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Los Angeles

Trehalose Glycopolymers and Hydrogels
for Enhancing Protein Stability

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

by

Juneyoung Lee

2014

ABSTRACT OF THE DISSERTATION

Trehalose Glycopolymers and Hydrogels for Enhancing Protein Stability

by

Juneyoung Lee

Doctor of Philosophy in Chemistry

University of California, Los Angeles, 2014

Professor Heather D. Maynard, Chair

Proteins are widely used in broad regions of interest from laboratories to industries and from reaction catalysts to therapeutic agents. However, the instability of proteins toward various environmental stressors both in storage and *in vivo* is a barrier to their general use. In this dissertation, development of trehalose glycopolymers and hydrogels to enhance protein stability and their pharmacokinetic properties is described. Traditional polymers used to prevent degradation by thermal stress and lyophilization are shown in Chapter 1.

Chapter 2 describes the synthesis of three trehalose-based monomers and polymers. Trehalose glycopolymers were synthesized using free radical polymerization with trehalose moieties as side chains. The resulting polymers were then used as excipients to stabilize

horseradish peroxidase, glucose oxidase, and β -galactosidase during heating and lyophilization processes. The protein activities were subsequently tested and found to be significantly higher when the polymers were present during the stress compared to no additive and to equivalent amounts of trehalose.

In Chapter 3, the conjugation of trehalose polymers to therapeutic proteins, including insulin and granulocyte colony-stimulating factor, to enhance stability and improve pharmacokinetic properties is described. First, the insulin structure was monitored by native gel before and after heating an insulin solution to 90 °C for 1 h. The structure of insulin was maintained when trehalose polymers were added as additives whereas insulin alone or with trehalose as an additive completely or partially lost its original structure. To prepare polymers with protein reactive end-groups, ketone and benzaldehyde end-group functionalized chain transfer agents were synthesized and used to mediate reversible addition-fragmentation chain transfer polymerization of trehalose monomers. The prepared polymers were covalently conjugated to insulin and granulocyte colony-stimulating factor through reductive amination. Pharmacokinetic studies using mice showed a dramatic increase in the half-life of insulin-trehalose polymer conjugates compared to native insulin and the effect was similar to that of insulin-poly(ethylene glycol) conjugates. Also, the synthesis of a trehalose polymer with aminoxy end-group that could form an oxime bond and modification of cell surface with ketone functional group are presented.

Chapter 4 demonstrates the preparation of trehalose and hyaluronic acid-based delivery agents. There are four different approaches discussed to enable stabilization and delivery of proteins. With the described technologies, the protein may be delivered passively or by triggered release.

In Chapter 5, we report the use of a trehalose polymer as a new resist that allows direct electron beam lithography writing of multiple proteins. First, trehalose glycopolymer was shown to effectively crosslink to surfaces as negative resists. Also, it was observed that proteins were stabilized during electron beam exposure and vacuum conditions when the trehalose glycopolymer was added into the protein solution. Using this technique we could directly pattern multiple proteins at the micrometer and nanometer scale without additional steps, such as preparing protein-reactive polymer or conjugating proteins to polymer patterns. Utilizing the high precision alignment capability of electron-beam lithography, surfaces with complex patterns of multiple proteins were successfully generated.

The dissertation of Juneyoung Lee is approved.

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2014

This dissertation is dedicated to my family and my lovely wife Heeju.

Thank you for all the supports and trusts.

Without you all this would not happen today.

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LIST OF ABBREVIATIONS

AIBN	Azobisisobutyronitrile
CDCl ₃	Deuterated Chloroform
CTA	Chain Transfer Agent
D ₆ DMSO	Deuterated Dimethyl Sulfoxide
DCM	Methylene Chloride
DMAP	4-Dimethylaminopyridine
DMF	<i>N, N'</i> -Dimethylformamide
DMSO	Dimethyl Sulfoxide
D-PBS	Dulbecco's Phosphate Buffered Saline
E-beam	Electron beam
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EtOAc	Ethyl Acetate
DCC	<i>N, N'</i> -Dicyclohexylcarbodiimide
Hex	Hexane
HPLC	High Performance Liquid Chromatography
MeOH	Methanol
M _n	Number Average Molecular Weight
MWCO	Molecular Weight Cut Off
NMR	Nuclear Magnetic Resonance
<i>p</i>	Confidence Interval
PDI	Polydispersity Index
PEG	Poly(ethylene glycol)
RAFT	Reversible Addition-Fragmentation Chain Transfer
THF	Tetrahydrofuran
TFA	Trifluoroacetic Acid

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PUBLICATIONS

- (4) Lee, J.; Lin, E.-W.; Lau, U. Y.; Hedrick, J. L.; Bat, E.; Maynard, H. D. Trehalose Glycopolymers as Excipients for Protein Stabilization, *Biomacromolecules* **2013**, *14*, 2561-2569.
- (3) Vázquez-Dorbatt, V.; Lee, J.; Lin, E.-W.; Maynard, H. D. Synthesis of Glycopolymers by Controlled Radical Polymerization Techniques and Their Applications, *ChemBioChem* **2012**, *13*, 2478-2487.
- (2) Grover, G. N.; Lee, J.; Matsumoto, N. M.; Maynard, H. D. Aminoxy and Pyridyl Disulfide Telechelic Poly(poly(ethylene glycol) acrylate) by RAFT Polymerization, *Macromolecules* **2012**, *45*, 4958–4965.
- (1) Mancini, R. J.; Lee, J.; Maynard, H. D. Trehalose Glycopolymers for Stabilization of Protein Conjugates to Environmental Stressors, *J. Am. Chem. Soc.* **2012**, *134*, 8474–8479.

PRESENTATIONS

- (7) UCLA Organic Chemistry Gradyation Symposium “Trehalose Glycopolymers and Hydrogels for Enhancing Protein Stability” Oral presentation, Los Angeles, CA, 06/07/2014
- (6) Glenn T. Seaborg Symposium, “Trehalose Glycopolymers for Protein Stabilization (PolyProtek)” Poster presentation, Los Angeles, CA, 10/26/2013
- (5) Gordon Research Conference-Polymer, “Trehalose Glycopolymers for Protein Stabilization” Poster presentation, South Hadley, MA, 06/12/2013
- (4) Cram Lecture, “Trehalose Glycopolymers for Protein Stabilization against Environmental Stressors” Poster presentation, Los Angeles, CA, 05/02/2013

(3) Orville L. Chapman Lecture, “Trehalose Glycopolymers for Protein Stabilization against Environmental Stressors” Poster presentation, Los Angeles, CA, 02/28/2013

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(1) Winstein 100th Birthday Symposium, “Trehalose Glycopolymers for Protein Stabilization” Poster presentation, Los Angeles, CA, 10/06/2012

PATENT

(1) Maynard, H. D.; Mancini, R. J.; Lee, J.; Lin, E.-W., “Stabilization of Biomolecules using Sugar Polymers,” International Patent Application PCT/US2013/23235, January 27, 2012

Chapter 1

Polymers: Powerful Tools for Improving the Stability of Biomolecules

1.1 Introduction

Proteins are used in a variety of fields ranging from catalysts for reactions to drugs for therapeutic applications.^{1,2} Even though proteins play key roles in biotechnology, the poor stability of proteins during storage and processing inhibits their applications in the field.³⁻⁶ There are a number of environmental factors that can degrade protein structure and reduce its bioactivity such as high and low temperatures, pH, and pressure.^{7,8} To maintain protein activity during long-term storage, lyophilization (freeze-drying) is most often employed.⁹ However, extreme cooling and low pressure during the lyophilization process can also decrease the activity of proteins. Additionally, most therapeutic proteins in the clinic are already prepared in solution for safety and injection convenience, which decreases their shelf life compared to the dried form. It is generally recommended that these protein drugs be kept away from heat and cold, which makes storage and use difficult for patients. There are excellent articles on protein degradation due to various environmental stressors during their preparation and processing for pharmaceutical or chemical purposes.¹⁰⁻¹⁴ Many techniques such as chemical modification, protein engineering, and addition of stabilizing reagents (excipients or additives) have been developed to address this problem of protein instability.

Among the above techniques, excipients are the easiest to implement and can be applied toward various proteins, lowering development costs.¹⁵ Many excipients have been developed to enhance protein stability under various storage conditions. Some amino acids stabilize proteins through preferential hydration, direct binding, or buffering capacities. Sugars are also well known protein stabilizers and are currently used in formulation of therapeutic proteins. Osmolytes also are excipients because of their preferential hydration mechanism. Proteins, such

as human serum albumin and gelatins, are also widely used as additives to improve stability of many proteins.

In this chapter, we will review the utilization of polymers for protein stabilization against heat and lyophilization stressors, which are the most common stressors responsible for degradation of proteins. Different classes of polymers such as PEG, polysaccharides, and nanoparticles will be discussed.

1.2 Thermal Stress

Temperature is one of the key factors that degrade proteins. Proteins have unique three-dimensional structures due to interactions between their amino acids such as hydrogen bonding, hydrophobic interactions, salt bridges and others. Elevated temperature is well known to break the hydrogen bonds and unfold the structure, leading to protein aggregation.¹⁶ To prevent protein aggregation and maintain activity, polymers such as PEG, polysaccharides, and nano- or microparticles have been developed.

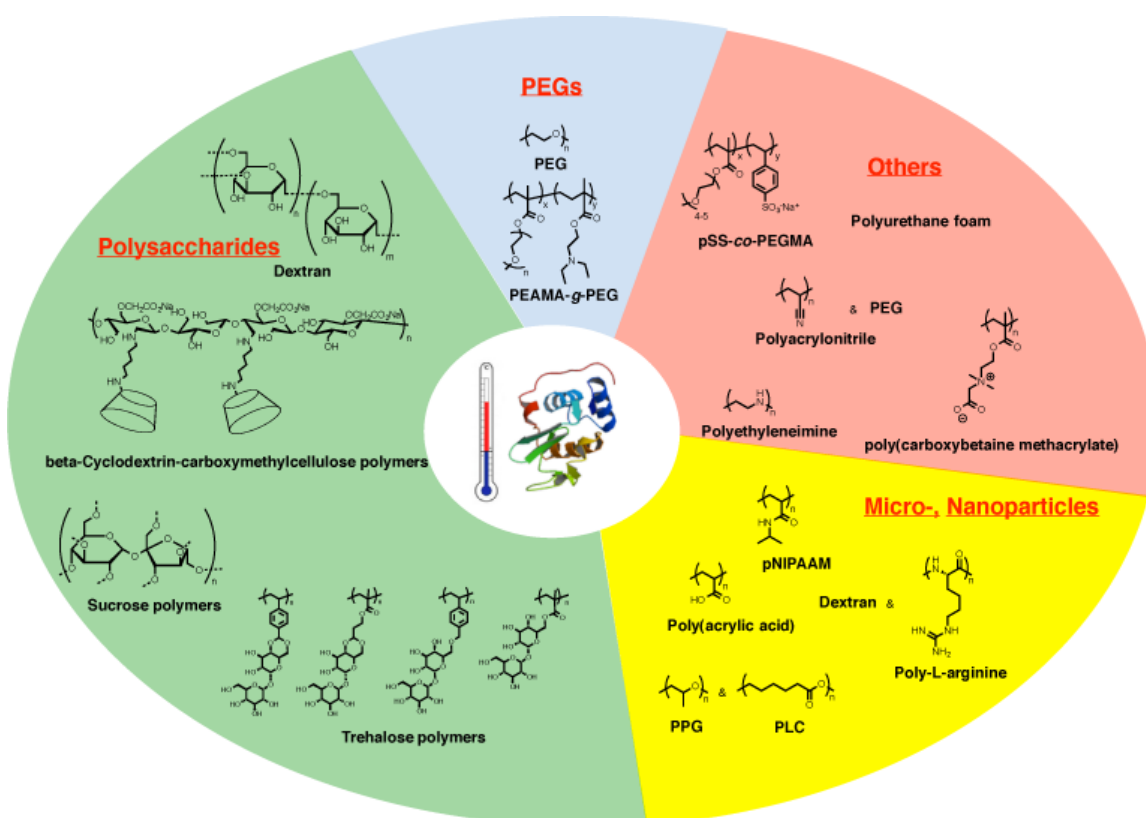


Figure 1-1. Various polymers used in protein stabilization against heat stress.

1.2.1 PEGs

PEG is widely used in the pharmaceutical industry in the form of protein-PEG conjugates to increase the half-life of therapeutic proteins *in vivo*.^{17,18} Since Abuchowski *et al.* showed superior immunogenic properties of PEGylated (PEG covalently attached) bovine serum albumin in 1977,¹⁹ more than ten PEGylated proteins have been approved by U.S Food and Drug Administration as of 2014. Researchers have also extensively studied the stability of PEGylated proteins *in vitro*. PEG has shown to increase the thermal stability of proteins, either as a covalently attached conjugate, or as an added excipient. Gaertner *et al.* have modified 80% of free amine on trypsin with PEG chains.²⁰ This PEGylated trypsin showed significant increase in heat resistance up to 80 °C compared to the native trypsin. They assumed that this enhanced stability was due to the formation of a highly hydrogen-bonded structure made up of the enzyme and the PEG chains. The Longo group has also demonstrated increased stability against heat in PEGylated enzymes.²¹ In their study, three enzymes including lysozyme, α -chymotrypsin, and *Candida rugosa* lipase were modified with PEG. PEGylation critically lowered the lysozyme activity and therefore lysozyme was not studied further. However, α -chymotrypsin and *Candida rugosa* lipase all showed enhanced thermal stabilities at 50 °C compared to unmodified proteins. The authors proposed that the amphiphilicity of PEG stabilized the tertiary structure of the proteins by interacting with the hydrophobic clusters on the enzyme surface, in addition to the increased rigidity from PEGylation that contributed to the stabilization. Yang *et al.* also utilized PEG to modify subtilisin, the family of enzymes which is important in food and detergent industries.²² The modification with the PEG increased thermostability of the subtilisin up to 50 °C. By combining activity results with differential scanning calorimetry (DSC), they concluded that PEGylation improved thermostability by increasing stability to autolysis rather

than by preventing unfolding of the protein. The Griebenow group reported enhancement in thermal stability of α -chymotrypsin at 45 °C by PEGylation.^{23,24} In 2008, the group reported increasing thermodynamic stability by reducing of the protein structural changes achieved by PEGylation. In their subsequent work, they varied PEG molecular weight and degree of protein substitution. They observed that enzyme activity decreased upon conjugation of PEG for all tested molecular weights, while thermal resistance increased with increasing PEG molecular weight. Even though the above results showed enhanced thermostability by PEGylation, the decreased protein activity upon modification remained a significant challenge.

Alternatively, PEGs were also used as excipients to stabilize against heating. The Erarslan group tested different molecular weight PEGs in various concentrations for stabilizing penicillin G acylase.²⁵ In the study, the enzyme activity after exposure to 50 °C was nearly 20 fold greater in the presence of 4 kDa and 6 kDa PEGs than without any additives. Tomita, Nagasaki, and Shiraki studied PEG as an additive for preventing thermal inactivation of lysozyme and ribonuclease A at 98 °C.²⁶ They have shown that 1 kDa PEG could be used as an efficient additive since the PEG suppressed aggregation and intermolecular disulfide exchange by binding to heat-denatured proteins. PEG has been also been introduced in the form of copolymers. In 2010, the Nagasaki group utilized poly(*N,N*-diethylaminoethyl methacrylate)-graft-PEG (PEAMA-*g*-PEG) as an excipient to improve heat resistance of lysozyme and ribonuclease A (RNase A) against 98 °C.^{27,28} In the lysozyme study, PEAMA-*g*-PEG completely reduced protein activity at polymer concentrations over 10 mg/mL even without heating. However, interestingly upon addition of poly(acrylic acid) (PAA) the lysozyme recovered 80% of the activity before and after the heat stress.²⁷ The RNase A/PEAMA-*g*-PEG complex effectively stabilized the enzyme after heating by suppressing protein aggregation, while the

PEG homopolymer did not prevent the deactivation of RNase A.²⁸

1.2.2 Polysaccharides

Sugars are widely used and one of the most common and effective protein stabilizers. As such, research on developing polysaccharides that can use or amplify saccharide properties is becoming popular. Addition of dextran, a complex and branched polysaccharide that is enzymatically synthesized from sucrose,²⁹ often results in increased thermostability of proteins. Marshall and Rabinowitz have shown that dextran acts as a thermostabilizer for various proteins.³⁰⁻³² In their study, dextran was activated using cyanogen bromide, which can then be covalently crosslinked to primary amines on the protein of interest. Since then, many groups have contributed to the research on using dextran for protein stabilization. In 1994, Ichishima and coworkers published their work on dialdehyde dextran that can be crosslinked to a serine alkaline proteinase.³³ Non-site-specific modification of the polymer resulted in decreased activity of the enzyme, yet the modification increased stability at high temperature. Kazan *et al.* reported the stabilization of penicillin G acylase after crosslinking dextran aldehyde polymer to the protein.^{15,34} In their work, dextran showed superior performance than glucose, sucrose, and various PEGs. Abdel-Naby also used the same strategy for stabilization of cellobiase (β -glucosidase) against heating.³⁵ Although large molecular weight dextran conjugates showed decrease enzyme activity, the dextran was an effective stabilizer upon heating. The Singhal group have synthesized a dextran- α -amylase conjugate in order to increase the enzyme half-life at 60-80 °C by increasing the activation energy for protein denaturation.³⁶ Recently in 2013, the same group screened the effect of various polysaccharides on the thermostability of α -amylase.³⁷ In the study, they separately conjugated nine polysaccharides to the α -amylase and observed that only dextran induced significant thermostability as well as imparting antimicrobial properties without additional preservatives. Research on thermostability of proteins with dextran added (not

crosslinked) has also been studied. Altikatoglu *et al.* studied dextran as an excipient that formed non-covalent complexes with horseradish peroxidase and glucose oxidase.^{29,38} Their results showed that dextran was an effective additive which increased heat resistance as well as storage lifetime at 4 °C.

Cyclodextrin, produced from starch by enzymatic conversion,³⁹ is also used as a protein stabilizer in polymeric form. Cyclodextrin forms complexes with hydrophobic guests such as hydrophobic amino acid side chains on the surface of proteins, which can enhance protein thermostability.^{39,40} The Villalonga group has studied the use of cyclodextrin polymers as thermostabilizers.⁴⁰⁻⁴⁴ A prepared β -cyclodextrin-carboxymethylcellulose polymer was conjugated to α -amylase.⁴⁰ This modification slightly decreased the amylolytic activity of the enzyme while enhancing its thermal stability. Interestingly, the introduction of 1-adamantanol, which forms stable complexes with cyclodextrin, decreased the stabilization effect. This indicates that supramolecular interactions between cyclodextrin and the protein are directly involved in the stabilization mechanism. The same cyclodextrin polymer also showed functional stabilization of trypsin upon conjugation.⁴² The Villalonga group has also studied the effects of β -cyclodextrin-dextran polymers in the stabilization of trypsin.⁴¹ Upon adding the polymer, trypsin thermostability and the activation free energy of thermoinactivation were greater than with no additive. However, there was no enhanced thermostability of trypsin when equimolar amounts of β -cyclodextrin and dextran were added together. *O*-carboxymethyl-poly- α -cyclodextrin has also been used to modify α -chymotrypsin and trypsin.^{43,44} In both studies, modified proteins showed increased heat resistance, thereby allowing the enzymes to catalyze reactions at higher temperatures in order to improve substrate conversion and reduce microbial contamination.

Polymeric sucrose can also enhance the thermostability of proteins. Sundaram and

coworkers have oxidized polymeric sucrose with sodium metaperiodate.⁴⁵ This oxidized sucrose polymer was then conjugated to the cysteine protease papain. In the study, papain showed increased thermotolerance after being modified with the polymer. They observed that increase in the activation free energy of inactivation and activation enthalpy prevented structural unfolding of the protein. They also demonstrated increased thermal stability of α -chymotrypsin.⁴⁶ They compared the sucrose polymer carboxymethyl cellulose to dextran. All of the tested polymers showed stabilization effect under heating, but the oxidized sucrose polymer maintained the highest activity. Jayaraman and Narayanan also reported improved thermodynamic stability of subtilisin Carlsberg by covalently attaching oxidized sucrose polymers.⁴⁷ Sucrose polymers and polyglutaraldehydes of various molecular weights were tested for stabilization of subtilisin, and sucrose polymers were shown to be more effective in protecting against heating.

Polysaccharides may also be prepared by the polymerization of synthetic monomeric units. The Klein group prepared three different vinylsaccharide monomers, which are neutral, anionic, and cationic.⁴⁸ Cationic polymers with trimethylammonium chloride and anionic polymers with sulfate groups were effective in stabilizing horseradish peroxidase at 55 °C; however, the authors mentioned that this trend cannot be directly generalized to other enzymes. Our group has reported the synthesis of trehalose-based monomers and polymers.^{49,50} Trehalose, which is one of the most widely used sugars in preserving protein structure, was modified with various vinyl groups such as styrenyl or methacrylate moieties, using either ether or acetal linkages between the vinyl group and trehalose. In the first report, we covalently conjugated lysozyme to the trehalose polymer, which was prepared by reversible addition-fragmentation chain transfer (RAFT) polymerization, and demonstrated a superior stabilization effect against thermal stress compared to trehalose or PEG as excipients.⁴⁹ Trehalose polymers with varying

molecular weights were tested and all were effective. In our next study, we have shown synthesis of a series of trehalose polymers with different trehalose-based monomers through free radical polymerization (Chapter 2).⁵⁰ In this work, all the polymers were separately added (not conjugated) to horseradish peroxidase and glucose oxidase solution to increase their thermostability. The polymers displayed no cytotoxicity up to concentrations of 8 mg/mL, suggesting their potential use for medical applications.

1.2.3 Nano-, Micro-particles

Encapsulating proteins in nano- or micro-sized particles also enhances their thermostability. Suh and coworkers have developed a new strategy to stabilize protein against heating by microencapsulation.⁵¹ In the study, papain was encapsulated inside a microcapsule composed of hydrophobic poly(propylene glycol) (PPG) interlayers and poly(ϵ -caprolactone) (PCL) walls. This microencapsulation increased the storage time at 40 °C without loss of enzymatic activity. The Shi group have used the thermoresponsive poly(N-isopropylacrylamide) (pNIPAAm) polymer for protein stabilization upon heating.⁵² Carbonic anhydrase B was encapsulated with pNIPAAm using a reverse microemulsion technique. When the protein-polymer complex was heated to a high temperature the polymer formed nanoscale aggregates, which adsorbed denatured carbonic anhydrase B through hydrophobic interactions. This process prevented intermolecular aggregation of the protein and led to recovery of enzymatic activity upon cooling. The Kumar group reported the synthesis of a hemoglobin-poly(acrylic acid) conjugated nanoparticle.⁵³ The hemoglobin was active even after exposure to steam sterilization conditions (122 °C, 40 min, 17-20 psi). The authors observed that the nanoparticle improved thermodynamic stability as well as aiding in refolding of the denatured hemoglobin. Konrad and coworkers have prepared biocompatible, multilayered polyelectrolyte microcapsules for stabilization of L-asparaginase.⁵⁴ Using a layer-by-layer technique, the microcapsule was synthesized with two or three polydextran/poly-L-arginine-based bilayers and then coprecipitated with the enzyme. This microcapsule effectively inhibited thermo inactivation at 37 °C, prolonging the enzyme's *in vivo* half-life.

1.2.4 Others

There are other polymers that have been shown to increase protein stability during heating stress. Russell and coworkers have reported the immobilization of phosphotriesterase in a polyurethane foam matrix.⁵⁵ The immobilized enzyme exhibited lower activity than the original enzyme, however, its half-life was increased more than 100-fold at 50 °C. The same group also demonstrated the thermostabilization of diisopropylfluorophosphatase using the same matrix.⁵⁶ The enzyme quickly lost 90% of its original activity, but became hyperstable at elevated temperatures with the support of polyurethanes. The Hatti-Kaul group investigated the effect of adding the cationic polymer polyethyleneimine as an excipient.^{57,58} The polymer was effective in increasing the long-term stability of lactate dehydrogenase, alcohol dehydrogenase, β -galactosidase, and trypsin at 36 °C. Circular dichroism (CD) results suggested that polyethyleneimine prevented the loss of protein secondary structure. The Godjevargova group utilized a mixture of hydrolyzed polyacrylonitrile and PEG to stabilize glucose oxidase at high temperatures.⁵⁹ The group studied the effect of each mixture on protein stabilization at 45 and 60 °C, with the highest stabilization effect achieved by using 3 wt % hydrolyzed polyacrylonitrile and 1 wt % PEG. The Jiang group has reported the synthesis of a poly(zwitterionic)-protein conjugate using poly(carboxybetaine methacrylate).⁶⁰ The conjugated α -chymotrypsin showed increased thermostability without sacrificing binding affinity or bioactivity. The poly(zwitterionic) conjugate was more effective than PEG at 50 and 55 °C. Additionally, our group has conjugated a heparin-mimicking polymer to basic fibroblast growth factor, which is important for diverse cellular functions.⁶¹ The polymer (pSS-*co*-PEGMA) was prepared with sodium 4-styrenesulfonate (SS) and PEG methyl ether methacrylate (PEGMA) using RAFT polymerization. The conjugated polymer demonstrated thermostabilization

comparable to addition of excess amounts of the natural stabilizer, heparin.

1.3 Lyophilization (Freeze-Drying)

In 1935, Flosdorf and Mudd developed a procedure and apparatus for lyophilization.⁹ Since then, lyophilization became the most common method to prepare solid forms of proteins for long-term storage.^{62,63} Moreover, lyophilization is often used in the preparation of delivery vehicles for nucleic acids such as DNA and RNA. However, lyophilization involves rapid freezing at low temperature, low pressure, and drying steps, all of which can stress proteins by causing ice crystal formation, concentration changes, and pH changes, leading to protein or nucleic acid denaturation.^{62,64} To prevent biomolecule denaturation during lyophilization, polymers such as PEG and dextran have been used as excipients,⁶⁵ and the development of polymer lyoprotectants is an active field of research.

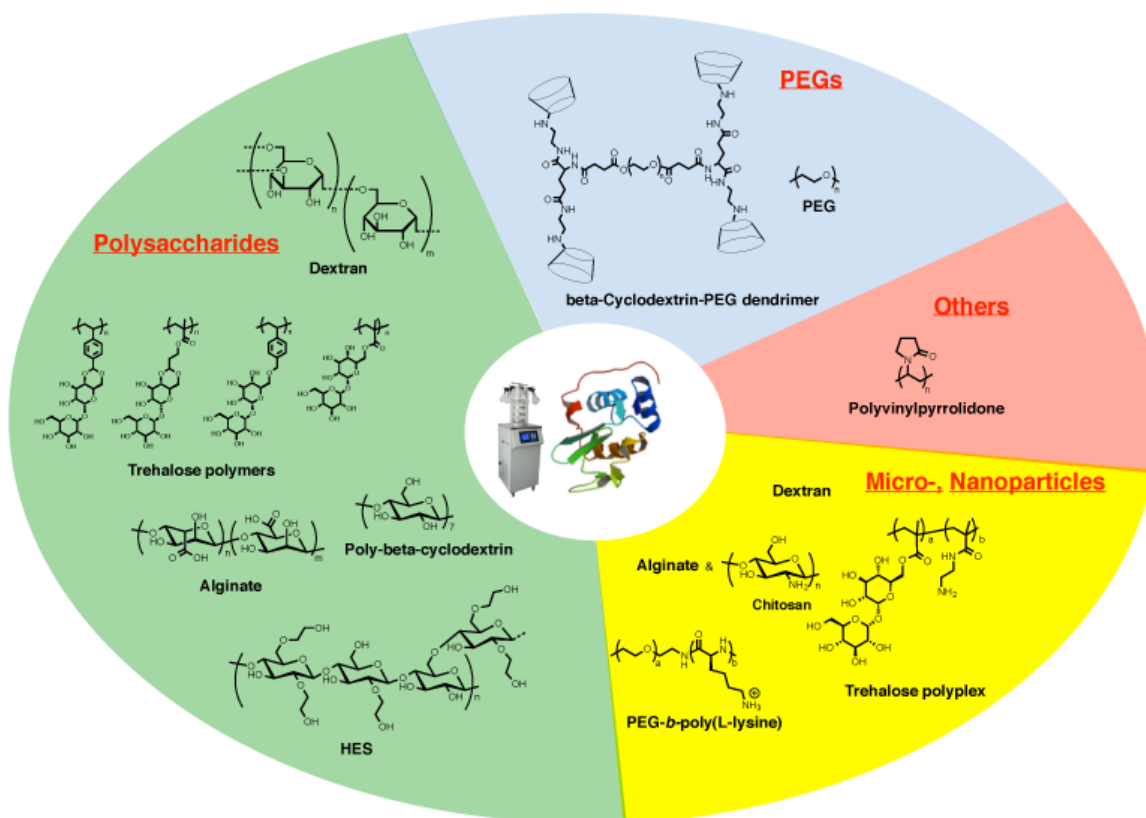


Figure 1-2. Different polymers used as lyoprotectants to stabilize proteins and nucleic acids.

1.3.1 PEGs

The Griebenow group reported that PEG can be used as stabilizer and emulsifying agent to prevent aggregation and inactivation during the lyophilization process.⁶⁶ When γ -chymotrypsin was encapsulated in a poly(lactic-co-glycolic) acid microsphere, lyophilization led to a 50% loss in activity. However, co-lyophilization with PEG resulted in only 8% loss in activity, demonstrating that the PEG excipient is an effective stabilizer for lyophilization. The group also has shown the effect of PEGylation during lyophilization.⁶⁷ Various conjugates of α -chymotrypsin and PEG (5 kDa) were prepared (PEG : protein molar ratios from 0.4 : 1 to 96 : 1), encapsulated into poly(lactic-co-glycolic) acid microspheres, and lyophilized. Highly modified α -chymotrypsin demonstrated low activity even before lyophilization, but proteins modified with

1-8 PEG chains displayed enhanced stability. The Sahoo group has studied the effect of PEG as an excipient on the conformational stability of bovine serum albumin (BSA).⁶⁸ Different molar ratios of PEGs were added to the protein and co-lyophilized. The optimal molar ratio of PEG was added to form protein particles, which had higher stability against lyophilization. Characterization with CD showed conformational changes of the protein after interaction with PEG. Walle and coworkers also used PEG as an additive for the preparation of freeze-dried bacteriophages.⁶⁹ To maintain stability of lyophilized bacteriophages, high concentrations of PEG (6 kDa) and sucrose additives were required. However, gelatin did not protect the bacteriophage against freeze-drying stress. Even though a high excipient concentration prevented denaturation and aggregation during lyophilization, it also accelerated inactivation of the bacteriophage in the dried form; however, complete attenuation of activity did not occur. Buera and coworkers have synthesized a low generation PEG dendrimer functionalized with β -cyclodextrin.⁷⁰ They designed the dendrimer using PEG as a cryoprotectant and β -cyclodextrin as a supramolecular stabilizer. The dendrimer was shown to be the best stabilizer for the tested enzyme in freeze-thawing stress while trehalose was the most effective additive against freeze-drying. However, the dendrimer sufficiently protected the enzyme and had synergistic behavior when compared to PEG or β -cyclodextrin added alone.

1.3.2 Polysaccharides

Sugars such as trehalose, glucose, and sucrose are well known to protect proteins against freezing and low pressure and are therefore widely used as lyoprotectants.^{63,71} Polysaccharides have also been used to prevent loss of protein activity during the freeze-drying process. Müller and coworkers studied low molecular weight dextran as an additive for aviscumine.⁷² In the study, dextran (1 kDa) increased protein activity by 20% compare to no additive after the lyophilization. The authors attributed this effect to the steric hindrance from large dextran polymer chains. Additionally, increasing the concentration of dextran resulted in increased interactions between the polymer and the protein, such as hydrogen bonding. The addition of 8 wt% dextran was able to maintain 100% protein activity after lyophilization. The Burea group also studied the effect of three biopolymers– alginate, dextran, and β -cyclodextrin-branched alginate– on stabilization during freeze-drying of protein.^{73,74} In their first report, the three polymers were used as excipients alone or combined with trehalose to stabilize the enzyme invertase.⁷³ After freeze-drying, the protein activities from samples treated with alginate and dextran were comparable to the samples only with trehalose. However, β -cyclodextrin-branched alginate was not as effective as the others even though the polymer was designed to combine the protective effects of both alginate and β -cyclodextrin. They then tested poly- β -cyclodextrin and carboxymethyl-poly- β -cyclodextrin as additives.⁷⁴ In both β -cyclodextrin-based polymers, glass transition temperature (T_g) of the invertase increased more than when only trehalose was added. This greater increase in T_g stabilized the structure of the enzyme. However, poly- β -cyclodextrin retained only 85% original activity compared to trehalose as an excipient, whereas carboxymethyl-poly- β -cyclodextrin lost 80% of the original activity, implying that increased structural stability does not directly correlate to activity. Trehalose-based polymers from our

group also demonstrated lyoprotection effects, both when conjugated or added as an excipient.^{49,50} When the trehalose glycopolymer was conjugated to lysozyme, the conjugate maintained 100% activity even after 10 cycles of lyophilization, while the native protein lost over 80% of its original activity.⁴⁹ This stabilization effect was superior to addition of either trehalose or PEG excipients. The trehalose polymers also functioned as excipients, preventing loss of β -galactosidase activity during multiple cycles of lyophilization (Chapter 2).⁵⁰

1.3.3 Nano-, Micro-particles

Kataoka and coworkers have prepared polyplex micelle polyion complexes from pDNA and thiolated PEG-poly(L-lysine) block copolymers for *in vivo* gene delivery.⁷⁵ Significant lyoprotection was observed when the thiols were crosslinked in the core compared to the non-crosslinked polyplex micelles. By crosslinking, the micelles showed excellent stability in the absence of other lyoprotectants. Wu *et al.* have encapsulated glucose oxidase in alginate/chitosan microspheres using a method of emulsification-internal gelation, glucose oxidase adsorption, and polyelectrolyte coating.⁷⁶ After lyophilization of the glucose oxidase encapsulated microspheres and storage at -20 °C for a month, 90% of the original glucose oxidase activity was observed. The Jin group synthesized polysaccharide glassy microparticles based on dextran with bovine serum albumin, myoglobin, and β -galactosidase.⁷⁷ Protein-loaded polysaccharide particles were 1-4 μm in diameter and the existence of a glassy state was confirmed by DSC and X-ray diffraction (XRD). This microspheres preserved protein activity after lyophilization. The Gilani group used chitosan as a protecting agent while spray-drying or freeze-drying plasmid DNA (pDNA).⁷⁸ Chitosan is an attractive polysaccharide for gene delivery because of its availability, biocompatibility, inherent positive charge and permeability enhancing capability. In the report, low molecular weight chitosan was used to form nanocomplexes with pDNA and the activity of pDNA and the particle size were tested after lyophilization stress. The results suggested that chitosan can efficiently maintain its plasmid supercoiled structure as well as its transfection capability during freeze-drying and spray-drying. Interestingly, even in the presence of leucine, which is known to destabilize complexes between DNA and positively charged molecules, the supercoiled structure was still retained. The stability of siRNA with a trehalose polyplex was also investigated by the Reineke group.⁷⁹ A prepared siRNA-loaded polyplex was lyophilized

and resuspended in water to yield nanoparticles that retained their size and shape. After lyophilization, the siRNA-mediated gene down-regulation activity was not decreased, even in the absence of other lyoprotectants.

1.3.4 Others

The Carpenter group stabilized lactate dehydrogenase during the freeze-drying process.⁸⁰ By protecting quaternary structures in the frozen state, they argued that polymers could protect multimeric proteins. BSA and polyvinylpyrrolidone (PVP) were tested and both inhibited dissociation of lactate dehydrogenase in the initial step of lyophilization, therefore preventing loss of activity. Kawai and coworkers have examined xanthine oxidase stabilization by the addition of disaccharide-polymer mixtures.⁸¹ In the study, xanthine oxidase was freeze-dried in the presence of sucrose, trehalose, BSA, and dextran (separately or mixed) and the percent original activity was measured. The results showed that the mixture of BSA and either sucrose or trehalose improved protein stability synergistically; however, dextran did not stabilize BSA either alone or in combination with the disaccharides.

1.4 Conclusion

Most research on stabilizing proteins has focused on the addition of small molecules such as saccharides or amino acids. However, because of their flexibility toward easy modification and multiple functionalities, polymers have become increasingly used to achieve the same goal. Various polymers can be used to enhance thermal and lyo-stability of proteins in the form of protein-polymer conjugates, excipients, or nano- and micro-particles. PEG and polysaccharides are mainly studied in this field. Developing appropriate polymers for protein stabilization can lower cost and simplify bulk processes compared to protein engineering. Yet in many cases, decreased protein activity was observed after conjugation or addition of polymer. Additionally, the possible toxicity of the polymer must be addressed for applications in the pharmaceutical industry. Thus, there are many research opportunities available to develop non-toxic and effective polymers for the stabilization of proteins against environmental stressors.

1.5 References

- (1) Stennicke, H. R.; Ostergaard, H.; Bayer, R. J.; Kalo, M. S.; Kinealy, K.; Holm, P. K.; Sorensen, B. B.; Zopf, D.; Bjorn, S. E. *Thromb. Haemost.* **2008**, *100*, 920-928.
- (2) Bordusa, F. *Chem. Rev.* **2002**, *102*, 4817-4867.
- (3) O'Fagain, C. *Enzyme. Microb. Technol.* **2003**, *33*, 137-149.
- (4) Rathore, N.; Rajan, R. S. *Biotechnol. Prog.* **2008**, *24*, 504-514.
- (5) Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. *Pharm. Res.* **2003**, *20*, 1325-1336.
- (6) Polizzi, K. M.; Bommarius, A. S.; Broering, J. M.; Chaparro-Riggers, J. F. *Curr. Opin. Chem. Biol.* **2007**, *11*, 220-225.
- (7) Patro, S. Y.; Freund, E.; Chang, B. S. *Biotechnol. Annu. Rev.* **2002**, *8*, 55-84.
- (8) Wang, W. *Int. J. Pharm.* **2005**, *289*, 1-30.
- (9) Flosdorf, E. W.; Mudd, S. *J. Immunol.* **1935**, *29*, 389-425.
- (10) Chang, B. S.; Yeung, B. In *Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals*; John Wiley & Sons, Inc.: 2010, p 69-104.
- (11) Shahrokh, Z.; Sluzky, V.; Cleland, J. L.; Shire, S. J.; Randolph, T. W. *Therapeutic Protein and Peptide Formulation and Delivery, Copyright, Advisory Board, Foreword*; American Chemical Society, 1997; Vol. 675.
- (12) Carpenter, J. F.; Manning, M. C. *Rational Design of Stable Protein Formulations*, 2002; Vol. 13.
- (13) Bhatnagar, B. S.; Bogner, R. H.; Pikal, M. J. *Pharm. Dev. Technol.* **2007**, *12*, 505-523.
- (14) Cao, E.; Chen, Y.; Cui, Z.; Foster, P. R. *Biotechnol. Bioeng.* **2003**, *82*, 684-690.
- (15) Kazan, D.; Erarslan, A. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 1157-1164.

- (16) Hamada, H.; Arakawa, T.; Shiraki, K. *Curr. Pharm. Biotechnol.* **2009**, *10*, 400- 407.
- (17) Bailon, P.; Berthold, W. *Pharm. Sci. Technol. To.* **1998**, *1*, 352-356.
- (18) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polymer Chemistry* **2011**, *2*, 1442-1448.
- (19) Abuchowski, A.; Vanes, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3578-3581.
- (20) Gaertner, H. F.; Puigserver, A. J. *Enzyme. Microb. Technol.* **1992**, *14*, 150-155.
- (21) Longo, M. A.; Combes, D. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 25-32.
- (22) Yang, Z.; Domach, M.; Auger, R.; Yang, F. X.; Russell, A. J. *Enzyme. Microb. Technol.* **1996**, *18*, 82-89.
- (23) Rodriguez-Martinez, J. A.; Sola, R. J.; Castillo, B.; Cintron-Colon, H. R.; Rivera- Rivera, I.; Barletta, G.; Griebenow, K. *Biotechnol. Bioeng.* **2008**, *101*, 1142-1149.
- (24) Rodriguez-Martinez, J. A.; Rivera-Rivera, I.; Sola, R. J.; Griebenow, K. *Biotechnol. Lett.* **2009**, *31*, 883-887.
- (25) Kazan, D.; Erarslan, A. *Appl. Biochem. Biotechnol.* **1997**, *62*, 1-13.
- (26) Tomita, S.; Nagasaki, Y.; Shiraki, K. *Biotechnol. Bioeng.* **2012**, *109*, 2543-2552.
- (27) Ganguli, S.; Yoshimoto, K.; Tomita, S.; Sakuma, H.; Matsuoka, T.; Shiraki, K.; Nagasaki, Y. *J. Am. Chem. Soc.* **2009**, *131*, 6549-6553.
- (28) Ganguli, S.; Yoshimoto, K.; Tomita, S.; Sakuma, H.; Matsuoka, T.; Shiraki, K.; Nagasaki, Y. *Macromol. Biosci.* **2010**, *10*, 853-859.
- (29) Altikatoglu, M.; Kuzu, H. *Polish Journal of Chemical Technology* **2010**, *12*, 12- 16.
- (30) Marshall, J. J.; Rabinowitz, M. L. *Arch. Biochem. Biophys.* **1975**, *167*, 777-779.
- (31) Marshall, J. J.; Rabinowitz, M. L. *Biotechnol. Bioeng.* **1976**, *18*, 1325-1329.
- (32) Marshall, J. J.; Rabinowitz, M. L. *J. Biol. Chem.* **1976**, *251*, 1081-1087.

- (33) Yamagata, Y.; Arakawa, K.; Yamaguchi, M.; Kobayashi, M.; Ichishima, E. *Enzyme. Microb. Technol.* **1994**, *16*, 99-103.
- (34) Kazan, D.; Ertan, H.; Erarslan, A. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 191-197.
- (35) Abdel-Naby, M. A. *Microbiol. Res.* **1999**, *154*, 213-218.
- (36) Jadhav, S. B.; Singhal, R. S. *Carbohydr. Polym.* **2012**, *90*, 1811-1817.
- (37) Jadhav, S. B.; Singhal, R. S. *Carbohydr. Polym.* **2013**, *92*, 1724-1729.
- (38) Altikatoglu, M.; Basaran, Y. *Prot. J.* **2011**, *30*, 84-90.
- (39) Szejtli, J. *Chem. Rev.* **1998**, *98*, 1743-1753.
- (40) Darias, R.; Herrera, I.; Fragoso, A.; Cao, R.; Villalonga, R. *Biotechnol. Lett.* **2002**, *24*, 1665-1668.
- (41) Fernandez, M.; Villalonga, M. L.; Caballero, J.; Fragoso, A.; Cao, R.; Villalonga, R. *Biotechnol. Bioeng.* **2003**, *83*, 743-747.
- (42) Villalonga, M. L.; Fernandez, M.; Fragoso, A.; Cao, R.; Villalonga, R. *Prep. Biochem. Biotechnol.* **2003**, *33*, 53-66.
- (43) Fernandez, M.; Villalonga, M. L.; Fragoso, A.; Cao, R.; Banos, M.; Villalonga, R. *Process Biochem.* **2004**, *39*, 535-539.
- (44) Villalonga, M. L.; Reyes, G.; Fragoso, A.; Cao, R.; Fernandez, L.; Villalonga, R. *Biotechnol. Appl. Biochem.* **2005**, *41*, 217-223.
- (45) Rajalakshmi, N.; Sundaram, P. V. *Protein Eng.* **1995**, *8*, 1039-1047.
- (46) Sundaram, P. V.; Venkatesh, R. *Protein Eng.* **1998**, *11*, 699-705.
- (47) Srimathi, S.; Jayaraman, G.; Narayanan, P. R. *Enzyme. Microb. Technol.* **2006**, *39*, 301-307.
- (48) Kuhlmeier, C.; Klein, J. *Enzyme. Microb. Technol.* **2003**, *32*, 99-106.
- (49) Mancini, R. J.; Lee, J.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 8474-8479.

- (50) Lee, J.; Lin, E.-W.; Lau, U. Y.; Hedrick, J. L.; Bat, E.; Maynard, H. D. *Biomacromolecules* **2013**, *14*, 2561-2569.
- (51) Kim, J. W.; Jung, M. O.; Kim, Y. J.; Ryu, J. H.; Kim, J.; Chang, I. S.; Lee, O. S.; Suh, K. D. *Macromol. Rapid Commun.* **2005**, *26*, 1258-1261.
- (52) Tao, Q.; Li, A.; Liu, X.; Ma, R.; An, Y.; Shi, L. *Phys. Chem. Chem. Phys.* **2011**, *13*, 16265-16271.
- (53) Mudhivarthi, V. K.; Cole, K. S.; Novak, M. J.; Kipphut, W.; Deshapriya, I. K.; Zhou, Y.; Kasi, R. M.; Kumar, C. V. *J. Mater. Chem.* **2012**, *22*, 20423-20433.
- (54) Karamitros, C. S.; Yashchenok, A. M.; Moehwald, H.; Skirtach, A. G.; Konrad, M. *Biomacromolecules* **2013**, *14*, 4398-4406.
- (55) LeJeune, K. E.; Mesiano, A. J.; Bower, S. B.; Grimsley, J. K.; Wild, J. R.; Russell, A. J. *Biotechnol. Bioeng.* **1997**, *54*, 105-114.
- (56) Drevon, G. F.; Hartleib, J.; Scharff, E.; Ruterjans, H.; Russell, A. J. *Biomacromolecules* **2001**, *2*, 664-671.
- (57) Andersson, M. A.; Hatti-Kaul, R. *J. Biotechnol.* **1999**, *72*, 21-31.
- (58) Mazzaferro, L.; Breccia, J. D.; Andersson, M. M.; Hitzmann, B.; Hatti-Kaul, R. *Int. J. Biol. Macromol.* **2010**, *47*, 15-20.
- (59) Godjevargova, T.; Vasileva, N.; Letskovska, Z. *J. Appl. Polym. Sci.* **2003**, *90*, 1393-1397.
- (60) Keefe, A. J.; Jiang, S. *Nature Chem.* **2012**, *4*, 59-63.
- (61) Nguyen, T. H.; Kim, S.-H.; Decker, C. G.; Wong, D. Y.; Loo, J. A.; Maynard, H. D. *Nature Chem.* **2013**, *5*, 221-227.
- (62) Wang, W. *Int. J. Pharm.* **2000**, *203*, 1-60.
- (63) Randolph, T. W. *J. Pharm. Sci.* **1997**, *86*, 1198-1203.

- (64) Chang, B. S.; Beauvais, R. M.; Dong, A.; Carpenter, J. F. *Arch. Biochem. Biophys.* **1996**, *331*, 249-258.
- (65) Barbieri, D. M.; Heller, M. C.; Randolph, T. W.; Carpenter, J. F. In *Therapeutic Protein and Peptide Formulation and Delivery*; Shahrokh, Z., Sluzky, V., Cleland, J. L., Shire, S. J., Randolph, T. W., Eds. 1997; Vol. 675, p 90-108.
- (66) Castellanos, I. J.; Crespo, R.; Griebenow, K. *J. Controlled Release* **2003**, *88*, 135- 145.
- (67) Castellanos, I. J.; Al-Azzam, W.; Griebenow, K. *J. Pharm. Sci.* **2005**, *94*, 327-340.
- (68) Rawat, S.; Suri, C. R.; Sahoo, D. K. *Biochem. Biophys. Res. Commun.* **2010**, *392*, 561-566.
- (69) Puapermpoonsiri, U.; Ford, S. J.; van der Walle, C. F. *Int. J. Pharm.* **2010**, *389*, 168-175.
- (70) Santagapita, P. R.; Florencia Mazzobre, M.; Garcia Cruz, A.; Corti, H. R.; Villalonga, R.; Pilar Buera, M. *Biotechnol. Prog.* **2013**, *29*, 786-795.
- (71) Anchordoquy, T. J.; Izutsu, K.; Randolph, T. W.; Carpenter, J. F. *Arch. Biochem. Biophys.* **2001**, *390*, 35-41.
- (72) Gloger, O.; Witthohn, K.; Muller, B. W. *Int. J. Pharm.* **2003**, *260*, 59-68.
- (73) Santagapita, P. R.; Brizuela, L. G.; Mazzobre, M. F.; Ramirez, H. L.; Corti, H. R.; Santana, R. V.; Buera, M. P. *Biomacromolecules* **2008**, *9*, 741-747.
- (74) Santagapita, P. R.; Gomez Brizuela, L.; Florencia Mazzobre, M.; Ramirez, H. L.; Corti, H. R.; Villalonga Santana, R.; Pilar Buera, M. *Carbohydr. Polym.* **2011**, *83*, 203-209.
- (75) Miyata, K.; Kakizawa, Y.; Nishiyama, N.; Yamasaki, Y.; Watanabe, T.; Kohara, M.; Kataoka, K. *J. Controlled Release* **2005**, *109*, 15-23.
- (76) Liu, Q.; Rauth, A. M.; Wu, X. Y. *Int. J. Pharm.* **2007**, *339*, 148-156.

- (77) Yuan, W.; Geng, Y.; Wu, F.; Liu, Y.; Guo, M.; Zhao, H.; Jin, T. *Int. J. Pharm.* **2009**, *366*, 154-159.
- (78) Mohajel, N.; Najafabadi, A. R.; Azadmanesh, K.; Vatanara, A.; Amini, M.; Moazenil, E.; Rahimi, A.; Gilani, K. *Daru-Journal of Pharmaceutical Sciences* **2012**, *20*.
- (79) Sizovs, A.; Xue, L.; Tolstyka, Z. P.; Ingle, N. P.; Wu, Y.; Cortez, M.; Reineke, T. M. *J. Am. Chem. Soc.* **2013**, *135*, 15417-15424.
- (80) Anchordoquy, T. J.; Carpenter, J. F. *Arch. Biochem. Biophys.* **1996**, *332*, 231-238.
- (81) Srirangsan, P.; Kawai, K.; Hamada-Sato, N.; Watanabe, M.; Suzuki, T. *Food Chem.* **2010**, *119*, 209-213.

