m, 2H), 3.316 (ddd, 1H, 3.8, 5.6, 9.4Hz), 3.203 (ddd, 1H, 4.8, 5.4, 9.3Hz). $^{13}$C NMR (500 MHz D$_6$DMSO) $\delta$: 138.4, 138.3, 137.2, 127.6, 126.7, 115.8, 101.6, 101.5, 95.3, 94.8, 82.4, 73.6, 73.1, 72.4, 70.9, 70.5, 69.2, 63.3, 61.6. MS (ESI-MS) calc. for C$_{21}$H$_{28}$O$_{11}$Na$: 479.15 observed: 479.15, IR: $\delta = 3356, 2921, 1629, 1455, 1376, 1148, 1073, 976, 833, 800$ cm$^{-1}$. UV/Vis (H$_2$O) $\lambda = 225, 259$ nm.
CHAPTER 4

PROTEIN REACTIVE TREHALOSE

SIDE-CHAIN GLYCOPOLYMERS
INTRODUCTION

There are ubiquitous occurrences of carbohydrates that exist as polysaccharides in nature. Examples range from polymers employed for physical properties such as cellulose, chitosan, and hyaluronate to macromolecules involved in cellular function such as heparin, xanthan gum, and other polysaccharides utilized in signaling pathways. Although a wide-range of carbohydrate sources exist, biosynthetically, many polysaccharides are produced from UDP-glucose combined with various glucose phosphates as is the case with trehalose.

While less common, endogenous production of trehalose based polysaccharides are known, for example in the cord factors of tuberculosis bacteria. It is possible that these polymers could play a role in evading immune response to the infection in the host organism. Similar natural trehalose based macromolecules occur in bacterial cell walls as trehalose mono and di-mycolates. This particular family of glycolipids consist of the parent disaccharide esterified to mycolic acid at the 6 or 6’ positions. The trehalose mycolate family has been implicated in bacterial signaling, and synthetic analogs of trehalose-mycolates are known.

A myriad of synthetic polysaccharides have been reported and these carbohydrate based polymers are produced for diverse applications including foods, hydrogels, and cell culture. Several trehalose based materials have been produced as cross-linked polymer networks such as poly-substituted trehalose vinylbenzyl ether thermo-set resins. Achieving trehalose linear polymers has been challenging as the anomeric centers are relatively un-reactive due to the 1,1 glycosidic linkage. Therefore, typical
synthetic routes to produce trehalose based monomers contain several protection and deprotection steps, use symmetrical bifunctional monomers targeting the 6,6’ positions, or produce mixtures of regio-isomers that are not well defined. For example, a simple strategy for synthesizing a trehalose linear polymer was first reported in 1979, but selectivity to form linear polymer versus the branched ones was unclear at that time.\textsuperscript{647} Polymerization of diamino-type trehalose was explored to overcome the issue of branching, but the over-all process was more complicated.\textsuperscript{648} Acetalization,\textsuperscript{649} enzymes,\textsuperscript{650} Diels-Alder reactions,\textsuperscript{651} and click chemistry\textsuperscript{366,652} have been exploited to synthesize trehalose-based linear polymers, with the later study being extended to biological systems. However, most incorporate trehalose into the polymer backbone rather than as a side chain. To date, a well-defined trehalose polymer synthesized by controlled radical polymerization has not been reported, and trehalose based protein-glycopolymer conjugates have yet to be realized. Herein we report the synthesis of such protein reactive glycopolymers including polymerization of a trehalose monomer that has been esterified at the 2-hydroxyl position with methacryloyl chloride (3.2) as well as our recently reported glycomonomer 3.4.\textsuperscript{505}
RESULTS AND DISCUSSION

**Polymerization of 2-methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose**

The controlled radical polymerization technique reversible addition-fragmentation chain transfer polymerization (RAFT) was the method of choice employed in the polymerization of both protected trehalose glycomonomer 2-methacryl-4,6,4',6'-O-dibenzylidene-α,α-trehalose (3.2) and 4,6-O-(4-vinylbenzylidene)-α,α-trehalose (3.4) as RAFT polymerization readily provides well-defined polymers with protein-reactive end-groups.\(^{653}\) It was envisioned that the protein-reactive glycopolymer could be prepared first in each case followed by attachment to proteins of interest in a grafting to approach. Therefore, the known chain transfer agent (CTA 1) was prepared with a pyridyl disulfide group, because this functionality has been frequently reported as an effective moiety for reaction with free cysteines in proteins.\(^{253, 267}\) The synthesis of CTA 1 was accomplished by esterification of 2-(ethyl sulfanylthiocarbonyl sulfanyl)-propionic acid with 2-thioethanol previously activated with Aldrithiol to afford CTA 1 in 76 % yield (Figure 4.1).

![Figure 4.1: Synthesis of thiol-reactive CTA 1 via carbodiimide mediated coupling with 2-thioethanol that has been activated with Aldrithiol.](image)

Polymerization was attempted with glycomonomer 3.2 in protected form as it was expected that the ester moieties in the polymer side-chain could withstand hydrogenolysis conditions after the methacrylate olefin was reacted. Therefore, RAFT polymerization commenced at 80°C in D\(_6\)DMSO. The polymerization was found to progress slowly, reaching
55% conversion after 24 hours (Figure 4.2). The polymer was purified by dialysis against water (MWCO 3 kDa, 3 days). Although analysis by GPC demonstrated that a well defined polymer had been synthesized (Figure 4.3), characterization by NMR was challenging due to significant line broadening in the carbohydrate ring systems. Nevertheless, end-group and vinyl signals could be compared to dibenzylidene groups in the side-chain allowing for \( M_n \) and end-group retention to be observed by NMR.

![Figure 4.2: 2-methacryl-4,6,4',6'-O-dibenzylidene-\( \alpha,\alpha \)-trehalose (3.2) was polymerized by RAFT utilizing thiol-reactive CTA 1 and azobisisobutyronitrile (AIBN) initiator in D\(_6\)DMSO at 80°C to form a polymer with \( M_n(GPC) = 7,100 \) and PDI of 1.23.]

![Figure 4.3: Gel Permeation Chromatography (GPC) trace of protected glycopolymer 4.1 illustrating a unimodal peak shape and narrow polydispersity.]

**Figure 4.2:** 2-methacryl-4,6,4',6'-O-dibenzylidene-\( \alpha,\alpha \)-trehalose (3.2) was polymerized by RAFT utilizing thiol-reactive CTA 1 and azobisisobutyronitrile (AIBN) initiator in D\(_6\)DMSO at 80°C to form a polymer with \( M_n(GPC) = 7,100 \) and PDI of 1.23.

**Figure 4.3:** Gel Permeation Chromatography (GPC) trace of protected glycopolymer 4.1 illustrating a unimodal peak shape and narrow polydispersity.
Once the protected glycopolymer was obtained, deprotection steps were attempted using the acidic hydrogenolysis conditions described previously. Although removal of the benzylidene protecting groups was observed by NMR post-dialysis, line broadening of each signal in the pyranoside ring systems excluded cogent assignment of signals. This made conclusive elucidation of deprotected 4.1 elusive.

**Polymerization of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose**

The low yield of monomer 3.2 made further exploration of polymer 4.1 prohibitive. Glycomonomer 3.4 was obtained via a much higher yielding synthesis and was thus pursued. RAFT polymerization of 4,6-O-(4-vinylbenzylidene)-α,α-trehalose with the thiol reactive CTA 1 was performed similarly at 80°C in DMF (Figure 4.4). The ratio of reagents used for polymerization was [CTA]:[monomer]:[AIBN] = 1:29:0.2, with a concentration of 0.8 M monomer. After 6 h, the polymerization was stopped by immersion in liquid nitrogen to obtain 77% conversion. The polymer was dialyzed against aqueous sodium bicarbonate (MWCO 3 kDa, 3 days). The molecular weight of the polymer was analyzed by 1H NMR spectroscopy and was found to be 9.6 kDa by comparing the integration of end-group pyridine peaks to the aromatic ring from styrene (Figure 4.5). The PDI by GPC was 1.07, demonstrating that a well-defined polymer was formed (Figure 4.6). The [CTA] : [monomer] : [AIBN] ratios were then altered to obtain other molecular weights ranging from 4.2 to 19 kDa (Table 4.1) with narrow PDIs obtained in all cases.
**Figure 4.4:** Polymerization of glycomonomer 3.4 with the known thiol reactive CTA 1 to produce thiol-reactive trehalose side-chain glycopolymers of varied molecular weights and well-defined polydispersities (Poly 1-4).

**Figure 4.5:** $^1$H NMR trehalose side-chain glycopolymer synthesized by RAFT polymerization with CTA 1 and 4,6-O-(4-vinylbenzylidene)-α,α-trehalose. End-group retention is confirmed by the peak at 8.5 ppm, and $M_n$ is calculated by integrating this signal to the benzyl group in the side-chain.
Figure 4.6: Gel Permeation Chromatography (GPC) of Poly 1-3. A high molecular weight shoulder and slight increase in polydispersity is observed at increased molecular weight.

<table>
<thead>
<tr>
<th>Entry</th>
<th>CTA:M : AIBN</th>
<th>Target ( M_n )</th>
<th>Conv (%)</th>
<th>( M_n ) NMR</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly 1</td>
<td>1:21:0.2</td>
<td>7,000</td>
<td>72</td>
<td>4,200</td>
<td>1.05</td>
</tr>
<tr>
<td>Poly 2</td>
<td>1:29:0.2</td>
<td>9,600</td>
<td>77</td>
<td>9,400</td>
<td>1.07</td>
</tr>
<tr>
<td>Poly 3</td>
<td>1:35:0.2</td>
<td>13,800</td>
<td>84</td>
<td>14,700</td>
<td>1.11</td>
</tr>
<tr>
<td>Poly 4</td>
<td>1:50:0.2</td>
<td>20,000</td>
<td>85</td>
<td>19,000</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Table 4.1: Polymerization conditions and resultant \( M_n \) for polymerization reactions of the 4,6-\( O \)-(4-vinylbenzylidene)-\( \alpha,\alpha \)-trehalose glycomonomer with thiol reactive CTA 1.

Synthesis of Novel Chain Transfer Agents

Although well defined polymers with high end-group retention could be obtained using the known thiol-reactive CTA 1, it was thought that conjugation yield to proteins would be low as the carbohydrate side-chain of the polymer would prevent access to the protein reactive end-group. Therefore, two new CTAs were synthesized to contain triethylene glycol spacers. The first CTA was devised to contain a hydrophilic triethylene glycol spacer between the trithiocarbonate and pyridyl disulfide end-group. Tri(ethylene glycol) (TEG) was modified by tosylation followed by refluxing with thiourea. Upon addition of base, the resulting 1-
mercapto triethylene glycol was treated with Aldrithiol to obtain \(\alpha\)-pyridylisulfide-\(\omega\)-hydroxy tri(ethylene glycol) (4.2). An amine reactive CTA was similarly sought by carbodiimide mediated coupling of levulinic acid to TEG to produce \(\alpha\)-levulinyl-\(\omega\)-hydroxy tri(ethylene glycol) (4.4). Subsequent coupling of each alcohol moiety of 4.2 or 4.4 to the acid functionality on 2-(ethyl sulfanyltiocarbonyl sulfanyl)-propionic acid (4.3) afforded the thiol (CTA 2) and amine reactive (CTA 3) chain transfer agents in 62% and 49% yield respectively (Figure 4.7) with small amounts of 4.4 impurity observed by NMR for CTA 3.

**Figure 4.7:** Synthesis of CTA 2 and CTA 3. Aldrithiol is coupled to mercapto-triethylene glycol under acidic conditions (4.2) and levulinic acid is attached to triethylene glycol via carbodiimide coupling (4.4). 2-(ethylsulfanyltiocarbonyl sulfanyl)-propionic acid was prepared via known procedures and subsequently esterified with each intermediate to produce thiol reactive CTA 2 and amine reactive CTA 3.
Polymerization with Novel Chain Transfer Agents

Polymerization with CTA 2 was effected under comparable conditions at 80°C in DMF. RAFT polymerization of 3.4 in the presence of CTA 2 was shown to proceed for a range of molecular weights (Table 4.2). After purification by dialysis (1 kDa MWCO, H₂O, 3 days), these thiol reactive glycopolymers were characterized by NMR spectroscopy to confirm end-group retention and complete removal of monomer (Figure 4.8). Analysis by GPC confirmed that well-defined polymers were synthesized with slight deviation from narrow polydispersities obtained for the highest molecular weight attempted (Figure 4.9). For the highest molecular weight attempted, the increase in PDI is from lower molecular weight polymer chains indicating a process that terminates chain growth is occurring as opposed to radical recombination which would result in a higher molecular weight shift. Furthermore, polymer side-chain absorbance at 254 nm relative to trithiocarbonate absorbance at 310 nm was observed for varied molecular weights (Figure 4.10). The increase in absorbance at 254 nm indicates an increase in the concentration of the benzylidene backbone with increasing molecular weight as would be expected for increasing the chain length of the polymer.

<table>
<thead>
<tr>
<th>Entry</th>
<th>CTA:M : AIBN</th>
<th>Target Mₙ</th>
<th>Conv (%)</th>
<th>Mₙ NMR</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly 5</td>
<td>1:20:0.4</td>
<td>9,000</td>
<td>94</td>
<td>8,000</td>
<td>1.10</td>
</tr>
<tr>
<td>Poly 6</td>
<td>1:60:0.2</td>
<td>11,400</td>
<td>40</td>
<td>15,400</td>
<td>1.13</td>
</tr>
<tr>
<td>Poly 7</td>
<td>1:76:0.2</td>
<td>25,800</td>
<td>73</td>
<td>24,500</td>
<td>1.20</td>
</tr>
<tr>
<td>Poly 8</td>
<td>1:120:0.4</td>
<td>44,800</td>
<td>81</td>
<td>49,500</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Table 4.2: Polymerization conditions and resultant Mₙ for polymerization reactions of the 4,6-\(\text{O-(4-vinylbenzylidene)}-\alpha,\alpha\)-trehalose glycomonomer with thiol reactive CTA 2.
Figure 4.8: $^1$H NMR of trehalose side-chain glycopolymer Poly 5 synthesized by RAFT polymerization with CTA 2 and 3.4. End-group retention is confirmed by the peak at 8.5 ppm, and $M_n$ is calculated by integrating this signal to the benzyl group in the side-chain.

Figure 4.9: Gel Permeation Chromatography traces of Poly 5-8 demonstrating unimodal peaks with increase in polydispersities for larger molecular weights.
Glyco-Polymer UV/Vis

![Glyco-Polymer UV/Vis spectra](image)

**Figure 4.10:** UV/Vis absorbance spectra of Poly 5-8. The spectra are normalized to the absorbance of the trithiocarbonate moiety at 310 nm. An increase in side-chain absorbance at 260 nm is observed with increasing degree of polymerization.

Further polymerization was effected using CTA 3 in D$_6$DMSO to achieve an amine reactive glycopolymer at 77% conversion with PDI 1.42 after heating to 80°C for 20 h. The polymer was purified by dialysis (3 kDa MWCO, 20% MeOH in water, 3 days), and analyzed by GPC to confirm a well-defined polymer was obtained (Figure 4.11). Although the levulinyl end-group could be observed by NMR, integrations could not be completely correlated to polymer chains by NMR due to signal overlap with the end-group and glycopolymer chain. Good correlation was observed however between the styrene backbone at 7-6 ppm and the 6’ hydroxyl proton indicating that hydrolysis has been minimized during polymerization (Figure 4.12).
**Figure 4.11:** Gel Permeation Chromatography trace of Poly 9 demonstrating moderate polydispersity but unimodal peak shape.

**Figure 4.12:** $^1$H NMR of a trehalose side-chain glycopolymers synthesized by RAFT polymerization with CTA 3 and 3.4. $M_n$ could not be calculated due to signal overlap with CTA 3.
Implications of Synthesized Glycopolymers

Overall, 4 different types of trehalose side-chain polymers have been prepared by RAFT polymerization with 3 different protein reactive CTAs. Although the dibenzylidene protecting groups could not be removed from protected glycomonomer 3.2 without hydrolysis of the methacrylate ester, polymerization could be successfully effected in protected form. Trehalose glycopolymers were produced from 3.4 with levulinyl or pyridyl disulfide end-groups with a range of molecular weights obtained for the later functionality. Therefore, it has been shown that trehalose based glycopolymers may be produced with attachment to the polymer backbone at the 2 or 4,6 hydroxyl groups. In the case of glycopolymers prepared by 3.4 good correlations between the starting monomer and resulting glycopolymer were observed in the $^1$H NMR spectra. In particular, signals from acetal protons in the side-chain present near 5.4 ppm could be compared to the signal of the aromatic backbone present between 7.4-6.2 ppm to confirm that hydrolysis of the acetal linkage did not occur under any of the polymerization conditions attempted. This also demonstrates the general utility of the novel glycomonomer 3.4 as it may be produced in 1 step from known compounds in yields that match the monobenzylidene derivatives$^{503}$ thereby providing a facile route to produce a large quantities of glycopolymers for use as stabilizing agents.
EXPERIMENTAL

Materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. All other chemicals were purchased from Sigma-Aldrich.