Chapter 2

Trehalose Glycopolymers as Excipients for Protein Stabilization[‡]

2.1 Introduction

Proteins are widely used as reagents in laboratories and as therapeutics for many different diseases.^{1,2} However, the instability of proteins during storage and transport increases costs and reduces effectiveness of protein drugs. For example, temperature fluctuations,³ desiccation,⁴ and light⁵ can significantly reduce biological activity of proteins. Proteins endure additional stressors during production and processing. For example, lyophilization, also known as freeze-drying, is the most common method to form protein powders for long-term storage and handling.⁴ However, during the lyophilization process, the protein is exposed to extreme temperature and concentration changes, low pressures, pH changes and desiccation stresses, all of which can induce denaturation or aggregation.⁶⁻⁸ Also, even when stored as a powder, proteins denature over time and require the addition of excipients to maintain activity.^{4,9}

Many excipients (additives) have been used to invoke protein stability. The most common excipients include amino acids,^{10,11} osmolytes,¹²⁻¹⁴ sugars,¹⁵⁻¹⁸ proteins,^{19,20} polymers,²¹⁻²⁷ and salts,²⁸ which are used to prevent denaturation of pharmaceutical proteins. Amino acids, such as histidine, arginine, and glycine, stabilize proteins through preferential hydration, direct binding, and buffering capacities.^{10,11} Osmolytes are also known protein stabilizers because of their preferential hydration mechanism.¹² Sugars, such as sucrose, lactose and trehalose, are used in both lyophilized and liquid formations to stabilize proteins.⁹ Human serum albumin and gelatins are widely used in protein drugs.⁹ Also, polymers are employed as additives to prevent aggregation and denaturation of proteins. For example, polyethylene glycol (PEG) or polypropylene glycol added to insulin prevents aggregation of the protein.²⁹ Zwitterionic polymer conjugates also remarkably stabilized proteins²⁴ and nonionic amphipols were effective to solubilize membrane proteins in water and protect them from denaturation.²⁵⁻²⁷ Yet, despite

the addition of excipients, many proteins still exhibit instability. Thus, the development of novel, nontoxic additives to stabilize proteins is an important area of research.

In this chapter, we describe the synthesis and investigation of three new polymers for potential use as excipients. The polymers have pendent trehalose units. Trehalose is a natural non-reducing disaccharide formed by α,α -1,1-linked glucose units, which is generally regarded as safe (GRAS) by the U.S. Food and Drug Administration.³⁰ Interestingly, many reports have shown that trehalose plays a key role in protecting cells and proteins to allow organisms to tolerate anhydrobiosis (desiccation) and cryobiosis (low temperature).³⁰ Currently, trehalose is widely used in the food and cosmetic industries. Also, trehalose is employed as an excipient in protein-based drugs including Advate[®] (Baxter), Avastin[®] (Genentech), Lucentis[®] (Genentech), and Herceptin[®] (Genentech).

Recently, we reported the synthesis of monomer 4,6-*O*-(4-vinylbenzylidene)- α,α -trehalose, which was polymerized using reversible addition-fragmentation chain transfer (RAFT) polymerization.³¹ The resulting polymer had a pyridyl disulfide end-group to conjugate to free cysteines. When the polymer was conjugated to lysozyme, the stability of the protein against heat and lyophilization stress was remarkably increased. We noted that adding excess of the polymer (not conjugating the polymer) also stabilized the protein, which means the strategy might be applicable to excipients. In this chapter, we describe the preparation of three different polymers with trehalose side chains. The polymers were added to three different proteins, horseradish peroxidase (HRP), glucose oxidase (GOx), and β -galactosidase (β -Gal) and the proteins were stressed. The activities of the proteins after treatment were evaluated with and without polymer, and to a comparable amount of trehalose. Also, stabilization effect of trehalose polymer depending on protein concentration was also investigated using lysozyme (Lyz). The results

together suggest that the polymers are promising as additives for protein stabilization. The details of the experiments are discussed below.

2.2 Experimental Section

Materials

All the chemicals and proteins were purchased from Sigma-Aldrich and Fisher Scientific and were used without purification unless noted. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX) and dried with ethanol and kept under vacuum before use in synthesis. Trehalose used in stability studies was dried with ethanol, lyophilized, and kept in a closed vial prior to use. Azobisisobutyronitrile (AIBN) was recrystallized from acetone before using. Amplex[®] Red Glucose/Glucose Oxidase Assay kit and EnzChek[®] lysozyme activity assay was purchased from Invitrogen and was used following the manufacturer's instructions. 4,6-*O*-(4-Vinylbenzylidene)- α,α -trehalose (**SAT**) was synthesized according to a literature procedure.³¹ 3,3'-Diethoxypropyl methacrylate (**2**) was prepared by following a literature procedure.^{32,33}

Analytical techniques

NMR spectra were obtained on Bruker AV 500 and DRX 500 MHz spectrometers. ¹H NMR spectra were acquired with a relaxation delay of 2 s for small molecules and 30 s for polymers. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. Gel permeation chromatography (GPC) was conducted on a Shimadzu high performance liquid chromatography (HPLC) system with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μ m mixed D columns. Eluent was DMF with LiBr (0.1 M) at 40 °C (flow rate: 0.60 mL/min). Calibration was performed using near-monodisperse pMMA standards from Polymer Laboratories. HPLC purification of the monomers were conducted on a Shimadzu HPLC system

with a refractive index detector RID-10A and one Luna 5 μm C18(2) column with MeOH and H₂O as an eluent. ESI-MS data were gathered on a Waters LCT premier with ACQUITY LC. Circular Dichroism (CD) spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan). Differential scanning calorimetry (DSC) was performed on a PerkinElmer Diamond DSC. Approximately 2 mg samples of 1-3 mol % trehalose or **P3** in water were prepared in hermetically sealed aluminum pans. Two heating/cooling scans were performed over a temperature range of -40 °C to 25 °C with a heating/cooling rate of 10 °C/min. Reported values were determined from the second scan. Thermal Gravimetric Analysis (TGA) was performed on a PerkinElmer Diamond TG/DTA using platinum pans. Measurements were performed under 200 mL/min Ar flow over a temperature range of 30 °C to 600 °C at a heating rate of 10 °C/min.

Methods

Synthesis of 4,6-O-(3-propyl methacrylate)- α,α -trehalose (MAT). Trehalose (3.96 g, 1.16×10^{-2} mol) was dissolved in 100 °C DMF (72 mL). *p*-Toluenesulfonic acid (*p*-TsOH, 44 mg, 2.31×10^{-1} mmol) was added and then hydroquinone (12.73 mg, 1.16×10^{-1} mmol) was added to inhibit autopolymerization of the methacrylate. After stirring for 5 min, **2** (500 mg, 2.31 mmol) was added to the reaction slowly. After 3 h, the reaction was stopped and the solvent was removed *in vacuo*. The crude product was purified by HPLC (50% MeOH in H₂O) and lyophilized resulting in a white powder (403.6 mg) of **MAT** in 37% yield. ¹H NMR (500 MHz in D₆DMSO) δ : 6.04 (s, 1H), 5.68-5.67 (t, *J* = 1.59 Hz, 1H), 5.10-5.09 (d, *J* = 5.31 Hz, 1H), 4.93-4.89 (m, 3H), 4.83-4.82 (d, *J* = 3.50 Hz, 1H), 4.78-4.76 (m, 2H), 4.70-4.68 (t, *J* = 5.00 Hz, 1H), 4.36-4.33 (t, *J* = 5.95 Hz, 1H), 4.16-4.13 (m, 2H), 3.97-3.94 (m, 1H), 3.89-3.84 (m, 1H), 3.69-3.64 (m, 2H), 3.57-3.53 (m, 2H), 3.49-3.41 (m, 2H), 3.35 (1H), 3.26-3.22 (m, 1H), 3.17-3.12 (m, 2H), 2.01-1.88 (m, 5H). ¹³C NMR (500 MHz in D₆DMSO) δ : 166.9, 136.1, 126.2, 99.2, 94.5, 94.1, 81.4, 73.0, 73.0, 72.4,

71.8, 70.3, 69.8, 68.1, 62.7, 61.0, 60.6, 33.5, 18.3. IR: ν = 3365, 2934, 1713, 1636, 1305, 1148, 979 cm^{-1} . ESI-MS (± 1.0) observed (predicted): Na^+ 489.1577.

Synthesis of 6-O-(4-vinylbenzylidene)- α,α -trehalose (SET). Sodium hydroxide (NaOH, 4.44 g, 1.14×10^{-1} mol) was added to dimethyl sulfoxide (DMSO, 96 mL) and stirred for 5 min. Trehalose (4.86 g, 1.42×10^{-2} mol) was then added to the reaction flask. After all the trehalose had dissolved, 4-vinyl benzyl chloride (0.4 mL, 2.84×10^{-3} mmol) was slowly added and reaction solution changed to bright yellow. The reaction was stirred for 24 h at 25 °C and then precipitated into 4 L of dichloromethane (DCM) in an ice bath. The solid was dried *in vacuo* and purified by HPLC (50% MeOH in H_2O). After lyophilization, the product (261.1 mg) was obtained as a white powder with 20% yield. ^1H NMR (500 MHz in D_2O) δ : 7.47-7.45 (m, 2H), 7.35-7.33 (m, 2H), 6.77-6.71 (m, 1H), 5.84-5.80 (d, J = 17.69 Hz, 1H), 5.30-5.28 (d, J = 11.42 Hz, 1H), 5.23-5.22 (d, J = 3.68 Hz, 1H), 5.14-5.13 (d, J = 4.05 Hz, 1H), 4.69-4.61 (m, 2H), 3.91-3.51 (m, 10H), 3.45-3.39 (m, 2H). ^{13}C NMR (500 MHz in D_2O) δ : 137.5, 136.6, 136.2, 128.9, 126.4, 114.7, 93.5, 91.4, 78.7, 73.2, 72.4, 72.2, 72.0, 72.0, 71.0, 69.8, 69.2, 60.6, 60.1. IR: ν = 3369, 2931, 1642, 1408, 1368, 1147, 1083, 1044, 989 cm^{-1} . ESI-MS (± 1.0) observed (predicted): Na^+ 481.1692 (481.1686).

Free Radical Polymerization of SAT. SAT (426 mg, 9.33×10^{-1} mmol) was dissolved in dimethylformamide (DMF, 1.17 mL). AIBN (7.00 mg, 4.26×10^{-2} mmol) was added to the solution. Five cycles of freeze–pump–thaw were undertaken, and the reaction was initiated by immersion in an oil bath at 80 °C. The polymerization was stopped by cooling with liquid nitrogen after 17 h. By ^1H NMR spectroscopy 100% monomer conversion was achieved. The product was purified by dialysis against H_2O for 3 days (MWCO 3,500 g/mol) and dried using a lyophilizer resulting in **P1**. Number-average molecular weight (M_n) of the polymer was 19,500

g/mol and polydispersity index (PDI) was 2.01 by GPC. ^1H NMR (500 MHz in D_6DMSO) δ : 7.11, 6.39, 5.45, 5.23, 4.96, 4.86, 4.41, 4.12, 3.97, 3.78, 3.70, 3.62, 3.48, 3.17, 1.60, 1.24, 0.86. IR: $\nu = 3384, 2916, 1635, 1380, 1150, 1074, 983\text{ cm}^{-1}$.

Free Radical Polymerization of MAT. MAT (263.8 mg, 5.66×10^{-1} mmol) was dissolved in DMF (2.83 mL) and AIBN (4.22 mg, 2.57×10^{-2} mmol) was added to the reaction flask. Freeze–pump–thaw cycles were repeated five times, and the reaction was initiated by immersion in an oil bath at 65 °C. After 22 h, monomer conversion reached 100% by ^1H NMR, and the polymerization was stopped by cooling in liquid nitrogen. The polymer was dialyzed against H_2O (MWCO 3,500 g/mol) and lyophilized resulting in **P2** with $M_n = 23,400$ g/mol and PDI = 2.40. ^1H NMR (500 MHz in D_6DMSO) δ : 5.15, 4.94, 4.85, 4.67, 4.38, 3.98, 3.88, 3.69, 3.57, 3.48, 3.17, 1.90, 1.23, 0.83. IR: $\nu = 3374, 2932, 1716, 1638, 1384, 1273, 1146, 1047, 984\text{ cm}^{-1}$.

Free Radical Polymerization of SET. SET (193.6 mg, 4.22×10^{-1} mmol) and AIBN (3.15 mg, 1.92×10^{-2} mmol) were dissolved in H_2O (1.59 mL) and DMF (2.12 mL), respectively. Both solutions were added to a reaction flask and after five cycles of freeze–pump–thaw, the polymerization started by immersing the flask into a 80 °C oil bath. After 30 h, the polymerization was stopped by cooling with liquid nitrogen. The resulting polymer was dialyzed against H_2O (MWCO 3,500 g/mol) for 3 days for purification and lyophilized to obtain **P3** in white powder. M_n was 14,600 g/mol and PDI was 1.77 by GPC. ^1H NMR (500 MHz in D_2O) δ : 7.04, 6.48, 5.04, 4.55, 3.82, 3.70, 3.60, 3.50, 3.35, 1.48, 1.14. IR: $\nu = 3341, 2932, 1654, 1510, 1424, 1367, 1147, 1080, 1046, 987\text{ cm}^{-1}$.

Horseradish Peroxidase Heating Studies. Horseradish peroxidase (HRP, 150 $\mu\text{g/mL}$) solution was prepared by adding HRP into pH 7.0, 10 mM sodium phosphate buffer. Stock solutions of

80, 50, 25, and 1 weight equivalent (wt equiv) of **P1**, **P2**, and **P3** to HRP were prepared. Trehalose was added to be equivalent to the trehalose amount in **P1** for each weight equivalent. 2 μ L aliquots of the HRP solution were mixed with 2 μ L aliquots of each polymer or trehalose solution to give final concentration of 75 μ g/mL of HRP. Samples were incubated at 70 °C for 30 min and a non-heated control sample was stored at 4 °C until the activity assay was performed. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as a substrate and 1 M H₂SO₄ solution as the stop solution. To assess the activity, absorbance at 450 nm was monitored. The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Glucose Oxidase Heating Studies. Glucose oxidase (GOx, 2 mg/mL) solution was prepared by adding GOx to pH 7.0 10mM sodium phosphate buffer. Stock solutions of **P1**, **P2**, and **P3** (20 wt equiv of trehalose units to GOx) and trehalose were also prepared in pH 7.0, 10 mM sodium phosphate buffer. 2 μ L aliquots of the GOx solution were mixed with 2 μ L aliquots of each polymer or trehalose solution to give final concentration of 1 mg/mL GOx. Samples were heated to 50 °C for 30 min and the non-heated control was stored at 4 °C until the activity assay was performed. The activity was measured using Amplex[®] Red Glucose/Glucose Oxidase Assay kit following the manufacturer's procedure with six repeats repeated overall two times (n=12).

β -Galactosidase Lyophilization Studies. β -Galactosidase (β -Gal, 0.4 mg/mL) solution was prepared by adding β -Gal to pH 7.0 10 mM sodium phosphate buffer. Each 25 μ L aliquot of β -Gal solution was mixed with 75 μ L of H₂O (the control) or 75 μ L of stock solution each containing 10, 5, or 1 wt equiv of **P1**, **P2**, **P3**, and trehalose equivalent to the amount in **P1** for each wt equiv to β -Gal in H₂O to result in a 0.1 mg/mL protein solution. Aliquots of each sample were frozen by immersion in liquid nitrogen before solvent removal via lyophilization. The samples were lyophilized for 12 h for one cycle under 1 mbar. After the first cycle, 100 μ L of

H₂O was added and lyophilization was repeated for the next cycles. The control sample was stored at 4 °C until the activity assay was conducted. After the desired number of cycles, the final lyophilized samples were dissolved into 50 µL of H₂O. 30 µL of *o*-nitrophenyl-β-D-galactoside (ONPG, 4 mg/mL) was added to the samples. The samples were incubated in dark for 5 min and the reaction stopped with 50 µL of 1 M Na₂CO₃. Activity was ascertained by reading the absorbance at 405 nm.³⁴ The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Effect of Polymer Molecular Weight on Protein Stabilization. 11,900 g/mol, 19,500 g/mol, and 40,800 g/mol molecular weight **P1** were synthesized. For the HRP stabilization, 25 wt equiv of each **P1** to HRP was added, and the stabilization effect was studied as described before. Also, the same polymers were tested for β-Gal stabilization against lyophilization. β-Gal with 2.5 wt equiv of each **P1** were lyophilized for three cycles and assayed as described above. The assay was repeated in total twelve times (n=12, two separate experiments with six repeats each).

Synthesis of benzyl trehalose (Benzyl T). NaOH (1.83 g, 4.68×10⁻² mol) was stirred with DMSO (40 mL) for 5 min. Trehalose (2 g, 5.84×10⁻³ mol) was added to DMSO and benzyl chloride (0.13 mL, 1.19×10⁻³ mol) was slowly added after trehalose had dissolved. The reaction was stirred for 14 h at 25 °C and precipitated in 2 L of DCM. The precipitate was collected and purified by HPLC (MeOH : H₂O = 1 : 1). ¹H NMR (500 MHz in D₂O) δ: 7.49-7.46 (m, 5H), 5.21-5.12 (m, 2H), 4.01-3.37 (m, 14H). ESI-MS (± 1.0) observed (predicted): Na⁺ 455.1456 (455.15).

Synthesis of poly(benzyl ether poly(ethylene glycol) methyl ether) (p(Benzyl PEG)). NaOH (1.12 g, 2.86×10⁻² mol) added into THF. After stirring for 5 min, PEG methyl ether (average M_n

= 350, 5.71×10^{-3} mol) was added to the reaction flask. Then, 4-vinyl benzyl chloride was added drop-wise. After stirring 20 h at 25 °C, silica gel column (EtOAc : MeOH = 7 : 3) was used to purify and obtain styrenyl PEG methyl ether. Styrenyl PEG methyl ether (500 mg, 1.03×10^{-3} mol) was polymerized using AIBN (8.52 mg, 5.19×10^{-2} mmol) as an initiator with 2.07 mL of DMF at 80 °C. After 21 h, the polymerization was stopped by cooling with liquid nitrogen and dialyzed against H₂O (MWCO 3,500) for 2 days, resulting in a bimodal polymer with M_n of 187,800 g/mol (PDI = 1.86) and M_n of 5,700 g/mol (PDI = 1.45). ¹H NMR (500 MHz in D₂O) δ : 7.04, 6.48, 4.38, 3.49, 3.20, 1.51, 0.97, 0.82.

Effect of Trehalose Derivatives and Polymer Analogue on Protein Stabilization. The stabilization of HRP and β -Gal against heating and lyophilization were tested with the following additives: **P3**, trehalose (**T**), **SET**, **Benzyl T**, and **p(Benzyl PEG)**. 80 and 10 wt equiv of each of the small molecules and polymers were added to HRP and β -Gal, respectively and activities were tested after heating and lyophilization (n=12, two separate experiments with six repeats each).

Lysozyme Lyophilization Studies Depending on Lysozyme Concentration. For the lysozyme stabilization studies 21,800 g/mol **P1** was used. Different concentration of lysozyme solutions (1,000 U/mL, 5,000 U/mL, and 10,000 U/mL) were prepared in pH 7.4, Dulbecco's phosphate-buffered saline (D-PBS). Into each of the lysozyme solutions 1 molar equiv **T**, 100 molar equiv **T**, 1 molar equiv **P1**, or 100 molar equiv **P1** was added. Each solution was frozen in liquid nitrogen before lyophilization (<1 mbar, 4 hours). The resulting powders were reconstituted by addition of Milli-Q water (20 μ L), and this procedure was repeated for a 6 and 10 lyophilization cycles. The lysozyme activity was checked by EnzChek® lysozyme activity assay before and after the desired number of lyophilization cycle. The experiment was repeated six times.

Circular Dichroism Experiments with HRP and β -Gal. Measurements in the far-ultraviolet region (190-260 nm) were performed on protein solutions (0.075 mg/mL in pH 7.0, 10 mM sodium phosphate buffer for HRP and 1.5 mg/mL in pH 7.0, 37.5 mM sodium phosphate buffer for β -Gal) employing a 1.0 mm-path-length cell at 25 and 70 °C. The CD spectra were recorded under the same conditions running two scans for each sample, corrected by subtracting the appropriate blank runs.

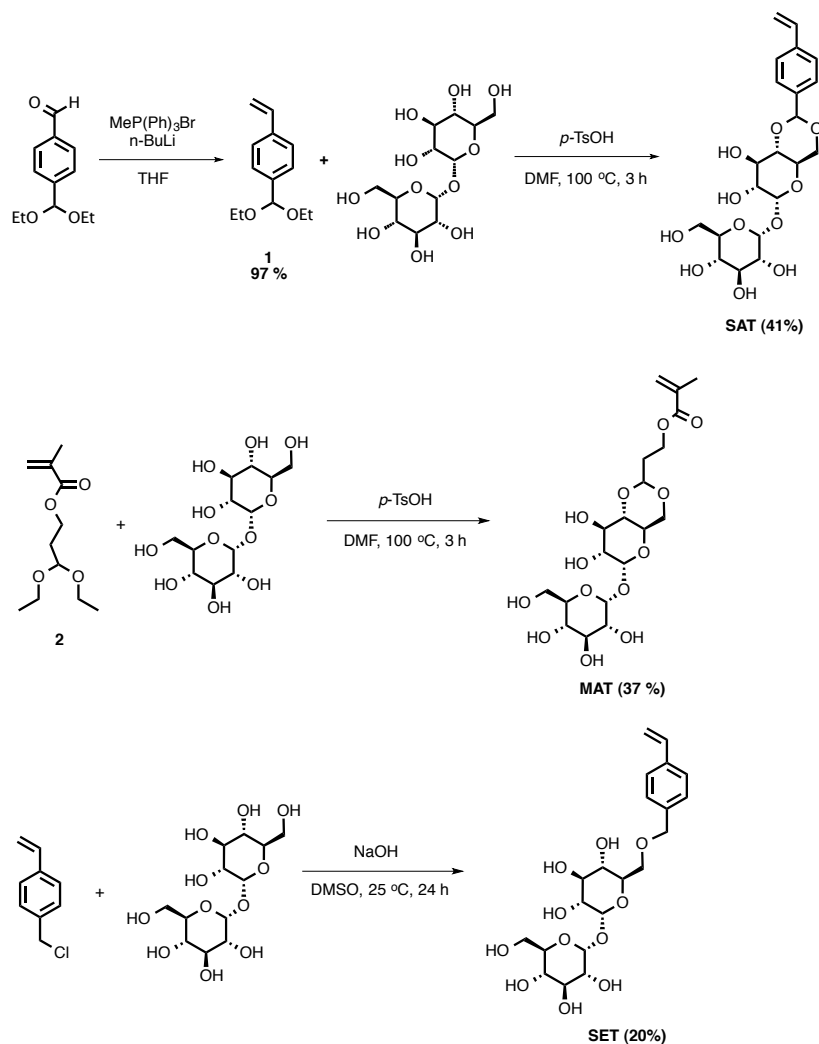
Glycopolymer Degradation Studies. **P1** and **P2** (8 mg/mL) solutions were prepared by dissolving **P1** and **P2** in pH 7.0 10 mM sodium phosphate buffer. The solutions were incubated at 70 °C for 30 min, and were then lyophilized for 12 h. The samples were evaluated by ^1H NMR spectroscopy and IR. Another set of **P1** and **P2** solutions were incubated at 37 °C for 48 h, followed by determination of any aldehyde formation with Purpald assay. The samples were added to the Purpald reagent according to the manufacturer's protocol, and the absorbance at 550 nm was monitored.

Statistical Analysis. All the p -values were calculated using the independent Student's t -test assuming unequal variances.

2.3 Results and Discussion

Synthesis of Trehalose Monomers and Glycopolymers

Scheme 1-1. Synthesis of trehalose monomers **SAT**, **MAT**, and **SET**.



In order to explore the protein stabilization capability of trehalose glycopolymers, we designed and synthesized three different monomers, styrenyl acetal trehalose (**SAT**), methacrylate acetal trehalose (**MAT**), and styrenyl ether trehalose (**SET**) (Scheme 1-1), and used free radical polymerization to prepare the polymers, **P1-P3**. The position of linkage was either through the 4, 6 (**SAT** and **MAT**) or 6 (**SET**) hydroxyl of trehalose, the chemistries of linkages

were an acetal (**SAT** and **MAT**) or ether (**SET**), and the polymer backbone was polystyrene (**SAT** or **SET**) or polymethacrylate (**MAT**). We anticipated that this set would allow us to ascertain the effect of polymer backbone or chemistry of linkage on the protein stabilization capability.

4,6-*O*-(4-Vinylbenzylidene)- α,α -trehalose (**SAT**)³¹ and 4,6-*O*-(3-propyl methacrylate)- α,α -trehalose (**SET**) were synthesized using styrenyl diethyl acetal (**1**) and 3,3'-diethoxypropyl methacrylate (**2**) as starting materials, respectively (Scheme 1-1).³¹⁻³³ Under acidic conditions with *p*-TsOH and trehalose, **SAT** and **MAT** were made in 41 and 37% yield. Another monomer, 6-*O*-(4-vinylbenzylidene)- α,α -trehalose (**SET**), was prepared in one step in 20% yield by adding sodium hydroxide and 4-vinyl benzyl chloride to a solution of trehalose. All the monomers were characterized using ¹H and ¹³C NMR and 2-D NMR spectra (Figure 2-1 to 2-8).

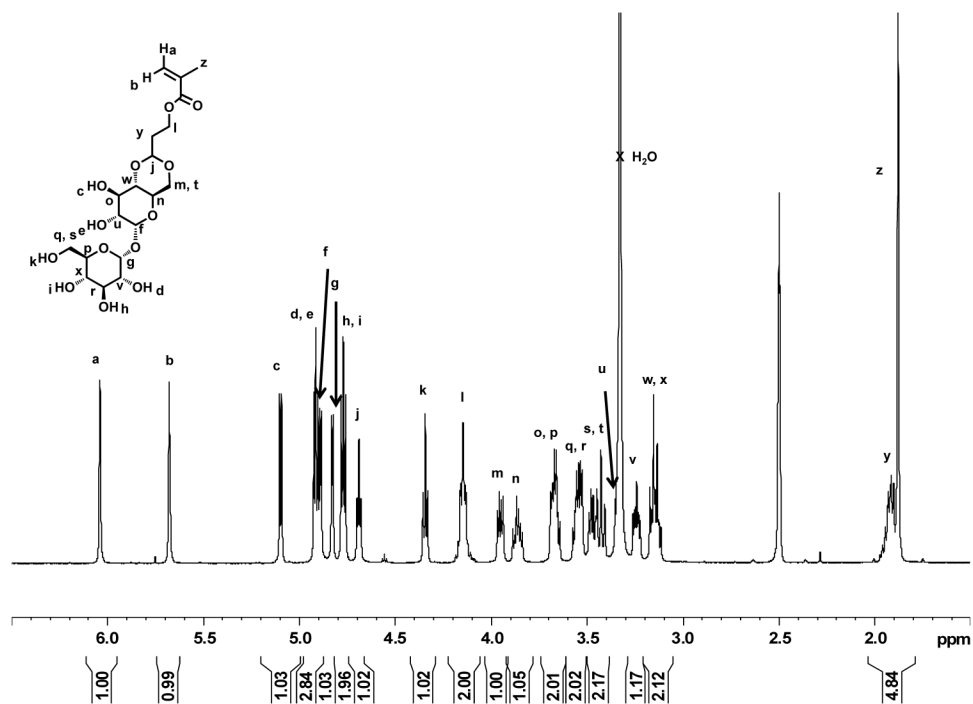


Figure 2-1. ^1H NMR spectroscopy of **MAT** (in D_6DMSO).

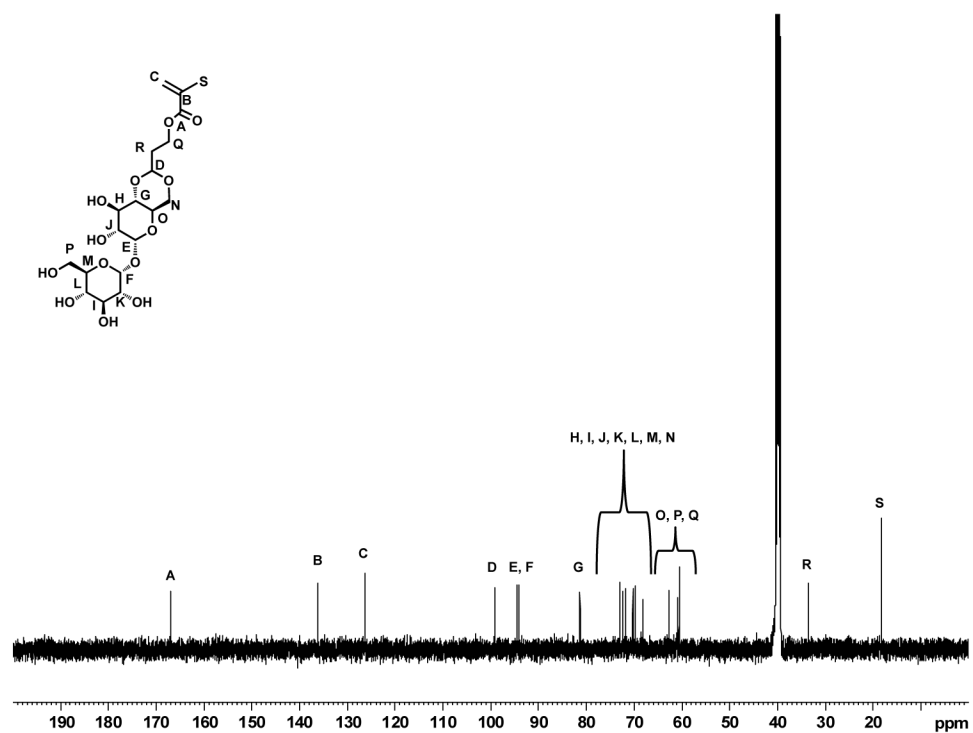


Figure 2-2. ^{13}C NMR spectroscopy of **MAT** (in D_6DMSO).

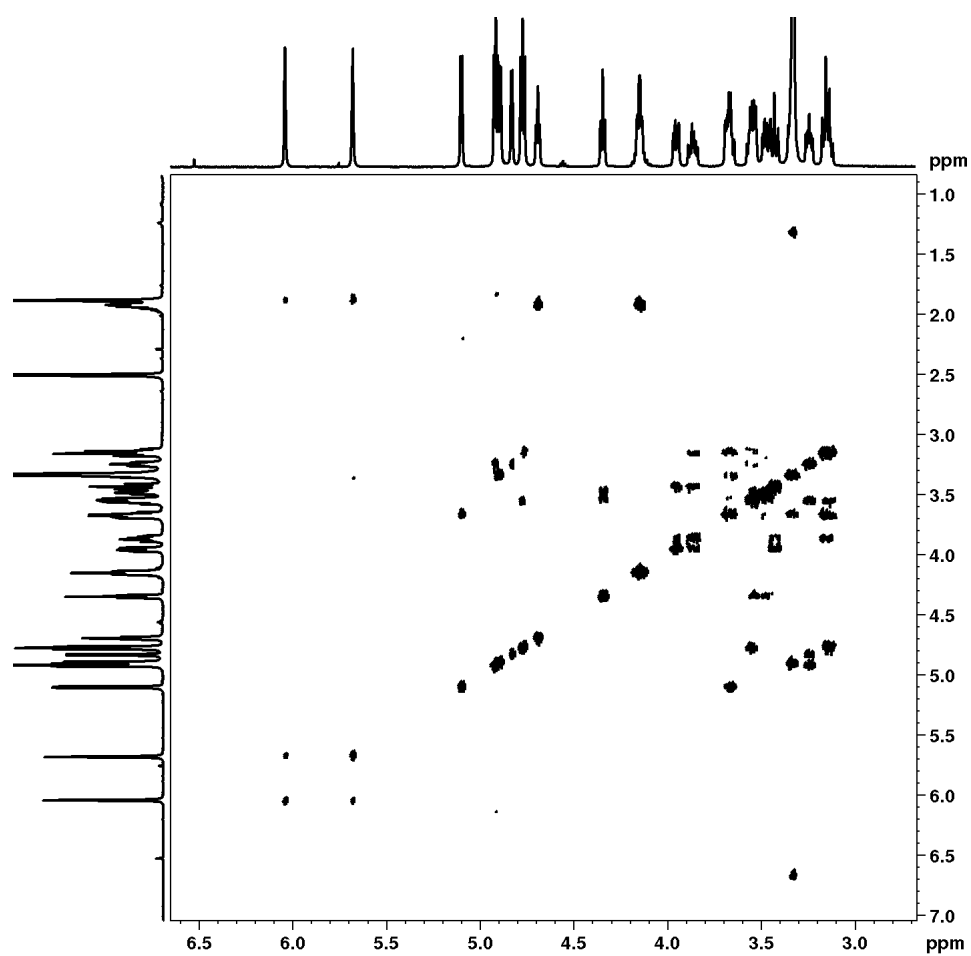


Figure 2-3. COSY spectroscopy of MAT (in D₆DMSO).

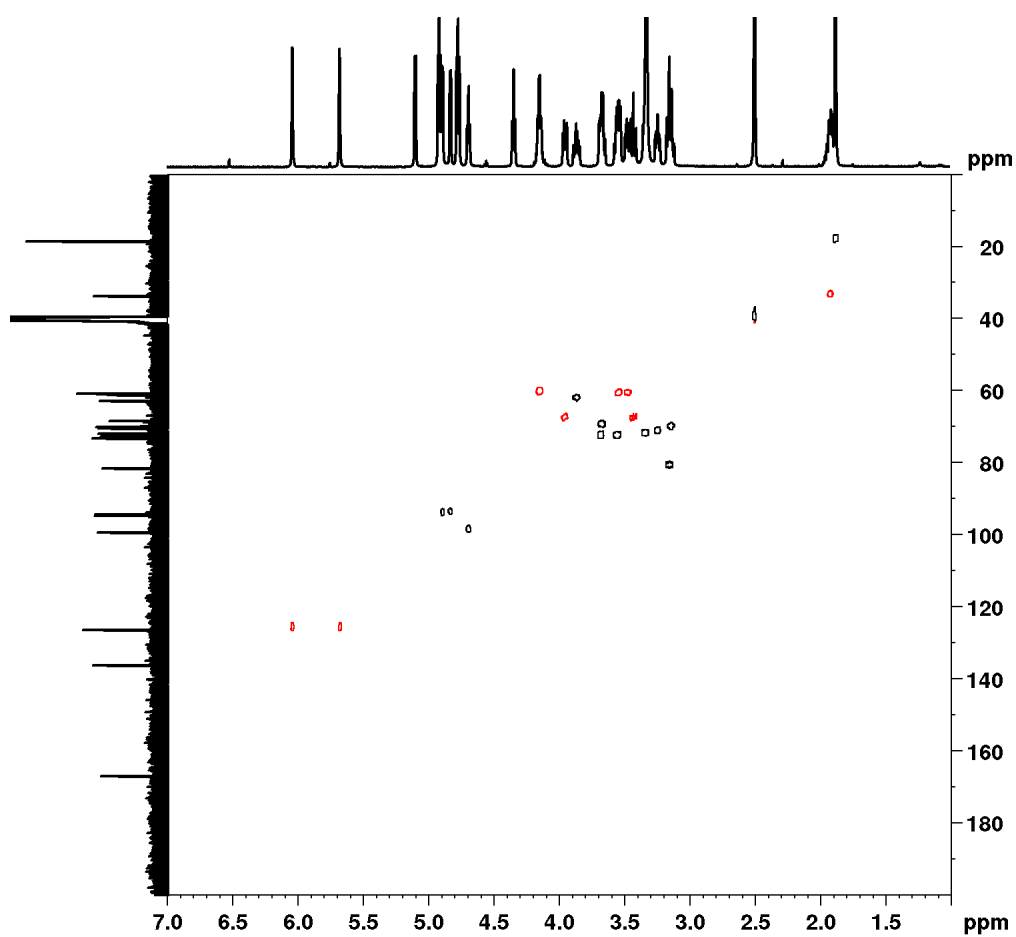


Figure 2-4. HSQC spectroscopy of MAT (in D₆DMSO).

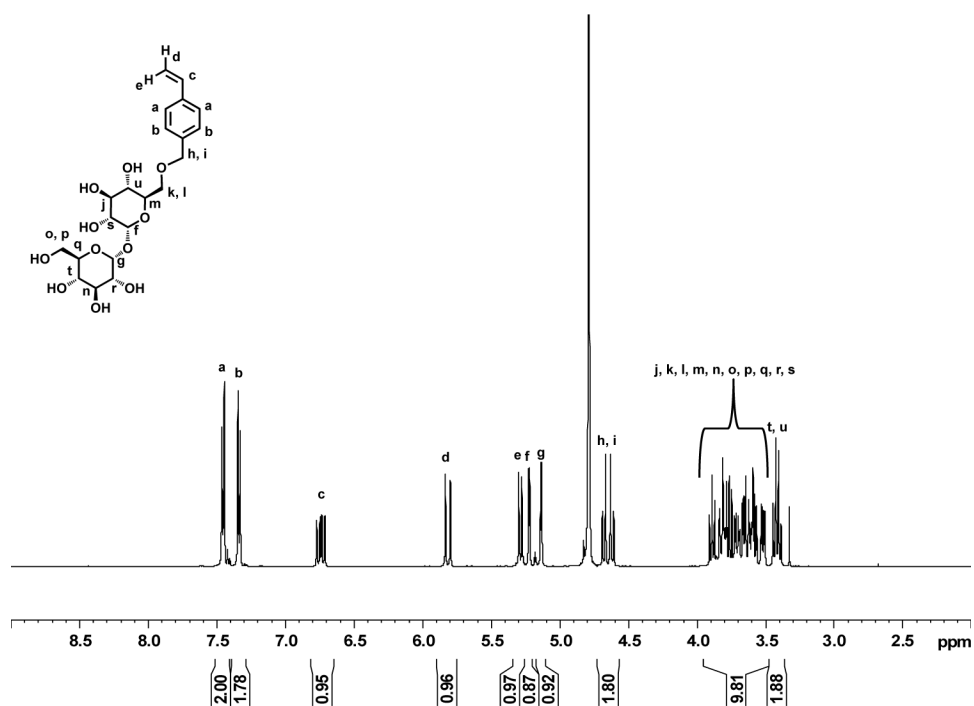


Figure 2-5. ¹H NMR spectroscopy of SET (in D₂O).

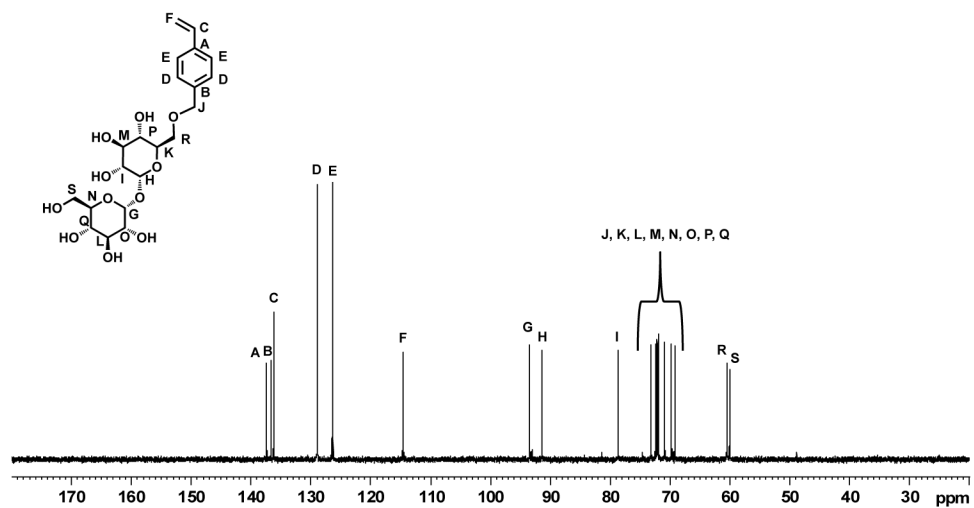


Figure 2-6. ¹³C NMR spectroscopy of SET (in D₂O).

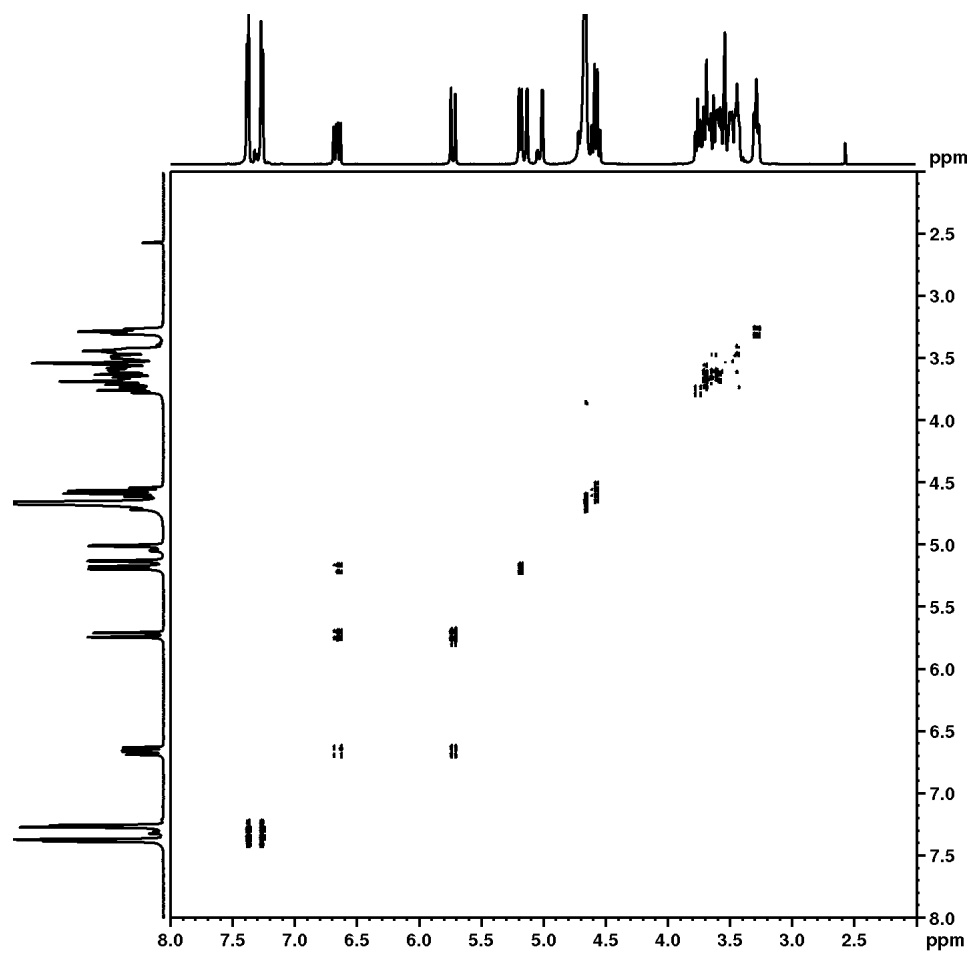


Figure 2-7. COSY spectroscopy of SET (in D₂O).

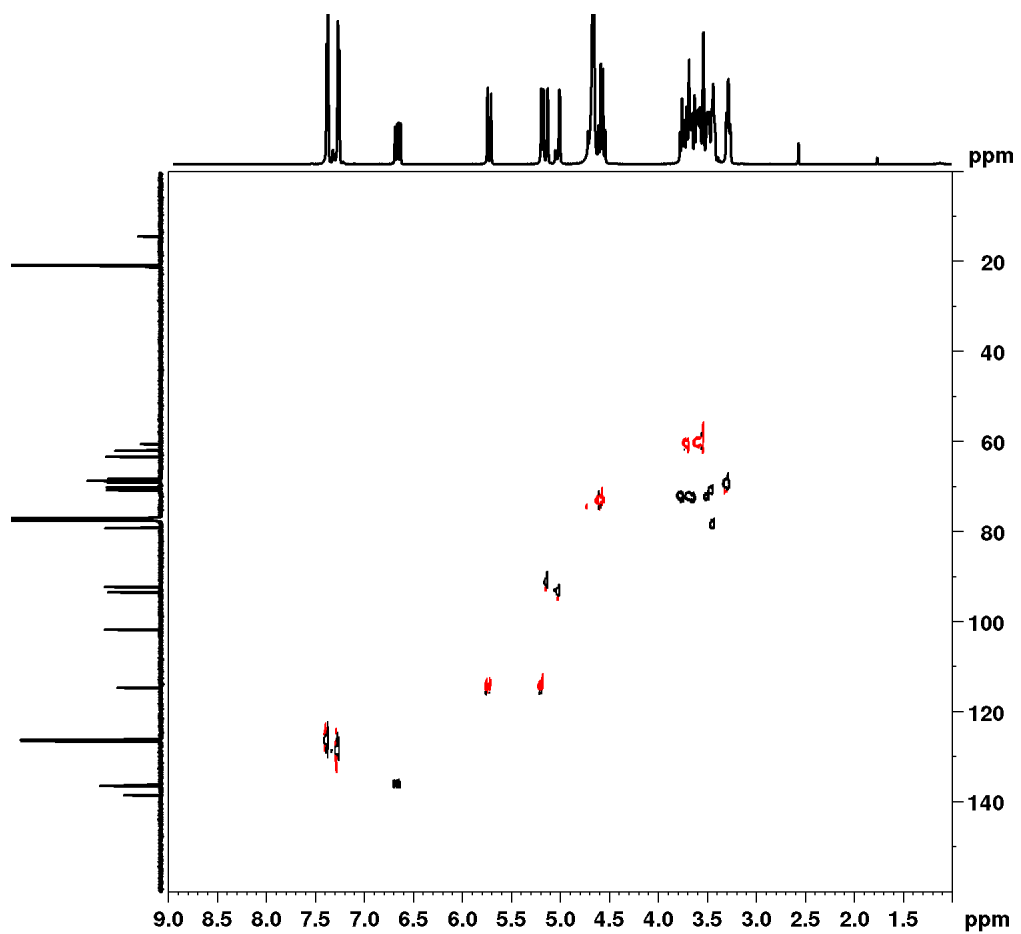
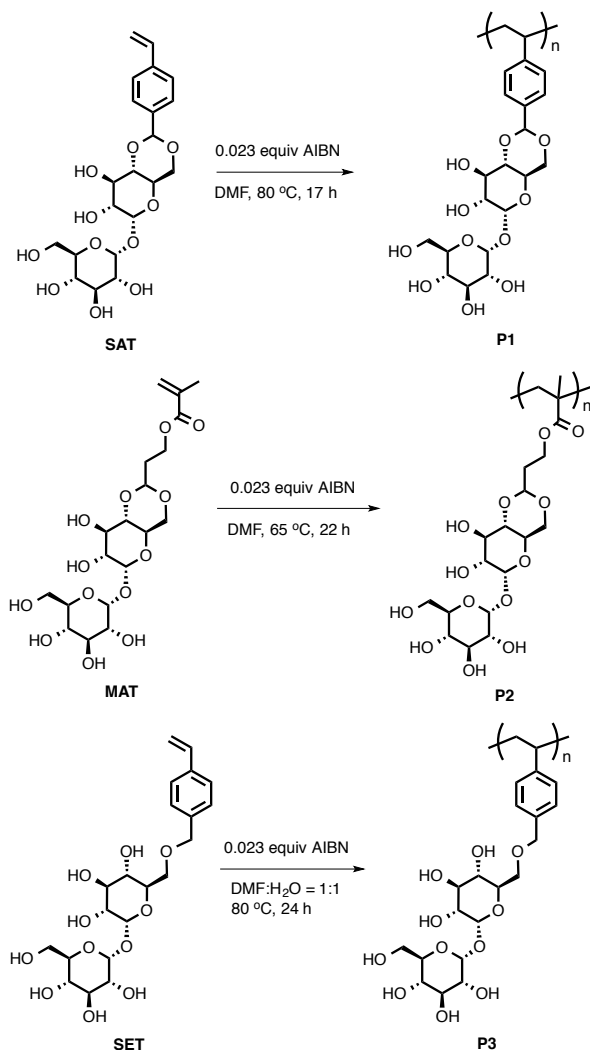


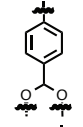
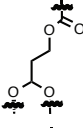
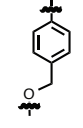
Figure 2-8. HSQC spectroscopy of SET (in D₂O).

Scheme 2-2. Synthesis of **P1-P3** through free radical polymerization mediated by AIBN.



The polymers were all prepared by free radical polymerization utilizing AIBN as an initiator. The polymerization temperature was 80 °C for the styrenyl-based monomers and 65 °C for the methacrylate. The resulting polymers **P1**, **P2**, and **P3** (Scheme 2-2) were prepared with number average molecular weights (M_n) between 14,600 g/mol and 23,400 g/mol (^1H NMR spectra of the polymers in Figure 2-9 to 2-11 and GPC traces in Figure 2-12). The M_n and polydispersity index (PDI) for all the polymers are summarized in Table 2-1.

Table 2-1. Molecular weight and PDI data of the trehalose polymers.