Analytical Techniques

NMR spectra were recorded on a Bruker Avance 500 or 600 MHz spectrometer. Gel permeation chromatography (GPC) was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A and two Polymer Laboratories PLgel 5 µm mixed D columns (with guard column). Lithium bromide (0.1 M) in N,N-dimethylformamide (DMF) at 40 °C was used as the solvent (flow rate: 0.6 mL/min). Near-monodisperse poly(methyl methacrylate) standards (Polymer Laboratories) were employed for calibration. UV-Visible spectroscopy was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments). Mass spectra were acquired using a Thermo Finnigan LCQ Deca Ion Trap MS. Infrared spectra were obtained with a Perkin-Elmer Spectrum One instrument equipped with a universal ATR accessory.
The synthesis was performed according to a literature procedure.\textsuperscript{50} 2-(Pyridin-2-yldisulfanyl)ethanol (97.94 mg, 0.52 mmol) and 2-(ethylsulfanylthiocarbonylsulfanyl)-propionic acid (100 mg, 0.48 mmol) were dissolved in 9.60 mL of dry dichloromethane (DCM). After the reaction was stirred at 0 °C for 20 min, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 273.41 mg, 1.43 mmol) and 4-(dimethylamino) pyridine (DMAP, 11.62 mg, 0.10 mmol) were added. The reaction color changed from yellow to orange. The ice bath was removed after 5 h, and the reaction was stopped after 24 h. The solution was washed with 30 mL of H\textsubscript{2}O three times and the organic layer was dried over MgSO\textsubscript{4}. After silica gel column chromatography (Hex : EtOAc = 1 : 1), 117 mg of product was collected (70\% yield) as yellow oil. \textsuperscript{1}H NMR (500 MHz, in CDCl\textsubscript{3}) δ: 8.48-8.47 (m, 1H), 7.70-7.63 (m, 2H), 7.11-7.09 (m, 1H), 4.81 (q, J = 7 Hz, 1H), 4.43-4.35 (m, 2H), 3.39-3.34 (q, J = 7 Hz, 2H), 3.09-3.03 (m, 2H), 1.61-1.59 (d, J = 7 Hz, 3H), 1.37-1.34 (t, J = 7 Hz, 3H). \textsuperscript{13}C NMR (500 MHz in CDCl\textsubscript{3}) δ: 221.8, 170.9, 159.7, 149.8, 137.2, 121.0, 120.0, 63.5, 47.9, 37.3, 31.5, 16.7, 12.8.
$^1$H NMR of CTA 1 (500 MHz in CDCl$_3$)

$^{13}$C NMR of CTA 1 (500 MHz in CDCl$_3$)
Synthesis of 2-(2-(pyridin-2-yl-disulfanyl)ethoxy)ethoxy)ethanol (4.2)

In a flame dried round bottom flask Aldrithiol (1.99 g, 9.03 mmol, 3 eq) was dissolved in methanol (24 mL) and acetic acid (1 mL). Next, 1-mercapto-tri(ethylene glycol) (500 mg, 3.01 mmol, 1 eq) was added drop wise over 10 min. The resulting yellow reaction mixture was stirred for 24 h at 25 °C before removal of solvent in vacuo. The crude reaction product was dissolved in DCM, washed with water, dried with magnesium sulfate, and concentrated by rotary evaporation. The product was purified by column chromatography (100% EtOAc) to produce a yellow oil (613 mg, 2.22 mmol, 74% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ: 8.46-8.45 (m, 1H), 7.77-7.64 (m, 2H), 7.10-7.08 (m, 1H), 3.75-3.58 (m, 10H), 3.01-2.98 (m, 2H), 2.14 (s, 1H). $^{13}$C NMR (500 MHz CDCl$_3$) δ: 149.44, 137.00, 120.57, 119.61, 72.40, 70.32, 70.25, 68.91, 61.69, 38.17. UV/Vis (MeOH) λ = 256, MS (ESI-MS) calc. for C$_{11}$H$_{17}$O$_3$NS$_2$Na$^+$: 298.055 observed: 298.061, IR: ν = 3666, 2973, 2927, 2875, 1735, 1703, 1606, 1575, 1509, 1444, 1419, 1389, 1370, 1334, 1299, 1211, 1176, 1092, 1033, 1019, 1000, 918, 869, 846, 809, 720, 695 cm$^{-1}$. 
$^1$H NMR of 4.2 (500 MHz, CDCl$_3$)

$^{13}$C NMR of 4.2 (500 MHz, CDCl$_3$)
In a flame dried round bottom flask 4 (500 mg, 1.82 mmol, 1.1 eq) and 2-(ethyl sulfanylthiocarbonyl sulfanyl)-propionic acid (347 mg, 1.65 mmol, 1 eq) were dissolved in 16.5 mL of anhydrous DCM. The reaction was stirred for 20 min at 0 °C before addition of EDC (759 mg, 3.96 mmol, 2.4 eq) and DMAP (40 mg, 0.33 mmol, 0.2 eq). After additional stirring at 0 °C for 5 hours the reaction was allowed to warm to room temperature and was stirred for an additional 24 h. The crude reaction product was washed 3 times with water and the organic layer was dried over MgSO₄. The solution was concentrated before purification by column chromatography (100% EtOAc) to isolate 5 as a yellow oil (478 mg, 1.02 mmol, 62% yield). ¹H NMR (500 MHz, CDCl₃) δ: 8.46-8.45 (m, 1H), 7.78-7.64 (m, 2H), 7.10-7.07 (m, 1H), 4.83 (q, J = 7Hz, 1H), 4.32-4.29 (m, 2H), 3.74-3.57 (m, 8H), 3.35 (q, J = 8 Hz, 2H), 3.00 (t, J = 6 Hz, 2H), 1.60 (d, J = 7 Hz, 3H), 1.34 (t, J = 7 Hz, 3H). ¹³C NMR (500 MHz, CDCl₃) δ : 221.8, 171.1, 160.4, 149.6, 137.1, 120.6, 119.6, 77.3, 77.0, 76.8, 70.6, 70.4, 69.5, 69.0, 69.0, 68.9, 65.0, 64.9, 64.4, 47.9, 39.9, 38.5, 31.5, 21.6, 16.9, 16.8, 13.0. UV/Vis (50% DMF (aq)) λ = 252, 297, IR: ν = 3043, 2866, 1732, 1573, 1445, 1417, 1374, 1352, 1298, 1250, 1163, 1138, 1112, 1080, 1026, 1042, 985, 875, 812, 760, 717, 616 cm⁻¹. MS (ESI) calc for C₁₇H₂₅NO₄S₅H: 468.0465, Found: 468.0466.
$^1$H NMR of CTA 2 (500 MHz, CDCl$_3$)

$^{13}$C NMR of CTA 2 (500 MHz, CDCl$_3$)
In a typical synthesis: To a flame dried Schlenk tube was added 2 (218.1 mg, 0.48 mmol), 5 (11 mg, 2.4×10⁻² mmol) and AIBN (1.58 mg, 9.62 µmol, 1 eq) and DMF (0.60 mL) before placing the mixture under argon. Oxygen was removed from the resulting solution by five freeze-pump-thaw cycles and the solution was heated to 80 °C to 94% monomer conversion. The polymerization was stopped by immersing the reaction vessel in liquid nitrogen, and the polymer was purified by dialysis (MWCO 1 kDa, 1mM NaHCO₃(aq), 3 days). Solvent was removed from the resulting polymer before standard characterization. NMR, GPC (Figure 4.9) UV/Vis (Figure 4.10), Molecular weights by ¹H NMR were calculated by comparing the ratio of end-group with the styrene side chains of the polymer. The weight of the CTA (end-groups) was added in each case.
Poly 5

$^1$H NMR (500 MHz, D$_6$DMSO) δ: 8.42 (1H), 7.80 (2H), 7.56-6.25 (m, 66H), 5.49, 5.20, 4.95, 4.79, 4.38, 4.11, 3.99, 3.78, 3.70, 3.58, 3.48, 3.43, 3.18, 2.99, 1.66, 1.25, 0.83 ppm. UV/Vis $\lambda_{\text{max}}$ 310 nm ($\varepsilon = 18939$ cm$^{-1}$M$^{-1}$). IR: ν = 3354, 2929, 1638, 1419, 1375, 1211, 1147, 1098, 1072, 1046, 977, 929, 826, 800, 722, 662 cm$^{-1}$. $M_n$ (NMR) 8.0 kDa. $M_n$ (GPC) 9.6 kDa. PDI (GPC) 1.10.

Poly 6

$^1$H NMR (500 MHz, D$_6$DMSO) δ: 8.47 (1H), 7.79 (2H), 7.40-6.16 (m, 130H), 5.46, 5.20, 4.95, 4.88, 4.38, 4.08, 3.96, 3.76, 3.69, 3.57, 3.47, 3.40, 3.15, 2.95, 1.48, 1.21, 1.08, 0.85 ppm. UV/Vis $\lambda_{\text{max}}$ 261 nm ($\varepsilon = 68804$ cm$^{-1}$M$^{-1}$). IR: ν = 3371, 2930, 1655, 1376, 1212, 1385, 1148, 1098, 1073, 1046, 977, 930, 826, 800, 722, 662 cm$^{-1}$. $M_n$ (NMR) 15.4 kDa. $M_n$ (GPC) 12.1 kDa. PDI (GPC) 1.13.