Polymer	Monomer	Linker to Trehalose	M _n by GPC (kDa)	PDI
P1	M1		19.5	2.01
P2	M2		23.4	2.40
P3	M3		14.6	1.77

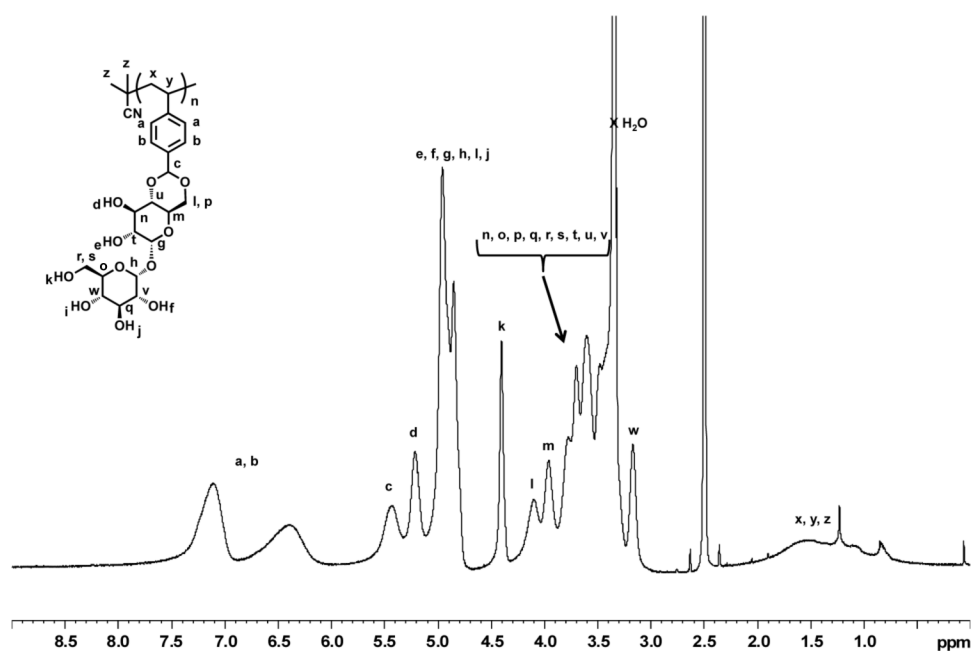


Figure 2-9. ¹H NMR spectroscopy of **P1** (in DMSO-D₆).

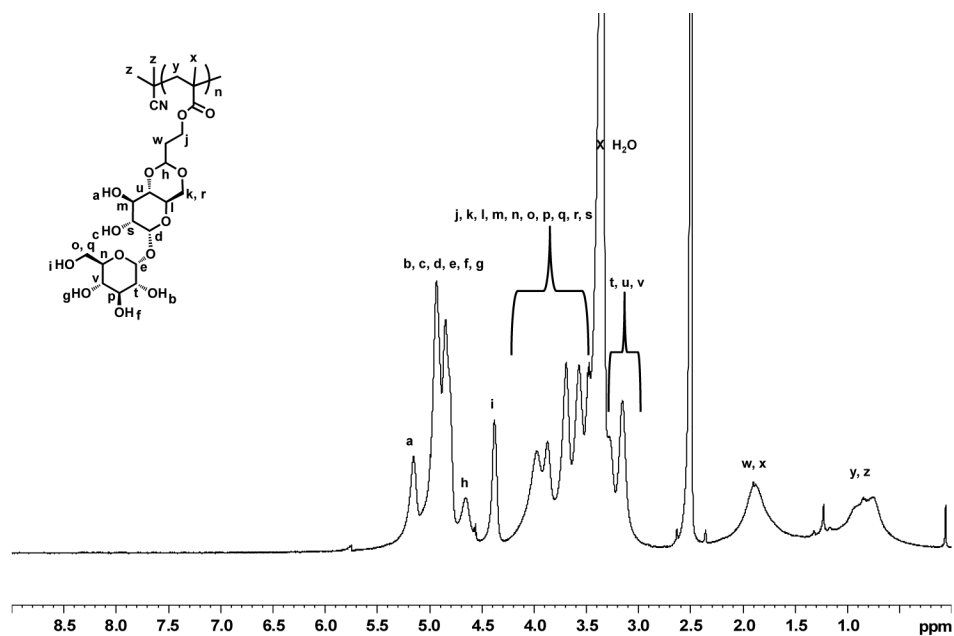


Figure 2-10. ^1H NMR spectroscopy of **P2** (in DMSO-D_6).

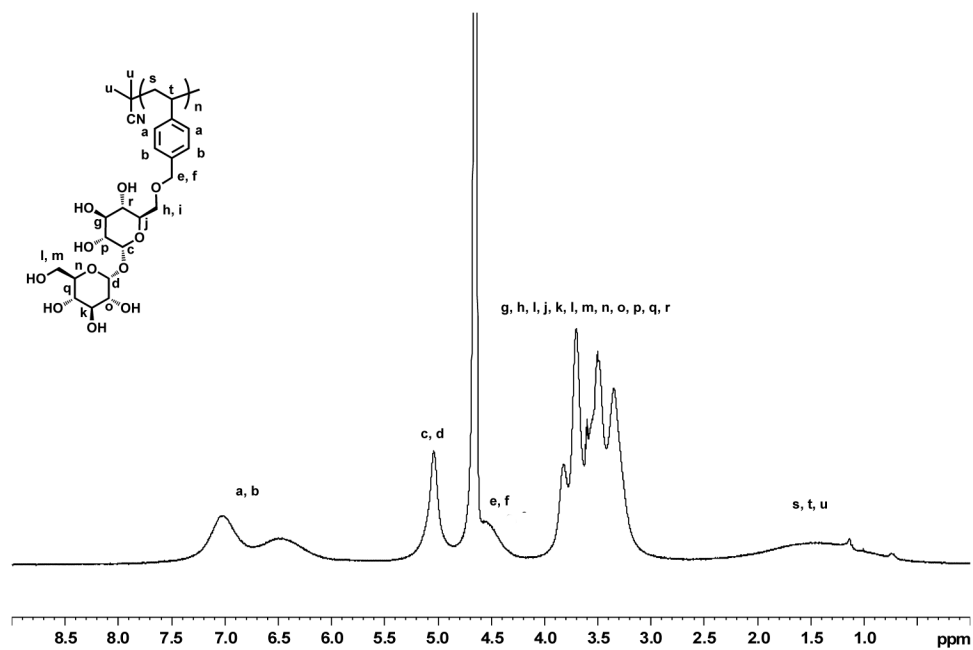


Figure 2-11. ^1H NMR spectroscopy of **P3** (in D_2O).

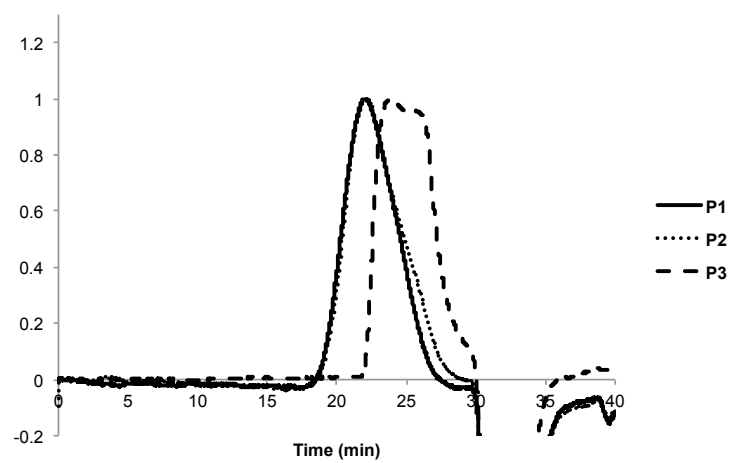


Figure 2-12. GPC traces of P1-P3.

Heat Burden Studies of Proteins with the Polymers

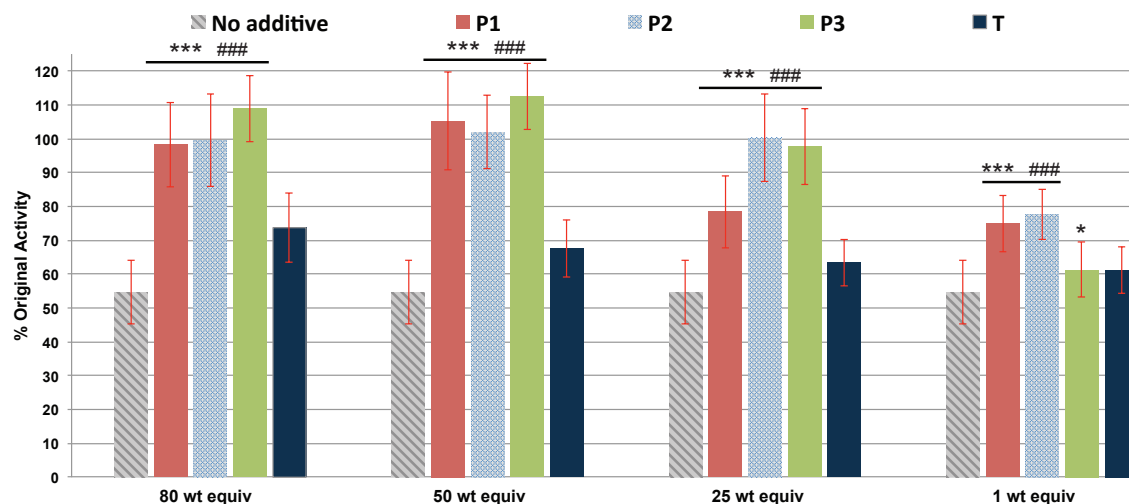


Figure 2-13. Activity of HRP (75 µg/mL) after 30 min incubation at 70 °C, with no additive, **P1**, **P2**, **P3**, and **T**. 80, 50, 25, and 1 wt equiv of each polymer were added to the protein. Trehalose was added to be equivalent to the amount of trehalose in **P1** for each set. Data shown as the average of n=12 with standard deviation. *** = $p < 0.001$ and * = $p < 0.05$ relative to no additive. #### = $p < 0.001$ and ## = $p < 0.01$ relative to trehalose.

The activity of HRP after exposure to 70 °C for 30 minutes with and without additives was assayed. HRP is an important heme-containing enzyme, which has been used in biotechnology and bioremediation. However, thermostability of the HRP limits its industrial application.³⁵ The activity of HRP was tested by addition of TMB, which produces a blue by-product in the presence of HRP. HRP samples were prepared without any additive, with **P1-P3**, or trehalose. To determine effectiveness of the polymers, 1 wt equiv to 80 wt equiv of each polymer relative to HRP was tested. Weight equivalents were utilized so that the same amount of trehalose was added in each set regardless of which polymer were used, and any inaccuracies or differences in molecular weight of the polymers would not be a factor. Trehalose was added such

that the amount was equivalent to the trehalose repeating units in **P1** for each set; in this way, trehalose and the polymer were directly comparable.

Samples that had been heated were tested for remaining HRP activity, and the percent original activities were calculated using the same concentration of HRP without heating and additive (Figure 2-13). Only 55% of the original activity was observed when HRP itself was heated to 70 °C. Yet, activity was completely retained (100%) for all of the glycopolymers (**P1-P3**) when 50 or 80 wt equiv were added. At 25 wt equiv, differences between the polymers were observed; **P1** stabilized better than no additive (80%) but less than the other polymers that retained HRP activity at 100%. With 1 equiv, all the polymers stabilized the protein slightly better than no additive, while **P3** had a weaker effect compared to the other polymers. The same equivalents of trehalose also stabilized HRP, but the HRP activity was lower compared to the polymers. Increasing the concentration of trehalose up to 4000 wt equiv, improved the stabilization observed (74%, Figure 2-14a) but the value was still well below that observed for the polymers. As a control, the polymers alone were tested. **P1-P3** were not substrates for the assay; there was no observed activity in the absence of the enzyme (Figure 2-14b).

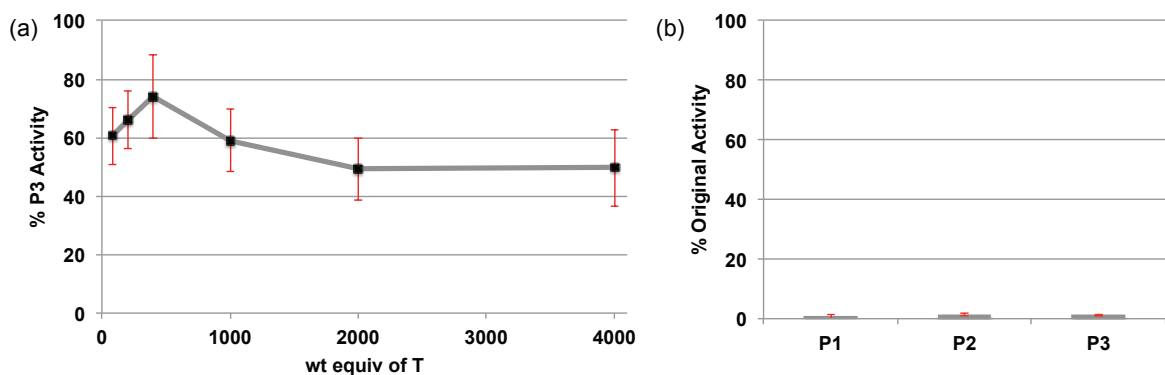


Figure 2-14. (a) Activity of HRP with 80, 200, 400, 1000, 2000, and 4000 wt eq of **T**. Results are displayed % activity in addition of **P3**. The average and standard deviation of 8 repeats normalized to % HRP activity with highest concentration of **P3** tested in each case (shown as a dashed line at 100 %) are shown. (b) Activity assays of polymers only (no protein) for HRP. Data shown are based on 6 repeats.

Circular dichroism investigations were undertaken in addition to the activity assays. CD spectra were taken at 70 °C with and without **P2** and also after cooling back to room temperature; the results were compared to pristine HRP. The data showed a similar trend as the activity studies. Loss of some secondary structure was observed for both HRP and HRP with **P2** (Figure 2-15a). However after cooling, the HRP heated in the presence of **P2** regained its secondary structure to a greater extent. This result suggests that denaturation was reversible and that the polymer may help the protein refold (Figure 2-15b).

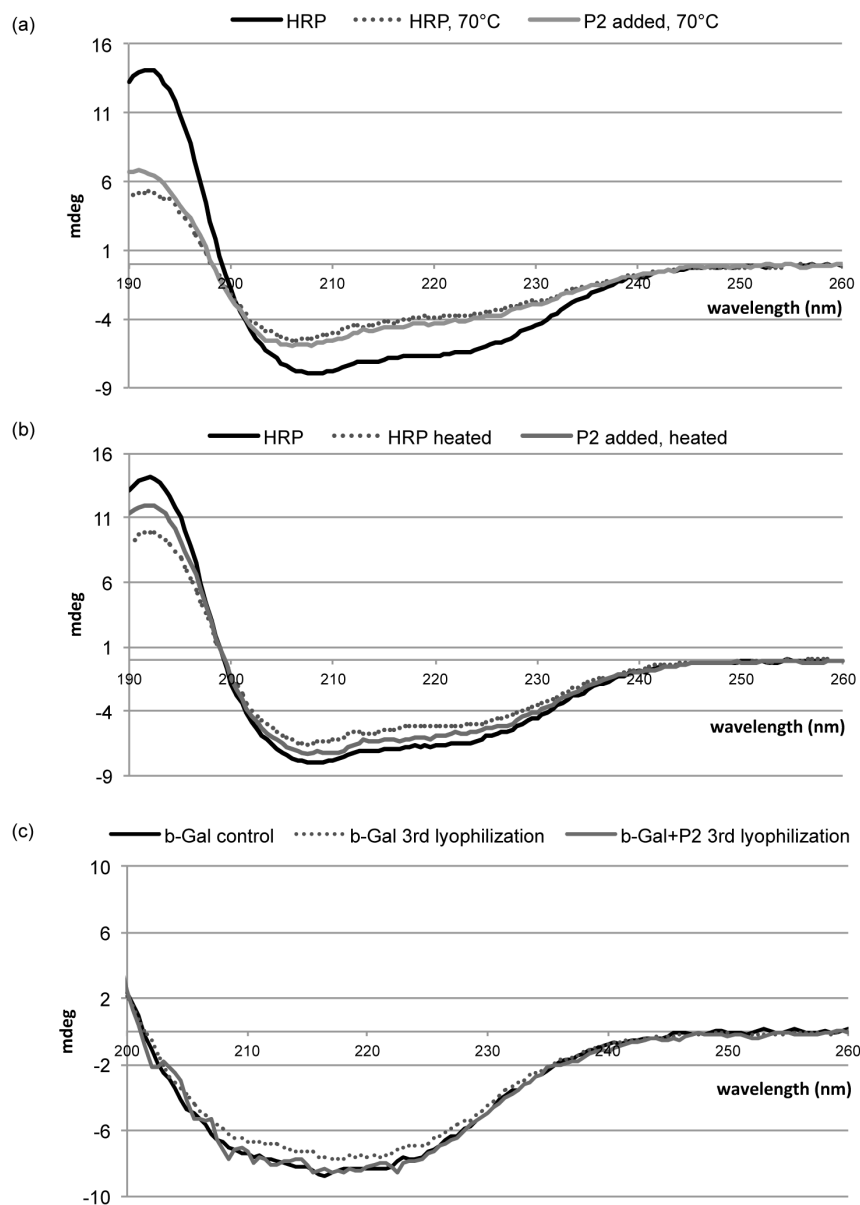


Figure 2-15. Circular dichroism data of (a) HRP control (without heating), HRP after incubation for 30 min at 70 °C, and after incubation with **P2** for 30 min at 70 °C taken at 70 °C (b) HRP control, HRP after incubation for 30 min at 70 °C, and after incubation with **P2** for 30 min at 70 °C taken at 25 °C after 3 h cooling, and (c) β -Gal control (before lyophilization), β -Gal after three cycles of lyophilization, and after three cycles of lyophilization with **P2**.

To test an additional enzyme to heat, GOx was also investigated. GOx is also an

interesting protein with industrial importance that is currently used in applications ranging from fiber bleaching, food preservatives, to glucose detection kits.³⁶ However, its structural instability is problematic. It was found that 20 wt equiv of trehalose glycopolymers **P1** through **P3** stabilized GOx to 50 °C heat for 30 min better than no additive; however, the effect was not as dramatic because the protein itself was relatively stable at these conditions (Figure 2-16a). In this case trehalose itself did not stabilize the protein and was statistically the same as no additive at all. In this case the polymers did not affect the assay result as well (Figure 2-16b).

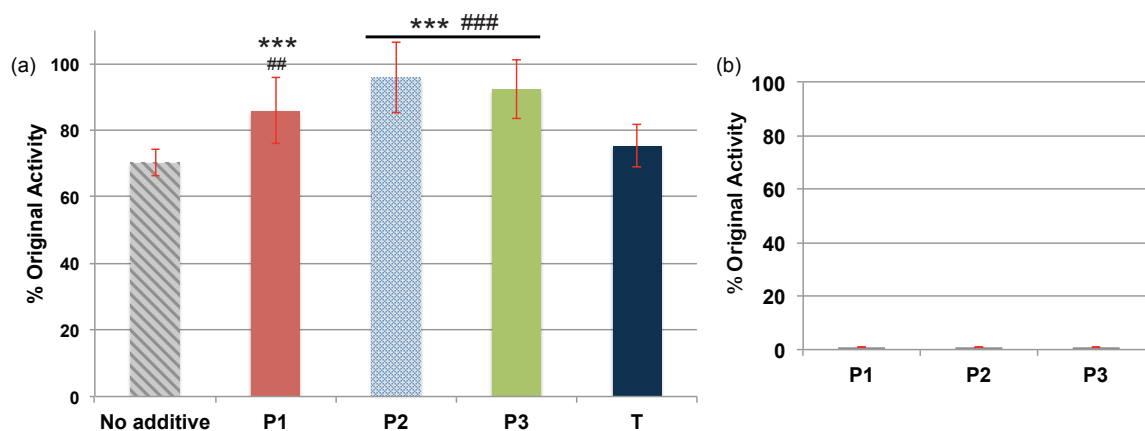


Figure 2-16. (a) Activity of GOx (1 mg/mL) after 30 min incubation at 70 °C, with no additive, **P1**, **P2**, **P3**, **P4**, and **T** to the GOx. Average and standard deviation values of 12 repeats are shown. *** = $p < 0.001$ and ** = $p < 0.01$ compared to no addition, ### = $p < 0.001$ and ## = $p < 0.01$ compared to **T**. (b) Activity assays of polymers only (no protein) for GOx (6 repeats).

Lyophilization Study

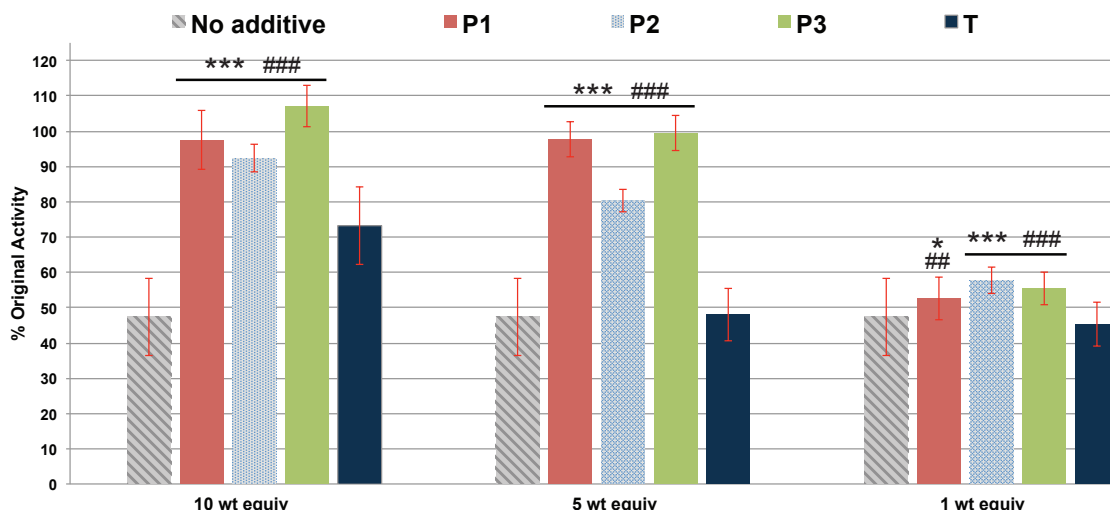


Figure 2-17. Activity of β -Gal (100 μ g/mL) after 3 cycles of lyophilization with no additive, **P1**, **P2**, **P3**, and **T** added to the β -Gal. 10, 5, and 1 wt equiv of each polymer were added to the protein. Trehalose was added to be equivalent to the amount of trehalose in **P1** for each set. Data shown as the average of $n=12$ with standard deviation. *** = $p < 0.001$, * = $p < 0.05$ relative to no additive. #### = $p < 0.001$ and ## = $p < 0.01$ relative to trehalose.

The stabilization of β -galactosidase (β -Gal) was also tested after multiple lyophilization cycles. β -Gal is an important enzyme that catalyzes hydrolysis of β -galactosides including lactose. Furthermore, a recent study shows that it could be used in gene replacement therapy.³⁷ However, β -Gal is known to be easily denatured by lyophilization or freeze-thaw cycles.³⁴ The activity of β -Gal was measured by reacting with ONPG, which releases *o*-nitrophenol, a yellow colored product that absorbs light at 405 nm. After 3 cycles of lyophilization, β -Gal had only 47% remaining activity, while 10 wt equiv **P1** through **P3** maintained activity from 85 to 108%, depending on the polymer and amount added (Figure 2-17). It has been reported that for GOx adding trehalose results in an increase in affinity of the substrate for the enzyme.³⁶ This was

proposed to be due to the sugar enhancing the protein/substrate binding or stabilizing that complex.³⁶ Thus, it is not unprecedented that greater than 100% activity was observed. The result showed that the polymers outperformed trehalose, since the trehalose maintained only 73% of the original activity maximum. At 5 wt equiv, the polymers maintained activity 80 to 100%, while trehalose was the same as no additive. At 1 wt equiv, the protein was only marginally stabilized by **P1**, **P2** and **P3**.

From this data the styrenyl polymers **P1** and **P3** provided the best stabilization, and were better than trehalose. Unlike the HRP heat burden study, when higher concentrations of trehalose were tested (100, 500, and 1000 wt equiv) β -Gal was completely stabilized (Figure 2-18a). However, by using the trehalose polymers, a much smaller amount was required for stabilization. To ensure that the polymers themselves did not have activity in the assay giving erroneous results, the polymers themselves were tested (Figure 2-18b). Again, the polymers were not substrates for the assay and no activity was observed.

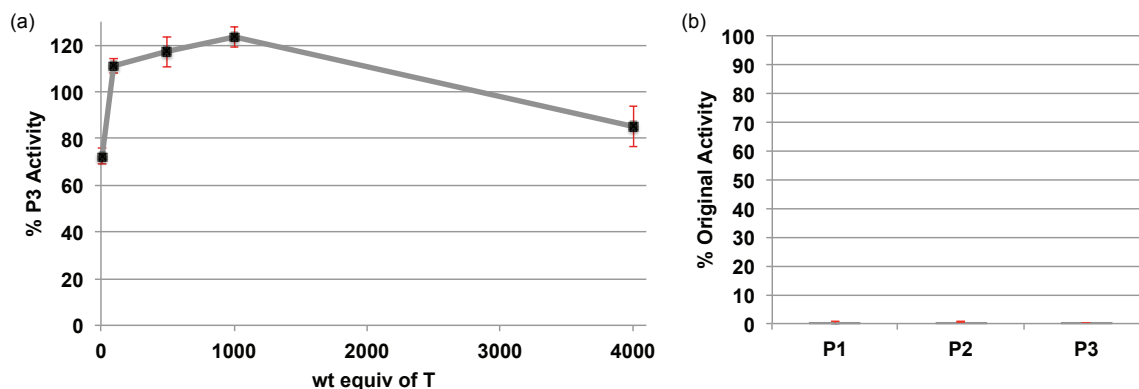


Figure 2-18. (a) Activity of β -Gal with 80, 200, 400, 1000, 2000, and 4000 wt eq of **T**. Results are displayed % activity in addition of **P3**. The average and standard deviation of 8 repeats normalized to % β -Gal activity with highest concentration of **P3** tested in each case (shown as a dashed line at 100 %) are shown. (b) Activity assays of polymers only (no protein) for β -Gal. Data shown are based on 6 repeats.

Finally, to rule out inaccuracies in the weight equivalent used due to differences in water, thermogravimetric analysis (TGA) of the polymer and trehalose was undertaken (Figure 2-19). The polymer contained more water (2.4%) than trehalose (1.4%). Interestingly, there was one regime of water loss for the polymer, while for trehalose there was a typically loosely held phase (0.8%) and tightly held water (0.6%). The latter is reported to be due to crystallization of trehalose.³⁸ Regardless, the TGA data clearly showed that the polymer weight was underestimated in these experiments. Thus water content was also not a factor, and the polymers did stabilize at lower concentrations based on trehalose content.

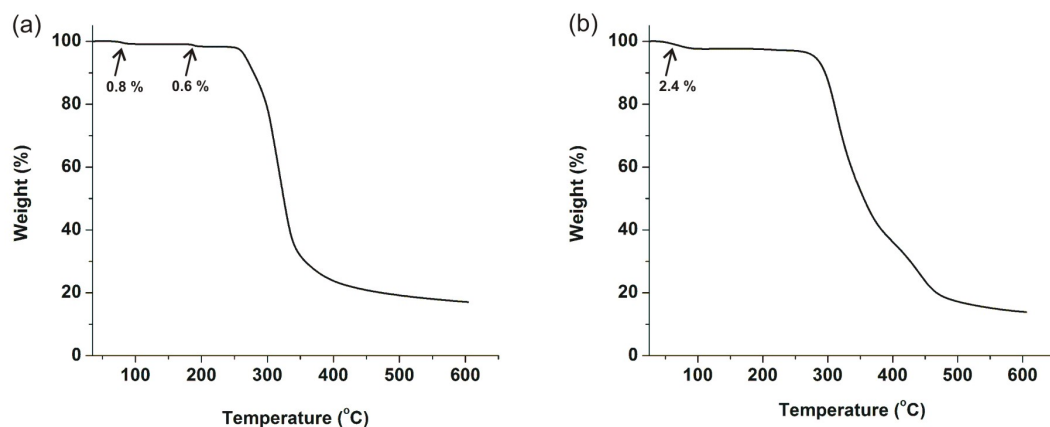


Figure 2-19. TGA curves of (a) **T**, (b) **P1** showing percentage mass loss with respect to temperature.

Effect of Polymer Molecular Weight on Protein Stabilization

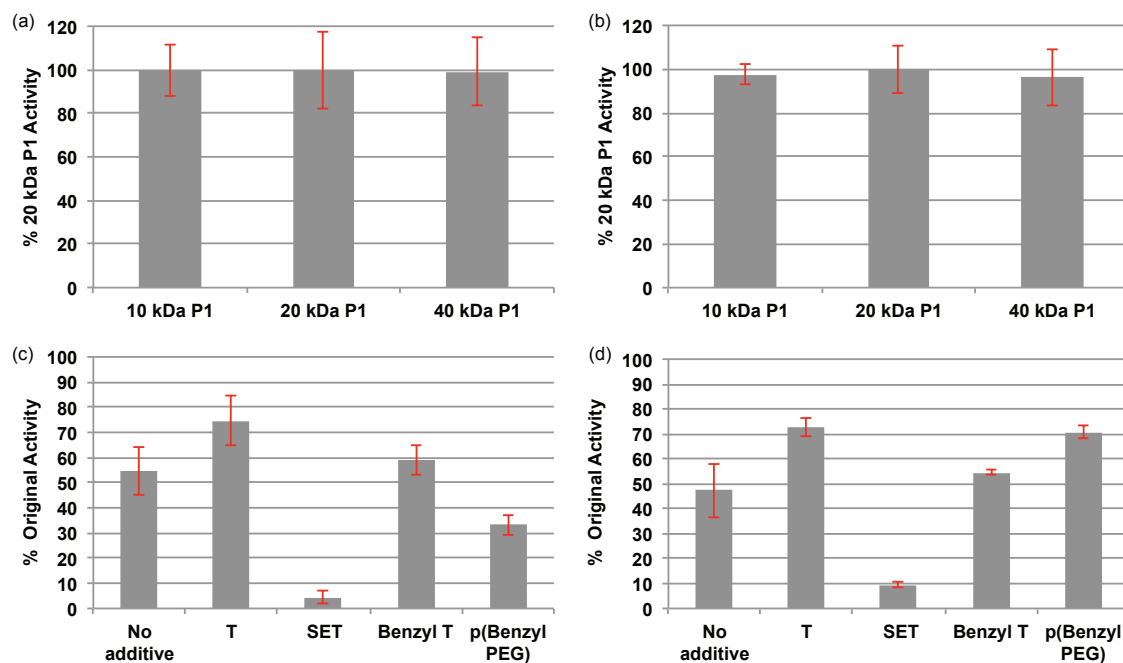


Figure 2-20. Activity of (a) HRP (75 µg/mL) after 30 min heating at 70 °C with 25 wt equiv of 10, 20, 40 kDa **P1**, (b) β-Gal (100 µg/mL) after 3 cycles of lyophilization with 2.5 wt equiv of 10, 20, 40 kDa **P1**, (c) HRP (75 µg/mL) after 30 min heating at 70 °C with 80 wt equiv of **T**, **SET**, **Benzyl T**, **p(Benzyl PEG)**,

Benzyl T, and **p(Benzyl PEG)**, and (d) β -Gal (100 μ g/mL) after 3 cycles of lyophilization with 10 wt equiv of **T**, **SET**, **Benzyl T**, and **p(Benzyl PEG)**.

To investigate the effect of polymer molecular weight on protein stabilization, **P1** was synthesized with a M_n of 11,900 g/mol (PDI = 2.50) and 40,800 g/mol (PDI = 2.80), and these in addition to the 19,500 g/mol **P1** that was used previously were tested. The polymers were added to HRP and β -Gal at 25 wt equiv and 2.5 wt equiv, respectively, prior to heating and lyophilization stresses. The remaining activity after the experiments was same regardless of the M_n or both HRP and β -Gal (Figure 2-20a and b) showing that the molecular weight had no effect, at least in this regime.

Investigation of Stabilization Properties

During lyophilization, protein denaturation can be observed due to ice formation, a change in concentration of solutes as a result of crystallization (which can lead to osmotic shock or large changes in pH values), or due to cold denaturation.³⁹ Trehalose is known to disrupt the hydrogen bonded network of water molecules causing depression of ice formation.⁴⁰ This leads to a decrease in the enthalpy of melting and crystallization of water and also to a reduction of the temperature of crystallization (T_c).⁴¹ To evaluate whether or not the polymers have the same properties, differential scanning calorimetry (DSC) was utilized. Trehalose or **P3** (1, 2 and 3 mol % based on trehalose) were added to water. It was found that for both trehalose and the polymer the enthalpy of melting and crystallization were reduced to a similar extent as was reported in literature (Table 2-2).⁴¹ The depression in enthalpy values increased with increasing concentration of trehalose and **P3**. In addition, the T_c was also depressed for both trehalose and the polymer, the relative amount of which depended on the mol %. These results show that the

polymer has similar properties in this respect to trehalose and suggest that the trehalose glycopolymers are capable of stabilizing proteins against ice formation induced denaturation or solute crystallization during lyophilization through a mechanism similar to that of trehalose.

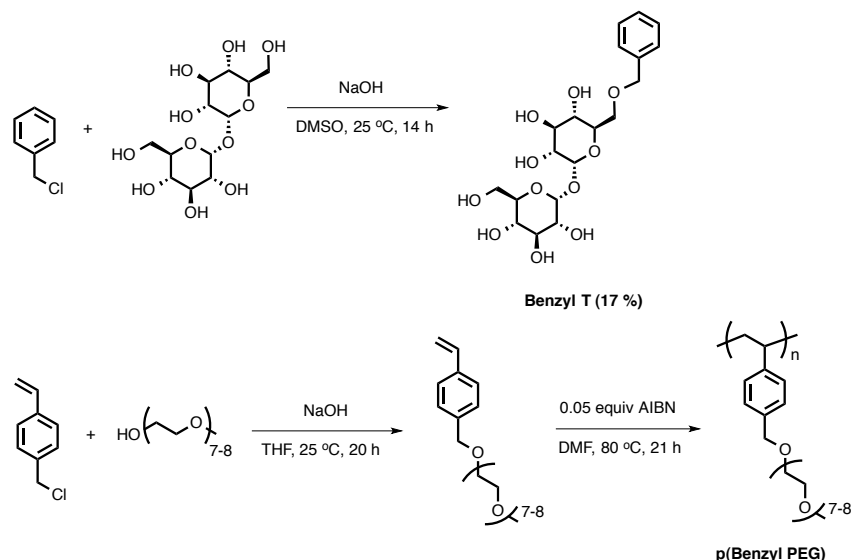
Table 2-2. Change in melting and crystallization enthalpy of water upon addition of 1-3 mol % trehalose or trehalose repeating units in **P3**.

Sample	H_m (J/g)	H_c (J/g)	DH_m (J/g)	DH_c (J/g)
Water	303.6	-270.8		
1 % Trehalose	207.6	-199.5	-96.0	71.3
2 % Trehalose	158.3	-154.5	-145.3	116.3
3 % Trehalose	100.4	-112.1	-203.2	158.7
1 % Trehalose in P3	220.7	-205.4	-82.9	65.4
2 % Trehalose in P3	153.4	-139.5	-150.2	131.3
3 % Trehalose in P3	107.6	-104.5	-196.0	166.3

The polymers all have hydrophobic backbones attached to the trehalose, meaning that they can be considered nonionic surfactants. To investigate the effect of hydrophobizing trehalose, the stabilization ability of the trehalose monomers and other analogues were determined. For this study, benzyl trehalose (**Benzyl T**) was synthesized, which is a **SET** analogue lacking the vinyl group. Poly(benzyl ether poly(ethylene glycol) methyl ether) (**p(Benzyl PEG)**) with styrenyl backbone and hydrophilic PEG side chains was also prepared in order to understand whether the trehalose component is required (Scheme 2-3). Interestingly, after heating and lyophilization, **SET** itself decreased HRP activity to 4 % and β -Gal activity to

10 % (Figure 2-20c and 2-20d). This may be due to reaction of the monomer unit with the proteins. In both experiments **Benzyl T** slightly stabilized protein, but less than trehalose itself. In the heat burden study **p(Benzyl PEG)** decreased the activity of HRP compared to having no additive. On the other hand, the stabilization effect of **p(Benzyl PEG)** was similar to trehalose in the case of lyophilization of β -Gal. Neither the **Benzyl T** or **p(Benzyl PEG)** stabilized as well as the polymers, suggesting that having hydrophilic-hydrophobic character in the polymer was not the only factor in stabilization.

Scheme 2-3. Synthesis of **Benzyl T** and **p(Benzyl PEG)**.



P1 and **P2** both contain acetal linkages, which are susceptible to cleavage under slightly acidic conditions. If the acetals did hydrolyze, then polymeric aldehydes would form. Polyaldehydes such as glutaraldehyde are known to stabilize proteins. To determine this, the polymers were subjected to conditions utilized in the activity studies, namely heating to 70 °C for 30 minutes, followed by lyophilization or were incubated for 37 °C for 48 h. The samples were then characterized by ¹H NMR spectroscopy, IR, and the Purpald assay. No aldehyde

formation was detected in any case. The hydrophobic backbone of the polymers likely protects the acetal from cleavage.

Effect of Protein Concentration

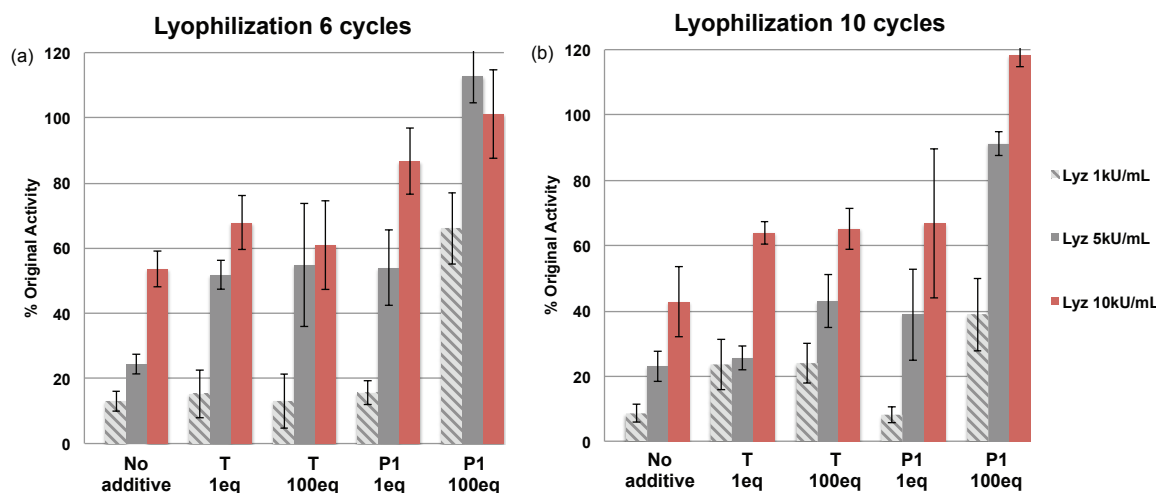


Figure 2-21. Activity of Lyz with no additive, 1 mol equiv **T**, 100 mol equiv **T**, 1 mol equiv **P1**, and 100 mol equiv **P1** after (a) 6 cycles and (b) 10 cycles of lyophilization.

To investigate the effect of protein concentration in preservation of activity, 1 kU/mL, 5 kU/mL, and 10 kU/mL Lyz solutions were prepared. Each solution was treated with no additive, 1 mol equiv **T**, 100 mol equiv **T**, 1 mol equiv **P1**, or 100 mol equiv **P1**. The Lyz solutions were lyophilized for six or ten cycles and the activities were compared to the activity of Lyz before lyophilization. As expected, the highest concentration, 10 kU/mL Lyz solution, showed the least degradation under all conditions, followed by the 5 kU/mL and 1 kU/mL Lyz solutions. After both six and ten cycles of lyophilization the stabilization effect of 1 molar equiv of **P1** was comparable to trehalose; however, 100 molar equiv **P1** was superior than other additives under all conditions tested. This data confirms that **P1** is a stabilizer for Lyz, regardless of the

concentration utilized. The effect was greatest when 100 mole equivalents of polymer were utilized.

Discussion

Together, the above results show that trehalose glycopolymers are good candidates for use as additives to stabilize proteins. In each of the three proteins studied, the trehalose glycopolymers stabilized the protein significantly better than no additive. Trehalose is currently used as one of the excipients in high concentrations (10-100 mg/mL) in protein-based therapeutics, such as Advate[®], Avastin[®], Lucentis[®], and Herceptin[®]. It may be possible to utilize the trehalose glycopolymers as alternative excipients, especially since the polymers stabilized the proteins better than trehalose to each stress. In addition, enzymes such as HRP, GOx, and β -Gal are used in industry and some have potential use in therapeutic applications. Thus, stabilization of these proteins to stresses which inactivate them is important.

The exact mechanism of how trehalose protects proteins or cells is not known. However, there are three main theories that have been proposed for this osmolyte. These are vitrification,⁴² water replacement,^{43,44} and water entrapment.⁴⁵ DSC results have demonstrated that the polymers are similar to trehalose with respect to suppression of the enthalpy of melting and reduction of the crystallization temperature. However, the polymers stabilized at a lower concentration (based on trehalose to trehalose units) than the sugar itself to lyophilization and were superior all together for heat stabilization. Thus, additional stabilization mechanisms must be in effect. The polymers all have hydrophobic backbones and hydrophilic (trehalose) side chains. Thus, they are nonionic surfactants. Nonionic surfactants are known excipients and are proposed to stabilize proteins by preventing proteins from adsorbing onto hydrophobic surfaces, directly interacting with hydrophobic residues of proteins, and may even act as chaperones for protein unfolding.⁹ To begin to look at the effect of hydrophobic moieties on trehalose, **Benzyl T** (an analogue of the **SET**) and **p(Benzyl PEG)** (which has the polystyrene backbone, but no

trehalose) were compared. Neither had the stabilizing ability of the trehalose polymers. The exact mechanisms responsible for the superior stabilization of the trehalose polymers remains to be determined. Yet it is clear that the polymers combine two important classes of excipients, namely the sugars and surfactants.

Different polymers were tested in order to ascertain the importance of attachment site, chemistry, and polymer backbone. **SAT**, **MAT**, and **SET** were easily prepared in one to two steps without requiring protecting group strategies. Although the yields were low in each case, trehalose is readily available as a food additive and is inexpensive. The polymers were all polymerized by simple free radical polymerization. **P1-P3** could be utilized directly after synthesis. **P1** and **P2** contained six, and **P3** had seven hydroxyl groups. All polymers were highly water-soluble. All the polymers were fairly similar in their ability to stabilize HRP, GOx, and β -Gal, especially at high concentrations, although there were slight differences that were observed as lower concentrations were used. There did not appear to be differences with regard to the site of attachment or chemistry of the linkages; the polymer backbone was the dominant effect. For heat treatment of HRP, the polymethacrylate backbone polymers, **P2**, stabilized best at lower concentrations. For lyophilization of β -Gal **P1** and **P3**, the styrenyl polymers, were better. With these straightforward syntheses, it should be possible to find a trehalose polymer stabilizer for many different types of proteins and stresses, even at low concentrations of polymer.

2.4 Conclusions

In this report, we described the synthesis of three trehalose glycomonomers and their respective homopolymers. The three glycopolymers all stabilized HRP and GOx to heat and β -Gal during lyophilization, compared to no additive, as well as to same amount of trehalose. The results show that the trehalose glycopolymers should be further explored as excipients in the pharmaceutical industry to protect protein-based drugs from denaturation, to protect enzymes used industrially as reagents, and in protein research and development.

2.5 References

- (1) Leader, B.; Baca, Q. J.; Golan, D. E. *Nat. Rev. Drug Discovery* **2008**, 7, 21-39.
- (2) Drews, J. *Science* **2000**, 287, 1960-1964.
- (3) Oobatake, M.; Ooi, T. *Prog. Biophys. Mol. Biol.* **1993**, 59, 237-284.
- (4) Wang, W. *Int. J. Pharm.* **2000**, 203, 1-60.
- (5) Maity, H.; O'Dell, C.; Srivastava, A.; Goldstein, J. *Curr. Pharm. Biotechnol.* **2009**, 10, 761-766.
- (6) Carrasquillo, K. G.; Sanchez, C.; Griebenow, K. *Biotechnol. Appl. Biochem.* **2000**, 31, 41-53.
- (7) Kasper, J. C.; Friess, W. *Eur. J. Pharm. Biopharm.* **2011**, 78, 248-263.
- (8) Crowe, J. H.; Carpenter, J. F.; Crowe, L. M.; Anchordoguy, T. J. *Cryobiology* **1990**, 27, 219-231.
- (9) Kamerzell, T. J.; Esfandiary, R.; Joshi, S. B.; Middaugh, C. R.; Volkin, D. B. *Adv. Drug Delivery Rev.* **2011**, 63, 1118-1159.
- (10) Arakawa, T.; Tsumoto, K.; Kita, Y.; Chang, B.; Ejima, D. *Amino Acids* **2007**, 33, 587-605.
- (11) Chen, B.; Bautista, R.; Yu, K.; Zapata, G. A.; Mulkerrin, M. G.; Chamow, S. M. *Pharm. Res.* **2003**, 20, 1952-1960.
- (12) Arakawa, T.; Timasheff, S. N. *Biophys. J.* **1985**, 47, 411-414.
- (13) Auton, M.; Bolen, D. W.; Rosgen, J. *Proteins-Structure Function and Bioinformatics* **2008**, 73, 802-813.
- (14) Bolen, D. W. *Methods* **2004**, 34, 312-322.
- (15) Kaushik, J. K.; Bhat, R. *J. Biol. Chem.* **2003**, 278, 26458-26465.

- (16) Kendrick, B. S.; Chang, B. S.; Arakawa, T.; Peterson, B.; Randolph, T. W.; Manning, M. C.; Carpenter, J. F. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 11917- 11922.
- (17) Kadoya, S.; Fujii, K.; Izutsu, K.-i.; Yonemochi, E.; Terada, K.; Yomota, C.; Kawanishi, T. *Int. J. Pharm.* **2010**, *389*, 107-113.
- (18) Kim, Y. S.; Jones, L. S.; Dong, A.; Kendrick, B. S.; Chang, B. S.; Manning, M. C.; Randolph, T. W.; Carpenter, J. F. *Protein Sci.* **2003**, *12*, 1252-1261.
- (19) Chuang, V. T. G.; Kragh-Hansen, U.; Otagiri, M. *Pharm. Res.* **2002**, *19*, 569-577.
- (20) Thyagarajapuram, N.; Olsen, D.; Middaugh, C. R. *J. Pharm. Sci.* **2007**, *96*, 3363- 3378.
- (21) Yoshioka, S.; Aso, Y.; Kojima, S. *Pharm. Res.* **1997**, *14*, 736-741.
- (22) Garzon-Rodriguez, W.; Koval, R. L.; Chongprasert, S.; Krishnan, S.; Randolph, T. W.; Warne, N. W.; Carpenter, J. F. *J. Pharm. Sci.* **2004**, *93*, 684-696.
- (23) Gombotz, W. R.; Pankey, S. C.; Phan, D.; Drager, R.; Donaldson, K.; Antonsen, K. P.; Hoffman, A. S.; Raff, H. V. *Pharm. Res.* **1994**, *11*, 624-632.
- (24) Keefe, A. J.; Jiang, S. *Nat. Chem.* **2012**, *4*, 59-63.
- (25) Sharma, K. S.; Durand, G.; Giusti, F.; Olivier, B.; Fabiano, A.-S.; Bazzacco, P.; Dahmane, T.; Ebel, C.; Popot, J.-L.; Pucci, B. *Langmuir* **2008**, *24*, 13581-13590.
- (26) Bazzacco, P.; Sharma, K. S.; Durand, G.; Giusti, F.; Ebel, C.; Popot, J. L.; Pucci, B. *Biomacromolecules* **2009**, *10*, 3317-3326.
- (27) Bazzacco, P.; Billon-Denis, E.; Sharma, K. S.; Catoire, L. J.; Mary, S.; Le Bon, C.; Point, E.; Baneres, J.-L.; Durand, G.; Zito, F.; Pucci, B.; Popot, J.-L. *Biochemistry* **2012**, *51*, 1416-1430.
- (28) Zhang, Y.; Cremer, P. S. In *Annu. Rev. Phys. Chem.* 2010; Vol. 61, p 63-83.
- (29) Loughheed, W. D.; Albisser, A. M.; Martindale, H. M.; Chow, J. C.; Clement, J. R. *Diabetes* **1983**, *32*, 424-432.

- (30) Teramoto, N.; Sachinvala, N. D.; Shibata, M. *Molecules* **2008**, *13*, 1773-1816.
- (31) Mancini, R. J.; Lee, J.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 8474-8479.
- (32) Zabransky, J.; Houska, M.; Kalal, J. *Makromolekulare Chemie-Macromolecular Chemistry and Physics* **1985**, *186*, 223-229.
- (33) Li, R. C.; Broyer, R. M.; Maynard, H. D. *Journal of Polymer Science Part a-Polymer Chemistry* **2006**, *44*, 5004-5013.
- (34) Pikal-Cleland, K. A.; Carpenter, J. F. *J. Pharm. Sci.* **2001**, *90*, 1255-1268.
- (35) Asad, S.; Torabi, S.-F.; Fathi-Roudsari, M.; Ghaemi, N.; Khajeh, K. *Int. J. Biol. Macromol.* **2011**, *48*, 566-570.
- (36) Paz-Alfaro, K. J.; Ruiz-Granados, Y. G.; Uribe-Carvajal, S.; Sampedro, J. G. *J. Biotechnol.* **2009**, *141*, 130-136.
- (37) Salehi, S.; Eckley, L.; Sawyer, G. J.; Zhang, X.; Dong, X.; Freund, J.-N.; Fabre, J. W. *Hum. Gene Ther.* **2009**, *20*, 21-30.
- (38) McGarvey, O. S.; Kett, V. L.; Craig, D. Q. M. *J. Phys. Chem. B* **2003**, *107*, 6614- 6620.
- (39) Bhatnagar, B. S.; Bogner, R. H.; Pikal, M. J. *Pharm. Dev. Technol.* **2007**, *12*, 505-523.
- (40) Lerbret, A.; Bordat, P.; Affouard, F.; Guinet, Y.; Hedoux, A.; Paccou, L.; Prevost, D.; Descamps, M. *Carbohydr. Res.* **2005**, *340*, 881-887.
- (41) Wang, G. M.; Haymet, A. D. J. *J. Phys. Chem. B* **1998**, *102*, 5341-5347.
- (42) Williams, R. J.; Leopold, A. C. *Plant Physiol.* **1989**, *89*, 977-981.
- (43) Crowe, J. H.; Madin, K. A. *Trans. Am. Microsc. Soc.* **1974**, *93*, 513-524.
- (44) Crowe, J. H. *Am. Nat.* **1971**, *105*, 563-573.
- (45) Lins, R. D.; Pereira, C. S.; Hünenberger, P. H. *Proteins: Struct., Funct., Bioinf.* **2004**, *55*, 177-186.

Chapter 3

Trehalose Polymer Conjugates for Enhancing Stability and Pharmacokinetic Properties of Therapeutic Proteins

3.1 Introduction

Since 1922, after human insulin has been successfully used for the treatment of diabetes, developments in recombinant protein technology have led to an increase in the number and use of therapeutic proteins.¹⁻⁶ The US Food and Drug Administration (FDA) approved the first recombinant insulin therapeutic in 1982 and as of 2008, there are more than 130 approved protein drugs, with many more therapeutics currently under development.^{7,8} Protein therapeutics are beneficial in the field of medicine, due to their high specificity and potency, as well as fewer adverse effects when compared to small molecule drugs. These benefits resulted in a \$99 billion global market value in 2009 for protein therapeutics.^{9,10} However, protein therapeutics have disadvantages such as immunogenicity, short half-life *in vivo*, low solubility, and poor stability during storage and transportation, which are all barriers for therapeutic applications.^{11,12} Recombinant DNA expression of proteins has been successfully applied to resolve immunogenicity and supply of some proteins.^{7,12} For example, when insulin was first purified from bovine and porcine pancreas in 1922, some patients showed immunological response to the animal insulin. Therefore, recombinant DNA technology was used by engineering *Escherichia coli* resulting in human insulin that was non-immunogenic.

To enhance the pharmacokinetic properties of therapeutic proteins, such as half-life and immunogenicity of the proteins, PEGylation, covalent attachment of PEG to protein, has been widely used.¹³⁻¹⁶ Increasing the size of the conjugates above the renal clearance threshold slows renal clearance of the protein drugs.^{17,18} There are currently more than ten PEGylated protein drugs used clinically.^{9,13} Neulasta® (Amgen), PEGylated granulocyte colony-stimulating factor (G-CSF), is one of the successful PEGylated protein therapeutics.¹⁹ The half-life of the G-CSF conjugate in the bloodstream is over ten times longer than native G-CSF, which translates to

decreased injection frequency for patients.^{20,21} However, PEGylation does not increase the stability of a protein against environmental stressors. Most protein therapeutics are currently injected rather than delivered orally, therefore, for the convenience and safety of the patients they are normally provided in the form of a dissolved solution. Most of therapeutic proteins including PEGylated proteins are recommended to be around 4 °C to prevent protein degradation from temperature fluctuations, which is inconvenient for patients and increases the cost of storage and transportation.^{22,23} In addition, some PEGylated proteins have illicit immune responses in humans.^{24,25} Therefore, there is an increasing interest in developing PEG alternatives. Conjugating hydroxyethyl starch (HES) to a protein, HESylation[®] (Fresenius Kabi), showed increased half-life *in vivo* for multiple therapeutic proteins including G-CSF and erythropoietin.^{26,27} However, HES has caused pruritus, kidney and liver dysfunction, and bone marrow suppression in human studies.²⁸

Herein, we describe the synthesis of protein-trehalose polymer conjugates for the enhancement of pharmacokinetic properties and temperature stability of therapeutic proteins. Trehalose is an α,α -1,1-linked glucose disaccharide which is commonly used as a natural stabilizer, allowing many plants and animals to endure dehydration stress as well as extreme temperature and pressure fluctuations.²⁹⁻³⁷ There are three main hypotheses how trehalose stabilizes the organisms that are not mutually exclusive: water replacement, water entrapment, and vitrification.³⁸⁻⁴¹ Much research has shown that trehalose allows animals and plants to survive extreme conditions by protecting cells and proteins from denaturation. For example, in dehydrating conditions, tardigrades (water bears), will accumulate up to 2.3% of their dry weight in trehalose and then go into a cryptobiotic state, which can be reversed when water is added.³⁴ Trehalose is generally regarded as safe (GRAS) by the FDA and is widely used in the cosmetic

and food industries.²⁹ Moreover, trehalose is employed as stabilizing excipient in protein-based therapeutics including Advate[®] (Baxter), Avastin[®] (Genentech), Lucentis[®] (Genentech), and Herceptin[®] (Genentech) to stabilize the protein.⁴²

There are some reports on the synthesis of trehalose-based glycopolymers, but because the 1,1-glycosidic linkage leads to relatively unreactive anomeric centers, previous syntheses of trehalose-based glycopolymers have focused on incorporating the trehalose unit into the polymer backbone rather than as a side chain.⁴³⁻⁴⁸ Miura group have synthesized acrylamide trehalose monomer.⁴⁹ However, the synthesis of the monomer included multiple protection steps leading total 8 steps from the trehalose. Our group reported trehalose side chain glycopolymers synthesized by controlled radical polymerization technique with trehalose side chain, which can be conjugated and stabilize lysozyme against extreme heat and multiple lyophilization cycles compared to native protein, trehalose, and similar molecular weight PEGs.²³ These conjugated proteins exhibited higher activity after environmental stressors when compared to native protein, trehalose as an excipient, and addition of similar molecular weight PEGs. We have also developed simple synthetic schemes for a series of trehalose-based polymers, which can be used as excipients for protein stabilization against environmental stressors (Chapter 2).²² More recently, the Reineke group has also reported a trehalose glycopolymer nanocomplex which stabilizes and efficiently delivers siRNA to cells.⁵⁰ However, an investigation of the pharmacokinetic properties of therapeutic protein-trehalose polymer conjugate as a PEG alternative has not yet been carried out.

We first targeted insulin was a therapeutic protein to be studied. As described above, insulin was the first FDA approved therapeutic protein and it remains the most effect treatment for type I and II diabetes. However, insulin has an extremely short half-life due to its relatively

small molecular weight (5.6 kDa). Currently, PEGylated insulin (Eli Lilly and Company, LY2605541) is undergoing a phase III clinical trial for FDA approval.^{51,52} We hypothesized that an insulin-trehalose glycopolymer conjugate would display improved pharmacokinetic properties while simultaneously stabilizing insulin against environmental stressors compared to the unmodified protein. First, we prepared a trehalose polymer with a ketone end group through RAFT polymerization and conjugated the polymer to the insulin using reductive amination. The insulin-trehalose conjugate was then purified using fast protein liquid chromatography (FPLC). The pure conjugate was injected into mice and its clearance from the blood plasma was compared to that of PEGylated insulin and unmodified insulin.

We also attempted the conjugation of trehalose glycopolymers to granulocyte colony-stimulating factor (G-CSF). G-CSF is used to stimulate bone marrow precursors and form neutrophils in order to fight infection in patients undergoing chemotherapy. Neulasta[®], the PEGylated conjugate of G-CSF, generates over 3.3 billion of revenue each year.^{20,21} For the preparation of this conjugate, a benzaldehyde end-group trehalose was synthesized by RAFT polymerization. This benzaldehyde end-group was conjugated to the N-terminal methionine of the G-CSF and the resulting conjugate was characterized by native gel and western blot.

Lastly, a trehalose polymer was also made to modify the surfaces of cells. *N*-levulinoyl mannosamine (**ManLev**) was first synthesized to modify the cell surfaces by biosynthetic conversion to the corresponding cell surface-associated unnatural sialic acid, which contains ketone functionality. Aminoxy end-group trehalose polymer was synthesized using RAFT polymerization, which can form an oxime bond with the ketone functionality.

The results show that trehalose glycopolymers can be synthesized using RAFT polymerization with the retention of various end-groups and can be subsequently conjugated to

both therapeutic proteins. Additionally, the insulin-trehalose polymer conjugate dramatically delayed clearance from the blood plasma in mice with no visible signs of acute toxicity, suggesting the possibility that trehalose glycopolymer is a viable replacement for PEG.

3.2 Experimental Section

Materials

All solvents and chemicals were purchased from Fisher Scientific and Sigma-Aldrich and used without further purification unless otherwise noted. Trehalose was purchased from The Endowment for Medical Research (Houston, TX) and residual water was removed prior to use by drying with ethanol and stored under vacuum. Recombinant human insulin was purchased from Sigma-Aldrich and recombinant human G-CSF from R&D System; both were used without further purification. The insulin ELISA kit was purchased from ALPCO and used following the manufacturer's instructions. Aminooxy-biotin trifluoroacetate salt (10009350) was purchased from Cayman Chemical and Streptavidin-Alexa Fluor[®] 488 (S11223) from Life technologies. **mPEG-butyraldehyde** (10,000 g/mol) was purchased from Laysan Bio. All trehalose monomers and **P1-P3** were synthesized by following literature procedures unless described.²² **ManLev** was prepared by following literature procedures.⁵³ All work performed on animals was in accordance with and approved by the Institutional Animal Care and Use Committee of University of California, Los Angeles.

Analytical Techniques

NMR spectra were recorded on Bruker AV 500 and DRX 500 MHz spectrometers. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. 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 μ m 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. FPLC (BioLogic DuoFlow, Bio-Rad) was used for conjugate purification with SuperdexTM 75 10/300 GL column (GE Healthcare). Fluorescence microscope images were visualized by using Axiovert 200 microscope with AxioCam MRm camera and FluoArc mercury lamp.

Experimental

Heat Burden Studies of Insulin with Trehalose Glycopolymers as Excipients. Insulin solution (1 mg/mL) was prepared in pH 7.4 D-PBS. 2.95 mg (60 wt equiv with respect to insulin) of **P1** (34,300 g/mol), **P2** (30,100 g/mol), **P3** (14,600 g/mol), and trehalose (**T**) were separately added to the 50 μ L insulin solution. All of the samples were heated at 90 °C for 1 h.

Synthesis of 2-(2,2-dimethoxyethoxy)ethyl 2-(((ethylthio)carbonothioyl)thio)propanoate (acetal-CTA). Triethylene glycol (66.8 mL, 5.00×10^{-1} mol) and KOH (11.90 g, 2.12×10^{-1} mol) was added to the reaction flask. After all the KOH was dissolved, 2-bromo-1,1-dimethoxy ethane was added drop wise to the reaction over 30 min and stirred at 115 °C for 72 h. The reaction was cooled and 50 mL of H₂O was added to dissolve all the salts. The aqueous layer was extracted with methylene chloride. The organic layer was purified by passing through two silica gel columns (DCM : MeOH = 95 : 5 and ethyl ether : MeOH = 95 : 5) yielding **3** as a yellow oil with unpurified triethylene glycol remaining. The crude **3** (934 mg) was dissolved in 10 mL of MeOH and stirred at 0 °C for 10 min. **4** (165 mg, 7.84×10^{-1} mmol) was added to the reaction flask and EDC (505 mg, 2.63 mmol) and DMAP (95.8 mg, 7.84×10^{-1} mmol) were added. The reaction was warmed to 25 °C after 3 h and stirred for an additional 14.5 h. The mixture was then washed with

DCM and H₂O and the collected DCM layer was dried over MgSO₄ and purified by silica gel column chromatography (EtOAc : Hex = 1 : 1) to yield 56.4 mg (yield 17%) of **acetal-CTA** as a yellow oil. ¹H NMR (500 MHz in CDCl₃) δ: 4.84-4.80 (q, *J* = 7.46 Hz, 1H), 4.52-4.50 (t, *J* = 5.15 Hz, 1H), 4.29-4.27 (dd, *J* = 5.59, 4.10 Hz, 2H), 3.70-3.68 (m, 2H), 3.67-4.23.63 (m, 8H), 3.53-3.52 (d, *J* = 5.21 Hz, 2H), 3.38-3.33 (m, 8H), 1.60-1.59 (d, *J* = 7.36 Hz, 3H), 1.35-1.32 (t, *J* = 14.72 Hz, 3H), ¹³C NMR (500 MHz in CDCl₃) δ: 221.8, 171.1, 102.7, 70.1, 70.9, 70.7, 70.6, 70.6, 68.9, 64.9, 54.0, 47.9, 31.5, 16.9, 13.0. IR: ν = 2871, 1733, 1636, 1449, 1375, 1304, 1251, 1068, 1030, 969, 875, 815 cm⁻¹. ESI-MS (± 1.0) observed (predicted): H⁺ 475.1514 (475.1494).

RAFT Polymerization of SET Monomer Mediated by Acetal-CTA (P4). Acetal-CTA (2.27 mg, 5.27×10⁻³ mmol). SET (144.7 mg, 3.16×10⁻¹ mmol) was dissolved in 0.59 mL of H₂O and 0.59 mL of DMF and added to the reaction flask. Oxygen was removed by three cycles of freeze-pump-thaw and reaction was stirred at 75 °C for 5 h. The reaction was stopped by immersion in liquid nitrogen and the reaction mixture was precipitated into MeOH to yield **P4** as a white powder. ¹H NMR (500 MHz in D₂O) δ: 7.14, 6.57, 5.16, 3.85, 3.63, 3.49, 1.64. M_n = 36,200 g/mL (by GPC), PDI = 1.37.

Deprotecting Acetal Group from P4 (aldehyde-P4). **P4** (11.4 mg) was dissolved in H₂O (0.57 mL) and TFA (0.57 mL) was added drop wise to the reaction. The reaction was stirred at 25 °C for 48 h and dialyzed against H₂O for 3 days (MWCO 3,500 g/mol). ¹H NMR (500 MHz in CDCl₃) δ: 7.00, 6.50, 5.04, 3.70, 3.60, 3.57, 3.51, 3.34, 1.33.

Synthesis of Ketone-CTA. In a flame dried flask, **4** (1.10 g, 4.43 mmol) and **5** (1.03 g, 4.90 mmol) were dissolved in 25 mL DMF. The reaction was cooled to 0 °C and DCC (1.38 g, 6.69 mmol) and DMAP (0.07 g, 5.73×10⁻¹ mmol) were added. The reaction was stirred at 0 °C for 5 h

and then warmed to 25 °C and stirred an additional 5 h. The crude mixture was precipitated in cold EtOAc and the solid precipitate was removed by filtration. The filtrate was collected, concentrated under decreased pressure, and purified by silica gel chromatography (EtOAc : Hex = 3 : 7) to yield the product in yellow oil (49% yield). ¹H NMR (500 MHz in CDCl₃) δ: 4.86-4.81 (q, *J* = 7.49, 6.86 Hz, 1H), 4.30-4.29 (dd, *J* = 5.77, 3.53 Hz, 2H), 4.24-4.22 (m, 2H), 3.74-3.65 (m, 8H), 3.38-3.34 (q, *J* = 7.32, 7.67 Hz, 2H), 2.76-2.74 (m, 2H), 2.62-2.60 (m, 2H), 2.19 (s, 3H), 1.61-1.60 (d, *J* = 7.06 Hz, 3H), 1.37-1.33 (t, *J* = 9.07 Hz, 3H), ¹³C NMR (500 MHz in CDCl₃) δ: 221.8, 206.7, 172.8, 171.2, 70.7, 70.6, 69.2, 68.9, 68.7, 64.9, 63/8, 47.9, 37.9, 34.0, 31.5, 29.9, 27.9, 25.6, 25.0, 16.9, 13.0. IR: ν = 2926, 2850, 1732, 1625, 1570, 1449, 1409, 1363, 1309, 1242, 1159, 1069, 1032, 946, 197, 814 cm⁻¹. ESI-MS (± 1.0) observed (predicted): Na⁺ 463.0907 (463.0895).

RAFT Polymerization of SAT Monomer Mediated by Ketone-CTA (P5). Lev-CTA (6.69 mg, 1.52×10⁻² mmol), SAT (416 mg, 9.11×10⁻¹ mmol), and AIBN (1 mg, 6.09×10⁻³ mmol) were dissolved in DMF (1.13 mL). Three cycles of freeze-pump-thaw were performed to remove oxygen and the reaction was started by heating to 90 °C. The polymerization was stopped after ¹H-NMR showed 81% monomer conversion and the crude mixture was dialyzed against H₂O for 5 days to remove residual unreacted monomer (MWCO 3,500 g/mol). ¹H NMR (500 MHz in D₆DMSO) δ: 7.15, 6.45, 5.49, 5.23, 4.98, 4.43, 4.11, 3.98, 3.79, 3.70, 3.59, 3.49, 3.42, 3.17, 1.56. M_n = 17,300 g/mol (by GPC), PDI = 1.27.

Conjugation and Purification of Insulin with P5. Insulin (400 µg, 7.14×10⁻⁵ mmol) was dissolved in pH 3.5, 100 mM sodium acetate buffer (0.5 mg/mL concentration). To the insulin solution, NaBH₃CN (0.64 mg/mL final concentration) and **P5** (47.59 mg, 3.45×10⁻³ mmol) were added. The mixture was incubated at 4 °C and conjugation was monitored by native gel.

Conjugation and Purification of Insulin with mPEG-butyraldehyde. Insulin (500 µg, 8.93×10^{-5} mmol) was dissolved in 1 mL of pH 3.5, 100 mM sodium acetate buffer. NaBH₃CN (0.64 mg/mL final concentration) and **mPEG-butyraldehyde** (44.64 mg, 4.47×10^{-3} mmol) were added. The mixture was incubated at 4 °C and conjugation was monitored by native gel. The conjugate was purified by FPLC to remove free insulin.

Pharmacokinetics (PK) study of Insulin, Insulin-P5 Conjugate, and mPEG-butyraldehyde. Unmodified insulin, insulin-P5 conjugates, and insulin-mPEG-butyraldehyde were formulated in D-PBS (pH 7.0). Balb/c mice (5-6 wks, female) were used for the pharmacokinetic studies (n=5 per group). A single dose of either unmodified insulin or insulin conjugates (16 µg/kg of insulin in 0.4 µg/mL concentration) was injected through the tail vein and blood samples (30-50 µL) were taken from the lateral saphenous vein at 8, 15, 30 min and 1h after administration. Blood was collected from each mouse four times and the total amount of blood collection was less than 1.25% of the animal's body weight. Insulin concentrations of the blood plasma samples were determined by ELISA.

Synthesis of Benzaldehyde-CTA. To the flame-dried flask, **4** (500 mg, 2.38 mmol) and 4-hydroxybenzaldehyde (377.38 mg, 3.09 mmol) were added and dissolved in DCM (20 mL). The reaction flask was cooled with an ice bath, and EDC (911.38 mg, 4.75 mmol) and DMAP (58.08 mg, 4.75×10^{-1} mmol) were added. The reaction was stirred at 0 °C for 30 min and stirred at 25 °C for another 2 h. The reaction was washed with H₂O three times and the organic layer was collected, dried over MgSO₄ and purified by silica gel column chromatography (EtOAc : Hex = 1 : 1) to yield 466.7 mg of yellow **Benzaldehyde-CTA** (62% yield). ¹H NMR (500 MHz in CDCl₃) δ: 9.99 (s, 1H), 7.92-7.90 (d, *J* = 9.47 Hz, 2H), 7.29-2.27 (d, *J* = 8.84 Hz, 2H), 5.01-4.97 (q, *J* = 7.18, 7.40 Hz, 1H), 3.42-3.35 (m, 2H), 1.75-1.73 (d, *J* = 7.41 Hz, 3H), 1.38-1.35 (t, *J* =

7.62 Hz, 3H), ^{13}C NMR (500 MHz in CDCl_3) δ : 221.7, 190.9, 169.3, 155.2, 134.2, 131.3, 122.2, 47.8, 31.8, 16.3, 13.0. IR: ν = 2974, 2925, 2740, 1755, 1698, 1597, 1501, 1449, 1425, 1376, 1298, 1266, 1202, 1151, 1420, 1066, 1032, 1013, 970, 896, 857, 821 cm^{-1} . ESI-MS ($\pm$ 1.0) observed (predicted): Na^+ 337.0012 (337.0003).

RAFT Polymerization of SAT Monomer Mediated by Benzaldehyde-CTA (P6). Benzaldehyde-CTA (3.82 mg, 1.21×10^{-2} mmol), SAT monomer (362.3 mg, 7.29×10^{-1} mmol), and AIBN (0.80 mg, 4.87×10^{-3} mmol) were dissolved in 0.91 mL of DMF. The solution underwent three cycles of freeze-pump-thaw and polymerization was initiated by immersing the flask into 80 °C oil bath. The polymerization was stopped when the monomer conversion reached 54% by ^1H -NMR and purified by dialyzing against H_2O (MWCO 3,500 g/mol). ^1H NMR (500 MHz in CDCl_3) δ : 9.92, 7.59, 7.15, 6.52, 5.44, 5.17, 4.92, 4.79, 4.37, 4.08, 3.95, 3.75, 3.67, 3.55, 3.46, 3.14, 1.49. M_n = 24,500 g/mol (by GPC), PDI = 1.19.

Conjugation of G-CSF with P6. To 50 μg of G-CSF (100 $\mu\text{L}/\text{mL}$ in 10 mM acetic acid), NaBH_3CN (0.64 mg/mL final concentration) was added. Once all NaBH_3CN was dissolved, **P6** (311 μg , 100 molar equiv to G-CSF) was added. The conjugation reaction was incubated at 4 °C for 24 h.

Modification of Mouse Embryonic Fibroblast Cell Surface with ManLev. Mouse embryonic fibroblast cells (NIH 3T3, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were seeded in 48-well plates at a density of 4.5×10^3 cells per well. The cells were supplemented with a filtered-sterilized 100 mM solution of **ManLev** in phosphate-buffered saline (PBS) to a final **ManLev** concentration of 10 mM. Following incubation with

ManLev for 72 h at 37 °C and 5% CO₂, cells were washed two times with ligation buffer (PBS, pH 6.5, 5% FBS). Cells were then incubated with 2.5 mM aminooxy-biotin in ligation buffer for 4 h at 37 °C. The cells were washed twice with a labeling buffer (PBS pH 7.4, 0.1% FBS, 0.1% NaN₃), followed by incubation with Alexa Fluor 488-streptavidin for 20 min in the dark. The cells were washed twice and fluorescent images were captured.

Synthesis of Acetylated SET Monomer. A flame-dried flask was prepared and SET (195.3 mg, 4.28×10^{-1} mmol) and pyridine (34.47 mL, 4.28×10^{-1} mol) were added. Acetic anhydride (1.21 mL, 1.28×10^{-2} mol) was added to the mixture and stirred for 19.5 h at 25 °C. Pyridine was removed *in vacuo* and resulting mixture was extracted with DCM and acidic H₂O followed by H₂O. After silica gel column chromatography with EtOAc : Hex = 1 : 1 the product was collected in 57% yield (184.1 mg). ¹H NMR (500 MHz in CDCl₃) δ: 7.41-7.39 (m, 2H), 7.24-7.23 (m, 2H), 6.73-6.68 (m, 1H), 5.77-5.73 (m, 1H), 5.50-5.44 (m, 2H), 5.30-5.26 (m, 3H), 6.18-5.17 (m, 1H), 5.11-5.07 (t, *J* = 10.08 Hz, 1H), 5.03-5.00 (m, 1H), 4.97-4.93 (t, *J* = 10.08 Hz, 1H), 4.61 (s, 2H), 4.29-3.66 (m, 6H), 2.07-2.00 (m, 21H). ¹³C NMR (500 MHz in CDCl₃) δ: 170.6, 170.0, 169.9, 169.8, 169.6, 137.6, 136.4, 136.4, 136.3, 128.4, 128.1, 126.4, 126.2, 92.7, 92.6, 92.5, 92.3, 75.9, 74.5, 73.3, 71.9, 70.3, 70.2, 70.1, 70.0, 69.8, 69.7, 69.5, 69.4, 69.1, 68.5, 68.1, 68.0, 62.3, 61.8, 20.9, 20.7, 20.7, 20.7, 20.7, 20.6, 20.6, 20.5. IR: ν = 2957, 2870, 1744, 1513, 1434, 1367, 1212, 1163, 1136, 1033, 1016, 985, 902, 854, 828, 804 cm⁻¹. ESI-MS (± 1.0) observed (predicted): Na⁺ 775.2580 (775.2426).

Synthesis of BOC Aminooxy-CTA. In a flame-dried flask, **4** (79.41 mg, 3.78×10^{-1} mmol) and **6** (79 mg, 4.15×10^{-1} mmol) were dissolved in DCM (5 mL). After 10 min stirring at 0 °C, EDC (180.93 mg, 9.44×10^{-1} mmol) and DMAP (9.22 mg, 7.55×10^{-2} mmol) were added to start the reaction. The reaction was stirred at 0 °C for 2 h and warmed to 25 °C to stir an additional 2 h.

The resulting mixture was washed with H₂O three times and the DCM layer was dried with MgSO₄ and purified by passing through two silica gel columns (EtOAc : Hex = 2 : 1 and EtOAc : DCM = 1 : 2). The product (47 mg) was collected as a yellow oil in 33% yield. ¹H NMR (500 MHz in CDCl₃) δ: 7.42-7.36 (m, 2H), 4.76-4.72 (q, *J* = 7.32, 14.96 Hz, 1H), 3.89-3.86 (t, *J* = 5.71 Hz, 2H), 3.42-3.38 (q, *J* = 6.04, 6.24 Hz, 2H), 3.36-3.32 (q, *J* = 7.60, 7.41 Hz, 2H), 1.78-1.73 (m, 2H), 1.58-1.57 (d, *J* = 6.79 Hz, 3H), 1.47 (s, 9H), 1.35-1.32 (t, *J* = 7.19 Hz, 3H). ¹³C NMR (500 MHz in CDCl₃) δ: 223.2, 170.5, 157.3, 82.1, 74.8, 49.0, 37.1, 31.5, 28.2, 27.2, 17.0, 13.0. IR: ν = 3267, 3081, 2975, 2927, 2871, 1715, 1652, 1542, 1478, 1448, 1392, 1367, 1276, 1251, 1163, 1110, 1082, 1032, 947, 813 cm⁻¹. ESI-MS (± 1.0) observed (predicted): H⁺ 383.1076 (383.1133).

RAFT Polymerization of acetylated SET Monomer Mediated by BOC Aminoxy-CTA (Acetylated-P7). BOC aminoxy-CTA (1.56 mg, 4.08×10⁻³ mmol), acetylated SET (184.1 mg, 2.45×10⁻¹ mmol), and AIBN (0.2 mg, 1.22×10⁻³ mmol) were dissolved in 0.49 mL of DMF. After three cycles of freeze-pump-thaw, the reaction was started by heating to 80 °C. After monomer conversion reached 47% by ¹H-NMR, the reaction was stopped by immersion in liquid nitrogen. The resulting mixture was diluted with EtOAc and precipitated in cold ethyl ether. Resulting **P7** was M_n = 11,300 g/mol and PDI = 1.19. ¹H NMR (500 MHz in CDCl₃) δ: 7.06, 6.45, 5.44, 5.26, 5.09, 5.00, 4.89, 4.57, 4.24, 4.08, 3.92, 3.66, 2.00, 1.31. M_n = 11,300 g/mol, PDI = 1.19.

Deprotecting Acetate from Acetylated-P7 (P7). Acetylated-P7 (33.1 mg, 2.93×10⁻³ mmol) was dissolved in 2 mL CHCl₃/MeOH = 1/1. NaOMe (30% in MeOH, 2.67 μL, 1.48×10⁻² mmol) was added and the reaction was stirred under Ar (g) at 25 °C for 3 h. A white precipitate began to form, and the solid was collected and dissolved in H₂O. The polymer was dialyzed against H₂O (MWCO 1,000 g/mol) resulting in **P7** (M_n = 8,600 g/mol and PDI = 1.08). ¹H NMR (500 MHz in

D₂O) δ : 7.21, 6.68, 5.17, 3.95, 3.87, 3.61, 3.51, 1.66, 1.43. M_n = 8,600 g/mol, PDI = 1.08.

Fluorophore Labeling of P7 (Atto590-P7). P7 (1.06 mg, 1.23×10^{-4} mmol) was dissolved in degassed H₂O (0.4 mL). Ethanol diamine (0.15 μ L in 0.1 mL H₂O, 2.49×10^{-3} mmol) was added and the reaction was stirred for 20 min at 40 °C under Ar (g). The reaction was cooled to 25 °C and Atto590[®] (0.5 mg in 0.2 mL H₂O, 6.15×10^{-4} mmol) was added. Then the reaction was stirred at 25 °C for 12 h under Ar (g) with exclusion of light. After the reaction, the mixture was dialyzed against H₂O (MWCO 3,500 g/mol) and stored under vacuum until used.

3.3 Results and Discussion

Heat Burden Studies of Insulin with Trehalose Polymers

To investigate if trehalose-based polymers have the ability to stabilize insulin against environmental stressors, we heated solutions of insulin with no additive, 60 wt equiv of **P1** (34,300 g/mol), **P2** (30,100 g/mol), **P3** (14,600 g/mol), and trehalose (**T**) at 90 °C for 1 h. After the heating, the insulin samples were run in native gel to determine the effect of excipients.

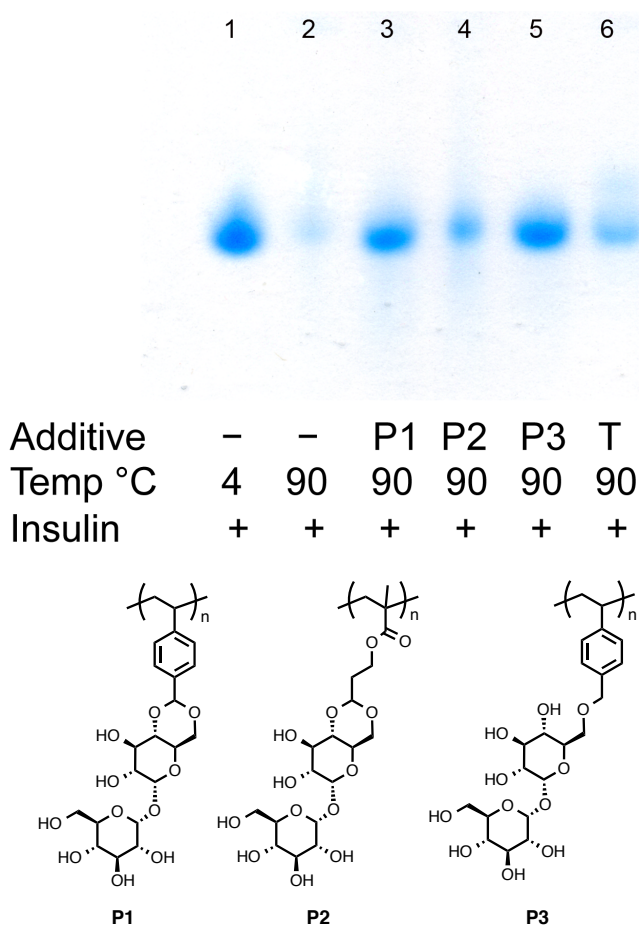


Figure 3-1. Native gel (coomassie stained) of lane 1: insulin without heating, lane 2: insulin heating, lane 3: insulin and **P1** heating, lane 4: insulin and **P2** heating, lane 5: insulin and **P3** heating, and lane 6: insulin and **T** heating (top) and structures of **P1-P3** (bottom).

In the native gel of insulin after heating at 90 °C for 1 h (lane 2 in Figure 3-1), the insulin band after coomassie staining became faint compared to the control (lane 1 in Figure 3-1), which was insulin stored at 4 °C. This result shows the heating is able to denature the insulin structure, which is important for activity. Insulin heated with **P2** (lane 4 in Figure 3-1) and trehalose (lane 6 in Figure 3-1) showed a slightly stronger band than with no additive; however, **P1** (lane 3 in Figure 3-1) and **P3** (lane 5 in Figure 3-1), which are both styrenyl based trehalose polymers (Figure 3-1 bottom), maintained the majority of the insulin structure. The strength of the insulin bands with **P1** and **P3** as excipients were comparable to the control band in lane 1. However, in the native gel no noticeable extra bands appeared, even though the original insulin band had disappeared. Both insulin and heated insulin without the addition of excipients were analyzed by matrix-assisted laser desorption/ionization (MALDI-TOF) to observe changes in the molecular ion peak after heating (Figure 3-2). Before heating, the m/z of insulin was shown to be the expected ~5.8 kDa whereas after heating the ~5.8 kDa peak was almost gone while peaks at ~2.3 kDa, ~2.9 kDa, and ~3.4 kDa became visible. This may be due to degradation of the insulin. Insulin has two disulfide bonds linking polypeptide chain A (2,377 Da) and chain B (3,431 Da); the latter corresponds to one of the peaks observed in the MALDI-TOF data (Figure 3-2), while two additional peaks were observed at 2,307 and 2,900 Da suggesting other degradation products. Therefore, the data suggests that trehalose polymers were able to stabilize the insulin to degradation under extreme heating stress.

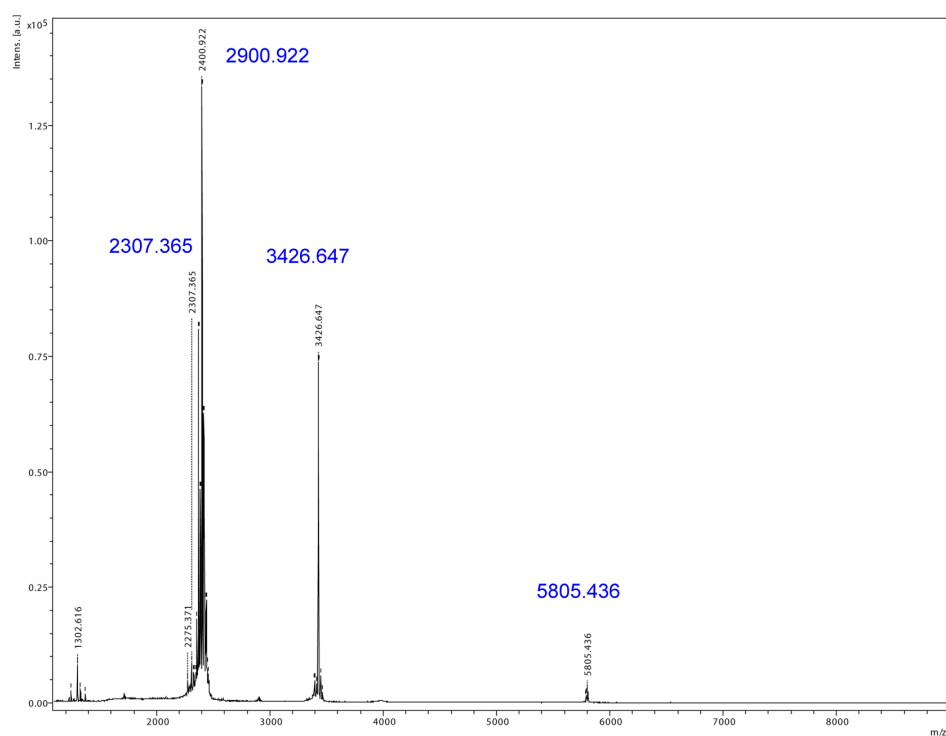
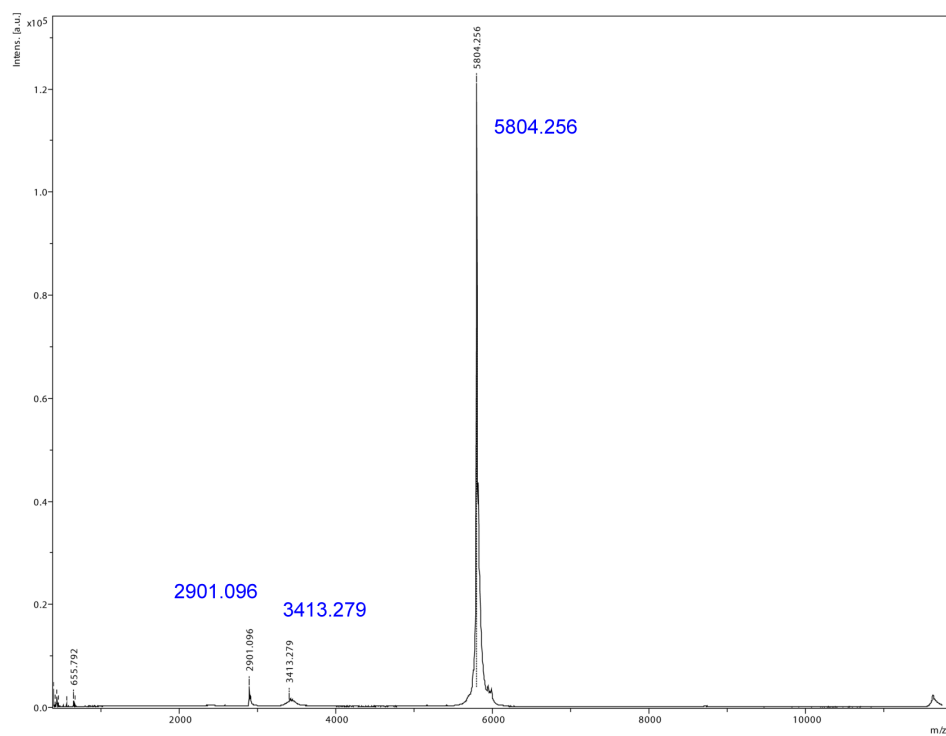
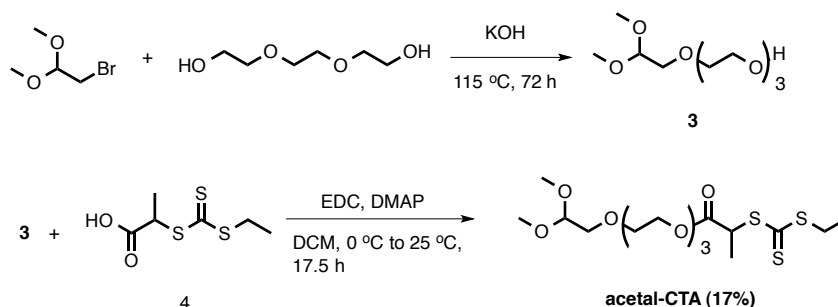


Figure 3-2. MALDI-TOF data of insulin before (top) and after (bottom) heating at 90 °C for 1 h.

Synthesis of Insulin Reactive Trehalose Polymers

Scheme 3-1. Synthesis of **Acetal-CTA**.



Next, we conjugated a trehalose polymer to insulin *via* reductive amination between a primary amine on insulin and an aldehyde or ketone end-group moiety of the trehalose polymer. First, to prepare the aldehyde end-group trehalose polymer, an acetal protected aldehyde end-group trithiocarbonate CTA was synthesized (Scheme 3-1). The **acetal-CTA** was synthesized through two steps by first attaching one of the hydroxyl groups of triethylene glycol to methyl acetal, followed by EDC coupling of the other hydroxyl with the carboxylic acid group of **4**. ¹H NMR spectroscopy of **3** showed excess triethylene glycol after purification, resulting in low yield (17%) in the coupling reaction between **3** and **4** (Figure 3-3 and 3-4).

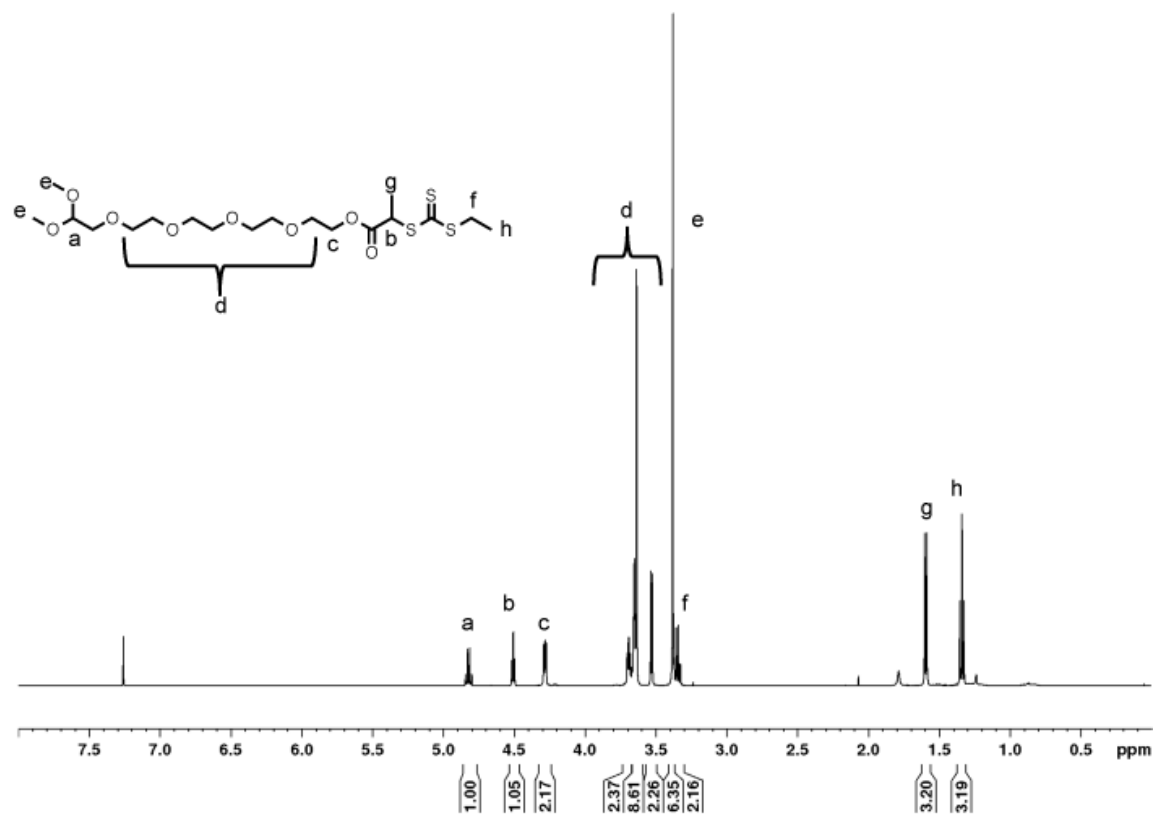


Figure 3-3. ^1H NMR spectroscopy of **4** (in CDCl_3).

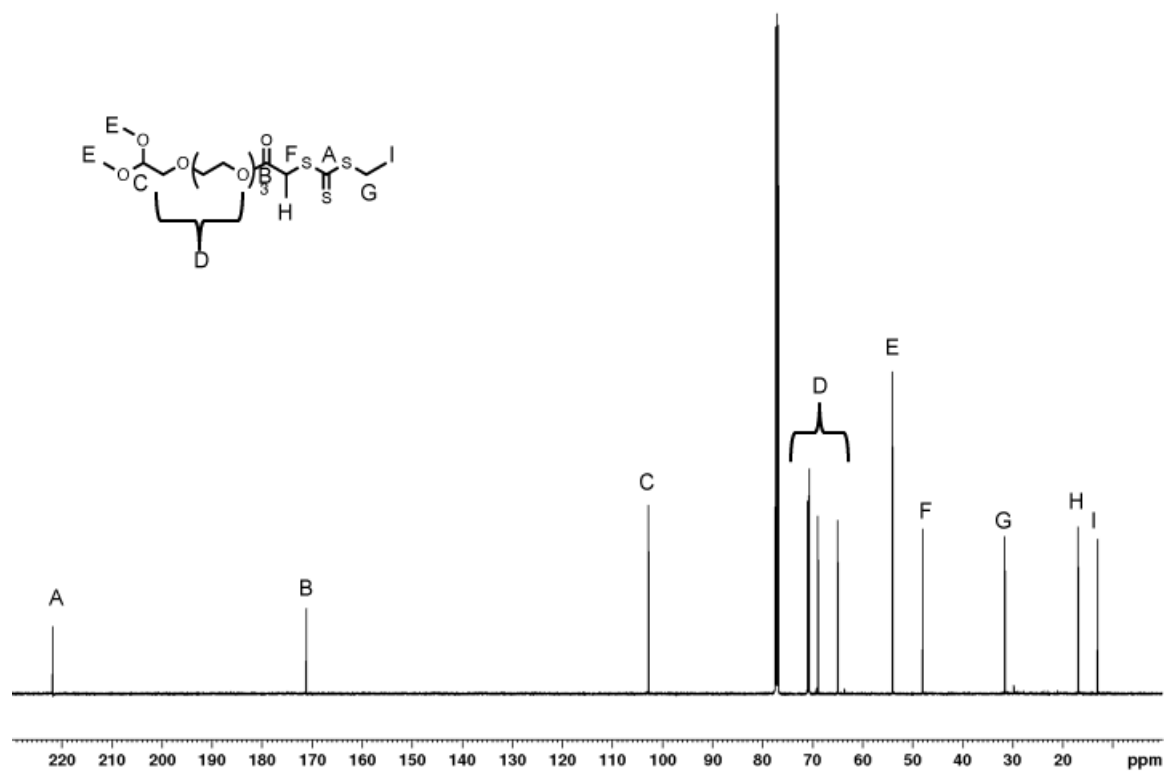
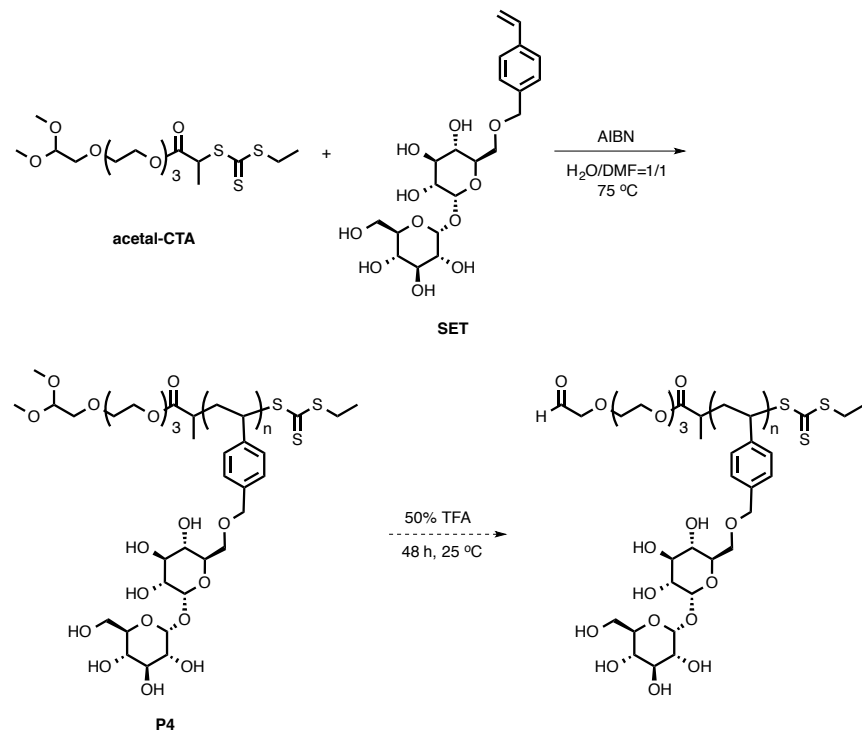


Figure 3-4. ^{13}C NMR spectroscopy of **4** (in CDCl_3).

Scheme 3-2. RAFT Polymerization of **SET** Monomer Mediated by **Acetal-CTA** and Deprotection of Acetal.



The previously synthesized **acetal-CTA** was used to mediate RAFT polymerization of the **SET** monomer. The **SET** monomer was selected instead of acetal linked trehalose-based monomers, because the TFA used to deprotect the acetal also cleaves the acetal link of trehalose monomers. The RAFT polymerization of **SET** resulted in **P4** with a M_n of 36,200 g/mol and PDI of 1.37 (Figure 3-5). **P4** was dissolved and stirred in 50% TFA aqueous solution to deprotect the acetal and yield the desired aldehyde end-group **P4**. However, after the reaction no aldehyde peak was detected by ^1H NMR spectroscopy.