Poly 7

$^1$H NMR (500 MHz, D$_6$DMSO) δ: 8.25 (1H), 7.82 (2H), 7.41-6.11 (m, 210H), 5.45, 5.22, 4.97, 4.87, 4.42, 4.13, 3.98, 3.80, 3.72, 3.60, 3.49, 3.43, 3.18, 1.65, 1.25, 0.87 ppm. UV/Vis $\lambda_{\text{max}}$ 261 nm ($\varepsilon = 38421$ cm$^{-1}$M$^{-1}$). IR: ν = 3353, 2919, 1618, 1376, 1212, 1148, 1098, 1073, 1046, 977, 929, 826, 800, 722, 662 cm$^{-1}$. $M_n$ (NMR) 24.5 kDa. $M_n$ (GPC) 18.8 kDa. PDI (GPC) 1.20.

Poly 8

$^1$H NMR (500 MHz, D$_6$DMSO) δ: 8.42 (1H), 7.80 (2H), 7.47-6.02 (m, 429H), 5.45, 5.22, 4.97, 4.85, 4.39, 4.10, 3.98, 3.78, 3.71, 3.60, 3.48, 3.43, 3.17, 1.49, 1.39, 0.85, 1.27 ppm. UV/Vis
$\lambda_{\text{max}}$ 261 nm ($\varepsilon = 59405 \text{ cm}^{-1}\text{M}^{-1}$). IR: $\nu = 3368, 2928, 1615, 1373, 1212, 1385, 1147, 1098, 1073, 1046, 977, 930, 825, 800, 722, 662 \text{ cm}^{-1}$. $M_n$ (NMR) 49.5 kDa. $M_n$ (GPC) 33.6 kDa. PDI (GPC) 1.47.

$^1\text{H NMR of Poly 5 (500 MHz, CDCl}_3)$
Synthesis of tri(ethylene glycol) levulinate (4.4)

In a flame dried round bottom flask levulinic acid (5.0 g, 43 mmol, 1.1 eq), anhydrous tri(ethylene glycol) (5.9 g, 39 mmol, 1 eq), and DCC (12.4 g, 60 mmol, 1.55 eq) were dissolved in 50 mL of anhydrous DCM. The reaction was cooled to 0 °C and DMAP (0.61 mg, 5.0 mmol, 0.13 eq) was added. The reaction was stirred at 0 °C for 5 hours then warmed to room temperature and stirred for an additional 5 hours. The crude reaction product was concentrated in vacuo before purification by column chromatography (EtOAc : DCM 1:1) to isolate the title compound 4.4 as a colorless oil (3.65 g, 38% yield). $^1$H NMR (500 MHz, D$_6$DMSO) $\delta$: 4.60 (t, 1H, J = 7 Hz), 4.13 (dd, 2H, J = 6, 7 Hz), 3.63-3.61 (m, 2H), 3.73-3.50 (m, 6H), 3.47-3.44 (m, 2H), 2.74 (t, 2H, J = 8Hz), 2.51 (t, overlap with DMSO, J = 8 Hz), 2.14 (s, 3H).

$^1$H NMR of 4.4 (500 MHz, CDCl$_3$)
Synthesis of amine reactive CTA 3

In a flame dried round bottom flask 4.4 (1.1 g, 4.3 mmol, 1 eq) and 2-(ethyl sulfonylsulfanylthiocarbonylsulfanyl)-propionic acid 4.3 (1 g, 4.3 mmol, 1.1 eq) were dissolved in 25 mL of anhydrous DCM. The reaction was cooled to 0 °C before addition of DCC (1.38 g, 6.7 mmol, 1.55 eq) and DMAP (0.07 g, 0.6 mmol, 0.1 eq). After additional stirring at 0 °C for 5 hours the reaction was allowed to warm to room temperature and was stirred for an additional 5 h. The crude reaction product was added to cold ethyl acetate, and the white precipitate was removed by filtration. The remaining solution was concentrated before purification by column chromatography (30% EtOAc in Hexanes) to isolate the title compound CTA 3 as a pale yellow solid (49 % yield). $^1$H NMR (500 MHz, CDCl$_3$) δ: 4.86, (t, 1H, 8 Hz), 4.32-4.30 (m, 2H), 4.26-4.23 (m, 2H), 3.73-3.62 (m, 8H), 3.40-3.38 (m, 2H), 2.76 (t, 2H, J = 6 Hz), 2.62 (t, 2H, J = 6 Hz), 2.20 (s, 3H), 1.62 (d, 3H, J = 8 Hz), 1.36 (t, 3H, J = 8 Hz).
$^1$H NMR of CTA 3 (500 MHz, CDCl$_3$)
RAFT Polymerization of Trehalose Monomer 3.4 with CTA 3 (Poly 9)

In a typical synthesis: To a flame dried Schlenk tube was added 3.4 (180 mg, 0.39 mmol, 100 eq), CTA 3 (8.7 mg, 0.02 mmol, 5 eq) and AIBN (0.65 mg, 0.4 µmol, 1 eq) and D6DMSO to a final concentration of 0.4M with respect to 3.4 before placing the mixture under argon. Oxygen was removed from the resulting solution by five freeze-pump-thaw cycles and the solution was heated to 80 °C to 77% monomer conversion. The polymerization was stopped by immersing the reaction vessel in liquid nitrogen, and the polymer was purified by dialysis (MWCO 3kDa, 20% MeOH in water, 3 days). Solvent was removed from the resulting polymer before standard characterization. NMR (600 MHz, D6DMSO) δ: 7.160-6.463 (m, 92H), 5.496, 5.241, 4.989, 4.882, 4.612, 4.598 (m, 1H), 4.585, 4.431 (m, 25 H), 4.140, 4.002, 3.741, 3.632, 3.520, 3.443, 3.323, 3.217, 2.740, 2.1566, 1.583, 1.158 ppm, GPC Mn = 18.4 kDa, PDI = 1.44.
$^1$H NMR of Poly 9 (600 MHz, D$_6$DMSO)

GPC trace of Poly 9

Mn = 18.4 kDa
PDI = 1.44
CHAPTER 5

CONJUGATION OF

TREHALOSE GLYCOPOLYMERS TO LYSOZYME
INTRODUCTION

Upon acquisition of purified protein-reactive glycopolymers, efforts were next focused on selection of proteins sensitive to degradation by environmental stressors. Essential attributes screened were: well-studied denaturation processes, current therapeutic use, as well as known activity measurement parameters. Initial emphasis was placed on the well characterized enzyme, lysozyme, as a model protein.

The primary sequence of egg-white lysozyme was first reported in 1962, with subsequent structural elucidation by X-ray analysis in 1965 along with a range of inhibitor complexes. Lysozyme has known activity by catalyzing degradation of N-acetyl-D-glucosamine containing oligomers. This occurs through hydrolysis about the 1,4-β glycosidic linkage; the enzyme preferentially cleaves hexamers or larger to tetramer and dimer sub units. Later activity studies showed further preference for alternating N-acetyl-D-glucosamine, N-acetylmuramic acid patterns, with specific binding sites proposed for each residue.

Lysozyme unfolding has been well-characterized and the enzyme is known to lose activity due to heat or lyophilization via complex multi-step processes. Thermal denaturation methods along with differential scanning calorimetry, infrared spectroscopy, circular dichroism and more recently, x-ray crystallography have all been used to probe lysozyme unfolding or denaturation. It has been shown that retention of α-helix character is essential for activity by a series of studies that refold denatured lysozyme to achieve a reactivated state. Interactions of lysozyme with trehalose has been studied computationally relative to other carbohydrates and lysozyme denaturation in the presence...
of various sugars and polyols has been investigated, though direct measurement of enzymatic activity was not reported in these cases.\textsuperscript{682, 683}

Lysozyme activity has been measured in solution via various methods. Historically, this was performed via turbidimetric assay measuring the decrease in absorbance of \textit{M. luteus} cells as a function of enzyme activity.\textsuperscript{684-686} Other methods have included highly sensitive radioimmunoassays,\textsuperscript{687, 688} and fluorescence polarization or fluorescence intensity with a fluorescent peptidoglycan substrate.\textsuperscript{689} Another method employs a combination of these techniques whereby \textit{M. Luteus} is covalently attached to fluorescent dyes.\textsuperscript{690} For our study, \textit{M. Luteus} labeled with FITC dye was purchased. The increase in fluorescence could be correlated to lysozyme activity as fluorescence quenching in the cell membrane was relieved when cell membrane was hydrolyzed in solution.