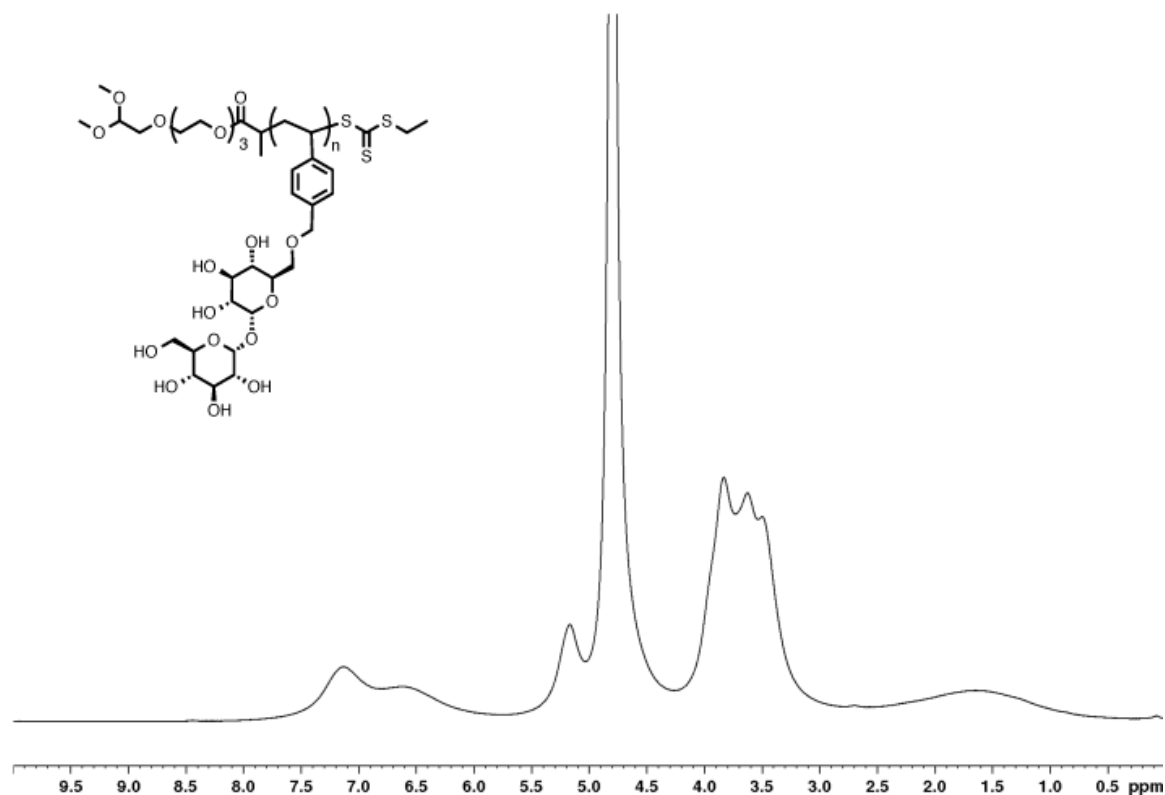
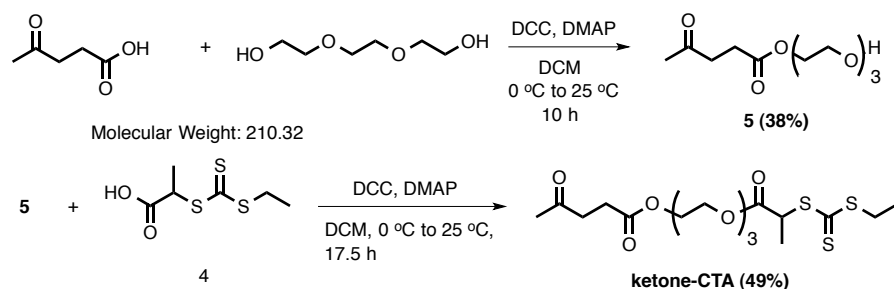


Figure 3-5. ^1H NMR spectroscopy of **P4** (in D_2O).

Scheme 3-3. Synthesis of **Ketone-CTA**.



Since acetal deprotection did not easily produce the aldehyde end-group, another amine reactive CTA containing a ketone end-group was prepared similarly by a carbodiimide mediated coupling reaction (Scheme 3-3). First, levulinic acid and triethylene glycol were coupled to produce triethylene glycol laevulinate **5** in 38% yield following already known procedure.⁵⁴ Next,

the alcohol moiety of **5** was coupled to the carboxylic acid moiety of **4** to obtain **ketone-CTA** in 49% yield (Figure 3-6 and 3-7).

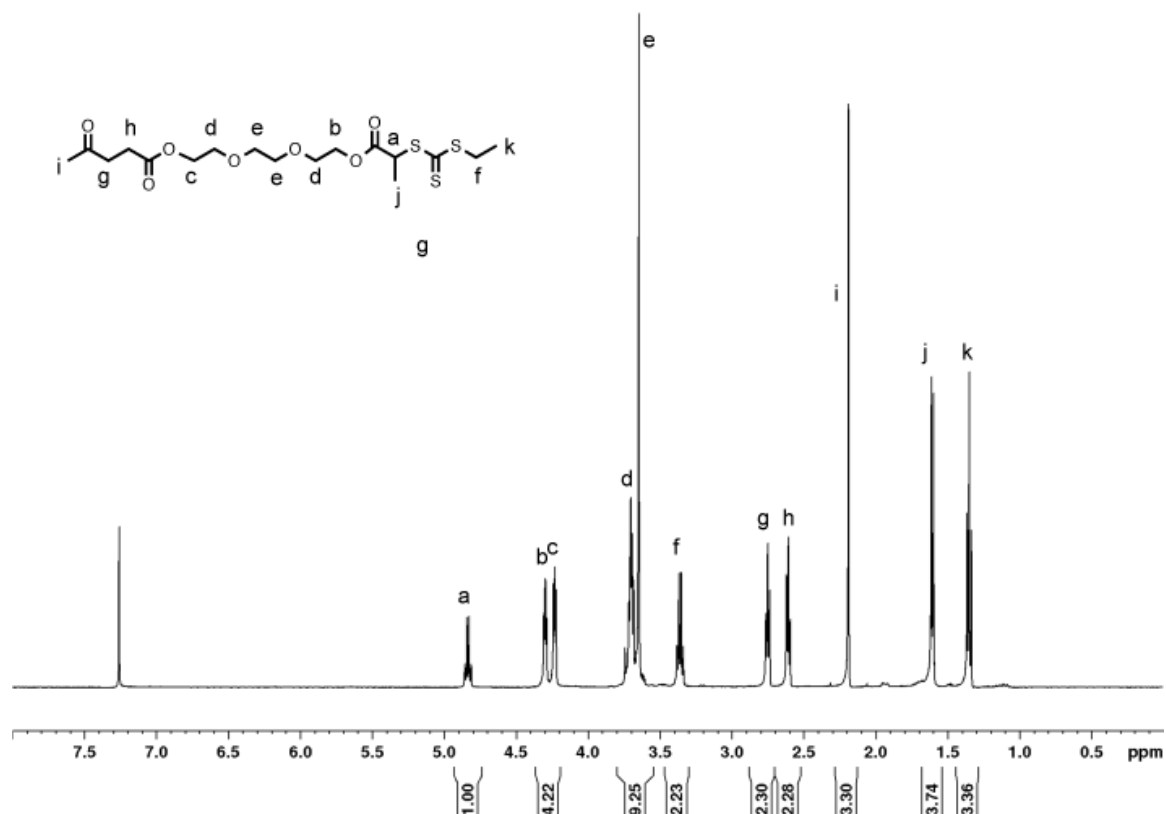


Figure 3-6. ^1H NMR spectroscopy of **ketone-CTA** (in CDCl_3).

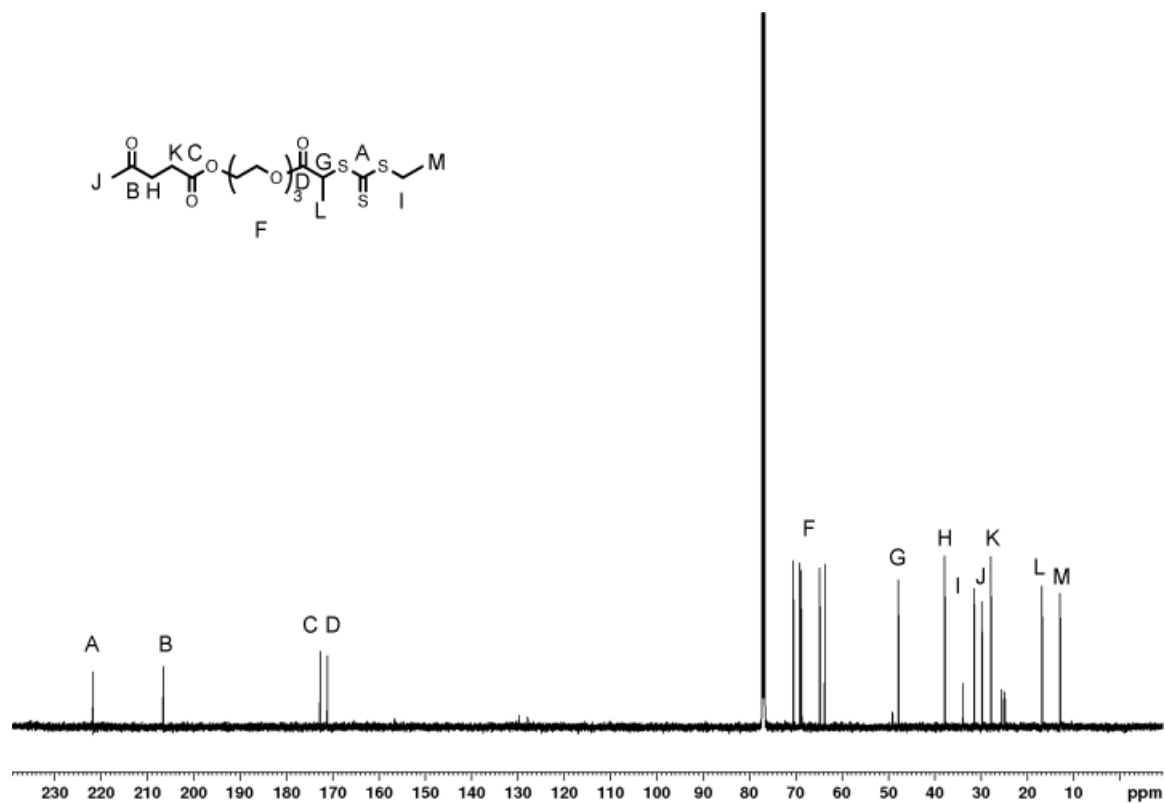
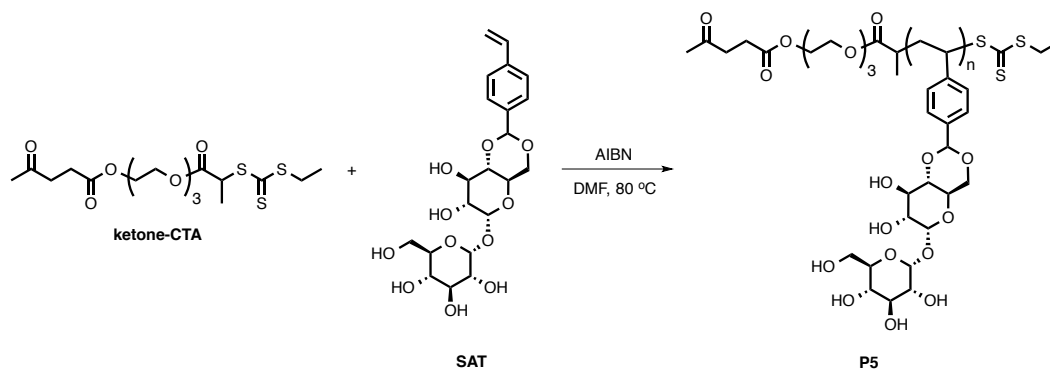


Figure 3-7. ^{13}C NMR spectroscopy of **ketone-CTA** (in CDCl_3).

Scheme 3-4. RAFT Polymerization of **SAT** Monomer Mediated by **Ketone-CTA**.



The **SAT** monomer was polymerized by RAFT polymerization mediated by the **ketone-CTA** to yield **P5** ($M_n = 17,300$ g/mol and $PDI = 1.27$, Figure 3-8). To control the molecular weight of the resulting polymer, polymerization was halted at different monomer conversion percentages monitored by $^1\text{H-NMR}$. With this approach, RAFT polymerization produced a series of **P5** polymers with molecular weights of 17,300 g/mol ($PDI = 1.27$), 23,600 g/mol ($PDI = 1.53$) and 31,500 g/mol ($PDI = 1.55$) (Figure 3-9).

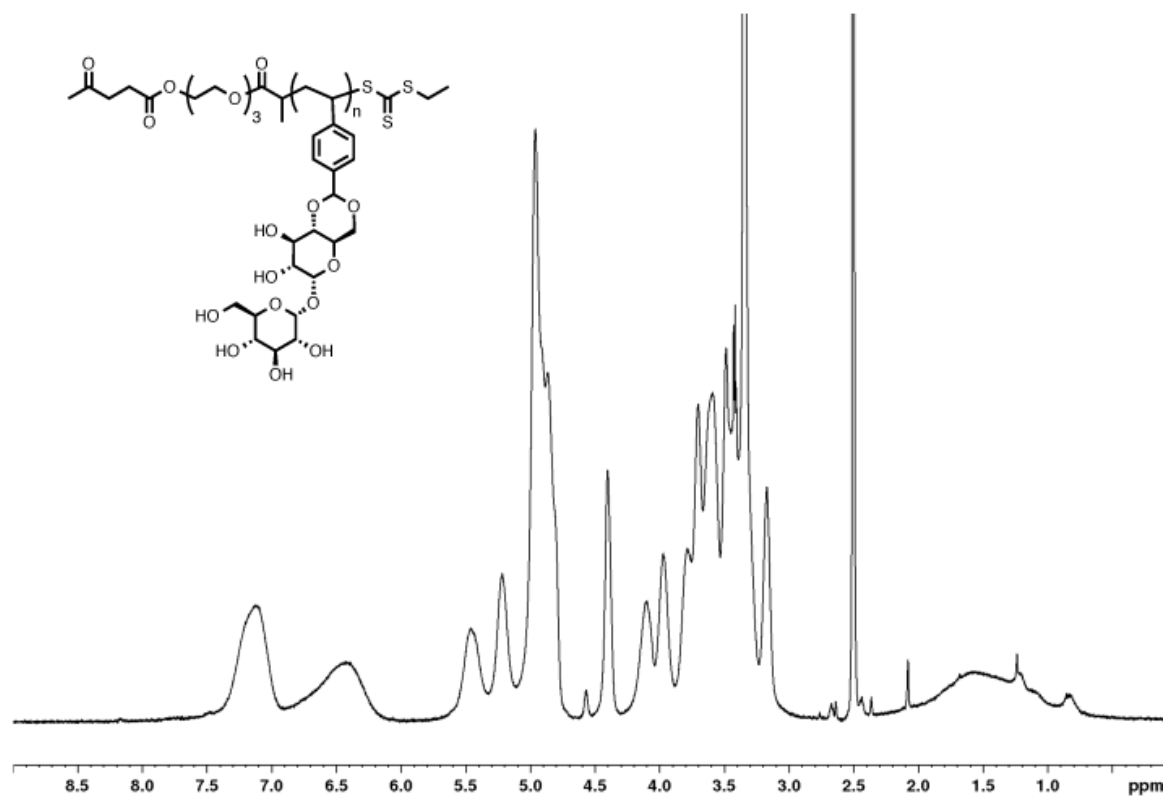


Figure 3-8. ^1H NMR spectroscopy of **P5** (in D_6DMSO).

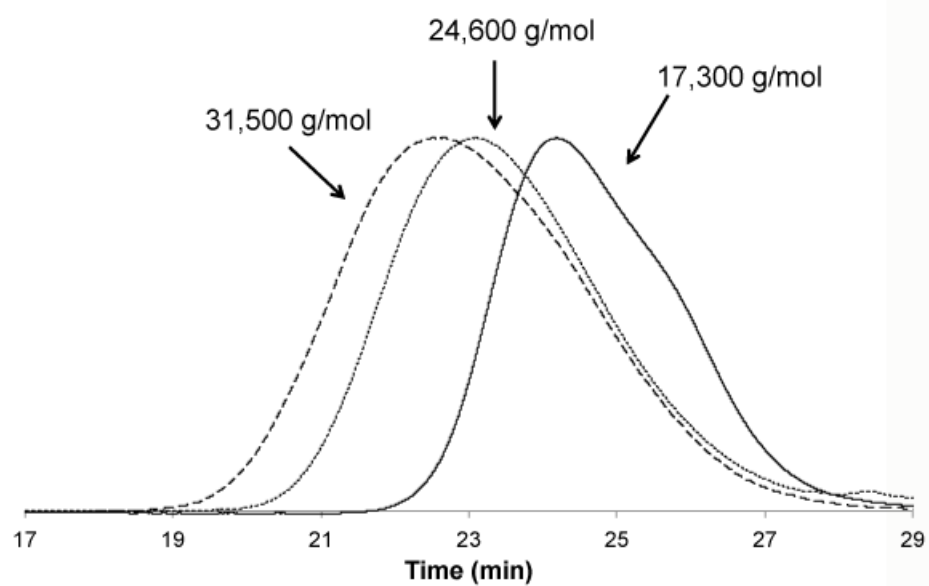
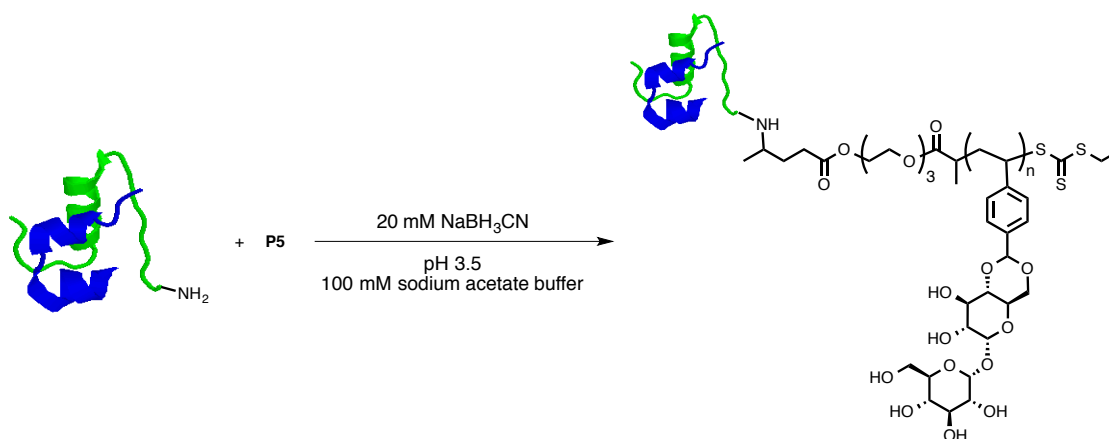


Figure 3-9. GPC traces of different M_n **P5s**.

Preparation of Insulin-Trehalose Polymer Conjugate

Scheme 3-5. Preparation of Insulin-**P5** Conjugate through Reductive Amination.



The **P5** polymer with weight of 17,300 g/mol was conjugated to the N-terminus amine of insulin through reductive amination (Scheme 3-5). To evaluate the efficiency of the conjugation, reactions with 1 : 1, 1 : 10, and 1 : 50 molar equiv of insulin to **P5** were attempted. Acidic buffer and NaBH_3CN were used to catalyze the imine formation and to reduce the imine to form a secondary amine. Existence of the conjugate and efficiency of the reaction was verified by native gel. As shown in Figure 3-10, coomassie staining revealed an extra band in conjugation reactions that did not correspond to either insulin or **P6**. This band was concluded to be from the conjugate. Increasing the ratio of **P5** resulted in higher yields of the insulin-**P5** conjugate.

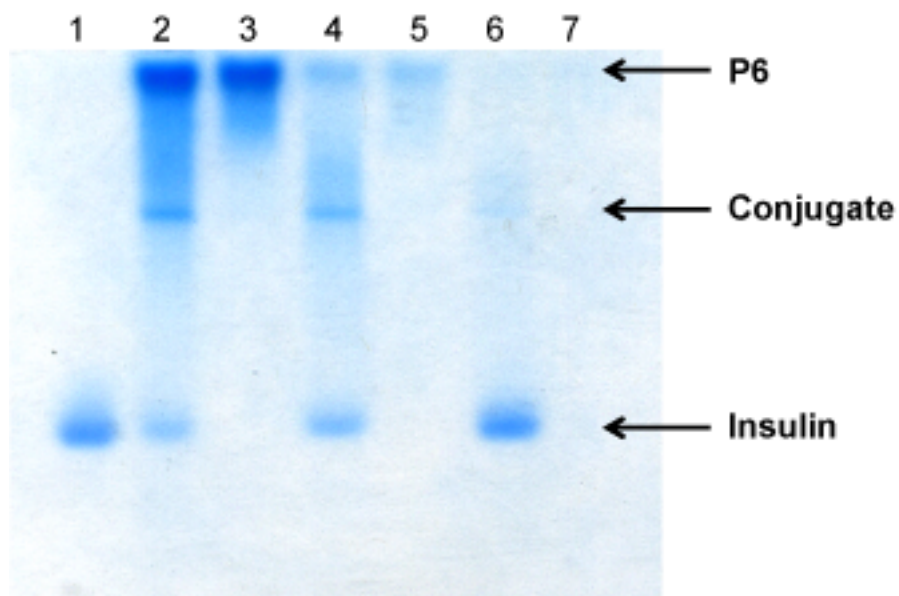


Figure 3-10. Native gel after coomassie staining; lane 1: insulin, lane 2: insulin : **P5** = 1:50 molar equiv, lane 3: **P5** (50 molar equiv) lane 4: 1:10, lane 5: **P5** (10 molar equiv) lane 6: 1:1, lane 7: **P5** (11 molar equiv).

The crude reaction of insulin with 50 molar equiv of **P5** was then purified by FPLC with D-PBS pH 7.0 as an eluent. The fractions from FPLC were run in native gel to verify the purification (Figure 3-11). Compared to the insulin control in lane 1, lanes 3 to 7 had no insulin band while lane 8 displayed an unconjugated insulin band, confirming separation of the conjugate from free insulin.

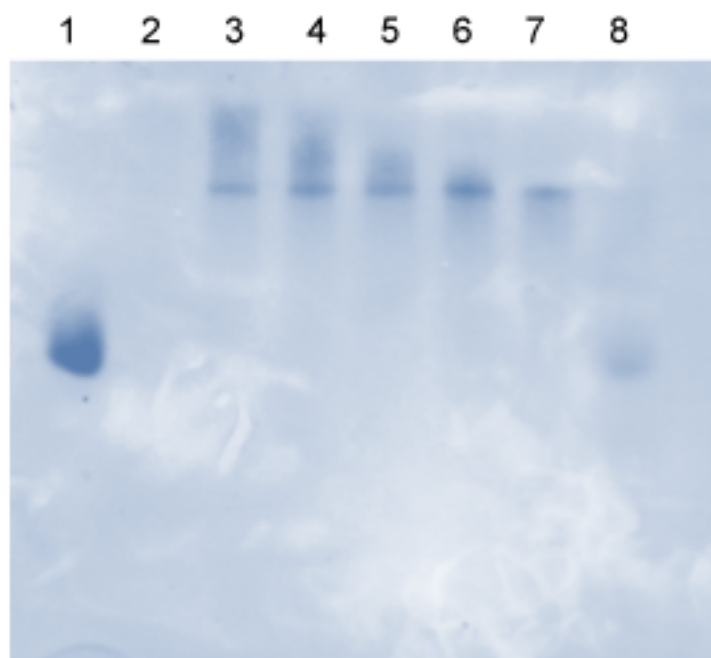


Figure 3-11. Native gel of FPLC fractions after coomassie staining; lane 1: insulin, lane 2-8: fraction 1-7 from FPLC purification.

Preparation of Insulin-mPEG Butyraldehyde Conjugate

Scheme 3-6. Preparation of Insulin-mPEG Conjugate.

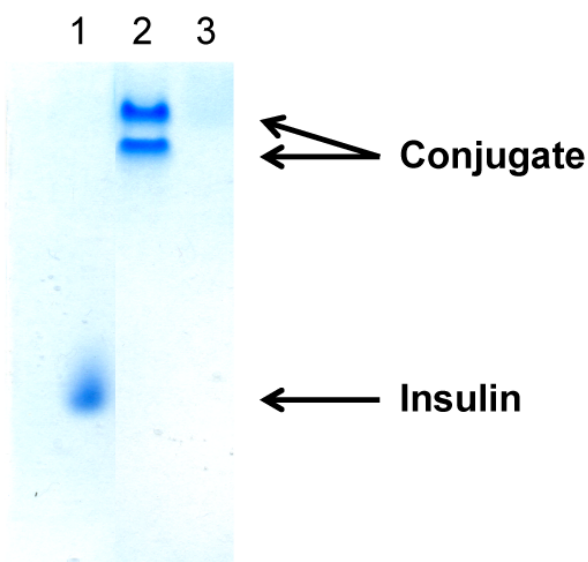
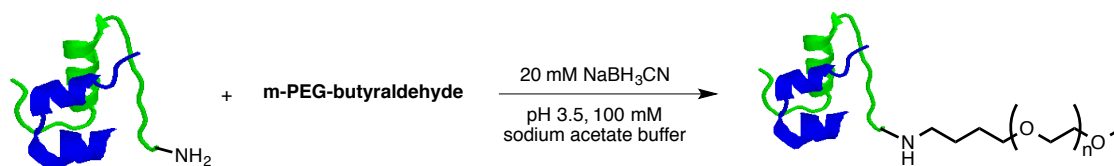


Figure 3-12. Native gel after coomassie staining; lane 1: insulin, lane 2: crude insulin-mPEG conjugate, lane 3: mPEG-butyraldehyde.

To compare the pharmacokinetics and stabilizing abilities of the insulin-trehalose polymer conjugate to a PEGylated conjugate, insulin-mPEG-butyraldehyde was also synthesized. The conjugation reaction was carried out in the same conditions as were used for conjugating **P5** to the insulin, with 50 molar equiv of the polymer, and identical concentrations of buffer and NaCNBH₃. The conjugation was again monitored by native gel and more efficient conjugation was observed than with **P5**. In the crude conjugation mixture, (Figure 3-12 lane 2)

there were no free insulin detected and two new bands were observed through coomassie staining. Since a control of **mPEG-butyraldehyde** was not stained (Figure 3-12 lane 3) the new bands must be due to insulin-**mPEG** conjugates.

Pharmacokinetic Study of Insulin and Insulin-Polymer Conjugates

For pharmacokinetic (PK) studies of the insulin, insulin-**P5** conjugate, and the insulin-**mPEG** conjugate, all samples were purified and their concentration was verified by enzyme-linked immunosorbent assay (ELISA). These samples containing 0.4 µg insulin were injected into each group of mice (n=5 per group) through the tail vein. After 3, 10, 20, 40 min and 1h of administration, blood samples were taken from the lateral saphenous vein. ELISA was used to identify the plasma concentrations of each injected sample.

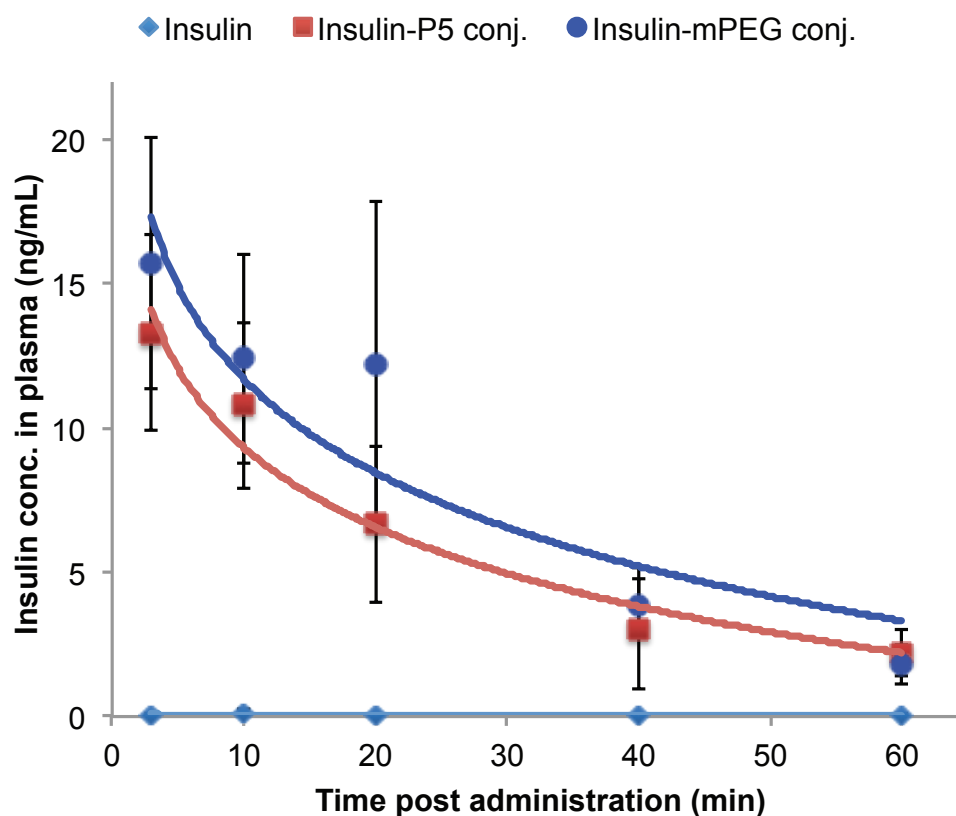
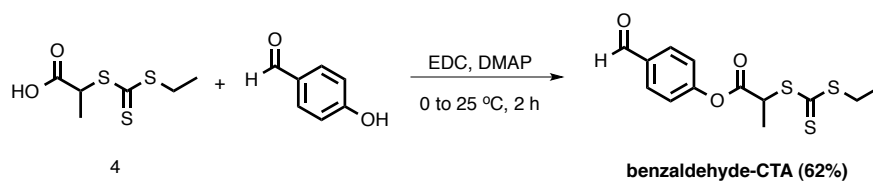


Figure 3-13. Insulin concentration in plasma of insulin-**P5** conjugate and insulin at 3, 10, 20, 40, and 60 min after the injection. Each data contains five individual mice.

The results clearly showed increased lifetime of both insulin-polymer conjugates in mice (Figure 3-13). Unmodified insulin was quickly cleared from the bloodstream within 5 min and no insulin was detectable in blood plasma. Free insulin was detectable when injected at 20 times higher dose (8 μ g) to mice. However, mice injected with the insulin-**P5** and insulin-mPEG conjugate each had approximately 5 ng/mL of insulin at the 5 min time point and the concentration slowly decreased. Even at 60 min after administration, insulin conjugates were still detectable by ELISA. The insulin-**P5** and insulin-mPEG conjugates showed similar trends in blood clearance and all the time points were statistically same (p value > 0.1).

Synthesis of Benzaldehyde End-Group Trehalose Polymer

Scheme 3-7. Synthesis of **Benzaldehyde-CTA**.



Another amine reactive aldehyde end-group CTA was prepared. EDC-mediated coupling of **4** and 4-hydroxybenzaldehyde yielded **benzaldehyde-CTA** in 62% yield (Scheme 3-7). The ^1H NMR and ^{13}C NMR spectra of **benzaldehyde-CTA** showed the retention of aldehyde end-group after the coupling reaction (Figure 3-14 and 3-15).

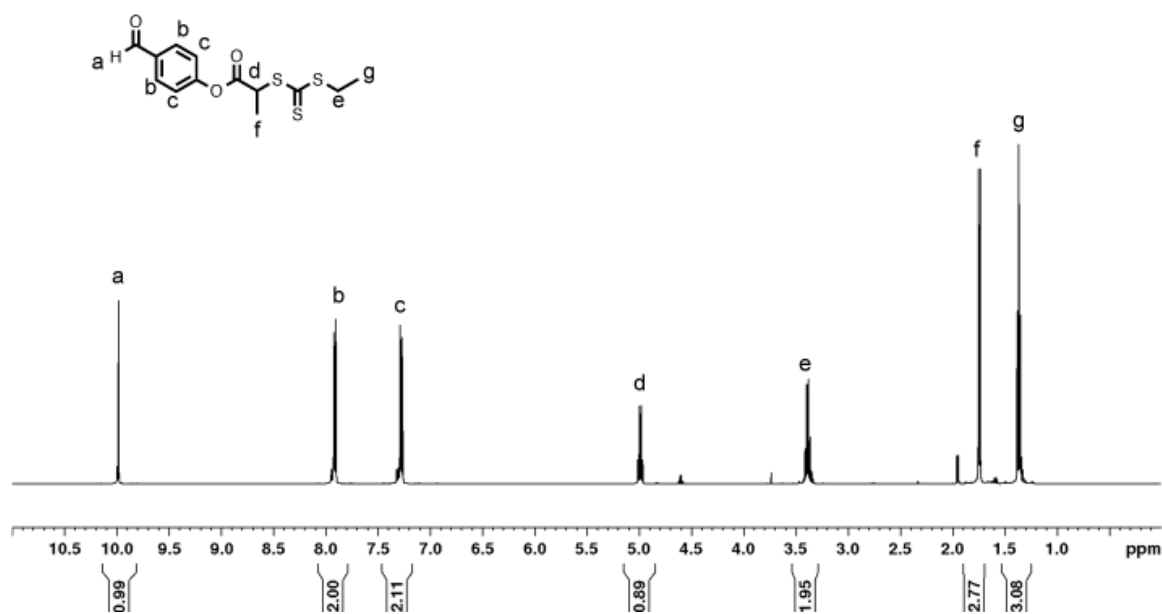


Figure 3-14. ^1H NMR spectroscopy of **benzaldehyde-CTA** (in CDCl_3).

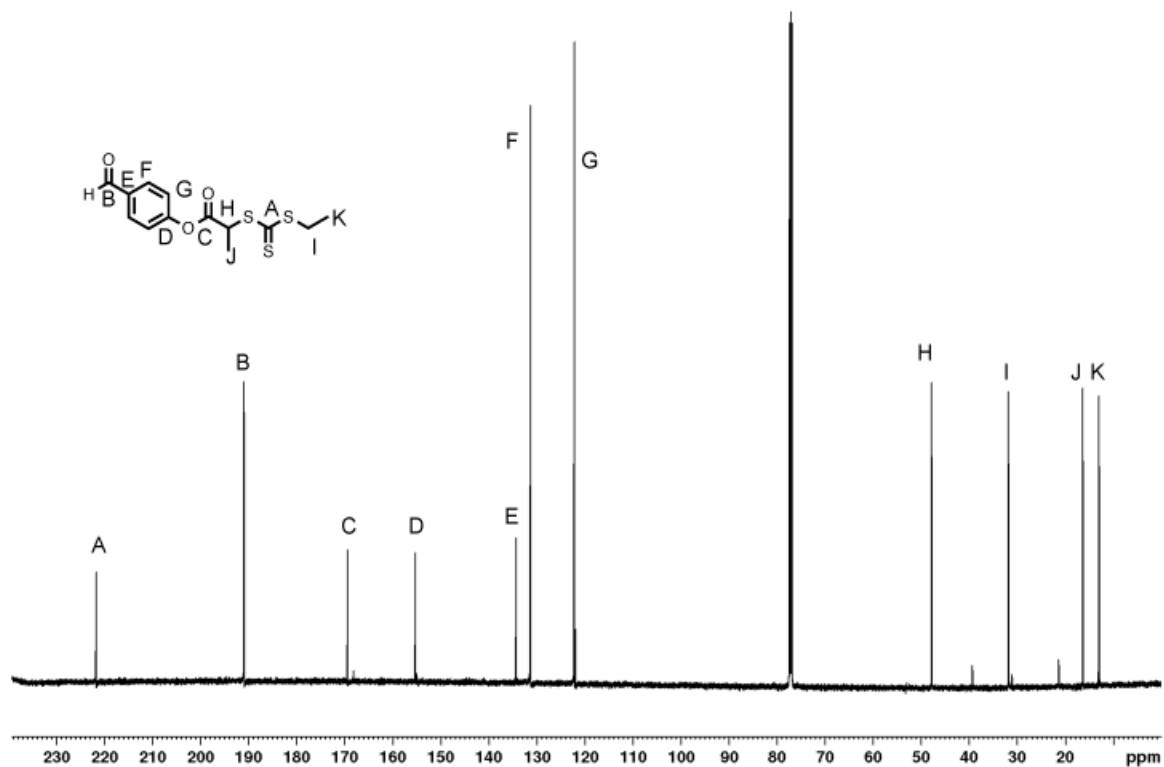
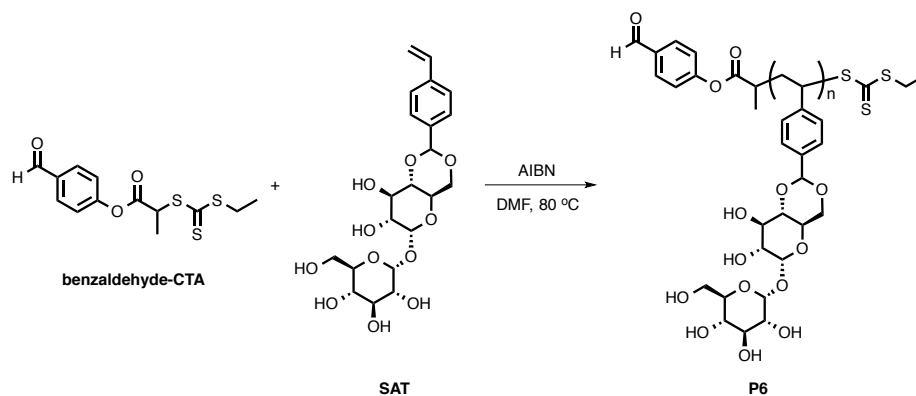


Figure 3-15. ^{13}C NMR spectroscopy of **benzaldehyde-CTA** (in CDCl_3).

Scheme 3-8. RAFT Polymerization of **SAT** Monomer Mediated by **Benzaldehyde-CTA**.



This **benzaldehyde-CTA** was used in the RAFT polymerization of **SAT** monomer with AIBN as an initiator. The crude polymer was purified by dialysis against H_2O resulting in **P6** ($M_n = 24,500$ g/mol and $\text{PDI} = 1.19$, Figure 3-16).

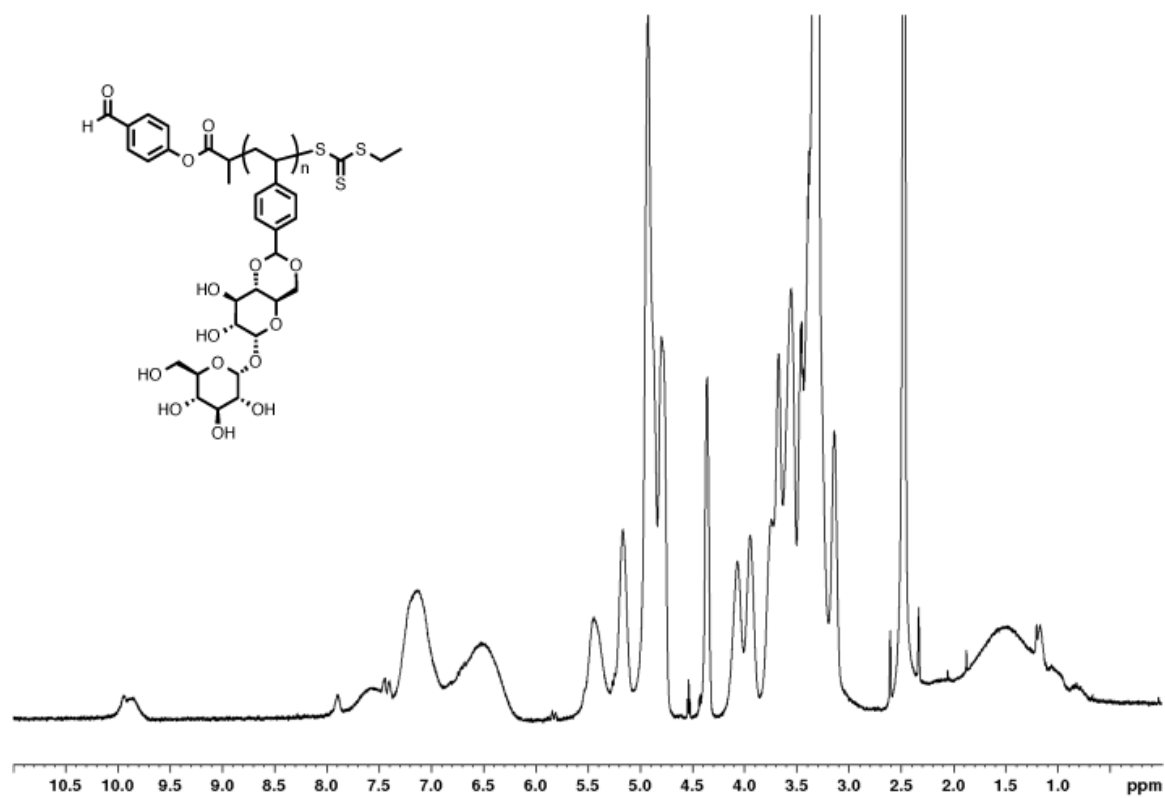
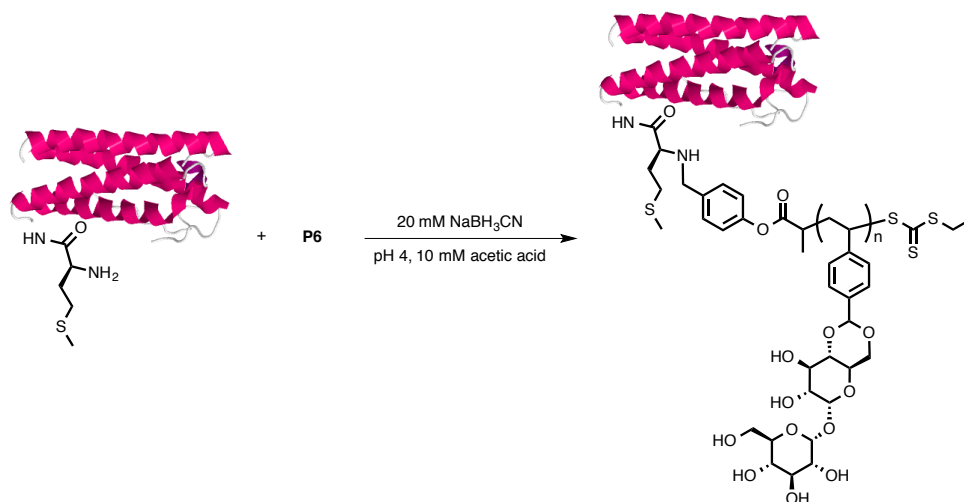


Figure 3-16. ^1H NMR spectroscopy of **benzaldehyde-CTA** (in D_6DMSO).

Preparation of G-CSF-Trehalose Polymer Conjugate

Scheme 3-9. Schematic Drawing of G-CSF-**P6** Conjugation.



The synthesized **P6** was conjugated to the N-terminal methionine of G-CSF in 10 mM pH 4 acetic acid in the presence of NaBH₃CN under similar conditions as the preparation of the insulin-trehalose polymer conjugate (Scheme 3-9). Silver staining of the native gel and western blot was used to verify that the conjugate formed during the reaction (Figure 3-17). In the native gel of the crude reaction, (Figure 3-17a lane 2) an additional band was stained at a slightly higher molecular weight than the unmodified G-CSF in lane 1. The polymer control lane (Figure 3-17a lane 3) showed no strong band in this region, indicating that the new band was the desired conjugate. Western blot was used to confirm that the extra band from silver stained native gel was G-CSF-**P6** conjugate (Figure 3-17b lane 2); **P6** alone did not stain at all. (Figure 3-17b lane 3). However, the new band was very faint. Although the shift to higher molecular weight in the gel suggests that a conjugate was formed, there was not enough material to confirm conjugation by FPLC or ELISA. In the future, a more optimal conjugation chemistry or longer linker to the sterically bulky polymer chain should be implemented to increase the yield of conjugation.

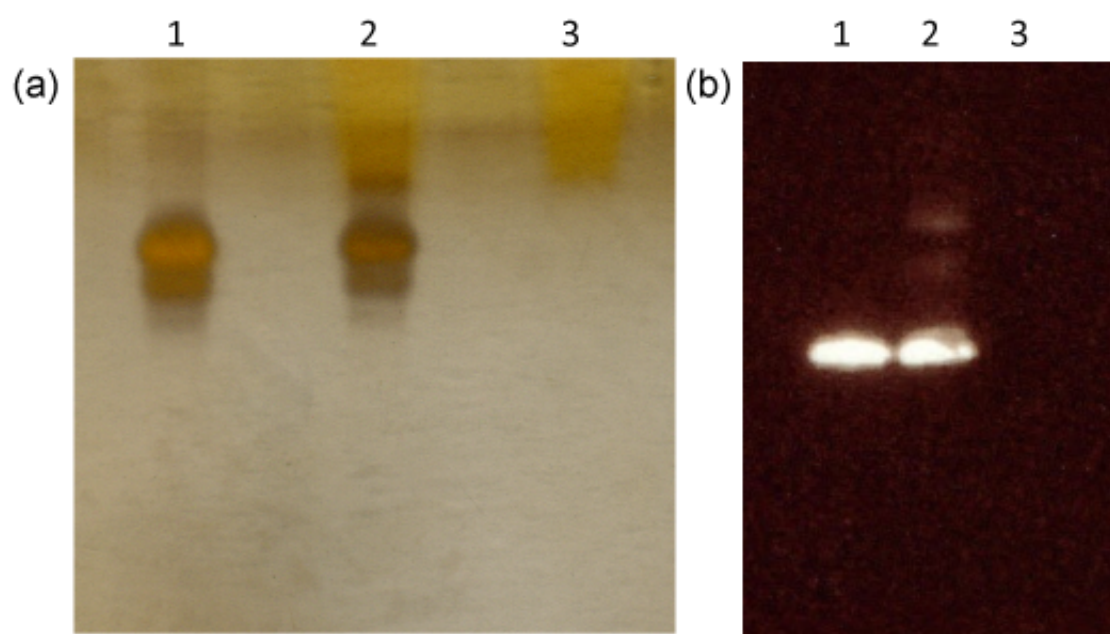
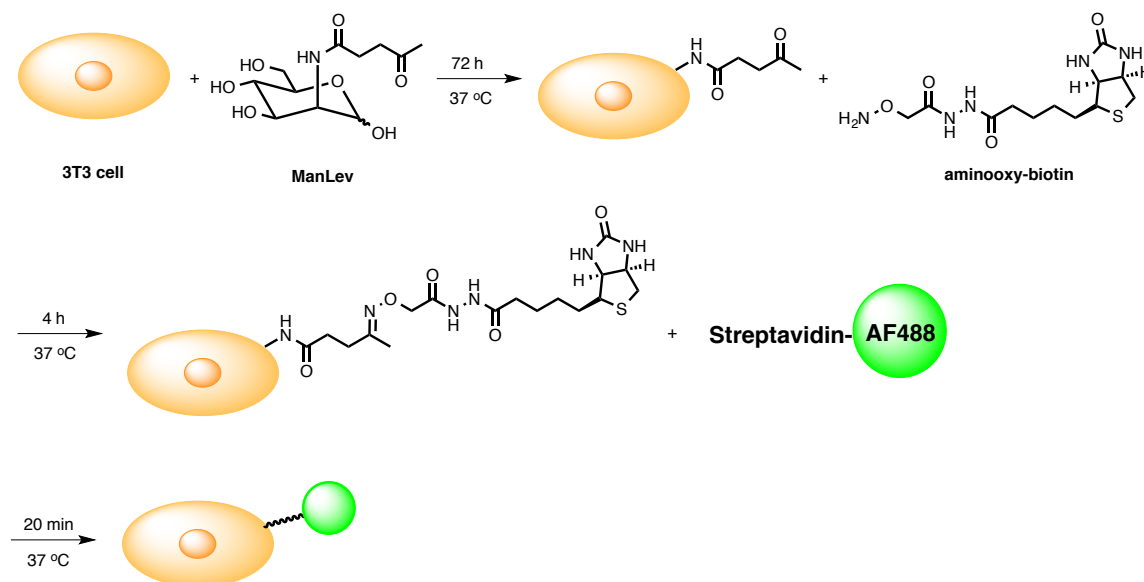


Figure 3-17. Native gel of the crude G-CSF-**P6** conjugate after (a) silver staining and (b) western blot; lane 1: G-CSF, lane 2: crude G-CSF-**P6** conjugate, lane 3: **P6**.

Modification of Cell Surface with ManLev

Scheme 3-10. Modification of Cell Surface with **ManLev**.



To modify cell surface with a desired functional group, a protocol published by Bertozzi was followed.⁵³ **ManLev** was synthesized following literature procedures and was then incubated with mouse embryonic fibroblast cells (NIH 3T3) for 72 h (Scheme 3-10).⁵³ To verify successful modification of the cell surface with the levulinic moiety, the biotin-streptavidine interaction was used and the fluorescent dye AF488 for visualization. Aminoxy-functionalized biotin was first incubated with the cell to form an oxime bond and the biotin group was then conjugated to streptavidin-AF488. After washing to allow removal of unconjugated materials, the AF488 modified cell was observed by fluorescent microscopy (Figure 3-18). In the absence of the **ManLev** modification, no signal could be detected (Figure 3-18 top). However, after incubation of the cells with **ManLev**, the cells could be clearly visualized, indicating the successful modification of the cell surface.

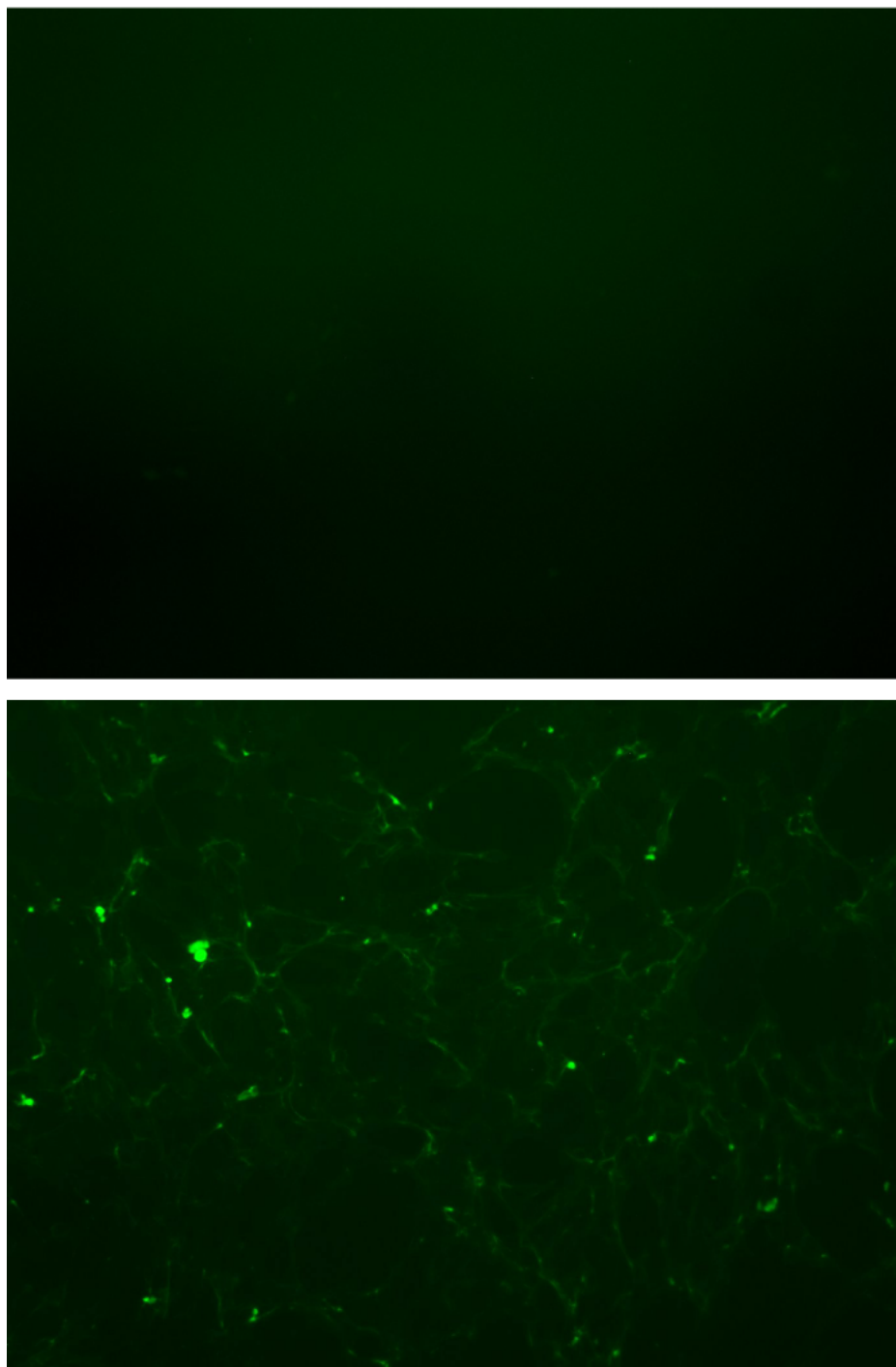
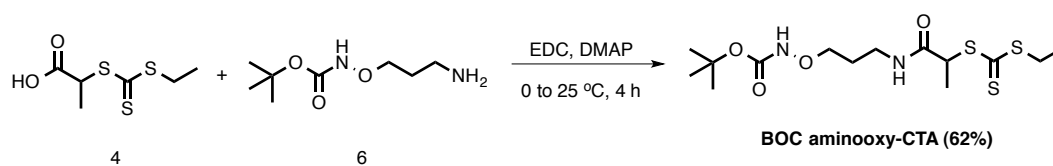


Figure 3-18. Fluorescent microscope images of 3T3 cells without incubation with **ManLev**, with aminooxy-biotin and AF488-Streptavidin (top) and with incubation of **ManLev**, aminooxy-biotin, and AF488-Streptavidin (bottom).

Synthesis of Aminoxy End-Group Trehalose Polymer

Scheme 3-11. Synthesis of **BOC Aminoxy-CTA**.



BOC aminoxy-CTA for RAFT polymerization was synthesized to conjugate the trehalose polymer to the levulinate cell surface through oxime click chemistry (Scheme 3-11). EDC coupling of the known product **6** with the carboxylic acid **4** yielded the desired product in 62% yield (Figure 3-19 and 3-20).

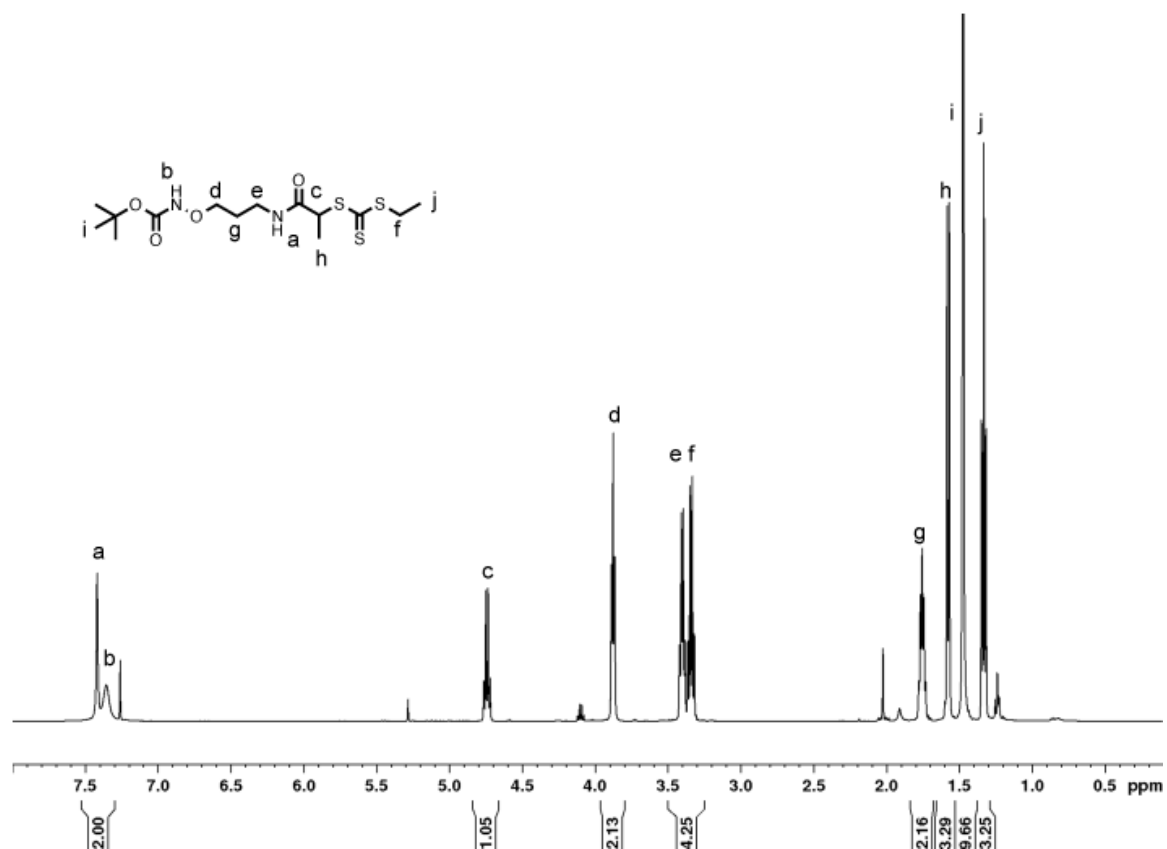


Figure 3-19. ¹H NMR spectroscopy of **BOC aminoxy-CTA** (in CDCl₃).

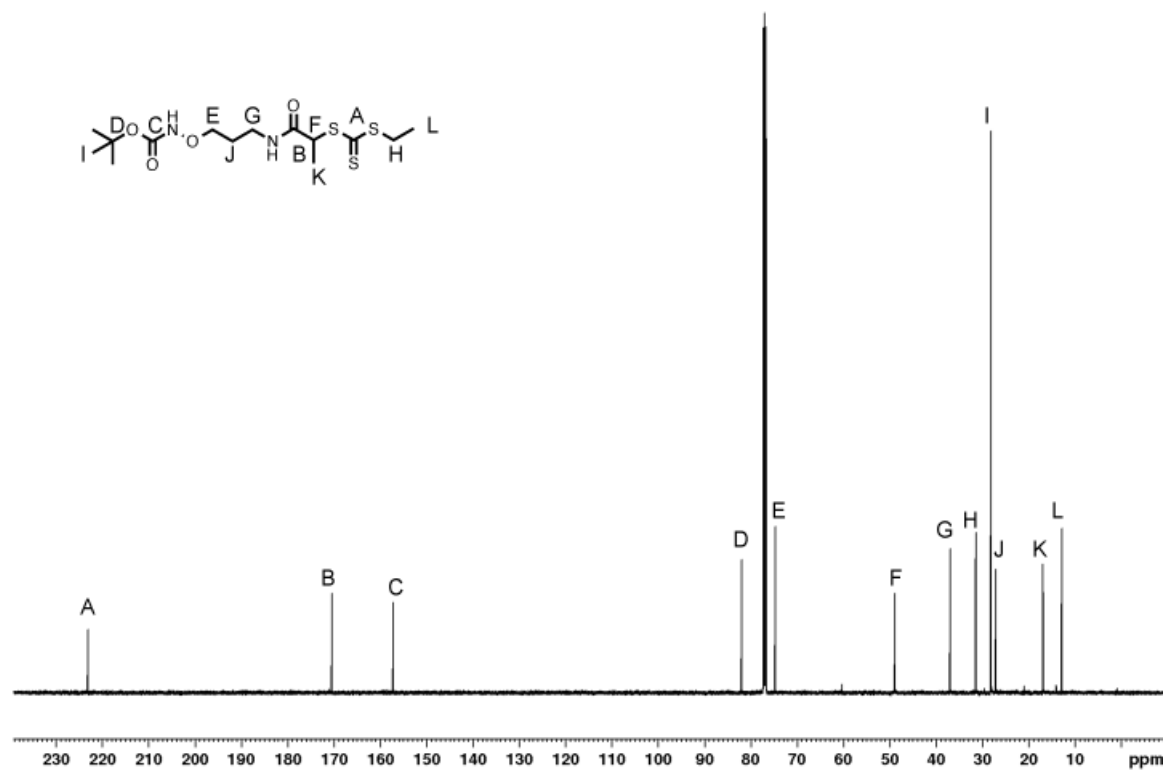
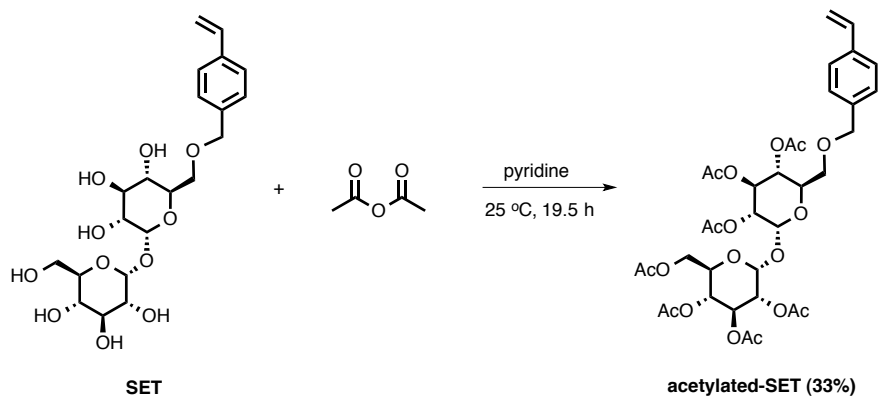


Figure 3-20. ^{13}C NMR spectroscopy of **BOC aminoxy-CTA** (in CDCl_3).

Scheme 3-12. Synthesis of **Acetylated-SET**.



Because the **SET** monomer has low solubility in organic solvents, an acetate-protected monomer was synthesized under basic condition using pyridine as a solvent in 33% yield (Figure 3-21).

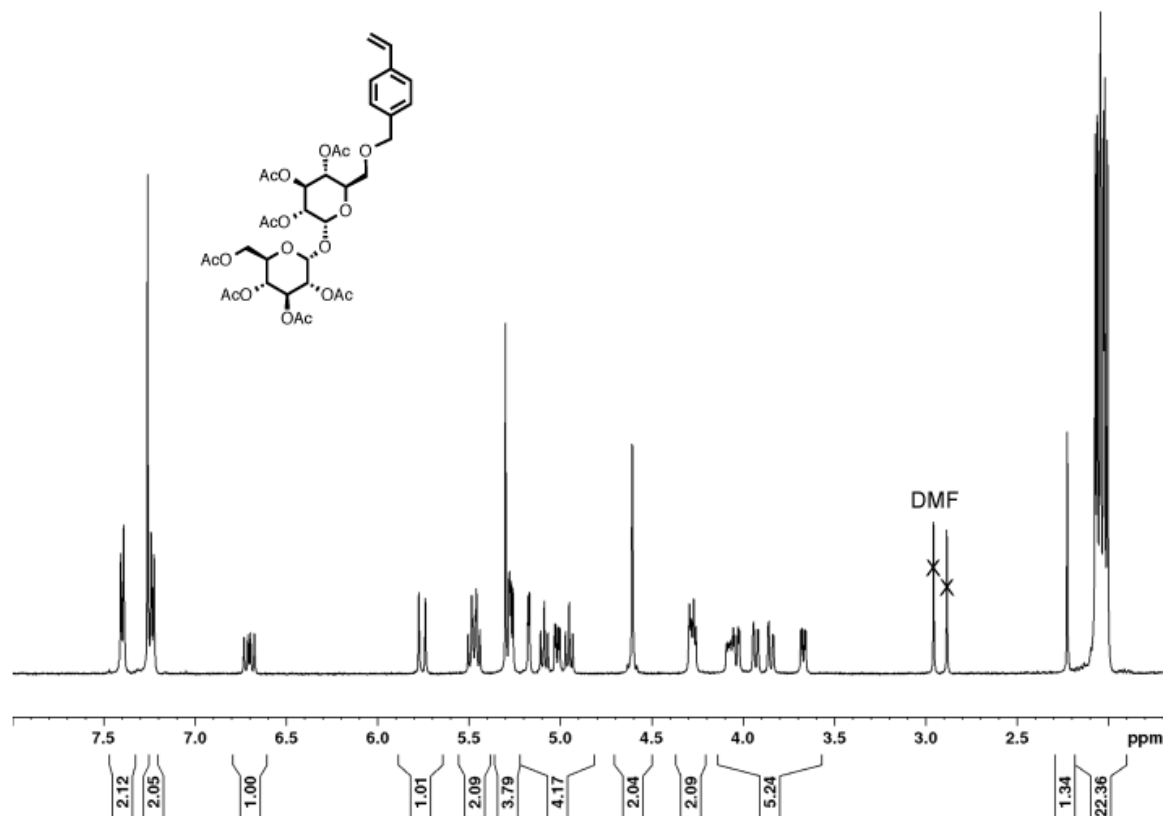
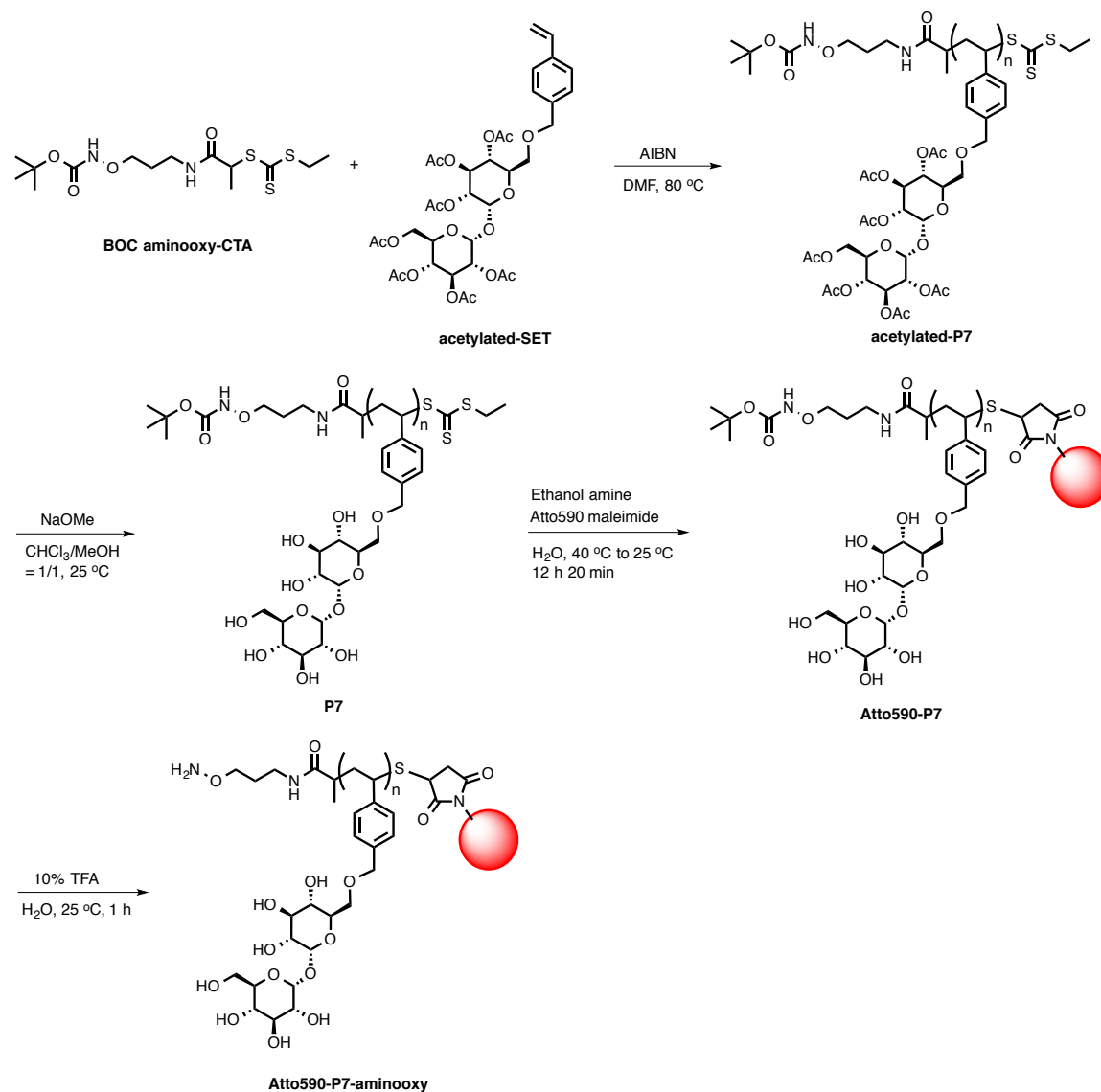


Figure 3-21. ¹H NMR spectroscopy of **Acetylated-SET** (in CDCl₃).

Scheme 3-13. Synthesis of **P7** and Fluorophore Labeled-**P7**.



The prepared **BOC aminooxy-CTA** and **acetylated-SET** were polymerized using RAFT polymerization with AIBN in DMF to yield **acetylated-P7** ($M_n = 11,300$ g/mol and PDI = 1.19). In the ¹H NMR spectrum (Figure 3-22), a large acetate peak from the polymer side chain was visible around 2 ppm. After treating **acetylated-P7** with NaOMe, the acetate peaks disappeared (Figure 3-23) indicating successful deprotection. GPC of the resulting **P7** revealed a lower molecular weight due to deprotection of the acetate group and a similar PDI as the precursor (M_n

= 8,600 g/mol and PDI = 1.08).

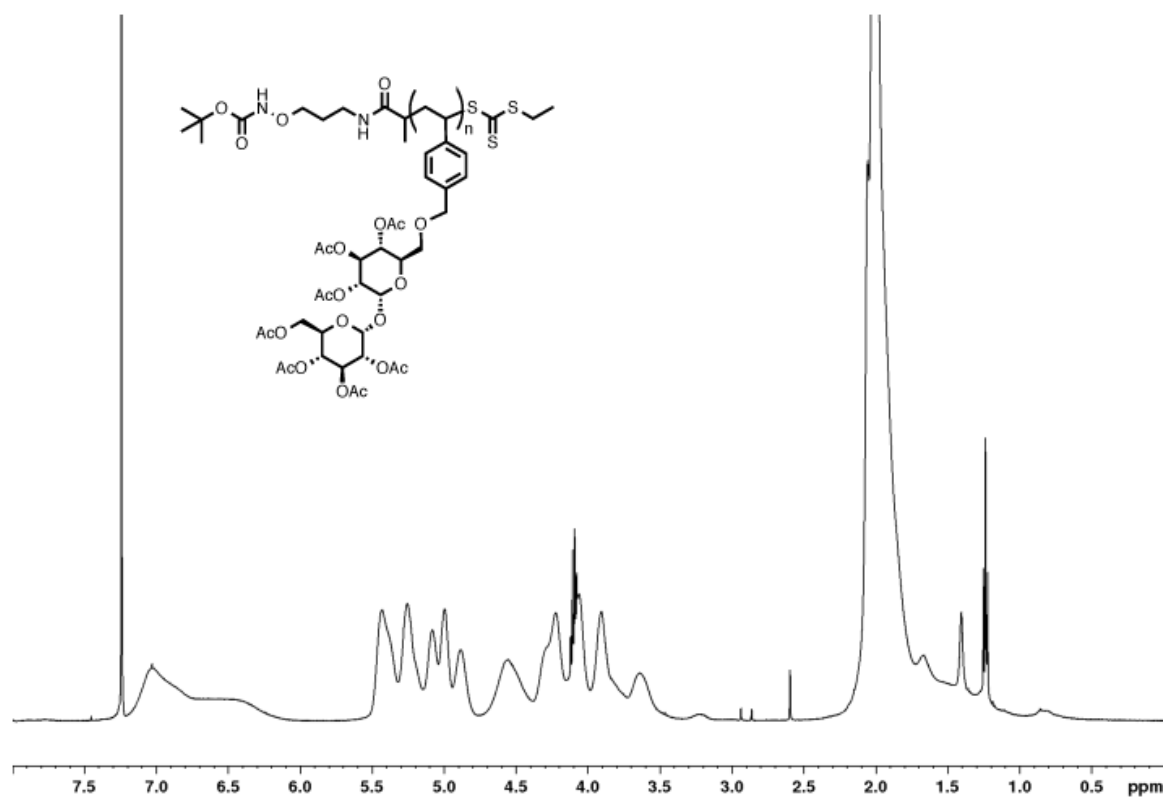


Figure 3-22. ^1H NMR spectroscopy of acetylated-P7 (in CDCl_3).

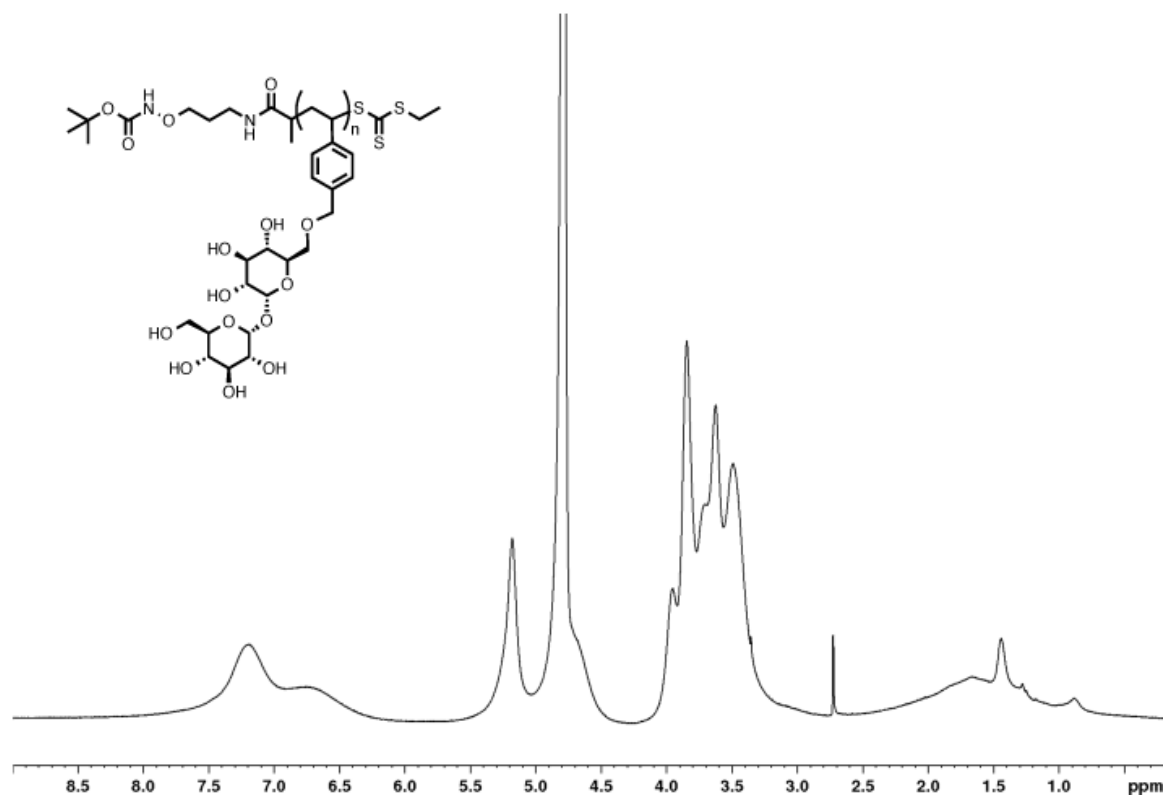


Figure 3-23. ^1H NMR spectroscopy of **P7** (in D_2O).

To visualize **P7** for further experiment, conjugation onto the **ManLev** modified cell surface, **P7** was also labeled with Atto590 dye (Scheme 3-13). The trithiocarbonate moiety of **P7** was transformed into a free thiol *via* aminolysis with ethanolamine. Atto590-maleimide could react with the free thiol and label the **P7**. This **Atto590-P7** was dissolved and treated with 10% TFA aqueous solution to deprotect the BOC group and obtain a free aminoxy functional group.

Discussion

The above data shows that trehalose polymers can enhance both the stability and pharmacokinetic properties of protein therapeutics. The heat burden study of insulin with polymers **P1-P3** indicated that trehalose polymers can be used as excipients for insulin stabilization against heating (Figure 3-1). We have already shown the stabilization effect of trehalose polymers as additives for multiple proteins.^{22,23} Interestingly, the native gel results indicate that trehalose polymers with polystyrene backbones were better stabilizers of the protein structure, whereas the methacrylate trehalose polymer had weaker effect, similar to trehalose. We have previously shown that styrenyl trehalose polymers (**P1** and **P3**) also showed higher stabilization effects than methacrylate trehalose polymer (**P2**) in horseradish peroxidase heating studies.²² However, further studies are needed to generalize differences in efficiency between the polymers.

Next the pharmacokinetic properties of insulin-trehalose polymer conjugates were studied. To prepare these conjugates, CTAs containing acetal protected aldehyde, ketone, and benzaldehyde end-groups, which can all be coupled to primary amines using reductive amination, were synthesized. First, the SET monomer was polymerized through RAFT mediated by an acetal protected aldehyde CTA. The SET monomer was chosen because in preliminary studies, styrenyl trehalose polymers showed better structural protection of insulin. Additionally, the SAT monomer contains an acetal bond between the styrene and trehalose moieties, which could be cleaved in the deprotection of the acetal end-group. Although the polymer was synthesized successfully, no aldehyde peak was detected in the ¹H NMR spectrum after the deprotection of the acetal end-group in 50% TFA aqueous solution. This absence of the aldehyde peak could be due to end-group loss during the deprotection reaction, potentially due to cleavage of the ester.

Therefore, we polymerized a ketone end-group CTA with the SAT monomer to prepare a different insulin reactive trehalose polymer. Three different molecular weight polymers (17,300 g/mol with PDI = 1.27, 23,600 g/mol with PDI = 1.53, and 31,500 g/mol with PDI = 1.55) were synthesized. However, the PDIs were broad in case of higher molecular weights, which needs to be optimized by changing polymerization condition. We have previously seen this trend using both RAFT or ATRP conditions in the synthesis of branched PEGs or trehalose polymer with a pyridyl disulfide CTA.²³ The insulin-trehalose polymer conjugate was prepared through reductive amination, which is currently the most general method for preparing protein-polymer conjugate in the pharmaceutical industry.¹³ Insulin has three primary amines, N-terminus glycine (A chain), phenylalanine (B chain), and lysine at B29. It is known that conjugating PEG chains to Phe B1 or Lys B29 does not decrease insulin bioactivity.⁵⁵ When monitoring the conjugation of insulin with the trehalose polymer, only one conjugate band was detected in the native gel, in contrast to the PEG conjugate which showed two conjugate bands (Figure 3-10 and 3-12). More studies on the insulin-trehalose polymer conjugate will be needed to verify the ratio between insulin and polymer as well as to confirm which amino acid moiety is conjugated to the polymer. The synthesized insulin-trehalose polymer was injected in mice (n=5) to compare the clearance time from the blood plasma to that of the unmodified insulin and insulin-mPEG conjugate. The results of insulin conjugates showed dramatic differences in insulin clearance rate in the bloodstream compared to free insulin (Figure 3-13). Unmodified insulin was undetectable within 5 min after administration, while insulin-trehalose polymer and insulin-mPEG conjugates were detectable by ELISA even at 60 min after injection. Both of the insulin conjugates were statistically same. These results suggest that the trehalose polymer can be used as a potential PEG alternative. No acute toxicity was observed in the mice; future studies will focus on toxicity, biodistribution, and biocompatibility of the conjugate.

G-CSF was selected as another therapeutic protein to be conjugated to the trehalose polymer. In previous studies, polymers **P1-P3** were screened to observation stabilization against various environmental stresses including lyophilization, heating and storage pH of G-CSF. Bioactivity of G-CSF was confirmed by proliferation of cells *in vitro*.⁵⁶ The study identified **P1** (containing styrenyl acetal trehalose side chain) as the most effective stabilizer of G-CSF over all conditions tested (data not shown). Therefore, a benzaldehyde end-group CTA was synthesized and used to mediate the RAFT polymerization of SAT monomer. The polymer was then conjugated to the *N*-terminal methionine of G-CSF, the location of PEGylation in Neulasta®. However, only a very weak band corresponding to a potential conjugate was observed in the native gel (Figure 3-17). These conjugation difficulties may be solved using more a concentrated G-CSF solution and more equivalents of the trehalose polymer.

Lastly, a trehalose polymer with an aminooxy end-group polymer was designed to conjugate to the surface of a ketone-modified cell. Oxime click chemistry is often chosen for engineering cell surface because of its benefits such as short reaction times, bioorthogonal reactions, physiological conditions, and non-toxic byproducts.⁵⁷⁻⁵⁹ For this study, we modified NIH 3T3 cell surface with **ManLev** by following a literature procedure.⁵³ Unnatural **ManLev** saccharide can be converted to the corresponding sialic acid and metabolically incorporated into the cell surface oligosaccharides to create ketone groups on the cell surface. Successful modification of the cell surface was then visualized with AF488 (Figure 3-18). First, aminooxy-biotin was added to form an oxime bond with the ketone group. Then, fluorescently labeled streptavidin-AF488 was added in order to visualize the cells through a biotin-streptavidin interaction. Without incubation with **ManLev**, no oxime bonds were formed, resulting in no interaction between aminooxy-biotin, streptavidin and the cells and therefore no signal in the

fluorescent microscope. Additionally, an aminoxy end-group trehalose polymer was prepared and modified with Atto590 dye to visualize the cell surface conjugation with trehalose polymer later. We expect that this cell-trehalose conjugate will be stabilized against freezing or drying for storage and shipping.

4.4 Conclusion

Herein, the synthesis of various end functionalized trehalose polymers to be used as stabilizers for proteins and cells were described. We have first verified by native gel that the trehalose polymer excipients are able to maintain the structure of insulin after heating. Interestingly styrenyl trehalose polymers outperformed the methacrylate trehalose polymer tested and trehalose. Next, the synthesis of CTAs and trehalose polymers that are able to react with proteins or modified cell surfaces were described. These polymers containing either aldehyde or ketone end-groups can react with the primary amine moieties of proteins through reductive amination. A trehalose polymers was conjugated to insulin, an important therapeutic proteins, and the protein-polymer conjugate was characterized by native gel and western blot. Furthermore, the purified insulin-trehalose polymer conjugate was injected in mice to study its pharmacokinetic properties compared to both unmodified insulin and PEGylated insulin. The insulin-trehalose polymer conjugate exhibited significantly longer biocirculation than insulin. Lastly, we have synthesized an aminooxy end-group trehalose polymer. This polymer will be utilized to conjugate to a 3T3 cell surfaces premodified with ketones using the oxime click reaction. Together, these results suggest the potency of trehalose polymer as a PEG alternative in therapeutic protein-polymer conjugates. It offers the possibility to enhance the pharmacokinetic properties of protein drugs with the additional advantage of improving protein stability against shipping and storage conditions.

4.5 References

- (1) Carter, P. J. *Exp. Cell Res.* **2011**, *317*, 1261-1269.
- (2) Goeddel, D. V.; Kleid, D. G.; Bolivar, F.; Heyneker, H. L.; Yansura, D. G.; Crea, R.; Hirose, T.; Kraszewski, A.; Itakura, K.; Riggs, A. D. *Proc. Natl. Acad. Sci. U. S. A.* **1979**, *76*, 106-110.
- (3) Schellekens, H. *Clin. Ther.* **2002**, *24*, 1720-1740.
- (4) Bhopale, G. M.; Nanda, R. K. *Curr. Sci.* **2005**, *89*, 614-622.
- (5) Breschkin, A. *Dev. Biol. Stand.* **1987**, *67*, 207-211.
- (6) Bristow, A. F. *Trends Biotechnol.* **1993**, *11*, 301-305.
- (7) Leader, B.; Baca, Q. J.; Golan, D. E. *Nat. Rev. Drug Discovery* **2008**, *7*, 21-39.
- (8) Ozgur, A.; Tutar, Y. *Protein Pept. Lett.* **2013**, *20*, 1365-1372.
- (9) Walsh, G. *Nat. Biotechnol.* **2010**, *28*, 917-924.
- (10) Duncan, R. *Nat. Rev. Drug Discovery* **2003**, *2*, 347-360.
- (11) Magnusson, J. P.; Saeed, A. O.; Fernandez-Trillo, F.; Alexander, C. *Polymer Chemistry* **2011**, *2*, 48-59.
- (12) Nechansky, A.; Kircheis, R. *Expert Opinion on Drug Discovery* **2010**, *5*, 1067-1079.
- (13) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polymer Chemistry* **2011**, *2*, 1442-1448.
- (14) Pasut, G.; Veronese, F. M. *J. Controlled Release* **2012**, *161*, 461-472.
- (15) Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003**, *2*, 214-221.
- (16) Pasut, G.; Sergi, M.; Veronese, F. M. *Adv. Drug Delivery. Rev.* **2008**, *60*, 69-78.
- (17) Modi, S.; Jain, J. P.; Domb, A. J.; Kumar, N. *Curr. Pharm. Des.* **2006**, *12*, 4785-4796.

- (18) Satchi-Fainaro, R.; Duncan, R.; Barnes, C. M. In *Polymer Therapeutics II: Polymers as Drugs, Conjugates and Gene Delivery Systems*; SatchiFainaro, R., Duncan, R., Eds. 2006; Vol. 193, p 1-65.
- (19) Molineux, G. *Curr. Pharm. Des.* **2004**, *10*, 1235-1244.
- (20) Morstyn, G.; Foote, M. A.; Walker, T.; Molineux, G. *Acta Haematol.* **2001**, *105*, 151-155.
- (21) Piedmonte, D. M.; Treuheit, M. J. *Adv. Drug Delivery. Rev.* **2008**, *60*, 50-58.
- (22) Lee, J.; Lin, E.-W.; Lau, U. Y.; Hedrick, J. L.; Bat, E.; Maynard, H. D. *Biomacromolecules* **2013**, *14*, 2561-2569.
- (23) Mancini, R. J.; Lee, J.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 8474-8479.
- (24) Richter, A. W.; Akerblom, E. *Int. Arch. Allergy Appl. Immunol.* **1984**, *74*, 36-39.
- (25) Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J.; Garratty, G. *Cancer* **2007**, *110*, 103-111.
- (26) Hey, T.; Knoller, H.; Vorstheim, P. In *Therapeutic Proteins*; Wiley-VCH Verlag GmbH & Co. KGaA: 2012, p 117-140.
- (27) Pisal, D. S.; Kosloski, M. P.; Balu-Iyer, S. V. *J. Pharm. Sci.* **2010**, *99*, 2557-2575.
- (28) Lameire, N.; Hoste, E. *Intensive Care Med.* **2014**, *40*, 427-430.
- (29) Teramoto, N.; Sachinvala, N. D.; Shibata, M. *Molecules* **2008**, *13*, 1773-1816.
- (30) Ohtake, S.; Wang, Y. J. *J. Pharm. Sci.* **2011**, *100*, 2020-2053.
- (31) Sakurai, M. *Water and Biomolecules: Physical Chemistry of Life Phenomena* **2009**, 219-240.
- (32) Kaushik, J. K.; Bhat, R. *J. Biol. Chem.* **2003**, *278*, 26458-26465.
- (33) Sundaramurthi, P.; Shalae, E.; Suryanarayanan, R. *Journal of Physical Chemistry Letters* **2010**, *1*, 265-268.
- (34) Westh, P.; Ramlov, H. *J. Exp. Zool.* **1991**, *258*, 303-311.

- (35) Hengherr, S.; Heyer, A. G.; Kohler, H. R.; Schill, R. O. *FEBS J.* **2008**, *275*, 281- 288.
- (36) Crowe, L. M.; Reid, D. S.; Crowe, J. H. *Biophys. J.* **1996**, *71*, 2087-2093.
- (37) Jain, N. K.; Roy, I. *Protein Sci.* **2009**, *18*, 24-36.
- (38) Crowe, J. H. *Am. Nat.* **1971**, *105*, 563-&.
- (39) Crowe, J. H.; Madin, K. A. *Trans. Am. Microsc. Soc.* **1974**, *93*, 513-524.
- (40) Lins, R. D.; Pereira, C. S.; Hünenberger, P. H. *Proteins: Struct., Funct., Bioinf.* **2004**, *55*, 177-186.
- (41) Williams, R. J.; Leopold, A. C. *Plant Physiol.* **1989**, *89*, 977-981.
- (42) Luyckx, J.; Baudouin, C. *Clinical ophthalmology (Auckland, N.Z.)* **2011**, *5*, 577-581.
- (43) Teramoto, N.; Arai, Y.; Shibasaki, Y.; Shibata, M. *Carbohydr. Polym.* **2004**, *56*, 1-6.
- (44) Park, O. J.; Kim, D. Y.; Dordick, J. S. *Biotechnol. Bioeng.* **2000**, *70*, 208-216.
- (45) Teramoto, N.; Arai, Y.; Shibata, M. *Carbohydr. Polym.* **2006**, *64*, 78-84.
- (46) Srinivasachari, S.; Liu, Y. M.; Zhang, G. D.; Prevette, L.; Reineke, T. M. *J. Am. Chem. Soc.* **2006**, *128*, 8176-8184.
- (47) Srinivasachari, S.; Liu, Y. M.; Prevette, L. E.; Reineke, T. M. *Biomaterials* **2007**, *28*, 2885-2898.
- (48) Kurita, K.; Masuda, N.; Aibe, S.; Murakami, K.; Ishii, S.; Nishimura, S. I. *Macromolecules* **1994**, *27*, 7544-7549.
- (49) Wada, M.; Miyazawa, Y.; Miura, Y. *Polymer Chemistry* **2011**, *2*, 1822-1829.
- (50) Sizovs, A.; Xue, L.; Tolstyka, Z. P.; Ingle, N. P.; Wu, Y.; Cortez, M.; Reineke, T. M. *J. Am. Chem. Soc.* **2013**, *135*, 15417-15424.
- (51) Bergenstal, R. M.; Rosenstock, J.; Arakaki, R. F.; Prince, M. J.; Qu, Y.; Sinha, V. P.; Howey, D. C.; Jacober, S. J. *Diabetes Care* **2012**, *35*, 2140-2147.
- (52) Caparrotta, T. M.; Evans, M. *Diabetes Obes. Metab.* **2014**, *16*, 388-395.

- (53) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. *Science* **1997**, 276, 1125-1128.
- (54) Mancini, R. J. *University of California, Los Angeles, Dissertation* **2012**.
- (55) Torosantucci, R.; Kukrer, B.; Mero, A.; Van Winsen, M.; Tantipolphan, R.; Jiskoot, W. J. *Pharm. Sci.* **2011**, 100, 2574-2585.
- (56) Lau, U. Y.; Lee, J. **Unpublished data**.
- (57) Grover, G. N.; Lam, J.; Nguyen, T. H.; Segura, T.; Maynard, H. D. *Biomacromolecules* **2012**, 13, 3013-3017.
- (58) Grover, G. N.; Braden, R. L.; Christman, K. L. *Advanced Materials* **2013**, 25, 2937-2942.
- (59) Xu, X.; Jha, A. K.; Harrington, D. A.; Farach-Carson, M. C.; Jia, X. *Soft matter* **2012**, 8, 3280-3294.

Chapter 4

Synthesis of Trehalose Hydrogels for Protein Delivery and Stabilization

In chapter 4, the development of trehalose and hyaluronic acid hydrogels for protein stabilization and delivery is discussed. Synthesis of glucose responsive trehalose-boronic acid hydrogels, pH responsive trehalose-acetal hydrogels, trehalose hydrogels for industrial animal feeds pelleting, and hyaluronic acid hydrogels through oxime bond formation will be presented.

4.1 Glucose Responsive Trehalose Hydrogel for Insulin Delivery

4.1.1 Introduction

The controlled *in vivo* delivery of biomolecules while maintaining stability is critical for their efficient therapeutic use. Interest in boronic acid containing hydrogels for applications in a wide variety of biomedical fields is growing.¹⁻³ Because boronic acids form reversible covalent complexes with 1,2- or 1,3-diols their incorporation into hydrogels results in glucose-responsive materials.⁴⁻⁷ Due to this glucose-responsive moiety, these hydrogels are commonly used as devices for insulin delivery.⁸⁻¹⁴ The majority of these insulin delivery boronic acid hydrogels were prepared by copolymerizing boronic acid and crosslinkable monomers to form a hydrogel that swells in the presence of glucose, thereby releasing insulin.

In this chapter, we propose the synthesis of a glucose responsive trehalose-based hydrogel for the stabilization and delivery of insulin. In 2011, Ellington, Anslyn, and Sessler have shown the formation of complexes between tetra-boronic acid DNA and saccharides including trehalose.¹⁵ However, to our knowledge, no hydrogel combining boronic acid and trehalose has been reported. Since we have already shown that trehalose polymers are effective stabilizers for proteins against lyophilization and heat,^{16,17} we expect the trehalose-based hydrogel can be used to deliver as well as stabilize insulin during storage. First, a trehalose polymer was synthesized following the literature procedure.¹⁷ 8arm PEG amine was modified

with phenyl boronic acid through reductive amination. The prepared trehalose polymer and boronic-acid functionalized PEG were then reacted at physiological pH to successfully form the trehalose-based hydrogel. Moreover, the glucose responsive properties of the hydrogel were tested by measuring the weight loss of the hydrogel after immersion into a glucose solution. The results showed a sharp decrease of weight in the presence of glucose due to the replacement of trehalose-boronic ester bonds with glucose-boronic esters. As expected, higher concentration glucose solutions de-crosslinked the hydrogel more rapidly.

4.1.2 Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich and Fisher Scientific. 8arm PEG amine was purchased from Jenkem Technology (Allen, TX). Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX), dried with ethanol and kept under vacuum before use. Azobisisobutyronitrile (AIBN) was recrystallized from acetone before use. Styrenyl ether trehalose monomer (**SET**) was prepared using our reported procedures as described in Chapter 2.¹⁷

Analytical techniques

NMR spectra were obtained on Bruker DRX 500 MHz spectrometers. ¹H NMR spectra were acquired with a relaxation delay of 30 s for polymers.

Methods

Synthesis of poly(styrenyl ether trehalose) (poly(SET)). AIBN (5.28 mg, 3.22×10^{-2} mmol) and **SET** (634 mg, 1.38 mmol) were dissolved in a mixture of DMF (2.31 mL) and H₂O (4.61 mL). Oxygen was removed by three cycles of freeze-pump-thaw and polymerization was initiated at 75 °C. The polymerization was stopped after 8.5 h by immersing the reaction into liquid nitrogen. The polymer was purified by dialysis against H₂O (MWCO 3,500) resulting in a polymer with M_n of 7.0 kDa and PDI of 1.28. ¹H NMR (500 MHz in D₂O) δ : 7.01, 6.45, 5.05, 3.81, 3.71, 3.59, 3.48, 3.36, 1.50.

Synthesis of 8Arm PEG Boronic Acid. 8-arm-PEG amine (400 mg, 10 kDa, 4×10^{-2} mmol) and 4-formyl boronic acid (96 mg, 6.40×10^{-1} mmol) were dissolved in 2.8 mL of MeOH. NaBH₃CN

(18.85 mg, 3.00×10^{-1} mmol) was added and the reaction was stirred at 25 °C. After 5 days the reaction solution was purified by dialysis against MeOH for 2 days and H₂O for 2 days. The sample was lyophilized and ¹H-NMR analysis showed 100% modification of amine end-groups in the PEG. ¹H NMR (500 MHz in D₂O) δ : 7.75, 7.41, 4.14, 3.69, 3.18.

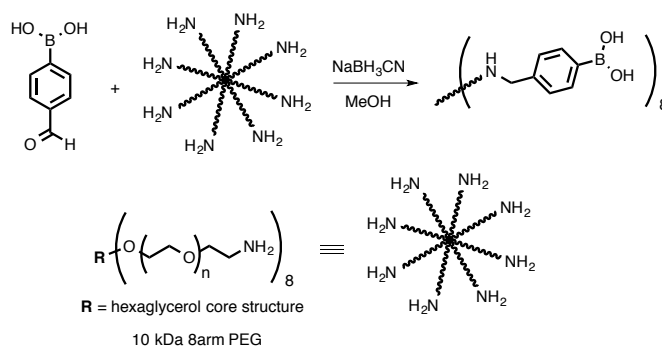
Synthesis of poly(SET)-Boronic Acid Hydrogel. Poly(SET) and 8 arm PEG boronic acid (10 kDa) were dissolved in pH 7.4 D-PBS buffer to concentrations of 500 mg/mL and 200 mg/mL respectively. 3 μ L of **poly(SET)** solution and 20.5 μ L of **8arm PEG amine** solution were mixed (1 : 1 = trehalose unit : boronic acid unit) to result in the hydrogel.

Glucose-Responsiveness Study. Fifteen separately prepared **poly(SET)-boronic acid hydrogels** were immersed into 150 μ L of pH 7.4 D-PBS buffer for 1 h. The hydrogels were air-dried for 15 min and their weights were measured. Each of the hydrogels were then immersed into 150 μ L of either pH 7.4 D-PBS buffer, 1 mg/mL, 5 mg/mL, 10 mg/mL, or 20 mg/mL glucose solution in pH 7.4 D-PBS buffer (three hydrogels per condition). Each time point was collected by air-drying hydrogels for 15 min and weighing the dried hydrogels.

4.1.3 Results and Discussion

Preparation of Boronic Acid Crosslinker and Trehalose Hydrogels

Scheme 4.1-1. Synthesis of 8arm PEG Boronic Acid through Reductive Amination.



The boronic acid crosslinker was synthesized through reductive amination, using 4-formyl boronic acid and 8arm PEG amine as starting materials (Scheme 4.1-1). Complete modification of 8arm PEG amines with phenylboronic acid was confirmed by ^1H NMR spectroscopy (Figure 4.1-1).

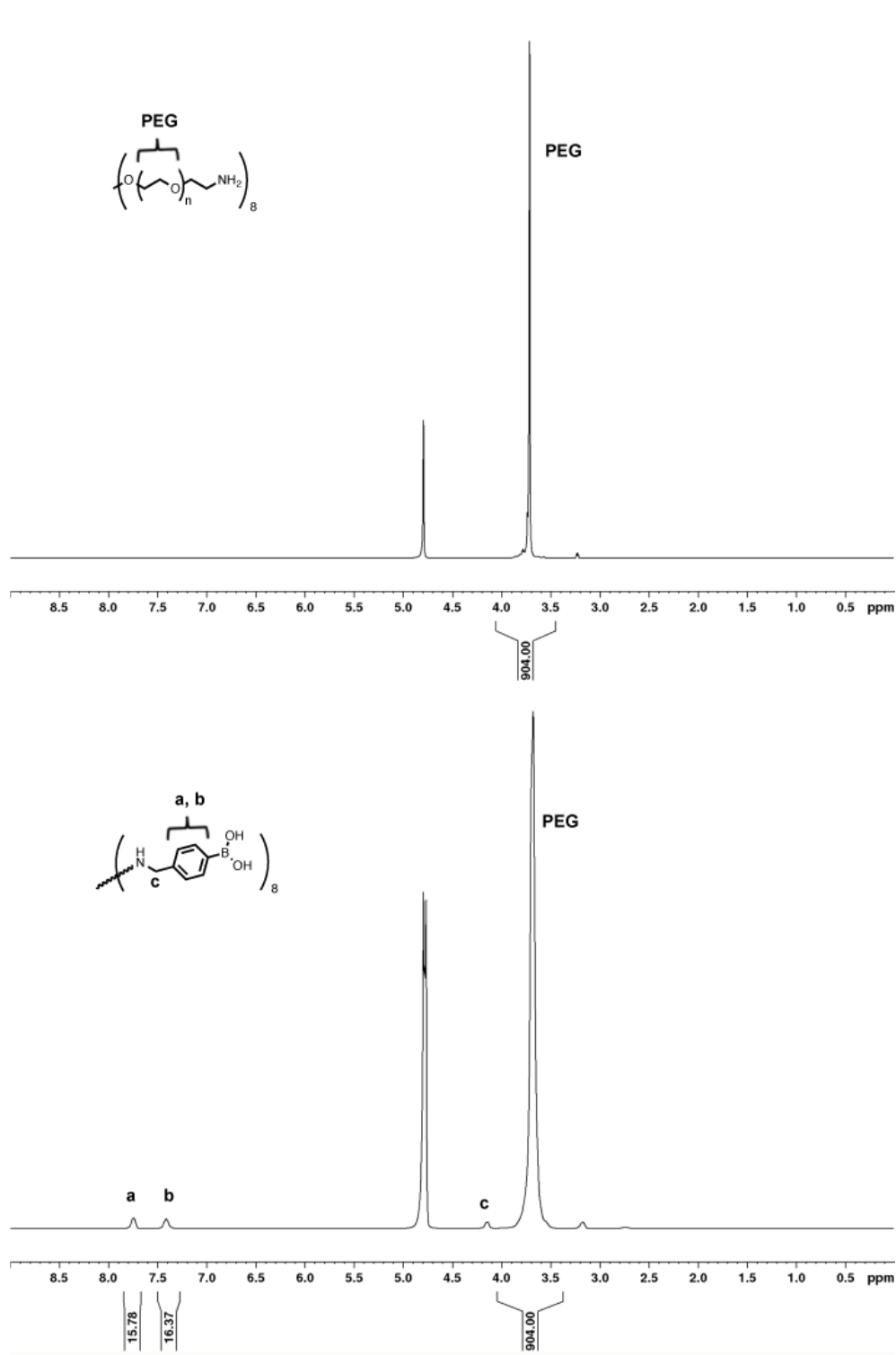


Figure 4.1-1. ^1H NMR spectra of **8arm PEG amine** (top) and **8arm PEG boronic acid** (bottom) (in D_2O).