These known stability, activity, unfolding, and denaturation parameters served as the rationale for the production of a lysozyme-glycopolymer conjugate. Lysozyme has been conjugated to several macromolecules including ubiquitin\textsuperscript{691} and chitosan.\textsuperscript{692} Lysozyme PEG interactions are also known,\textsuperscript{693, 694} and protein partitioning is observed under select conditions.\textsuperscript{695} Lysozyme-polymer conjugates have been reported,\textsuperscript{696-698} and it is known that lysozyme-polymer interactions are highly sensitive to the side-chain\textsuperscript{694} or end-group\textsuperscript{699, 700} of the polymer making lysozyme an intriguing choice for attachment to the novel trehalose side-chain glycopolymer. Here we demonstrate the protective effects conferred from trehalose side-chain glycopolymers.\textsuperscript{505}
RESULTS AND DISCUSSION

Attachment of Thiols to Hen Egg-White Lysozyme

As native lysozyme contains no free thiol groups, thiols were added to hen egg white lysozyme for subsequent conjugation to thiol-reactive glycopolymers. The thiol transfer agent succinimidyl S-acetyl-thiopropionate (5.1) was prepared in a one pot procedure starting from acrylic acid and thiol acetic acid (Figure 5.1). Once 5.1 was obtained, it was used in a procedure known to covalently attach thioacetate functionality to proteins via amide bonds (Figure 5.2). After deprotection with hydroxyl amine, excess 5.1 was removed by centriprep ultra-centrifugation (MWCO 3kDa). Free thiols were quantified on the thiolated lysozyme (LyzSH) by Ellman’s assay resulting in a thiol : protein ratio of 1:1.4 (71% thiol).

Figure 5.1: Synthesis of the thiol transfer agent N-hydroxysuccinimidyl-S-acetyltihiopropionate (5.1, SATP) via thiol-ene reaction with neat acrylic acid followed by carbodiimide mediated coupling to form the activated NHS ester.

Figure 5.2: Addition of free thiols to hen egg white lysozyme by treatment with 5.1 followed by deprotection with hydroxyl amine. Ellman’s assay confirmed 70% of the proteins contained a free thiol via this method.
Attachment of Trehalose Glycopolymers to Thiolated Lysozyme

Next, LyzSH was conjugated to various thiol-reactive trehalose polymers (Poly 5-8) by incubation in DPBS, pH 8.0 at 4°C for 3 h (Figure 5.3). After incubation with trehalose polymers, conjugates were seen by SDS-PAGE (Figure 5.4). The conjugation yield as observed by SDS-PAGE was lower for glycopolymers prepared with CTA 1 as compared to Poly 5-8. One possible explanation was that the triethylene glycol linker moves the thiol reactive end-group away from the bulky and hydrated trehalose side chain of the polymer improving access of the activated disulfide to the protein surface.

Figure 5.3: Conjugation of thiolated lysozyme (LyzSH) to thiol reactive glycopolymers to product lysozyme-glycopolymer conjugates (Lyz-Poly 5-8)
Figure 5.4: SDS-PAGE of conjugation reactions between LyzSH and Poly 5-8 before FPLC purification stained by a) iodine or b) coomassie. A significant amount of free lysozyme is observed in all reactions under both reducing (Lanes 2-6) and non-reducing (Lanes 7-11) conditions indicating the presence of excess un-conjugated lysozyme.

Purification of Lysozyme-Glycopolymer Conjugates

Lysozyme-glycopolymer conjugates (Lyz-Poly 5-8) were purified by fast protein liquid chromatography (FPLC) with subsequent runs of LyzSH and Poly 5-8 used to determine which fractions would be pure of starting materials (Figure 5.5). Although degree of conjugation studies were not able to be performed due to the low amounts of purified conjugate obtained, fractions were selected so as to minimize contamination with un-conjugated lysozyme or free glycopolymer (Figure 5.5, green boxes). These fractions were collected and utilized in subsequent studies. Additionally, SDS-PAGE was used to confirm isolation of purified conjugates as demonstrated by the absence of lysozyme bands under non-reducing conditions. Reappearance of the bands under reducing conditions indicated that the polymers were attached to lysozyme through reducible disulfide bonds (Figure 5.6). Activities of the purified Lyz-Poly 5-8 conjugate fractions were confirmed by observing active lysis of the FITC labeled
gram positive bacteria *Micrococcus luteus* in the EnzChek lysozyme activity assay. Determination of enzyme concentration based on activity is ambiguous in this case. This is because the lysozyme glycopolymer conjugate may lose activity due to conjugation or gain activity due to the addition of a polymer that contains carbohydrate in the side-chain. Regardless, the purified fractions obtained were standardized by dilution in DPBS, pH 7.4 to an activity near 1kU/mL for each sample. For each molecular weight conjugate these purified lysozyme-glycopolymer conjugates were used for further study in comparison to stabilizing effects conferred from trehalose, un-conjugated glycopolymer, or PEG. In the case of addition of unconjugated polymer, the enzyme concentration could be exactly determined thereby presenting a precise way to compare the glycopolymers relative to other excipients.

![Figure 5.5: Fast protein liquid chromatography (FPLC) trace of the conjugation of LyzSH to thiol-reactive glycopolymers Poly 5-8 monitored by conductivity (red), and UV absorbance at 254 nm (purple) and 280 nm (green) with corresponding FPLC traces of native lysozyme and free polymer. Fractions concentrated for use in subsequent experiments are highlighted by the green boxes.](image)
Figure 5.6: SDS-PAGE of Lyz-Poly 5-8 after FPLC purification viewed by a) iodine or b) coomassie. Disappearance of the lysozyme band under non-reducing conditions (Lane 8-11) relative to crude conjugation mixture (Figure 5.4, Lanes 7-11) indicates that un-reacted lysozyme has been removed by FPLC purification. An increase in the intensity of the lysozyme band under reducing conditions (Lanes 2-6) relative to non-reducing conditions (Lanes 8-11) indicates that the polymer is conjugated through a disulfide bond that is sensitive to reduction.
EXPERIMENTAL

Materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. All other reagents including hen egg white lysozyme was purchased from Sigma-Aldrich and used without further purification.

Analytical Techniques

NMR spectra were recorded on a Bruker Avance 500 or 600 MHz spectrometer. UV-Visible spectroscopy was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments). Mass spectra were acquired using a Thermo Finnigan LCQ Deca Ion Trap MS. Infrared spectra were obtained with a Perkin-Elmer Spectrum One instrument equipped with a universal ATR accessory. BioRad Fast Protein Liquid Chromatography with attached size exclusion column was used for FPLC separations operated at a flow rate of 0.1 mL/min in a buffer of 250 mM PB, pH 8.0.
Synthesis of N-hydroxysuccinimidy-S-Acetyltiopropionate (5.1)

This experiment was performed in one-pot relative to known literature procedures. Radical inhibitor was removed from acrylic acid immediately prior to use by passing the compound over a plug of alumina. In a flame dried round bottom flask, equal volumes of thiol acetic acid (3 mL) and acrylic acid (3 mL) were added and the reaction was stirred for 3 hours. A pale yellow precipitate formed and the excess acrylic acid was removed in vacuo to obtain 3 g of crude reaction product. The solid product (1.44 g, 9.72 mmol, 1eq) was then dissolved in anhydrous DCM (50 mL), and NHS (1.34 g, 11.66 mmol, 1.20 eq) was added. Next, the reaction was cooled to 0°C and EDC (2.05 g, 10.69 mmol, 1.10 eq) was added. DMAP (0.12 g, 0.97 mmol, 0.10 eq) was added and the reaction was allowed to warm slowly to room temperature over 1 hour before additional stirring at room temperature for 24 hours. The crude reaction was purified by column chromatography (50% EtOAc in Hexanes) to produce the product as a white solid in 48% yield. Analysis by NMR confirmed that product identical to literature procedure has been obtained. NMR (400 MHz, CDCl₃) δ: 3.18 (t, 2H, J = 7.0 Hz), 2.93 (t, 2H, J = 7.0 Hz), 2.83 (m, 4H), 2.33 (s, 3H) ppm.
$^1$H NMR of 5.1 (SATP) (400 MHz, CDCl$_3$)
Synthesis of S-Acetyl-Thiopropionyl Hen Egg White Lysozyme

Hen egg white lysozyme was reconstituted as received to a concentration of 10 mg/mL (50 mM phosphate buffer [PB], 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.5). Next, 10 µL of N-succinimidyl-S-acetylthiopropionate (16 mg/mL, 65 mM in DMF) was added and the solution was stored at 4 °C for 4 h. The resulting modified protein was purified by centriprep ultrafiltration (MWCO 3 kDa, 50 mM PB, 1 mM EDTA, pH 7.5) to a final volume of 1 mL.

Synthesis of Thiopropionyl Hen Egg White Lysozyme (LyzSH)

The protein was then deprotected by treatment with 100 µL of a 0.5 M hydroxyl amine solution (50 mM PB, 25 mM EDTA, pH 7.5). Purification of the final protein was performed by centriprep ultrafiltration (MWCO 3 kDa, 50 mM PB, 1 mM EDTA, pH 7.5) before quantification of free thiols by Ellman’s assay (1:1.4 thiol : lysozyme).
To a 1.5 mL Lo-Bind® centrifuge tube was added thiopropionyl hen egg white lysozyme (LyzSH) (70 µL, 10 mg/mL, DPBS, pH 8.0) and Poly 5-8 (0.71 µmol, 15 eq) and the entire reaction was diluted to a final volume of 500 µL (DPBS, pH 8.0) before storage at 4 °C for 3 hours. The resulting mixture was purified by FPLC (50 mM PB, 150 mM NaCl, pH 7.4), fractions (green boxes, Figure 5.5) were concentrated by centriprep ultrafiltration (MWCO 3 kDa, DPBS, pH 7.4) and stored at 4 °C before further characterization by SDS-PAGE (Figures S11, S12). The EnzChek lysozyme activity assay was employed confirm lysozyme activity of the conjugates. Purified fractions of each conjugate were concentrated by centriprep ultrafiltration (MWCO 3 kDa, DPBS, pH 7.4) to a final activity near 1 kU/mL.