The synthesized **8arm PEG boronic acid** was then mixed with **poly(SET)** in a ratio of boronic acid to trehalose unit 1 : 1 (Scheme 4.1-2). The gelation occurred rapidly within 3 min (Figure 4.1-2).

Scheme 4.1-2. Preparation of Trehalose-Boronic Acid Hydrogel.

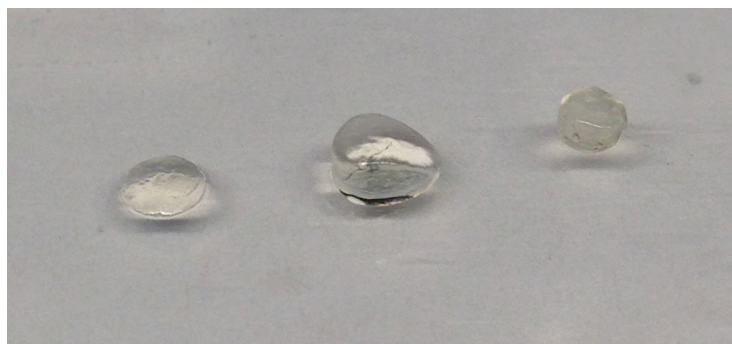
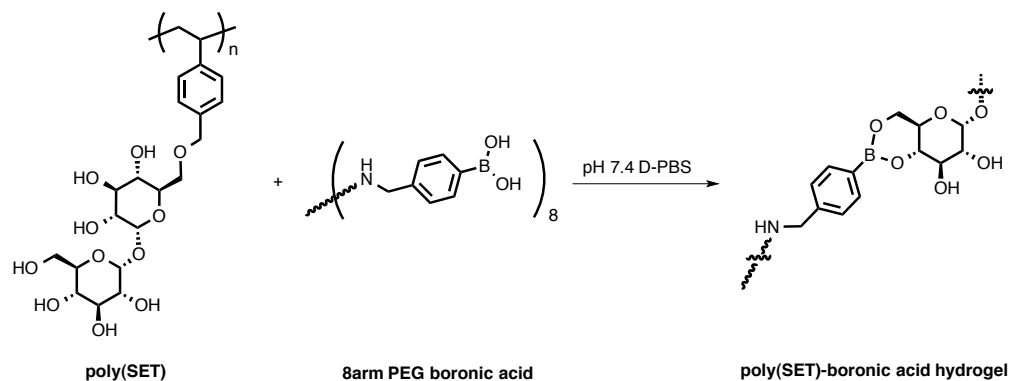


Figure 4.1-2. Synthesized **poly(SET)-boronic acid hydrogel**.

Glucose-Responsiveness Study of the poly(SET)-boronic acid hydrogel

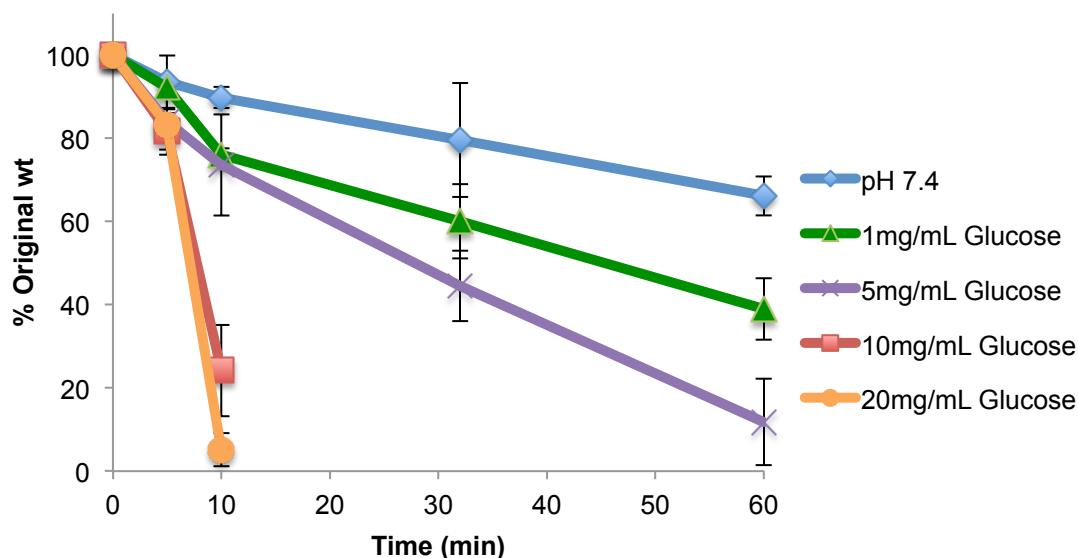


Figure 4.1-3. Weights of **poly(SET)-boronic acid hydrogels** after immersing into pH 7.4 D-PBS buffer, 1 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL glucose solution in pH 7.4 D-PBS buffer (n=3 per group).

The prepared **poly(SET)-boronic acid hydrogel** was then tested for glucose-responsiveness. Since the boronic ester bond from the trehalose-boronic acid complex is expected to have a significantly weaker binding affinity than that of glucose-boronic acid,^{18,19} glucose should replace the boronic ester bond between the trehalose polymer and the boronic acid crosslinker. This would de-crosslink the polymer chains and the boronic acid crosslinker reversing the hydrogel. As the polymer and the crosslinker are all water soluble and can therefore diffuse into the buffer, the hydrogel should lose weight during this process. As shown in Figure 4.1-3, the hydrogel lost 34% of its original weight after immersing the gel into pH 7.4 D-PBS buffer. This may be due to the diffusion of uncrosslinked polymer or crosslinker from the hydrogel.

However, when the hydrogels were placed into the buffer with glucose, their weight loss was clearly faster. There was a clear trend that higher concentration glucose solutions de-crosslinked the hydrogel more rapidly. The weights of the hydrogels immersed in 10 mg/mL and 20 mg/mL glucose solutions were unable to be measured after 10 minutes because they had dissolved into the solution, whereas hydrogels in 1 mg/mL and 5 mg/mL glucose solutions were still gels at 10 minutes.

Discussion

To date, hydrogels using trehalose and boronic acid binding have not yet been reported. The above data suggest that hydrogels can be prepared by utilizing a boronic ester bond between our previously reported trehalose polymers and a phenylboronic acid functionalized multi-arm PEG. The gelation was fast in physiological conditions (pH 7.4). Moreover, the resulting hydrogel was glucose-responsive. The addition of glucose led to de-crosslinking of the boronic ester bond between trehalose (polymer) and boronic acid (crosslinker) by competitive replacement of glucose-boronic acid complex due to the higher binding affinity of glucose to phenylboronic acid. As expected, higher glucose concentration buffers increased the rate of dissolution of the hydrogel. This suggests that these gels could be utilized for insulin delivery applications. Indeed, future work will involve studies of stabilization and release of insulin from these gels.

4.1.4 Conclusion

Herein, we have described the preparation of a hydrogel using a trehalose side chain polymer and 8arm boronic acid-functionalized PEG. In 1 : 1 ratio of trehalose unit to boronic acid unit, the hydrogel was formed within 3 min. By measuring the weight loss of the hydrogels after incubation in various conditions, deformation of the hydrogel was observed as expected. The higher the concentration of glucose, the faster the hydrogel dissolved. We expect that this trehalose-based hydrogel can be used for effective *in vivo* glucose-responsive insulin delivery, with the advantage of using trehalose polymer as an insulin stabilizer during storage before use.

4.2 pH Responsive Trehalose Hydrogels

4.2.1 Introduction

Hydrogels have been extensively used as drug delivery vehicles with biomedical applications.²⁰ “Smart hydrogels”, which respond to specific triggers, can be synthesized to deliver and release guest drugs into a specifically targeted site.²¹⁻²⁵ In particular, pH responsive hydrogels are frequently used in drug delivery because different cell types and compartments of cells have discrete pHs, which allows for site specific release of a payload. For example, the pH of the extracellular matrix (ECM) is typically around 7.4, while the cytosol has a lower pH and cancer cells are also more acidic than normal cells.^{26,27} Moreover, the pH in the stomach is between pH 2 and 4 depending on whether the stomach is empty or food has been ingested.²³ Therefore research on pH responsive hydrogels is an important field of interest. Significant research has been reported toward the oral administration of therapeutics using pH responsive hydrogels. These hydrogels target the stomach for site-specific delivery of antibiotic, therapeutic proteins, and peptides.²⁸⁻³⁴ Since the target site is the stomach, the hydrogels must only release their therapeutics in conditions more acidic than pH 3. This release occurs by changing the degree of swelling in the hydrogel or by cleaving the crosslinker.

In this chapter, we propose a unique pH responsive hydrogel based on trehalose. To our knowledge no pH responsive hydrogel based on trehalose have been reported. The trehalose is generally regarded as safe by US Federal Drug Administration (FDA) and act as a natural stabilizer for cells and proteins in organisms, which makes it to be a perfect candidate for synthesis of hydrogel for biomedical use.³⁵ We have already reported that trehalose side chain glypolymers help maintaining protein activity against heat and lyophilization (see Chapter 2).^{16,17}

Therefore, we expect trehalose hydrogels to act not only as a delivery vehicle but also as stabilizers against environmental stressors during storage and transportation. For hydrogel synthesis, a crosslinker was synthesized by bis-functionalizing trehalose with a polymerizable styrenyl group with an acid cleavable acetal linkage. Trehalose-based hydrogels were prepared using both free radical and redox polymerization. The solubility of the hydrogel was tested in different pH aqueous solutions. The hydrogel remained gelled in solutions greater than pH 5 and dissolved in 10% TFA.

4.2.2 Experimental Section

Materials

All the chemicals were purchased from Sigma-Aldrich and Fisher Scientific. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX), dried with ethanol and kept under vacuum before use. Azobisisobutyronitrile (AIBN) was recrystallized from acetone before use. 4-vinylbenzaldehyde diethyl acetal, styrenyl acetal trehalose monomer (SAT), styrenyl ether trehalose monomer (SET), and **poly(SAT)** were prepared using our reported procedures as described in Chapter 2.^{16,17}

Analytical techniques

NMR spectra were obtained on Bruker AV 500 and DRX 500 MHz spectrometers. ¹H NMR spectra were acquired with a relaxation delay of 2 s for small molecules and 30 s for polymers. Infrared absorption spectra were recorded using a PerkinElmer FT-IR equipped with an ATR accessory. ESI-MS data were gathered on a Waters LCT premier with ACQUITY LC.

Methods

Synthesis of Bis-Styrenyl Acetal Trehalose Crosslinker (bis-SAT). To the flame-dried reaction flask, trehalose (398 mg, 1.16 mmol) and DMF (4 mL) were added. *p*-TsOH (7.08 mg, 3.72×10⁻² mmol) was added and the reaction was stirred for 10 min immersed in a 100 °C oil bath. To the reaction 4-vinylbenzaldehyde diethyl acetal (600 mg, 2.91 mmol) was slowly added and the reaction was stirred at 100 °C for 2 h. After the reaction was complete, 80% of DMF was removed *in vacuo* and the remaining solution precipitated in benzene. The precipitate was filtered with saturated NaHCO₃ and washed with H₂O extensively. The filter cake was collected

and recrystallized in EtOH : H₂O = 2 : 1 resulting in 478.5 mg white powder with 72% yield. ¹H NMR (500 MHz in D₆DMSO) δ: 7.48-7.44 (m, 8H), 6.80-6.74 (m, 2H), 5.84-5.81 (d, *J* = 18.15 Hz, 2H), 5.56 (s, 2H), 5.29-5.27 (d, *J* = 10.37 Hz, 2H), 5.10-5.09 (m, 2H), 4.19-4.16 (m, 2H), 4.00-3.97 (m, 2H), 3.93-3.89 (m, 2H), 3.72-3.68 (t, *J* = 10.81 Hz, 2H), 3.58-3.53 (m, 2H), 3.47-3.44 (m, 4H), 3.32-3.30 (m, 2H). ESI-MS (± 1.0) observed (predicted): H⁺ 571.22 (571.22).

Preparation of SAT Hydrogel through Free Radical Polymerization. SAT (200 mg, 4.38×10⁻¹ mmol), **bis-SAT** (13.16 mg, 2.31×10⁻² mmol), and AIBN (0.72 mg, 4.38×10⁻³ mmol) were dissolved into 1 mL of DMF. Oxygen was removed by three freeze-pump-thaw cycles and polymerization was initiated by immersing the reaction flask into a 90 °C oil bath. Within 30 min the gel began to form and the reaction was stopped after 6 h by immersing the reaction flask into liquid nitrogen. The gel was washed with H₂O and MeOH to remove unreacted monomer and crosslinker.

Preparation of SAT Hydrogel through Redox Polymerization. SAT (20 mg, 4.38×10⁻² mmol) and **bis-SAT** (0.5 mg, 8.76×10⁻⁴ mmol) were separately dissolved in H₂O (150 μL) and DMF (50 μL), respectively. To the solution, TEMED (2.25×10⁻¹ μL, 1.5×10⁻³ mmol) and APS (50 μL in 2.28×10⁻³ mg/mL, 5.00×10⁻⁴ mmol) were added to start the gelation. A hydrogel was formed in 2 hours, and the resulting gel was purified by washing with H₂O and MeOH.

Preparation of SET Hydrogel through Free Radical Polymerization. SET (40.49 mg, 8.82×10⁻² mmol), **bis-SAT** (5 mg, 8.76×10⁻³ mmol) and AIBN (0.29 mg, 1.77×10⁻³ mmol) were dissolved in 0.11 mL DMF and 0.22 mL of H₂O. After three cycles of freeze-pump-thaw, the gelation was started at 80 °C and stopped after 4 h by cooling with liquid nitrogen. The resulting gel was washed with H₂O and MeOH to purify.

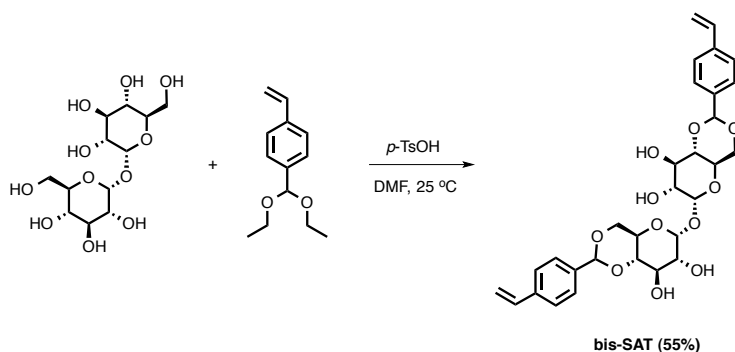
Hydrolysis Study of poly(SAT). 50 mg of **poly(SAT)** (33,700 g/mol, 1.48×10^{-3} mmol) was dissolved in pH 3, pH 4, pH 5, and 10% TFA aqueous solution. The reaction was stirred at 25 °C and dialyzed against H₂O (MWCO 3,500 g/mol) for three days and lyophilized.

Hydrolysis Study of SET Hydrogel. To the three SET hydrogels (0.3 mg each) 500 µL of pH 7.4 D-PBS, pH 5 PBS, and 10 % TFA solution was added. The solubility of each sample was monitored through the time.

4.2.3 Results and Discussion

Preparation of Trehalose Crosslinker and Trehalose Hydrogels

Scheme 4.2-1. Synthesis of **bis-SAT** Crosslinker.



To synthesize a trehalose crosslinker, we used the method previously reported for the synthesis of trehalose monomer.¹⁶ To increase the yield for the bis-functionalized crosslinker over the monomer, 2.2 molar equiv of 4-vinylbenzaldehyde diethyl acetal was added to the trehalose (Scheme 4.2-1). The **bis-SAT** crosslinker was prepared through transacetalization (Figure 4.2-1) in 72% yield.

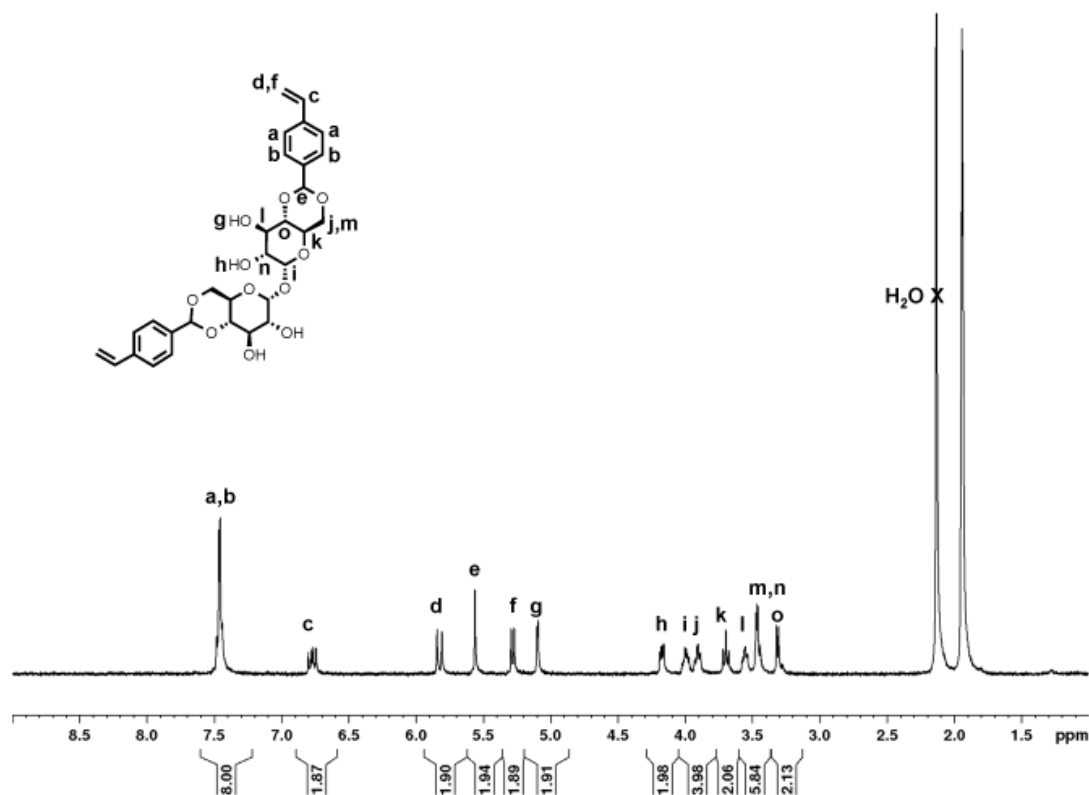
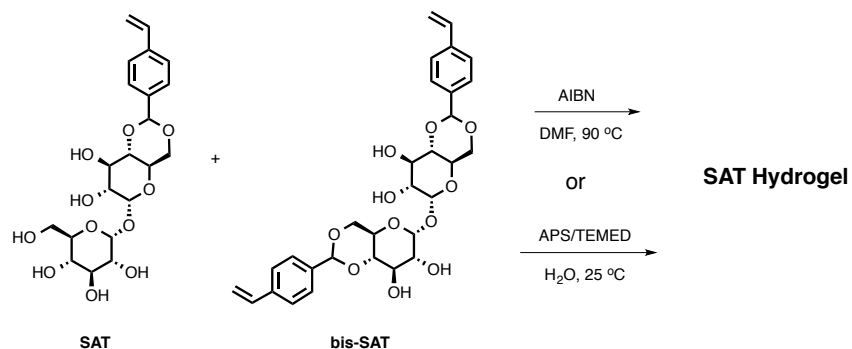


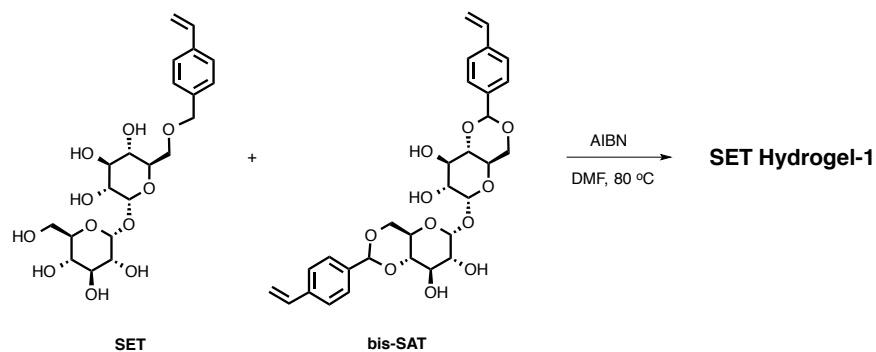
Figure 4.2-1. ¹H NMR spectroscopy of **bis-SAT** Crosslinker (in CD₃CN).

This **bis-SAT** crosslinker was copolymerized to form both SAT and SET hydrogels through heat initiated free radical polymerization and redox polymerization (Scheme 4.2-2 and 4.2-3). For the **SAT hydrogel**, both synthetic routes resulted in hydrogels. During the AIBN-mediated free radical polymerization, the hydrogel formation was observed within 30 min at 90 °C, whereas redox polymerization required 2 h to gel at 25 °C. In contrast, the SET hydrogel could only be obtained through free radical polymerization. During the free radical crosslinking in H₂O/DMF (= 2/1) co-solvent, the SET monomer was observed to precipitate out of the solution. All hydrogels were purified by washing with H₂O and MeOH to remove unreacted starting materials or uncrosslinked polymer chains.

Scheme 4.2-2. Synthesis of **SAT Hydrogels** through Free Radical Polymerization or Redox Polymerization.



Scheme 4.2-3. Synthesis of **SET Hydrogel-1** using Free Radical Polymerization.



Hydrolysis Studies of SAT Polymer and SET Hydrogel

To study acetal cleavage in the hydrogels, linear **poly(SAT)** was used as a model system. **Poly(SAT)** was dissolved in a series of acidic pHs to induce hydrolysis of the acetal linkage between trehalose and the pendant moiety in the polymer backbone. The resulting aldehyde was observed by ¹H NMR spectroscopy. In pH 3-5, all the trehalose peaks remained constant and no aldehyde peak was observed (data not shown). However, when the polymer was treated with 10% TFA, the ¹H NMR peaks corresponding to the trehalose protons (Figure 4.2-2 top, 3.0-5.5 ppm) disappeared and an aldehyde peak became visible. The ¹H NMR spectrum of the resulting

polymer appeared identical to the trace expected for a 4-benzaldehyde polymer (Figure 4.2-2 bottom).

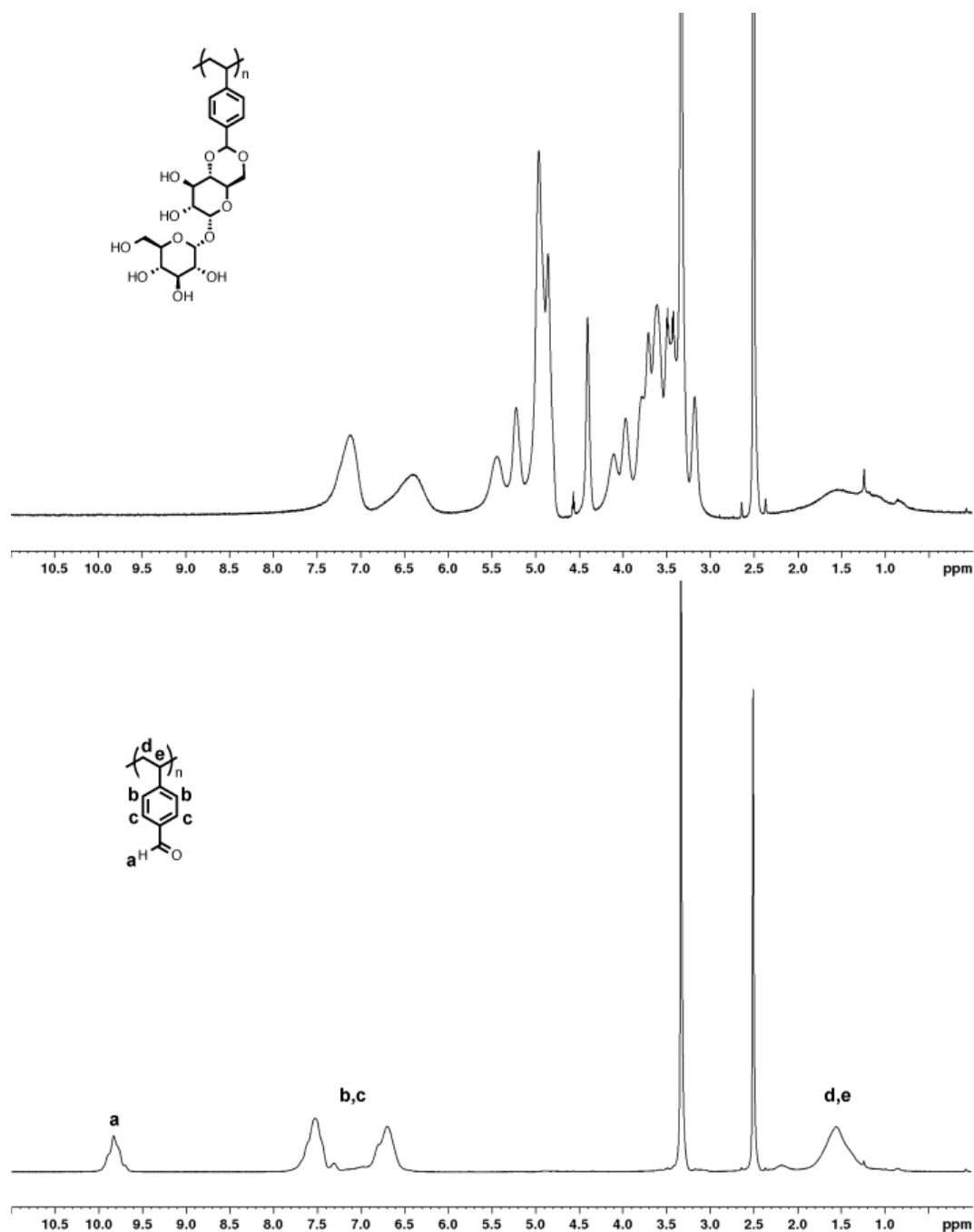


Figure 4.2-2. ^1H NMR spectroscopy of **poly(SAT)** before (top) and after (bottom) treatment with 10% TFA aqueous solution (in D_6DMSO).

Next, the **SET hydrogel-1**, where the polymer contains stable ether linkages and only the cross-linker has a reversible bond was treated with D-PBS, pH 7.4, PBS, pH 5, and 10% TFA aqueous solution. Because of the crosslinking, the hydrogel would not dissolve in aqueous buffer. Yet similar to the **poly(SAT)** hydrolysis study, the **SET hydrogel-1** in 10% TFA dissolved completely within 3 min. The gel remained at both pH 7.4 and pH 5 even after 48 h incubation at 25 °C suggesting a low pH is required to reverse the acetal crosslinker linkage (Figure 4.2-3).

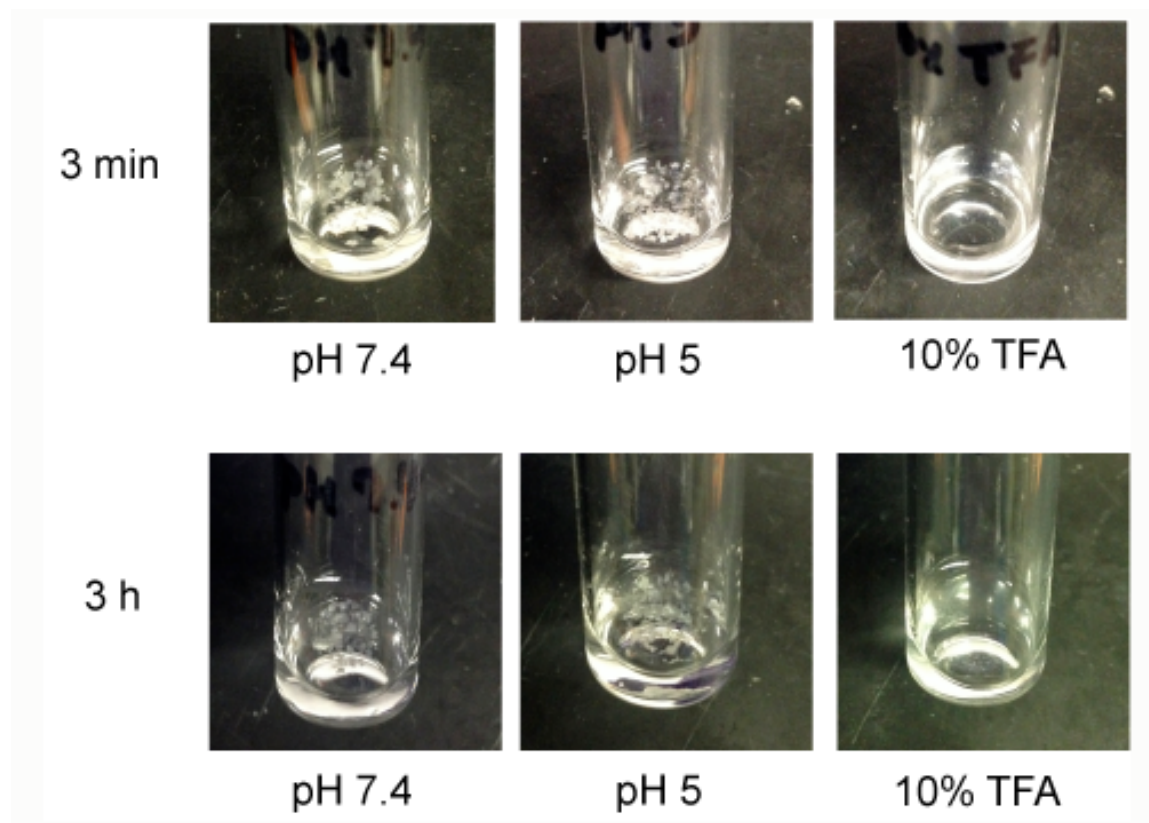


Figure 4.2-3. SET hydrogel-1 in pH 7.4 D-PBS, pH 5 PBS, and 10% TFA after 3 min (top) and 3 h incubation (bottom) at 25 °C.

Discussion

Together, the above data describe the development of acid-responsive trehalose-based hydrogels. First, a trehalose crosslinker was synthesized using identical chemistry as was used to prepare the **SAT** monomer. This resulted in a hydrogel containing trehalose moieties in the crosslinker as well as the backbone, which may increase the stabilization effect for encapsulated therapeutic proteins. Although **SAT** could form a hydrogel with **bis-SAT** using both free radical polymerization and redox chemistry, **SET** was not able to form a hydrogel with the same crosslinker. This is likely due to the differential solubility of **SET** and **bis-SAT** in 25 °C, H₂O and DMF respectively. A higher temperature is required to solubilize the monomer and crosslinker. A noticeable observation was that the acetal bond of **bis-SAT** (or **SAT**) could not be hydrolyzed even in pH 3. Only when 10% TFA was added was the acetal bond hydrolyzed to the aldehyde. This acid-stability was unexpected, since acetal bonds had previously been used as pH responsive crosslinkers in hydrogels.³⁶⁻³⁹ The surprising acid stability could be due to the polymer backbone, which is in the *para* position of the benzaldehyde acetal. The substituent in the *para* position is known to be important in influencing the acid lability of the acetal bond.^{38,40} In addition, the hydrophobic backbone may prevent water from reaching the acetal bond thereby preventing hydrolysis. When a 10% TFA solution was added to linear **poly(SAT)**, all the side chains were hydrolyzed releasing the trehalose; only the polybenzaldehyde backbone was left after purification. However, there were no difference in ¹H NMR spectra when the pH 3 to pH 5 aqueous solution was added. Also, when the **SET hydrogel-1** was treated with a 10% TFA solution, the gel solubilized suggesting that the **bis-SAT** hydrolyzed.

4.2.4 Conclusion

In this chapter, we have described the synthesis of various trehalose hydrogels using a bis-styrenyl acetal functionalized trehalose crosslinker. Two different trehalose monomers formed gels with this crosslinker through AIBN-mediated free radical polymerization. Hydrolysis of the acetal linkage was not detected until it added into a 10% TFA solution. We expect these acid cleavable trehalose hydrogels could be used as vehicles for delivery of protein or peptide therapeutics to the stomach or stabilizers for enzymes used in acid triggered chemical synthesis and water purification.

4.3 Trehalose Hydrogel for Stabilization of Phytase in Industrial Animal Feeds Pelletizing Process

4.3.1 Introduction

Currently in the animal feed industry, pelletizing is the most common process for preparing animal feeds since it improves their efficiency and reduces nutrient excretion compared to mashed forms.^{41,42} One of the important enzymes included in animal feeds is phytase. Phytase is a phosphohydrolytic enzyme that catalyzes the conversion of phosphate from phytate, the major form of phosphorus in plant-based feeds, to a usable form of inorganic phosphorus; it also enhances digestibility of calcium.⁴²⁻⁴⁵ This conversion of phytate is essential for simple-stomached species such as swine, poultry, and fish to decrease their phosphorus excretion.⁴³ As of 2011, phytase accounted for approximately 60% of the \$550 million global feed enzyme market.⁴⁶ However, the biggest challenge in the use of phytase in animal feeds is that steam heating at 71-90 °C during the pelletizing process degrades the phytase due to its low thermostability.^{42,43,47,48} Therefore, research on improving the thermostability of phytase to tolerate the pelletizing process on an industrial scale is continuously increasing.

There are two main strategies for developing an animal feed pellet that contains highly active phytase. The first method is avoiding heat exposure to phytase during the pelletizing by coating a thermotolerant on the phytase granules or by spraying a phytase solution onto the cooled pellet surface after pelletizing.^{43,48-50} However, these methods do not address the deactivation of phytase upon storage and transportation. Another strategy is to modify phytase to be more thermostable using protein engineering. Error-prone polymerase chain reaction (PCR), random mutation, rational design, a higher number of N-linked glycosylation sites, and the

exchange of secondary structural elements have been used to enhance the thermostability of phytase.^{43,51-58} However, protein engineering increases the cost of the final phytase product and may not be ideal on an industrial scale.

In this chapter, we propose a hydrogel system based on the natural disaccharide trehalose as an efficient excipient to enhance the thermostability of phytase at 90 °C for pelleting. This trehalose hydrogel can be prepared in only two steps from trehalose using simple purification steps, which can be directly applied industrially for stabilization of phytase at a lower cost than using protein engineering methods. Also, unlike protein engineering, which requires gene mutations and protein expression for each enzyme target, a trehalose hydrogel may be beneficial toward a broad range of enzymes requiring severe heat treatments for usage similar to the phytase.

4.3.2 Experimental Section

Materials

All the chemicals were purchased from Sigma-Aldrich, Thermo Scientific, and Fisher Scientific and were used without purification unless noted otherwise. Trehalose was purchased from The Endowment for Medical Research (Houston, TX) and dried with ethanol and kept under vacuum before use. Phytase was provided from Phytex LLC (Sheridan, IN) and stored at 4 °C as a dried powder until use. Alexa Fluor[®] 488 microscale protein labeling kit (A30006) was purchase from life technologies.

Analytical techniques

UV-Vis spectra were obtained using a Thermo Scientific Nanodrop 2000 Spectrophotometer. Confocal microscopy images were obtained from a Leical SP2 1P-FCS confocal microscope with axial resolution of 25 μm . Phytase activity assays were conducted by Phytex LLC (Sheridan, IN).

Methods

One Pot Reaction for Synthesis of 6-O-(4-vinylbenzylidene)- α,α -trehalose (SET monomer) and Crosslinkers (Crude SET). The one pot reaction for **crude SET** was prepared by modifying the previously reported literature procedure.⁵⁹ Sodium hydroxide (NaOH, 4.44 g, 1.14×10^{-1} mol) was added to dimethyl sulfoxide (DMSO, 96 mL). After stirring for 5 min, trehalose (4.86 g, 1.42×10^{-2} mol) was added to the reaction. After all the trehalose was dissolved, 4-vinylbenzyl chloride (0.4 mL, 2.84×10^{-3} mmol) was slowly added to the reaction and was stirred for 24 h at 25 °C. The crude product was then precipitated into 4 L of DCM to remove highly modified

trehalose. The resulting solid was dried *in vacuo* and used for gelation without further purification.

Synthesis of SET Hydrogel using Redox Polymerization in the Presence of Phytase. Phytase stock solution was prepared with a 36 mg/mL concentration in H₂O. 50 mg of **crude SET** was dissolved in different amount of phytase solution (phytase : **crude SET** = 1: 111, 1: 222, and 1: 444 wt equiv). After addition of tetramethylethylenediamine (TEMED, 0.25 μ L) samples were diluted to the same **crude SET** concentration (1 mg/ μ L). An aliquot of the crude mixture containing 90 μ g of phytase was taken for use as a control in the heat burden study. Ammonium persulfate (APS, **crude SET** : APS = 10 mg : 25 μ g) was added to rest of the mixture to initiate the gelation, and the reaction was incubated at 4 °C for 24 h. After gelation, the gel and the phytase mixture were lyophilized.

Synthesis of SET Hydrogel using Redox Polymerization and Adding Phytase. 50 mg of **crude SET** was dissolved in H₂O (50 μ L) and then treated with TEMED (0.25 μ L). The crude mixture was divided into 10 μ L aliquots and then 2.5 μ L of APS solution (10 mg/mL in H₂O) was added to each aliquot to initiate the gelation. The solution started gelling within 10 min at 25 °C. After 24 h, the gel was washed continuously with H₂O to remove unreacted trehalose, **crude SET**, TEMED, and APS. After confirming complete removal of excess small molecules by UV-Vis absorbance at Abs₂₅₄, the samples were lyophilized and phytase solutions of different concentration were added to the each dried gel (phytase : **crude SET** = 1: 111, 1: 222, and 1: 444 wt equiv) in 10 μ L volume. The gels were incubated at 4 °C with the phytase solution for 24 h and lyophilized to yield a white powder for testing in the heat burden study.

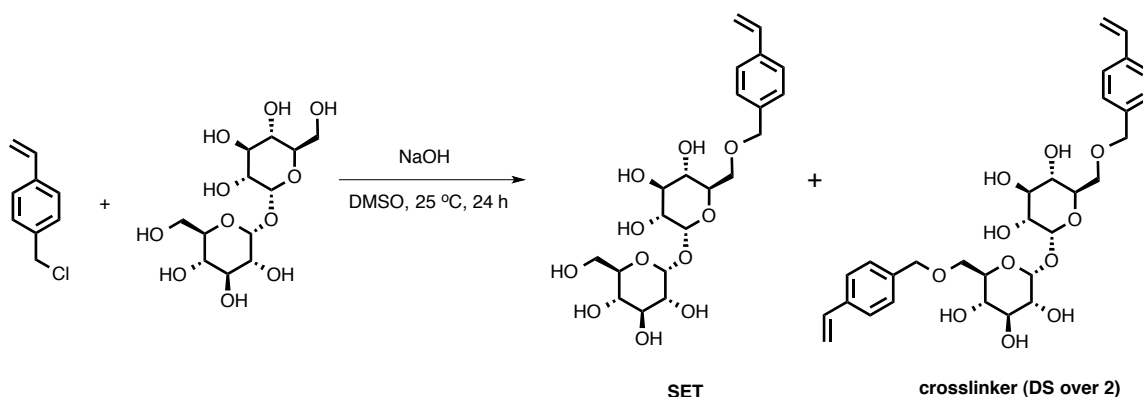
Heat Burden Studies of Phytase and SET Hydrogel. To the dried hydrogel and phytase mixture, 53 wt % of H₂O with respect to the phytase was added. The hydrogel was incubated at 4 °C for 24 h with gentle rocking to evenly distribute the H₂O. The hydrogel was then heated at 90 °C for 1 min before diluting with 0.1 M sodium acetate buffer, pH 5, for the activity assay.

Preparation of HRP-AF488 for Confocal Microscope. To the 100 µL horseradish peroxidase (HRP) solution (1 mg/mL) in pH 7.4 D-PBS, alexa Fluor[®] 488 TFP ester (AF488, 10 µL in 1 M sodium bicarbonate solution) was added. The reaction was incubated at 25 °C for 30 min and purified using centrprep tube (MWCO 3,000 g/mol). The degree of labeling of HRP-AF488 after purification was 5.24. The prepared SET hydrogel from above was immersed into 100 µL HRP-AF488 at 4 °C for 12 h and after short wash with H₂O, confocal microscope image was taken.

4.3.3 Results and Discussion

Synthesis of Trehalose Monomer and Crosslinker

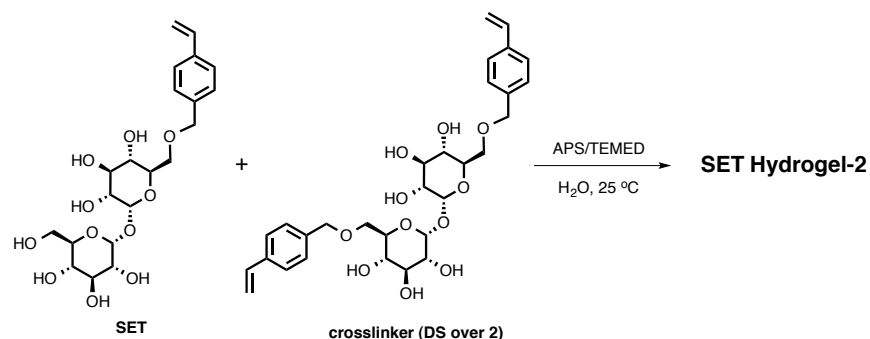
Scheme 4.3-1. Synthesis of **Crude SET**.



Simple synthesis and purification steps are one of the most important factors in industrial scale reactions. Originally, we purified the trehalose monomers by precipitating the reaction mixture into DCM followed further purification to remove all the side products, which have degree of substitution (DS) over two. However, we envisioned synthesis of trehalose-based hydrogel directly using these side products as crosslinkers. (Scheme 4.3-1). Due to the presence of crosslinker in the monomer, the product in this case would be a hydrogel rather than a linear polymer. , In this chapter, we describe hydrogel synthesis using **SET** monomer, since a crude mixture of monomer and crosslinker could be produced from the starting materials 4-vinylbenzyl chloride and trehalose in one step.⁵⁹ First, 4-vinylbenzyl chloride was reacted with excess trehalose under basic conditions. The resulting crude mixture was then precipitated into DCM and filtered to remove DMSO and trehalose with a high DS. The **crude SET** was then directly used for gelation.

Synthesis of SET Hydrogel

Scheme 4.3-2. Synthesis of **SET Hydrogel-2** by Redox Polymerization.



Redox polymerization with ammonium persulfate (APS) and tetramethylenediamine (TEMED) as radical initiators was used to form a **SET hydrogel-2** from the **crude SET** with and without enzyme present (Scheme 4.3-2). The **crude SET** was dissolved in H₂O at 1 mg/mL concentration and TEMED was added (Figure 4.3-1a). Initially the solution remained in the sol phase. However, after adding APS the solution started gelating within 10 min at 25 °C or 4 °C with or without the phytase (Figure 4.3-1b). The resulting hydrogel had the same yellow color as the crude mixture. The hydrogel network remained intact after lyophilization and re-immersion into H₂O (Figure 4.3-1c and d). Extensive washing with H₂O removed the yellow color, resulting in a colorless **SET hydrogel-2** (Figure 4.3-1e). Using a mortar, the purified **SET hydrogel-2** was then ground into a powder to increase the surface area for phytase stabilization and also for ease of handling.

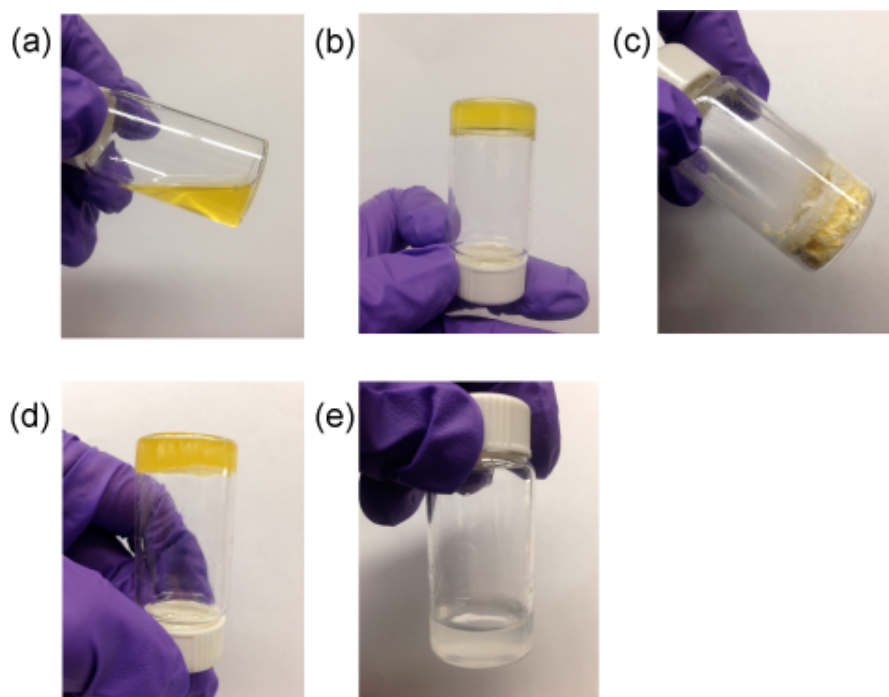


Figure 4.3-1. (a) Crude SET and TEMED dissolved in H₂O, (b) after adding APS, (c) after lyophilizing the hydrogel, (d) immersing lyophilized gel again into the H₂O, and (e) after washing the hydrogel (grounded after lyophilization and immersed in H₂O).

Heat Burden Studies of Phytase

Initially we attempted to form the hydrogel in the presence of phytase, as well as exposing phytase to an unwashed **SET hydrogel-2**. When phytase was present, the **SET hydrogel-2** was formed at 4 °C. The enzymatic activity of the phytase that had been exposed to hydrogel formation was compared to the set of samples in which the phytase solution was added to the unwashed hydrogel as well as to phytase alone. After incubating the samples at 4 °C for 24 h, all samples were lyophilized. Next, approximately 53 wt % of H₂O with respect to the phytase was added to simulate the pelleting process, during which phytase is exposed to steam and heat. After another incubation at 4 °C for 24 h to allow H₂O to evenly distribute, the samples were

heated at 90 °C for 1 min to mimic the heat burden during the industrial pelleting process. The samples with the unwashed **SET hydrogel-2**, regardless of whether the phytase was added before or after the hydrogel formation, lost enzymatic activity (data not shown). This result is not surprising, since in our previous report the **SET** monomer was shown to dramatically reduce the activity of both horseradish peroxidase and β -galactosidase.¹⁷ This might due to the vinyl group reacting with proteins, since the addition of benzyl trehalose (no alkene) did not have an adverse effect on protein activity.¹⁷

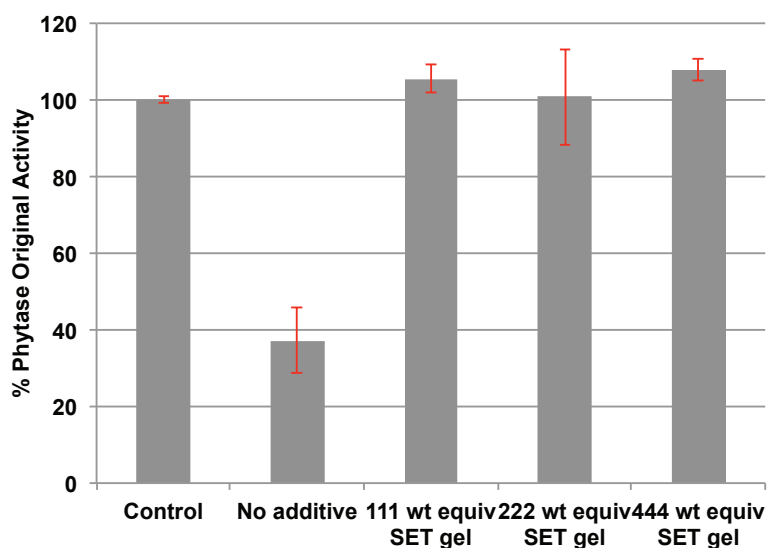


Figure 4.3-2. Activity of phytase before heating (control) and after heating at 90 °C for 1 min with no additive, 111 wt equiv, 222 wt equiv, 444 wt equiv of **SET hydrogel-2** to the phytase. $p < 0.006$ for heating with no additive compared to control and $p > 0.5$ for samples heated with the **SET hydrogel-2** compared to the control. Each condition was repeated three times.

To retain activity of the protein, the **SET hydrogel-2** was extensively washed to remove any unreacted **crude SET** prior to addition of the enzyme. The phytase solution was added to the purified and lyophilized **SET hydrogel-2**. The purified **SET hydrogel-2** containing phytase was

heated as described above after incubating for 24 h with ~53 wt % of H₂O added to the phytase to replicate steam heating process. Remarkably, 100 % of phytase activity was maintained after heating at 90 °C for 1 min for all the tested wt equiv of **SET hydrogel-2** while phytase activity decreased to 37% after heating in the absence of the hydrogel (Figure 4.3-2).