**Lysozyme-glycopolymer conjugate (Lyz-Poly 5-8)**
CHAPTER 6

STABILIZATION EFFECTS

OF TREHALOSE GLYCOPOLYMERS
INTRODUCTION

Protein Environmental Stressors

Stressors of proteins and protein based therapeutics are defined here as inputs that modulate activity. They can occur through physical insults such as heat, freezing, desiccation, and lyophilization, or chemical or photo-chemical means such as, pH, salt, and light. There is some debate over the correlation between protein stability to these different burdens, and for the purpose of this study stability towards heat and lyophilization were examined. Thermal denaturation is a process whereby a protein undergoes reversible or irreversible transition from the folded to unfolded states. This process has been shown to depend on several external factors such as protein concentration and heating rate with the reversibility of unfolding closely tied to thermal stability. Although some examples of protein denaturation by cooling exist, a more common cause of degradation is freeze-thaw cycling which has been shown to decrease activity. Freezing below the solution Tg is largely implicated in the mechanism of biologic degradation in this instance, and protein denaturation is thought to mostly occur at the solid-liquid interface. Desiccation can occur via a variety of methods including spray drying, spray freeze-drying, film-drying, and supercritical drying. Although the dehydrated product is often more robust, some loss of activity typically occurs during the process. It is standard practice for therapeutics to be distributed in a stabilized lyophilized form, and re-suspended immediately prior to use. However, lyophilization imparts several stressors to the protein in the form of freezing and dual-stage desiccation associated with the process. Therefore, the most significant stressors associated with protein
based therapeutics are thermal fluctuations imparted during storage, and the complex stress of lyophilization applied at the time the therapeutic is manufactured; these insults can combine to degrade pharmacological activity via a variety of pathways.

**Protein Degradation Mechanisms**

The degradation mechanisms of protein-based drugs include amino acid deamidation, isomerization, racemization, β-elimination, oxidation, N-terminal cyclization or addition, hydrolysis, and disulfide exchange among other reactions. In particular, Aparagine (Asn) deamidation is common and has been known to occur in α-helices, β-sheets, and β-turns. Isomerization of aspartic acid (Asp) is a second degradation pathway that is highly sensitive to pH and temperature, and this mechanism has been commonly reported to occur in the degradation of monoclonal antibodies. The Asp residue is also known to participate in hydrolysis. The three major hydrolysis degradation pathways are Asp, tryptophan (Trp), and hinge-region hydrolysis. This last type of hydrolysis is dependent on secondary and tertiary structure and is often accompanied by “V-shaped” pH dependent kinetics. Other mechanisms particularly attributed to Asp are racemization and β-elimination. Although racemization is typically slow, β-elimination is known to affect activity of therapeutic proteins such as interleukins and insulin on relatively shorter timescales. Protein N-termini have also been demonstrated to possess highly sensitive reactivity as cyclization to diketopiperazine or pyroglutamate adducts can occur under a variety of conditions. Oxidation of cysteine (Cys), histidine (His), methionine (Met), tyrosine (Tyr), or Trp amino acids by reactive oxygen species has been observed, and this is known to decrease
protein activity.\textsuperscript{813-817} Other oxidative mechanisms including photooxidation\textsuperscript{818-821} and metal catalyzed oxidation\textsuperscript{822-826} further complicate exposure to oxidative environments. Complimentary to oxidation is disulfide scrambling whereby proteins can lose activity or even aggregate through the formation of disulfide isoforms.\textsuperscript{827-831} Alternative degradation pathways are also plentiful including macroscopic modes such as aggregation and precipitation.\textsuperscript{832}

**Protein Drug Formulations and Stabilizing Excipients**

To mitigate the effects of chemical degradation caused by these environmental insults, several methods have been employed including chemical modification, mutagenesis, and addition of stabilizing excipients.\textsuperscript{778, 832-835} Typically, the stabilizing strategy of choice involves first augmenting the buffer formulation of the protein therapeutic to include salts such as acetate, citrate, phosphate, or tris(hydroxylaminomethane); as this is known to minimize hydrolysis\textsuperscript{803} or deamidation\textsuperscript{786, 836-840} degradation mechanisms which may occur upon storage. Optimization of buffer pH has been shown to damper oxidative effects,\textsuperscript{841-844} however this observation is largely protein specific.\textsuperscript{845-847} Addition of ionic materials may be used to impart stabilization\textsuperscript{848, 849} according to binding\textsuperscript{850-852} or Hofmeister effects\textsuperscript{853-855} and protein stabilization has also been demonstrated via addition of amino acid salts to the formulation buffer.\textsuperscript{856} Stabilization by salt concentration alone is often ineffective however, as increasing osmolarity increases the reaction kinetics of other degradation pathways such as N-terminal cyclization.\textsuperscript{857-860}

Addition of associative ligands that alter protein structure have also been used to drive protein stability.\textsuperscript{861} For example, retardation of Asn deamidation rates are observed upon
addition of excipients that induce α-helical structure.\textsuperscript{862} This technique was originally thought to proceed via exclusion of potentially denaturing solutes,\textsuperscript{863, 864} however binding to the denatured state can also accelerate denaturation,\textsuperscript{865, 866} thereby limiting efficacy of the protein once degradation has been initiated.

Other non-ionic surfactants and glycolipids have been used to prevent interfacial denaturation of proteins, particularly during freezing,\textsuperscript{867, 868} however, a strict balance of these ligands is required. For example, mixtures of polysorbates are often use to prevent aggregation and adsorption,\textsuperscript{869-871} however these mixtures are known to enhance the rate of photooxidation\textsuperscript{872} restricting use of these otherwise beneficial ligands to applications where the formulation can tolerate additional anti-oxidant excipients. Furthermore, although non-ionic surfactants may initially prevent protein aggregation,\textsuperscript{761} these additives have been shown to exhibit opposite effects in long-term stability studies.\textsuperscript{873}

Sugar based excipients have also been explored.\textsuperscript{874-881} For example it has been shown that sucrose increases chain flexibility thereby slowing deamidation;\textsuperscript{882} chain flexibility has been shown to be critical in mitigating other mechanisms such as hinge region hydrolysis\textsuperscript{883} and oxidative degradation as well.\textsuperscript{845} Cyclodextrans are known to impart stabilization to protein therapeutics\textsuperscript{884-887} and have also been used to re-fold denatured enzymes to the active state.\textsuperscript{888} Other polyols have been shown to mitigate deamidation effects by compacting protein structure in solution.\textsuperscript{889, 890} However, instances of glycation (redox addition of reducing carbohydrates to proteins) are common,\textsuperscript{891-900} and most sugars increase the rate of protein degradation at the solution-air interface,\textsuperscript{901, 902} specifically because they increase chain
flexibility.\textsuperscript{903, 904} Some carbohydrates also play a role in oxidative damage to proteins via unknown mechanisms.\textsuperscript{905}

Trehalose has been used in dried protein formulations\textsuperscript{906} as well as an excipient\textsuperscript{907, 908} with several reports demonstrating trehalose to be a superior lyoprotectant relative to other disaccharides.\textsuperscript{909-913} Although the mechanism has not been proven, continuous X-ray diffraction (XRD) of trehalose during lyophilization has revealed that the crystal state of trehalose in solution is rate, cycle, and temperature dependent.\textsuperscript{914} Trehalose is unique as an excipient in that it is a non-reducing sugar composed of two glucose units $\alpha,\alpha$ linked about the 1,1' positions, and this is likely the cause of its anomalous effect on protein structure as a stabilizing carbohydrate.\textsuperscript{348, 915} We sought to combine the advantages of polymeric and sugar excipients with the added stability and known protectant properties of trehalose.
RESULTS AND DISCUSSION

Lyophilization of Lysozyme and Trehalose Glycopolymers

The ability of the synthesized glycopolymers (Poly 5-8) to stabilize lysozyme to lyophilization was first verified, and the results were compared to trehalose at the same concentration relative to trehalose units in the side-chain. Poly 5-8 at 1 or 100 molar equivalents relative to protein was added (not conjugated) to wild-type lysozyme containing no free thiols. Samples with trehalose added at equivalent concentrations to the trehalose in the side-chains of the various polymers were also tested. The samples were exposed to 10 lyophilization cycles near a concentration of 26 µg/mL, and lysozyme activity was determined. Wild type lysozyme retained 16% activity after this treatment, while with a 100-fold excess of polymer, full retention (100%) of activity was observed (Figure 6.1). The same effect was seen regardless of the molecular weight tested (8 to 49.5 kDa). Trehalose at equivalent concentrations to that in the 100-fold polymers only stabilized the lysozyme between 18-31%. The results confirm that the polymers are able to protect the protein during lyophilization and that the protection conferred is greater than the native disaccharide alone. This indicates that stabilization effects of trehalose are enhanced by using a polymer where the entropic barrier to having several carbohydrate moieties organized around the protein has already been included in the excipient. The results suggest that trehalose polymers may be useful as a replacement for the parent disaccharide in formulations of unmodified biomolecules, particularly in instances where the additional materials properties imparted by the polymer would be advantageous.
Figure 6.1: Activity of lysozyme-glycopolymer conjugate, wild type lysozyme with glycopolymer (1 or 100 eq relative to lysozyme), or wild-type lysozyme with trehalose (1 or 100 eq relative to polymer side-chain units) as excipients exposed to 10 cycles of lyophilization. Data shown is repeated 6 times with p<0.01 for all polymer 100x and conjugate samples relative to wild-type.

To investigate the ability of the polymer to stabilize when conjugated to the protein, Lyz-Poly 5-8 conjugates were exposed to the same 10 lyophilization cycles. In this case, the conjugates exhibited between 59 and 100% retention of original activity compared to 16% retention by wild-type lysozyme (Figure 6.1). The smallest molecular weight Lyz-Poly 5 had the lowest activity, while the largest polymer conjugate Lyz-Poly 8 had full retention of activity. The activities of the medium sized polymers did not directly correlate to molecular weight. It is possible that this is due to differing numbers of polymer attached to the protein in the fractions analyzed. As mentioned in Chapter 5, the number of polymers attached in the collected fractions could not be accurately estimated by SDS PAGE due to the low concentrations involved, therefore, polymer degree of labeling remains ambiguous. Unconjugated polymer was observed in the crude reaction mixture in all cases, so it is likely that most conjugates had only 1 polymer attached; conversely, the largest M_w fractions were used in each case to ensure
complete removal of unreacted lysozyme and glycopolymer. Importantly, the conjugates were all significantly more stable compared to samples containing 1 eq of unattached polymer relative to lysozyme; addition of 1 eq of un-conjugated polymer resulted in only 18-47% retention of lysozyme activity with up to 100% retention of activity for lysozyme-glycopolymer conjugates. These results show that conjugating the polymer to the protein is advantageous with regard to environmental stability. This is likely due to the increase in local concentration as stabilization to lyophilization appears to be concentration dependent with respect to glycopolymer. Studies to determine the in vitro and in vivo stability of protein conjugates prepared from these polymers are underway in order to evaluate pharmacokinetic properties and potential use of the polymer in therapeutic conjugates.

**Heating Lysozyme and Trehalose Glycopolymers**

Trehalose is also known to stabilize biomolecules to increases in heat.\(^{916-918}\) Therefore, an identical array of lysozyme samples were also challenged by exposure to a heat burden of 90 °C for 1 hour (Figure 6.2). Although trehalose was found to confer marginal stabilization at the highest concentration tested here (31% activity retention for 100 eq relative to DP of Poly 8), the trehalose polymer confers superior protection to heat, both as a conjugate and when added unconjugated. Up to 81% retention of activity under these rigorous conditions was observed. In this particular study, the protective effect was not concentration dependent as adding 1 eq and 100 eq or conjugating the polymer gave similar retention of activity for all molecular weights tested.
Figure 6.2: Activity of lysozyme-glycopolymer conjugate, wild type lysozyme with glycopolymer (1 or 100 eq relative to lysozyme), or wild-type lysozyme with trehalose (1 or 100 eq relative to polymer monomer units) as excipients exposed to a heat burden of 90°C for 1 hour. Data shown is repeated 6 times with p<0.001 for all polymer and conjugate samples relative to wild-type.

Overall, the results demonstrate that the glycopolymers stabilize lysozyme to high temperatures. Furthermore the data suggest that the polymers should be investigated further as an excipient and conjugate to mollify the rigorous storage requirements that are typical for proteins, particularly during transport where large fluctuations in temperature may be experienced.

Effects of Trehalose Glycopolymers Relative to Poly(ethylene glycol)

Initial studies were also conducted to compare the trehalose glycopolymers to the commonly used excipient poly(ethylene glycol) (PEG). As such, PEG (Mₙ = 2–20 kDa) was combined with wild-type lysozyme at 1 or 100 molar equivalents and stressed by lyophilization or heat in an identical fashion to the lysozyme samples stabilized by trehalose glycopolymers. The lyoprotective effect of PEG (Figure 6.3 and 6.4) was examined in contrast to the trehalose glycopolymer with comparable stabilization observed for both excipients at 1 and 100 eq
relative to protein for all degrees of polymerization (DP) tested. Based on molecular weight, the lyoprotective effect of the glycopolymer does not appear to depend on molecular weight for 1 or 100 eq relative to lysozyme. For PEG, the lyoprotective effect is enhanced for select weights although a direct or inverse dependence on molecular weight is not observed. Significant (>60%) retention of activity was obtained for all glycopolymer samples at 100 eq and one PEG sample at 100 eq. Thermal stability of PEG relative to the glycopolymers was also investigated by exposing the relevant samples to a heat burden of 90°C for 1 h as before. Although PEG mediates thermal stress to a minimal extent, the trehalose glycopolymer outperforms PEG based on DP or $M_n$ for all samples tested (Figure 6.3 and 6.4). The data collectively indicates that trehalose side chain polymers are highly effective and superior to PEG alone as stabilizers of a representative protein, lysozyme, to heat stress and at least as good as PEG for stabilization to lyophilization stress.

![Figure 6.3](image)

**Figure 6.3.** Activity retention of lysozyme stabilized by addition of PEG (1 or 100 eq) or trehalose polymer (1 or 100 eq) versus degree of polymerization stressed by 10 cycles of lyophilization (top) or a heat burden of 90 °C for 1 hour (bottom). Error bars represent standard deviation for the average of 6 repeated trials with $p < 0.001$ for all heated trehalose polymer samples relative to PEG and significance indicated for lyophilized samples as follows: $^*_p < 0.01$ and $^*_p \Phi_p < 0.05$. 

136
Figure 6.4. Activity retention of lysozyme stabilized by addition of PEG (1 or 100 eq) or trehalose polymer (1 or 100 eq) versus molecular weight stressed by 10 cycles of lyophilization (top) or a heat burden of 90 °C for 1 hour (bottom). Error bars represent standard deviation for the average of 6 repeated trials with $p < 0.001$ for all heated trehalose polymer samples relative to PEG, and significance indicated for lyophilized samples as follows: $^* p < 0.01$ and $^\dagger p < 0.05$.

**Stability Observations and Mechanistic Implications**

Application of trehalose in polymeric form was advantageous as cooperativity dependent effects were enhanced; a feature that could prove beneficial if multiple coordination sites are required for protein stabilization as is typically seen with sugar-protein interactions. This effect also created a non-ionic surfactant which was in stoichiometric balance with the disaccharide moieties in the side-chain. It is likely that the carbohydrate and polymer backbone complemented each other: the non-ionic surfactant property of the polymer limited possible degradation due to the air-solution interface, and the antioxidant properties of trehalose mitigated any potential photo-oxidation effects of the surfactant while conferring the usual protective effects associated with trehalose.

Additionally, this particular glycopolymer was advantageous because proteins were immune to degradation via glycation reactions with the polymer due to the non-reducing nature of the glycopolymer.
There are three main hypotheses for the method by which trehalose confers stabilization to biomolecules: vitrification,\textsuperscript{390, 391} water-entrapment,\textsuperscript{394, 395} and water-replacement.\textsuperscript{392, 393} Though highly contested and not mutually exclusive,\textsuperscript{389} it is likely that not all are useful in describing the protective abilities of the glycopolymer to each stress individually. In the case of heat, concentration dependence was not observed when lysozyme was stressed at a concentration of 1 kU/mL, and no correlation to glycopolymer molecular weight could be inferred. In the case of lyophilization, concentration dependence was observed with seemingly little correlation to molecular weight. The two stressors tested, heat and lyophilization, will therefore be addressed individually.

**Heat Burden**

Although the trehalose glycopolymer presumably impacts the phase transitions of the protein solution, little concentration dependence was observed with regard to heat stabilization, as 1 eq of free trehalose glycopolymer stabilized lysozyme similarly to 100 eq of conjugate for all but the smallest molecular weight tested. This implies that vitrification in the disaccharide side-chain plays little or no role in stabilization, as the ability of side-chain trehalose moieties to interact with each other should be largely concentration and molecular weight dependent. Because the phase of the solution during the entire heat burden process was liquid, it is unlikely that different glycopolymer chains interact with each other to hold certain aspects of the protein tertiary structure in place via a vitrification mechanism.\textsuperscript{390, 391} There is some molecular weight dependence on the observed concentration effect (or lack thereof) and the presence of cooperativity among the stabilizing moieties could explain this
observed effect among different molecular weights. This is supported by the fact that trehalose alone does not stabilize lysozyme to heat as well as trehalose glycopolymer. It may then be inferred that the glycopolymer either directly interacts with protein or that water tightly bound by the trehalose “clam-shell” is responsible for resistance to denaturing under thermal insults. Therefore, a water-replacement or water-entrapment mechanism fits most closely with the results obtained from the heat burden experiments.

**Lyophilization Stress**

In the case of lyophilization at least three different stresses are actually present: freezing, main-phase drying, and second-phase drying. Trehalose, and therefore the corresponding glycopolymer, could protect via a different mechanism in each instance. Furthermore, the mechanism of glycopolymer protection is not necessarily congruent to the parent disaccharide in each case. Correlation of activity retention to glycopolymer molecular weight for the range of polymers that were examined in this study could not be inferred. This indicates that either chain length is not a factor in the lyoprotectant ability of the glycopolymer or, more likely, that it is only a factor in one of the stressors associated with lyophilization such as freezing. Desiccation during lyophilization operates over two steps, main-phase drying, and second-phase drying. During main phase drying the polymer must shield the protein from the increased concentration of buffer salts that occurs upon removal of excess solvent. At this stage the glycopolymer also likely keeps the protein hydrated with water. However, upon second-phase drying this water is removed. Therefore, the trehalose polymer must stabilize the protein through physical immobilization of protein tertiary structure (vitrification) or, more
likely, through direct interaction with the protein (water-replacement). The above are hypotheses based on the observations. However, more detailed mechanistic studies are underway in order to more precisely ascertain the exact mechanisms involved.
EXPERIMENTAL

Materials

All solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. Poly(ethylene glycol) was purchased from Sigma-Aldrich and used without further purification. The EnzChek lysozyme activity assay was purchased from Invitrogen and used according to manufacturer instructions.

Wild-Type Lysozyme

Wild-type lysozyme was prepared by reconstituting hen egg white lysozyme (0.1 mg/mL, 1 eq, DPBS, pH 7.4) as received without further purification. Samples were further diluted to 0.021 mg/mL (1 kU/mL) in 20 µL aliquots prior to application of lyophilization or thermal insults.

Wild-Type Lysozyme Stabilized by Trehalose Glycopolymer

Wild-Type lysozyme samples mixed with glycopolymer were prepared by adding Poly 5-8 (1 or 100 eq relative to lysozyme) to a solution of lysozyme (0.1 mg/mL, DPBS, pH 7.4) before storage at 4 °C. Samples were further diluted to a lysozyme concentration of 0.021 mg/mL (1 kU/mL) in 20 µL aliquots prior to application of lyophilization or thermal insult.

Wild-Type Lysozyme Stabilized by Trehalose

Wild-Type lysozyme samples mixed with trehalose were prepared by adding trehalose (1 or 100 eq relative to the number of trehalose monomer units in Poly 5-8) to a solution of lysozyme (0.1 mg/mL, DPBS, pH 7.4) before storage at 4 °C. Samples were further diluted to a
Lysozyme concentration of 0.021 mg/mL (1 kU/mL) in 20 µL aliquots prior to application of lyophilization or thermal insults.

**Wild-Type Lysozyme Stabilized by Poly(ethylene glycol) (PEG)**

Wild-Type lysozyme samples mixed with PEG were prepared by adding PEG (2 kDa, 8.5 kDa, or 20 kDa; 1 or 100 eq relative to lysozyme) to a solution of lysozyme (0.1 mg/mL, DPBS, pH 7.4) before storage at 4 °C. Samples were further diluted to a lysozyme concentration of 0.021 mg/mL (1 kU/mL) in 20 µL aliquots prior to application of lyophilization or thermal insults.

**Lysozyme Stabilized by Conjugation to Poly 5-8**

Lyz-Poly 5-8 conjugates were used following FPLC purification (Figure 5.5). Samples were concentrated by centriprep to a concentration of 1 kU/mL in DPBS, pH 7.4 and stored at 4 °C in 20 µL aliquots prior to application of lyophilization or thermal insults.

**Lyophilization stability assay**

Aliquots of each type were frozen by immersion in liquid nitrogen before solvent removal via lyophilization (<1 mbar, >4 hours). The resulting white solids were reconstituted by addition of Milli-Q water (20 µL), and this procedure was repeated for a total of 10 lyophilization cycles. Next, the EnzChek lysozyme activity assay was employed to measure lysozyme activity of the stressed samples relative to identical aliquots that were stored at 4 °C and not lyophilized. In a typical assay 50 µL of diluted sample was incubated with 50 µL of Micrococcus luteus labeled with FITC (1 mg/mL) at 37 °C for 1 hour. Subsequently, fluorescence
of lysed cell membrane and thus lysozyme activity was measured as FITC fluorescence (abs 480 nm/em 530 nm) and quantified relative to known standards. The results are provided for 6 repeats. Statistics to determine significance were calculated utilizing the Students t test; % confidence as +/- = t(standard deviation)/(number of trials)^{1/2} with p < 1-% confidence/100.

**Heat Burden Assay**

Aliquots of each type were stressed by heating to 90 °C for 1 hour before cooling to 4 °C. Next, all samples were assayed using of the EnzChek lysozyme activity assay as described above. Activities of stressed samples were measured relative to identical aliquots that were stored at 4 °C and not exposed to heat. The results are provided for 6 repeats. Statistics to determine significance were calculated utilizing the Students t test; % confidence as +/- = t(standard deviation)/(number of trials)^{1/2} with p < 1-% confidence/100.
CHAPTER 7

CONCLUSIONS
**Encapsulated Hydrogel Architectures by E-Beam Lithography**

Three different poly(ethylene glycol) based polymers have been synthesized via known procedures to contain alkyne, aminooxy, or biotin functionality. The polymers were subsequently cross-linked by electron beam lithography, and the reactive heterogeneity of multi-component PEG based hydrogels has been demonstrated by confocal microscopy. Microstructures have been fabricated by e-beam lithography such that AO-PEG was encapsulated as a core within a shell of Biotin-PEG, and Alkyne-PEG was patterned as a core, encapsulated by a contiguous shell of AO-PEG. The enzymes glucose oxidase and horseradish peroxidase have been functionalized with reactive moieties that are complimentary to polymer end-groups, and retention of activity has been confirmed for enzymes immobilized within the encapsulated hydrogel architecture. An enzyme cascade reaction with glucose and Amplex Red substrates has been carried out on enzymes immobilized in this encapsulated configuration, and activity has been shown to be comparable to each enzyme immobilized individually on the surface via identical chemistry.

**Trehalose Based Glycopolymers**

Two different monomers based on trehalose have been synthesized. One was obtained in the dibenzylidene protected form as 2-methacryl-4,6-O-dibenzylidene-α,α-trehalose along with the bis-functionalized cross-linker 2,2’-methacryl-4,6-O-dibenzylidene-α,α-trehalose. The benzylidene group has also been modified directly to contain polymerizable functionality through installation of a vinyl moiety at the 4 position of terephthaldehyde diethyl acetal to produce 4,6-O-(4-vinylbenzylidene)-α,α-trehalose. Protein-reactive CTAs were utilized to effect
RAFT polymerization of the glycomonomers resulting in well-defined polymers with pendant trehalose functionalities and protein-reactive end-groups.

Thiol-reactive glycopolymers of different molecular weights were conjugated to hen egg white lysozyme modified to contain a free thiol. It was found that a longer spacer length between the activated disulfide end group and polymer chain provided better conjugation yields. The resulting purified lysozyme-glycopolymer conjugates exhibited active cell lysis. When un-conjugated polymers were added to wild-type lysozyme significant increases in resistance to lyophilization and heat stresses were observed relative to wild type protein, or protein stabilized by trehalose. The glycopolymers were found to exhibit comparable lyoprotective effects relative to the excipient PEG, and the glycopolymer was superior at stabilizing lysozyme to heating relative to PEG for all molecular weights investigated. When the polymer was conjugated to lysozyme, stabilization to lyophilization was significantly enhanced relative to addition of un-conjugated polymer, and this effect was likely due to an increase in local concentration around the protein. These results suggest that trehalose glycopolymers could be potentially useful in advancing the proliferation of protein formulations through stabilization of biological therapeutics to environmental stressors.
REFERENCES


77. Haase, M., Blood 2002, 100, 4242-4242.


Medicine 2000, 343, 1673-1680.

1990, 322, 1430-1434.


104. Perrillo, R. P.; Schiff, E. R.; Davis, G. L.; Bodenheimer, H. C.; Lindsay, K.; Payne, J.; Dienstag, J. L.;


1608-1614.


Maricic, M.; Miller, P.; Moniz, C.; Peacock, M.; Richardson, P.; Watts, N.; Baylink, D., *American Journal of


Internal Medicine* **2005**, 143, 559-569.


154-161.


222.


711. Evans, P. R.; Bowler, K., Sub-Cellular Biochemistry 1973, 2, 91-95.