Discussion

The data demonstrate that the **SET hydrogel-2** can stabilize phytase against the extreme heat generated in the animal feed pelleting process. Linear trehalose polymer also showed some stabilization of phytase (data not shown); however, for industrial scale up, the **SET hydrogel-2** synthesis requires minimal and inexpensive purification steps. Another advantage of hydrogel formulation is its ease of removal. Since the hydrogel is not soluble in H₂O or organic solvents it can be separated from the mixture by simple filtration or centrifugation. Despite much research on the genetic engineering of phytase for its thermal stabilization, the use of native phytase would be advantageous with regard to simplicity. Moreover, since enzymes are very different from one another and often require multiple optimization iterations after modification, a mutation strategy successful in the stabilization of phytase will not necessarily be applicable to other enzymes. In contrast, the **SET hydrogel-2** may stabilize a wide range of enzymes and proteins that need to undergo harsh thermal treatment. Since our group has already demonstrated stabilization of various proteins against heating using linear trehalose polymers, the trehalose-based hydrogel described may be readily applicable to thermal stabilization of other industrially important enzymes or proteins.^{16,17}

Even though we have shown that the **SET hydrogel-2** can stabilize phytase against heating, we have yet to confirm whether phytase is located inside the gel or adsorbed on the surface. To begin to determine the location of the protein, we used HRP modified with Alexa Fluor[®] 488 tetrafluorophenyl ester (AF488) as a model system. The **SET hydrogel-2** with HRP-AF488 was prepared in a similar manner to the **SET hydrogel-2** that showed 100% stabilization of phytase. As shown in Figure 4.3-3, the preliminary confocal microscopy images indicated that the HRP-AF488 is present inside the hydrogel. Since HRP (44.2 kDa) is similar in molecular

weight to phytase (56 kDa) it may be possible that phytase permeates the hydrogel mesh even with addition of the phytase solution after hydrogel formation. However, studies need to be undertaken to determine the location of phytase within the hydrogel.

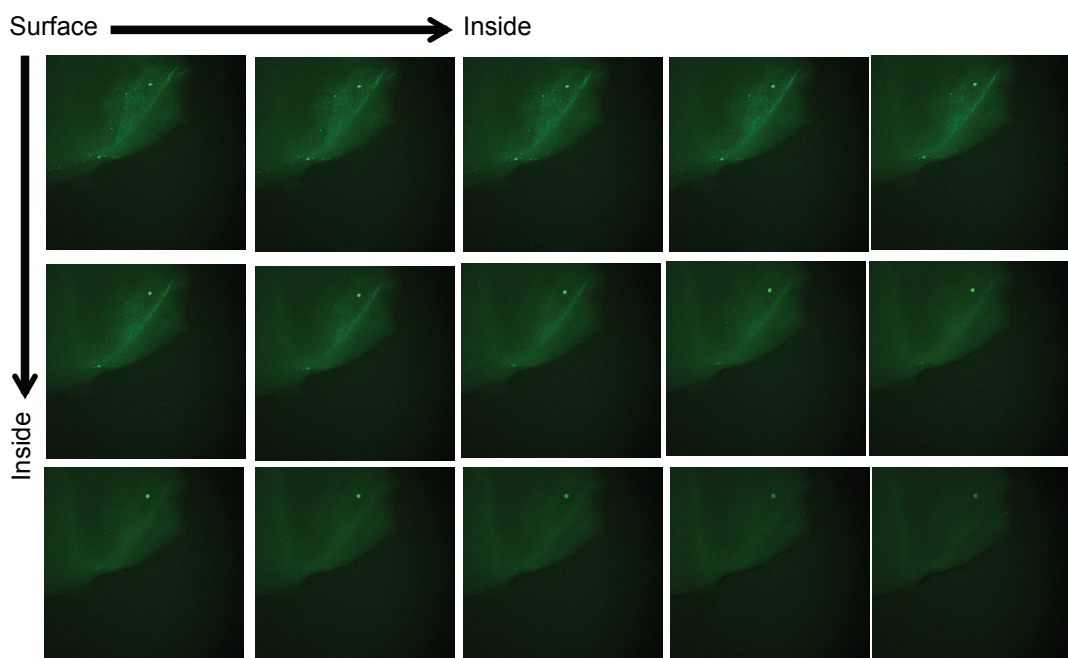


Figure 4.3-3. Axial confocal microscopy images (15 scans) of the edge of the **SET hydrogel-2** immersed in HRP-AF488 (green) solution (axial resolution of 25 μm) for 24 h and then briefly washed..

4.3.4 Conclusion

We have detailed the synthesis of a trehalose hydrogel for industrial-scale stabilization of proteins. This hydrogel can be prepared via simple synthesis and purification steps, which are among the most important considerations in industrial processes. The resulting trehalose hydrogel preserved phytase activity up to 100% under temperatures similar to those reached in the pelleting procedure for animal feed preparation. Currently, most enzymes in animal feeds lose the majority of their activity during this steam pelleting process. Although we have only tested the phytase enzyme in this chapter, the trehalose hydrogel is a promising system for stabilizing various enzymes or proteins against the pelleting procedure or other high-temperature processes.

4.4 Hyaluronic Acid Oxime Bond Hydrogel

4.4.1 Introduction

Click chemistry is one of the most popular strategies for the preparation of hydrogels, as it is highly efficient and tolerant of various functional groups. Additionally, click reactions work in aqueous solutions at room temperature or physiological pH, and are therefore ideal for preparation of hydrogels as biomaterials for use in cell and tissue engineering.^{60,61} In 2012, our group was the first to report the synthesis of a PEG hydrogel using oxime click chemistry.⁶¹ The resulting PEG hydrogel with integrin ligand arginine-glycine-aspartic acid (RGD) was biocompatible and successfully incorporated mesenchymal stem cells (MSC) with high cell viability and proliferation.

Hyaluronic acid (HA) is an anionic non-sulfated glycoaminoclycan that is present in the extracellular matrix (ECM) of connective, epithelial, and neural tissues.⁶²⁻⁶⁴ HA hydrogels are heavily used for tissue engineering because of their biocompatibility, biodegradability, non-immunogenicity, and non-thrombogenicity.⁶³ In cell and tissue engineering, cross-linking methods require, 1) short reaction times, 2) bioorthogonal reactions, 3) physiological conditions, and 4) non-toxic byproducts, all of which can be satisfied through click chemistry.^{61,63} Various click reactions have been used in crosslinking the hyaluronic acid. Diels-Alder reaction with furan modified HA and dimaleimide poly(ethylene glycol) formed a HA hydrogel.⁶⁵ Copper catalyzed azide-dialkyne click reaction was also used in HA hydrogel formation.^{66,67} HA hydrogel using thiol-Michael addition click chemistry with RGD or MMP peptide as a crosslinker.^{68,69}

In this chapter, we describe the preparation of a HA hydrogel through an oxime bond. The oxime click reaction occurs between an aldehyde or ketone and an aminooxy group, which are more stable than the thiol that are widely used for hydrogel crosslinking. In 2013, an oxime crosslinked injectable HA hydrogel was reported for the first time for use in catheter delivery.⁷⁰ However, the oxidation of HA diols to aldehydes for the subsequent reaction with an aminooxy-PEG crosslinker is hard to control. We propose an aminooxy modified HA as a platform for HA hydrogel using aldehyde crosslinkers. HA was modified through simple coupling reaction and a gel formed rapidly upon treatment with glutaraldehyde.

4.4.2 Experimental Section

Materials

All chemicals and proteins were purchased from Sigma-Aldrich and Fisher Scientific and were used without purification unless noted otherwise. Hyaluronic acid (60,000 g/mol) was provided from Professor Tatiana Segura's group in the Chemical and Biomolecular Engineering Department at UCLA. **1** was prepared according to the literature procedure.⁷¹

Analytical techniques

NMR spectra were obtained on Bruker AV 500 and DRX 500 MHz spectrometers. ¹H NMR spectra were acquired with a relaxation delay of 2 s for small molecules and 30 s for polymers.

Methods

Synthesis of tert-butyl (3-aminopropoxy)carbamate (2). **1** (500 mg, 1.56 mmol) was dissolved in 4 mL MeOH. Hydrazine (0.2 mL, 6.24 mmol) was added slowly to the reaction flask and stirred for 18 h at 25 °C. MeOH was removed *in vacuo* and 50 mL of CH₃Cl was added to filter the crude mixture. The filtrate was collected and dried yielding 212.6 mg (72% yield) of product. ¹H NMR (500 MHz in CDCl₃) δ: 3.95-3.93 (t, *J* = 6.31 Hz, 3H), 2.86-2.83 (t, *J* = 6.32 Hz, 2H), 1.78-1.75 (t, *J* = 6.32, 2H), 1.48-1.46 (m, 9H). ¹³C NMR (500 MHz in CDCl₃) δ: 156.7, 81.6, 75.1, 39.3, 31.5, 28.5.

Synthesis of BOC Protected Aminoxy HA. HA (60,000 g/mol, 100 mg, 1.67×10⁻³ mmol) was dissolved in pH 5, 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 72.82 mg, 2.63×10⁻¹ mmol) was added and stirred for 10 min. **2** (25.02 mg, 1.32×10⁻¹ mmol, 0.5 equiv to the number

of HA repeating unit) was separately dissolved in MeOH (2 mL) and added to the HA dropwise. The reaction was stirred at 25 °C for 24 h and purified by dialysis against H₂O (MWCO 3,500 g/mol) and lyophilized to result in fine white powder. ¹H NMR (500 MHz in D₂O) δ: 4.54, 4.49, 4.13, 4.10, 3.97, 3.89, 3.82, 3.75, 3.59, 3.53, 3.48, 3.34, 3.22, 3.16, 2.92, 2.01, 1.47.

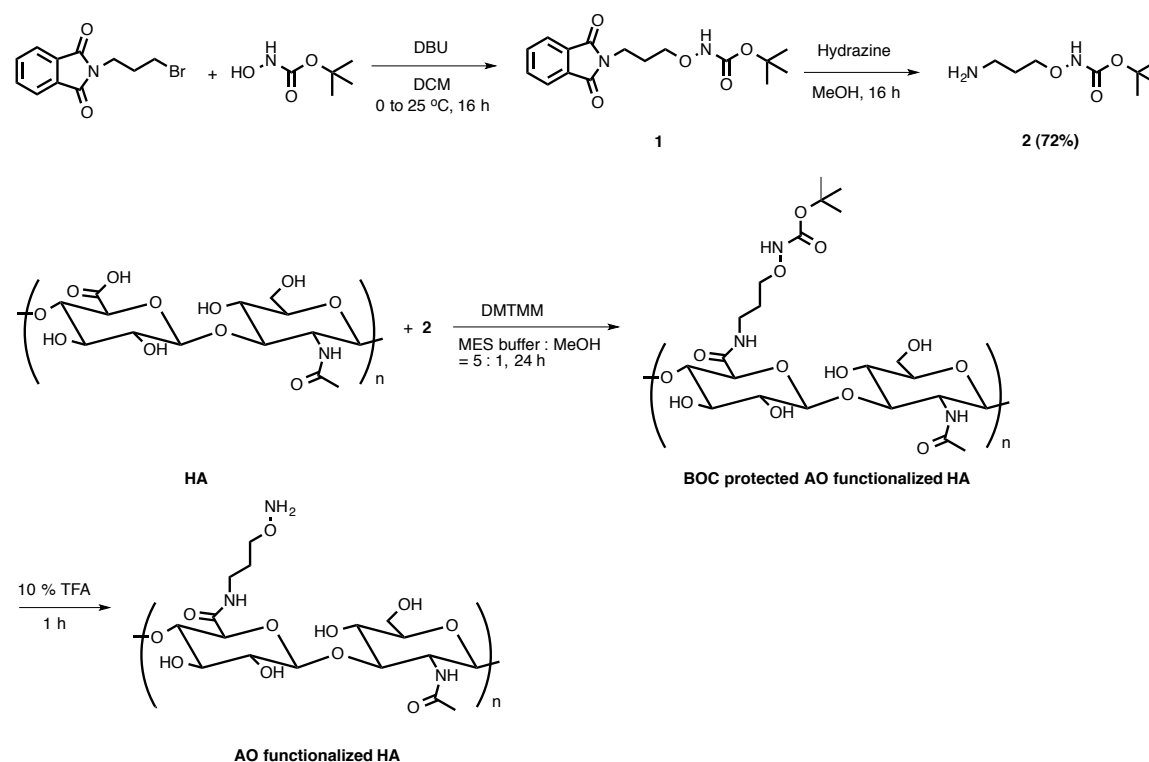
Deprotection of BOC Group for Aminoxy Functionalized HA. BOC protected aminoxy HA (20 mg, 3.33×10⁻⁴ mmol) was dissolved into H₂O (3 mL). 0.3 mL of TFA was slowly added to the reaction and stirred for 1 h. The reaction was dried *in vacuo*. ¹H NMR (500 MHz in D₂O) δ: 4.55, 4.13, 3.99, 3.90, 3.83, 3.73, 3.64, 3.55, 3.46, 3.36, 3.22, 3.13, 2.92, 1.99.

Preparation of HA Hydrogel with Glutaraldehyde as a Crosslinker. Aminoxy functionalized HA (4.82 mg, 8.03×10⁻⁵ mmol) was dissolved in 10% TFA aqueous solution (90.25 μL). Glutaraldehyde (1.8 μL, 1.91×10⁻⁵ mmol) was added to begin gelation. Aminoxy functionalized HA in 1.8 μL of 10% TFA aqueous solution in the absence of glutaraldehyde was the negative control.

4.4.3 Results and Discussion

Synthesis of HA Hydrogel

Scheme 4.4-1. Synthesis of Aminooxy Functionalized Hyaluronic Acid.



To prepare aminooxy functionalized HA, an amine with a BOC protected aminooxy group (**2**) was synthesized and coupled to the carboxylic acid moiety of the HA (Scheme 4.4-1 and Figure 4.4-1). We first attempted to use an alcohol as the nucleophilic coupling partner instead of the amine; however, the reaction was not efficient. DMTMM was used for HA-**2** coupling reaction, as it is known to possess superior reactivity compared to other coupling reagents. We previously used 1-ethyl-3-(3-dimethylamioethyl)carbodiimide (EDC) or N,N'-dicyclohexylcarbodiimide (DCC) coupling reagents but these did not produce the desired product, suggesting that the reactivity of the coupling reagent is important for HA modification.

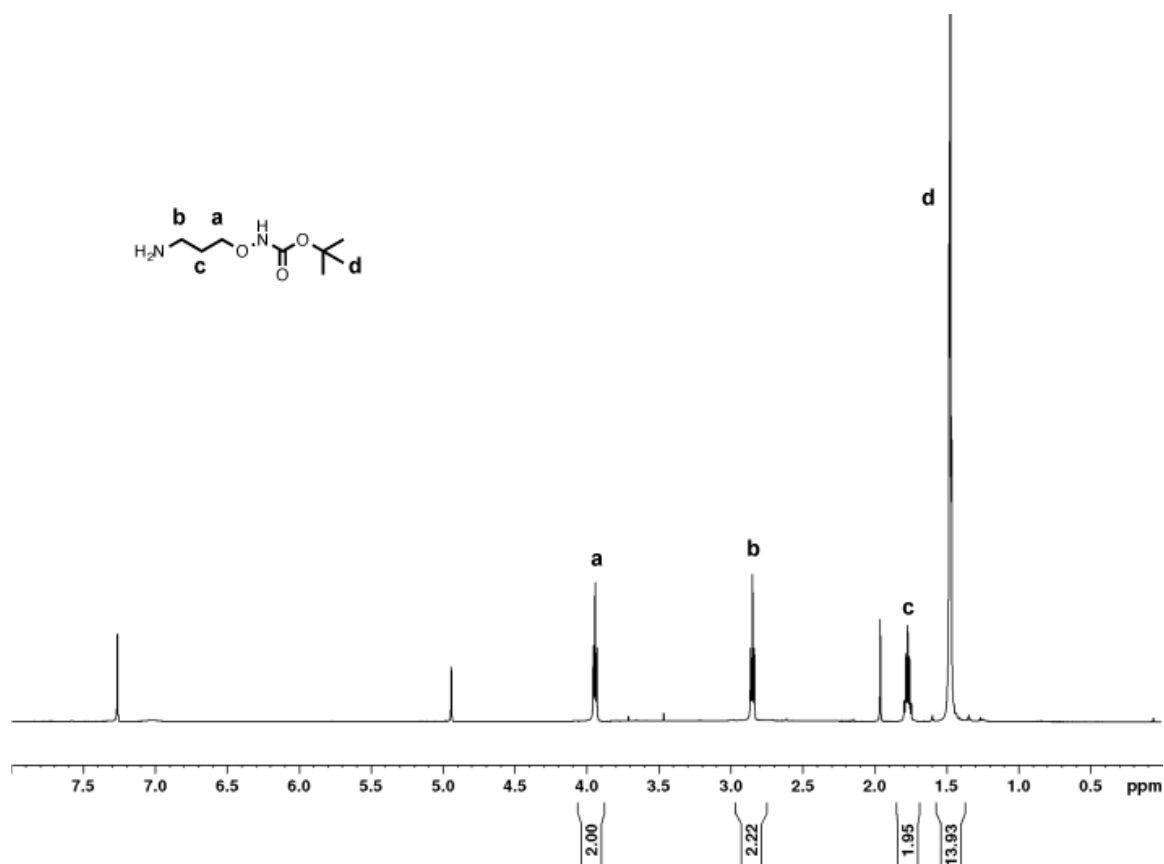


Figure 4.4-1. ¹H NMR spectroscopy of **2** (in CDCl₃).

For the HA modification, we first targeted 30% functionalization of the HA repeating unit. Accordingly, 0.5 molar equiv of **2** with respect to the number of HA repeating units was added. After purification, an additional peak from the BOC group was detected in the ¹H NMR spectra in the 1.4-1.5 ppm region (Figure 4.4-2, top and middle). Peak integration showed that 20% of the HA repeating units had been coupled with **2**. After deprotecting BOC group in a 10% TFA aqueous solution, the peak at 1.4-1.5 ppm disappeared, indicating the deprotection was complete (Figure 4.4-2, bottom).

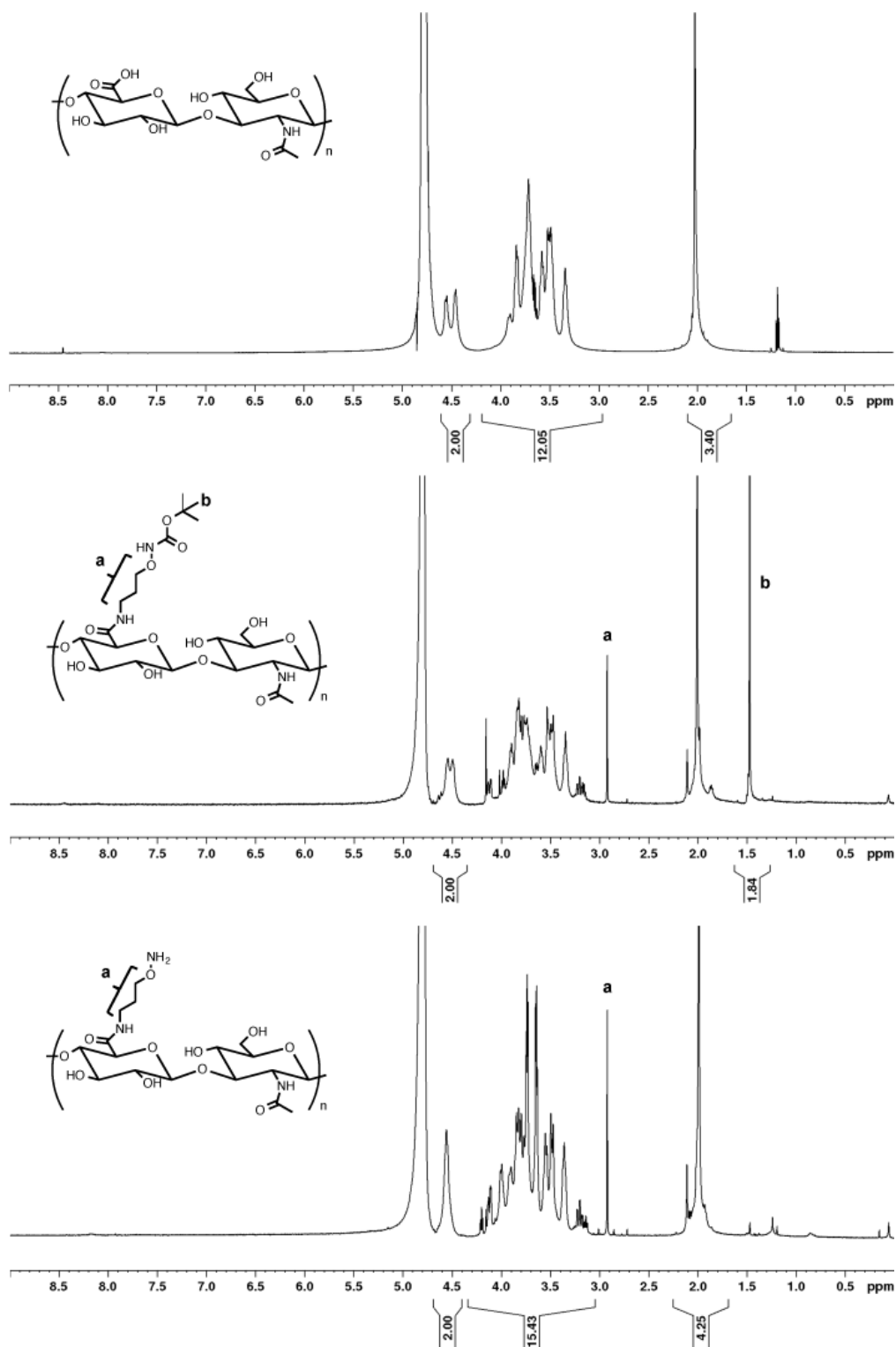
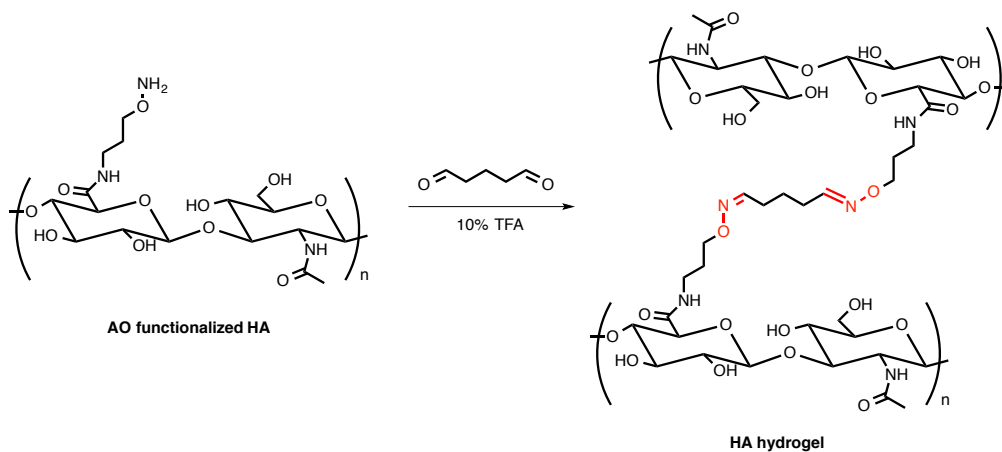


Figure 4.4-2. ^1H NMR spectroscopy of **HA** (top), BOC protected aminoxy functionalized **HA** (middle), and aminoxy functionalized **HA** (bottom) (in D_2O).

Scheme 4.4-2. Synthesis of Hyaluronic Acid Hydrogel through Oxime Click Chemistry.



To investigate the possibility of AO-functionalized HA as a platform for hydrogel synthesis through oxime bond formation, glutaraldehyde was used as a model crosslinker (Scheme 4.4-2). Within 10 min, the HA hydrogel was formed, whereas the control without glutaraldehyde remained in the non-viscous solution state (Figure 4.4-3). This result shows that glutaraldehyde can crosslink the HA through oxime click chemistry.

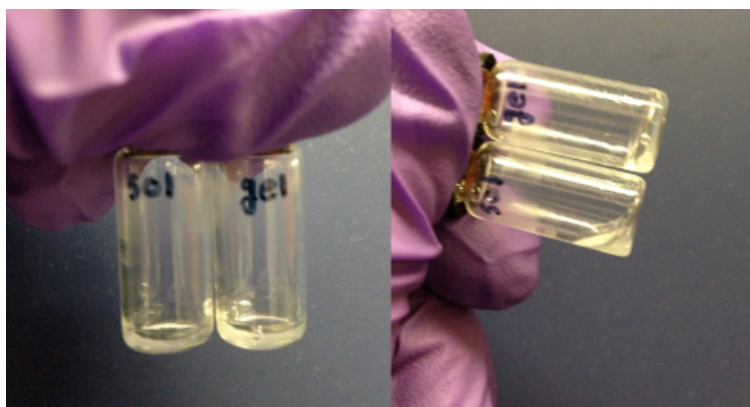


Figure 4.4-3. Hyaluronic acid in solution with (gel) or without (sol) glutaraldehyde.

Discussion

The above data shows that hyaluronic acid can be modified with an aminooxy group, which can then form an oxime bond by reacting with an aldehyde crosslinker and creating a HA hydrogel. Hyaluronic acid was coupled to BOC protected aminooxy amine when DMTMM was used as the coupling reagent, but not with EDC or DCC. We expect that varying the molecular equivalents of BOC-aminooxy amine in this simple coupling reaction would give greater control over the degree of modification of HA than oxidation of its diols; however, further study is required to confirm this hypothesis. Although we have confirmed that the gelation is mediated by oxime bond formation by observing hydrogel formation only in the presence of glutaraldehyde, kinetic studies of oxime bond formation between aminooxy functionalized HA and aldehyde crosslinker will be required in the future.

4.4.4 Conclusion

Herein, we have described the synthesis of a hyaluronic acid hydrogel through oxime bond formation. Aminoxy functionalization of hyaluronic acid was straightforward and gelation with glutaraldehyde through click reaction was rapid and effective. This aminoxy functionalized hyaluronic acid platform will be further investigated with various aldehyde or ketone modified peptide crosslinkers for tissue engineering applications.

4.5 References

- (1) Cambre, J. N.; Sumerlin, B. S. *Polymer* **2011**, *52*, 4631-4643.
- (2) Guan, Y.; Zhang, Y. *Chem. Soc. Rev.* **2013**, *42*, 8106-8121.
- (3) Ravaine, V.; Ancla, C.; Catargi, B. *J. Control. Release* **2008**, *132*, 2-11.
- (4) Kuivila, H. G.; Keough, A. H.; Soboczenski, E. J. *J. Org. Chem.* **1954**, *19*, 780-783.
- (5) Springsteen, G.; Wang, B. H. *Tetrahedron* **2002**, *58*, 5291-5300.
- (6) Yan, J.; Springsteen, G.; Deeter, S.; Wang, B. *Tetrahedron* **2004**, *60*, 11205-11209.
- (7) Barker, S. A.; Chopra, A. K.; Hatt, B. W.; Somers, P. J. *Carbohydr. Res.* **1973**, *26*, 33-40.
- (8) Matsumoto, A.; Yamamoto, K.; Yoshida, R.; Kataoka, K.; Aoyagi, T.; Miyahara, Y. *Chem. Commun.* **2010**, *46*, 2203-2205.
- (9) Wang, D.; Liu, T.; Yin, J.; Liu, S. *Macromolecules* **2011**, *44*, 2282-2290.
- (10) Ancla, C.; Lapeyre, V.; Gosse, I.; Catargi, B.; Ravaine, V. *Langmuir* **2011**, *27*, 12693-12701.
- (11) Matsumoto, A.; Ishii, T.; Nishida, J.; Matsumoto, H.; Kataoka, K.; Miyahara, Y. *Angewandte Chemie-International Edition* **2012**, *51*, 2124-2128.
- (12) Zhang, C.; Losego, M. D.; Braun, P. V. *Chem. Mater.* **2013**, *25*, 3239-3250.
- (13) Yuan, W.; Shen, T.; Wang, J.; Zou, H. *Polymer Chemistry* **2014**, *5*, 3968-3971.
- (14) Yang, T.; Ji, R.; Deng, X.-X.; Du, F.-S.; Li, Z.-C. *Soft Matter* **2014**, *10*, 2671-2678.
- (15) Hargrove, A. E.; Ellington, A. D.; Anslyn, E. V.; Sessler, J. L. *Bioconjug. Chem.* **2011**, *22*, 388-396.
- (16) Mancini, R. J.; Lee, J.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 8474-8479.

- (17) Lee, J.; Lin, E.-W.; Lau, U. Y.; Hedrick, J. L.; Bat, E.; Maynard, H. D. *Biomacromolecules* **2013**, *14*, 2561-2569.
- (18) Nagai, Y.; Kobayashi, K.; Toi, H.; Aoyama, Y. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2965-2971.
- (19) Vandenberg, R.; Peters, J. A.; Vanbekkum, H. *Carbohydr. Res.* **1994**, *253*, 1-12.
- (20) Roy, I.; Gupta, M. N. *Chem. Biol.* **2003**, *10*, 1161-1171.
- (21) Bajpai, A. K.; Shukla, S. K.; Bhanu, S.; Kankane, S. *Prog. Polym. Sci.* **2008**, *33*, 1088-1118.
- (22) Gupta, P.; Vermani, K.; Garg, S. *Drug Discov. Today* **2002**, *7*, 569-579.
- (23) Qiu, Y.; Park, K. *Adv. Drug Delivery Rev.* **2001**, *53*, 321-339.
- (24) Kiyonaka, S.; Sugiyasu, K.; Shinkai, S.; Hamachi, I. *J. Am. Chem. Soc.* **2002**, *124*, 10954-10955.
- (25) Mano, J. F. *Advanced Engineering Materials* **2008**, *10*, 515-527.
- (26) Ingber, D. E.; Prusty, D.; Frangioni, J. V.; Cragoe, E. J.; Lechene, C.; Schwartz, M. A. *J. Cell Biol.* **1990**, *110*, 1803-1811.
- (27) Wei, F.; Zhuyuan, W.; Shenfei, Z.; Hui, C.; Dan, Z.; Yuan, Z.; Yiping, C. *Biosens. Bioelectron.* **2014**, *57*, 10-15.
- (28) Lowman, A. M.; Morishita, M.; Kajita, M.; Nagai, T.; Peppas, N. A. *J. Pharm. Sci.* **1999**, *88*, 933-937.
- (29) Patel, V.; Amiji, M. *Pharm. Res.* **1996**, *13*, 588-593.
- (30) Besheer, A.; Wood, K. M.; Peppas, N. A.; Mader, K. *J. Control. Release* **2006**, *111*, 73-80.
- (31) Guo, B.-L.; Gao, Q.-Y. *Carbohydr. Res.* **2007**, *342*, 2416-2422.

- (32) Nho, Y. C.; Park, S. E.; Kim, H. I.; Hwang, T. S. *Nuclear Instruments & Methods in Physics Research Section B-Beam Interactions with Materials and Atoms* **2005**, 236, 283-288.
- (33) Sajeesh, S.; Sharma, C. P. *Journal of Biomedical Materials Research Part B-Applied Biomaterials* **2006**, 76B, 298-305.
- (34) Shantha, K. L.; Harding, D. R. K. *Int. J. Pharm.* **2000**, 207, 65-70.
- (35) Teramoto, N.; Sachinvala, N. D.; Shibata, M. *Molecules* **2008**, 13, 1773-1816.
- (36) Bachelder, E. M.; Beaudette, T. T.; Broaders, K. E.; Dashe, J.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2008**, 130, 10494-10495.
- (37) Li, R. C.; Broyer, R. M.; Maynard, H. D. *Journal of Polymer Science Part A: Polymer Chemistry* **2006**, 44, 5004-5013.
- (38) Murthy, N.; Thng, Y. X.; Schuck, S.; Xu, M. C.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2002**, 124, 12398-12399.
- (39) Chen, W.; Meng, F.; Cheng, R.; Zhong, Z. *J. Control. Release* **2010**, 142, 40-46.
- (40) Fife, T. H.; Jao, L. K. *The Journal of Organic Chemistry* **1965**, 30, 1492-1495.
- (41) Thomas, M.; van der Poel, A. F. B. *Anim. Feed Sci. Technol.* **1996**, 61, 89-112.
- (42) Nahm, K. H. *Crit. Rev. Environ. Sci. Technol.* **2002**, 32, 1-16.
- (43) Lei, X. G.; Weaver, J. D.; Mullaney, E.; Ullah, A. H.; Azain, M. J. *Annual Review of Animal Biosciences* **2013**, 1, 283-309.
- (44) Kuhn, I.; Partanen, K. *J. Anim. Sci.* **2012**, 90, 194-196.
- (45) Silversides, F. G.; Scott, T. A.; Bedford, M. R. *Poult. Sci.* **2004**, 83, 985-989.
- (46) Adeola, O.; Cowieson, A. J. *J. Anim. Sci.* **2011**, 89, 3189-3218.
- (47) Zhang, W.; Lei, X. G. *Appl. Microbiol. Biotechnol.* **2008**, 77, 1033-1040.

- (48) Slominski, B. A.; Davie, T.; Nyachoti, M. C.; Jones, O. *Livestock Science* **2007**, *109*, 244-246.
- (49) Hughes, K. P.; Soares, J. H., Jr. *Aquac. Nutr.* **1998**, *4*, 133-140.
- (50) Cao, L.; Wang, W.; Yang, C.; Yang, Y.; Diana, J.; Yakupitiyage, A.; Luo, Z.; Li, D. *Enzyme Microb. Technol.* **2007**, *40*, 497-507.
- (51) Wyss, M.; Pasamontes, L.; Remy, R.; Kohler, J.; Kuszniir, E.; Gadiant, M.; Muller, F.; van Loon, A. *Appl. Environ. Microbiol.* **1998**, *64*, 4446-4451.
- (52) Kim, M.-S.; Weaver, J. D.; Lei, X. G. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 751-758.
- (53) Garrett, J. B.; Kretz, K. A.; O'Donoghue, E.; Kerovuo, J.; Kim, W.; Barton, N. R.; Hazlewood, G. P.; Short, J. M.; Robertson, D. E.; Gray, K. A. *Appl. Environ. Microbiol.* **2004**, *70*, 3041-3046.
- (54) Zhang, W.; Mullaney, E. J.; Lei, X. G. *Appl. Environ. Microbiol.* **2007**, *73*, 3069- 3076.
- (55) Xiang, T.; Liu, Q.; Deacon, A. M.; Koshy, M.; Kriksunov, I. A.; Lei, X. G.; Hao, Q.; Thiel, D. J. *J. Mol. Biol.* **2004**, *339*, 437-445.
- (56) Rodriguez, E.; Wood, Z. A.; Karplus, P. A.; Lei, X. G. *Arch. Biochem. Biophys.* **2000**, *382*, 105-112.
- (57) Jermutus, L.; Tessier, M.; Pasamontes, L.; van Loon, A.; Lehmann, M. J. *Biotechnol.* **2001**, *85*, 15-24.
- (58) Bei, J.; Chen, Z.; Fu, J.; Jiang, Z.; Wang, J.; Wang, X. *J. Biotechnol.* **2009**, *139*, 186-193.
- (59) Teramoto, N.; Shibata, M. *J. Appl. Polym. Sci.* **2004**, *91*, 46-51.
- (60) Nimmo, C. M.; Shoichet, M. S. *Bioconjug. Chem.* **2011**, *22*, 2199-2209.
- (61) Grover, G. N.; Lam, J.; Nguyen, T. H.; Segura, T.; Maynard, H. D. *Biomacromolecules* **2012**, *13*, 3013-3017.
- (62) Lei, Y.; Gojgini, S.; Lam, J.; Segura, T. *Biomaterials* **2011**, *32*, 39-47.

- (63) Xu, X.; Jha, A. K.; Harrington, D. A.; Farach-Carson, M. C.; Jia, X. *Soft Matter* **2012**, *8*, 3280-3294.
- (64) Bulpitt, P.; Aeschlimann, D. *J. Biomed. Mater. Res.* **1999**, *47*, 152-169.
- (65) Nimmo, C. M.; Owen, S. C.; Shoichet, M. S. *Biomacromolecules* **2011**, *12*, 824- 830.
- (66) Testa, G.; Di Meo, C.; Nardecchia, S.; Capitani, D.; Mannina, L.; Lamanna, R.; Barbetta, A.; Dentini, M. *Int. J. Pharm.* **2009**, *378*, 86-92.
- (67) Piluso, S.; Hiebl, B.; Gorb, S. N.; Kovalev, A.; Lendlein, A.; Neffe, A. T. *The International journal of artificial organs* **2011**, *34*, 192-197.
- (68) Gojgini, S.; Tokatlian, T.; Segura, T. *Mol. Pharm.* **2011**, *8*, 1582-1591.
- (69) Tokatlian, T.; Cam, C.; Siegman, S. N.; Lei, Y.; Segura, T. *Acta Biomater.* **2012**, *8*, 3921-3931.
- (70) Grover, G. N.; Braden, R. L.; Christman, K. L. *Adv. Mater.* **2013**, *25*, 2937-2942.
- (71) Salisbury, C. M.; Maly, D. J.; Ellman, J. A. *J. Am. Chem. Soc.* **2002**, *124*, 14868-14870.

Chapter 5

Trehalose Glycopolymer Resists Allow Direct Write of Protein Patterns by E-beam Lithography[‡]

5.1 Introduction

Direct-write patterning of biomolecules at micrometer and nanometer length scales is of tremendous interest in the lithography field as it has the potential to enable pattern generation with less fabrication steps and to obviate the need to use harmful chemicals that are usually not compatible with biomolecules. Moreover, direct-write approaches facilitate generation of geometrically complex multicomponent patterns at the micrometer and nanometer scale. Such protein patterns are important for a myriad of applications.¹⁻⁸ For example, miniaturized biomolecule arrays are valuable in the development of rapid and highly sensitive diagnostic assays, biosensors, and in discovery of novel drugs, biomarkers, or drug targets.¹⁻⁶ In addition, they are powerful tools in studying, elucidating, and modulating cell behaviour on bioactive surfaces that are crucial for tissue engineering and regenerative medicine.⁷⁻¹³ Most of these applications require multiple proteins to be immobilized at high resolution and in close proximity with precision. However, this is still a challenge, particularly at the nanoscale.

Many techniques have been used to pattern biomolecules on surfaces.^{3,14} These include microcontact printing,¹⁵ microfluidics,¹⁶ electron beam lithography (EBL),^{17,18} ink-jet printing,¹⁹ and scanning probe microscopy based techniques such as polymer-pen nanolithography or dip-pen nanolithography (PPL or DPN).²⁰⁻²² To date, however, only a few studies have shown direct writing of proteins at the submicron scale.²²⁻²⁴ Mirkin and co-workers used DPL to obtain protein nanoarrays.²² Yet this approach still has various drawbacks including the inability to achieve nanometer inter-feature spacings between different proteins or limitations in the shapes that can be made. In the other two studies EBL was used for patterning. EBL is a maskless patterning technique that generates user designed complex patterns at high resolution. Although a serial technique, EBL offers nanometer scale alignment capability, which enables inter-feature

spacings that are so small that different protein features may be touching or arrayed one on top of the other.¹⁷ This is important when high density arrays or complex multicomponent features are desired. Thus far mostly indirect approaches have been used to pattern proteins by EBL,^{17,18,25-27} because the processing conditions necessitate high vacuum and high energy radiation that inactivate proteins. Indeed, the harsh conditions required have been exploited to pattern by selective ablation of proteins upon e-beam exposure.^{25,26} In two earlier work of direct protein patterning by EBL, bacteriorhodopsin and green fluorescent protein were patterned using poly(acrylic acid) and silk as resists.^{23,24} In both studies, the authors noted that the used proteins had exceptionally stable structure, which enabled them to be stable under harsh conditions of EBL. Silk has been shown to stabilize horseradish peroxidase to vacuum conditions of SEM but its stability under e-beam irradiation has not been shown. Herein, we describe a trehalose glycopolymer resist material that can stabilize proteins to vacuum and exposure to electron beams allowing for direct write of biologically active proteins.

Trehalose is a disaccharide that is accumulated in large amounts by many plants and animals under desiccation stress. The disaccharide dramatically increases the resistance of these organisms, as well as cells and biomolecules to such environmental stresses.²⁸⁻³² We recently reported that polymers with trehalose side chains provide superior protection to proteins against environmental stresses such as heating and freeze-drying than trehalose does.^{33,34} The polymers combine the advantageous properties of the osmolyte and non-ionic surfactant class of stabilizers into one material.

Herein, a new water-soluble resist that protects proteins during electron beam exposure under high vacuum conditions and its application in direct-write patterning of multiple proteins at the micrometer and nanometer scale by electron beam lithography are presented. Polymers

with pendant trehalose units were shown to effectively cross-link to surfaces as negative resists while at the same time providing stabilization to proteins during the vacuum and electron beam irradiation steps. In this manner, arbitrary patterns of several different classes of proteins such as enzymes and immunoglobulins were realized. Utilizing the high precision alignment capability of electron-beam lithography, surfaces with complex patterns of multiple proteins were successfully generated.

5.2 Experimental Section

Materials

Silicon wafers were obtained from Cemat Silicon S.A., Poland. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX). Sheep anti-glucose oxidase antibody was obtained from Abcam. Human, chicken, and mouse IgGs and the fluorescent antibodies AF488 goat anti- HRP, AF488 donkey anti-human IgG, AF594 donkey anti-chicken IgG were purchased from Jackson ImmunoResearch. AF350 donkey anti-mouse IgG, AF488 donkey anti-rabbit IgG, AF488 goat anti-sheep IgG were bought from Invitrogen. 6-O-(4-vinylbenzylidene)- α,α -trehalose and **poly(SET)** was prepared by following our literature procedure.³⁴ All the other chemicals were obtained from Sigma-Aldrich and Fisher Scientific unless otherwise stated. For generating multicomponent patterns, silicon chips with gold alignment marks were fabricated by standard photolithography, metal evaporation and lift-off techniques.¹⁷

Methods

Effect of Protein and Polymer Concentration. Silicon substrates were cleaned with freshly prepared piranha solution (3:1 H₂SO₄/H₂O₂, *Caution! Piranha solution reacts violently with organic materials.*). After washing with 10×200 mL of MilliQ water and drying under a stream of air, the cleaned substrates were spin-coated (500 rpm, 5s; 1000 rpm, 5 s; 2000 rpm, 20 s; or 4000 rpm for 10 s) with a 30 μ L solution containing **poly(SET)** and HRP. To investigate the effect of protein concentration, 25 μ L of **poly(SET)** (0.5 wt % in H₂O) was mixed with 5 μ L of protein solution (ranging from 0.60 μ M to 600 mM in D-PBS) leading to final protein concentrations ranging from 0.1 mM to 100 μ M. **poly(SET)**-HRP patterns were generated using

a JC Nabity e-beam lithographic system (Nanometer Pattern Generation System, version 9.0) modified from a JEOL JSM-6610 scanning electron microscope. The pattern files for EBL were designed using DesignCAD Express 16 software. An accelerating voltage of 30 kV was used, with a beam current of ~ 15 pA, a spot size of 30-40 nm. 10 mm square patterns were written at a dose range from 5 to 80 mC/cm². After e-beam exposure, any non-crosslinked polymer on the chips was washed excessively with D-PBS containing 1 mM of Tween 20 (PBS-Tween) and then stained with AF488 goat anti-HRP antibody (10 mg/mL in PBS-Tween).

To investigate the effect of polymer concentration, 25 μ L of **poly(SET)** (0.3-2 wt % in H₂O) was mixed with 5 μ L of protein solution (60 mM in D-PBS) leading to final protein concentration of 10 μ M. Patterns were written at doses ranging from 3 to 96 mC/cm² and stained as described above.

Single Protein Patterning with Different Excipients. A dose test was performed for each protein-excipient pair (HRP, GOx, sheep IgG, or SAv with **poly(SET)**, PEG (M_n = 14000 g/mol), trehalose, or no additive) to determine the optimal area dose, the dose that resulted in highest signal-to-noise ratio after antibody staining. The spin-coating solutions contained 25 μ L of **poly(SET)** (0.5 wt % in H₂O, or molar equivalents of PEG or trehalose (molar equivalent to trehalose in **poly(SET)**) and 5 μ L of protein (60 μ M in D-PBS) leading to a final protein concentration of 10 μ M. On each substrate, a 4 $\times$ 4 array of 100 mm² squares were written four times at doses ranging from 5 to 80 mC/cm² (n = 3). After antibody staining was performed as described above, signal-to-noise ratios were calculated for each dose and the optimal area dose was determined for each protein-excipient. Once the optimal dose was determined for each protein-excipient, a total of 64 squares measuring 100 mm² squares were written on each substrate at the predetermined optimal dose (n=3) and then stained with respective antibodies. To

compare the different excipients, signal-to-noise ratios were calculated for 3×64 squares that had been written for each protein-excipient.

Micro- and Nanopatterning of Multiple Proteins and Immunostaining. Silicon substrates with gold alignment marks were piranha cleaned and spin-coated with a 30 μL solution of **poly(SET)** (25 μL , 0.5 wt % in H_2O) and protein (5 μL of protein 30 μM in D-PBS) as described above. After the first pattern had been written with human IgG, the substrates were washed with PBS-Tween followed by a very brief rinsing with Milli-Q H_2O . After alignment to the first layer, a complementary pattern was written with chicken IgG. The third layer was written with mouse IgG. The generated patterns were visualized by immunofluorescence staining. The substrates were sequentially incubated with fluorescently labeled donkey anti-human, donkey anti-chicken, and donkey anti-mouse IgGs (30 min, 10 $\mu\text{g}/\text{mL}$ in PBS-Tween) for 30 min. In between the incubation steps, the substrates were washed with PBS-Tween solution.

Fluorescence Microscopy. The immunostained patterns were visualized by fluorescence microscopy using a Zeiss Axiovert 200 fluorescent microscope equipped with an AxioCam MRm monochrome camera, and pictures were acquired and processed using AxioVision LE 4.6 software. NIH ImageJ software was used to calculate the signal-to-noise ratio as (signal – background)/standard deviation of background.

5.3 Results and Discussion

Effect of Protein and Polymer Concentration

We first investigated whether the trehalose glycopolymers could be employed as resists and stabilize proteins to EBL conditions. We utilized a polymer synthesized from a styrenyl ether trehalose monomer (**poly(SET)**, Figure 5-1). Our initial experiments showed that, upon exposure to e-beams, this polymer was crosslinked and covalently bound to the silicon/silicon dioxide surfaces. This is to be expected since **poly(SET)** has a polystyrene backbone, which is known as a negative-tone EBL resist.³⁵ The polymer likely cross-links to the surface and to other polymer chains by a radical cross-linking mechanism similar to that observed for other polymers such as poly(ethylene glycol) (PEG).^{17,18} The process for generating protein patterns is outlined in Figure 5-1. An aqueous solution containing the protein to be patterned and **poly(SET)** is first spin-coated onto silicon substrates. Then, **poly(SET)**-protein patterns are generated by EBL. Multicomponent patterns are obtained by subsequent development, spin-coating, alignment and writing steps.

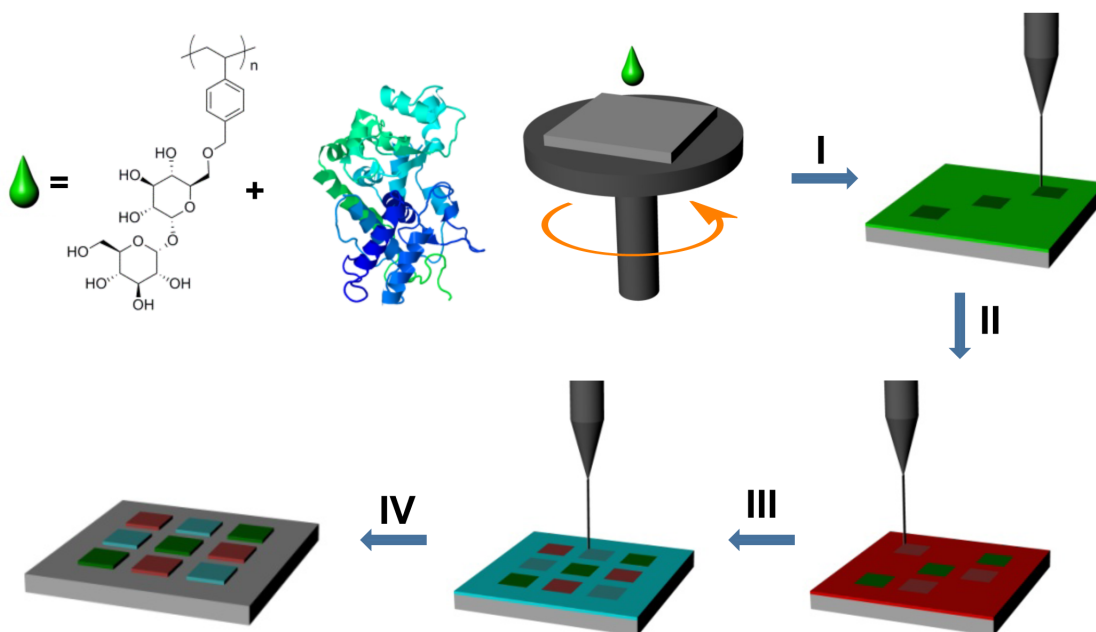


Figure 5-1. Electron beam lithography process for the generation of multiple protein patterns. **I**, Spin-coating with **poly(SET)-protein-1** solution and writing of the first layer. **II**, Rinsing of the unexposed **poly(SET)-protein-1** followed by spin-coating **poly(SET)-protein-2**, alignment to the first layer, and writing of the second layer. **III-IV**, Multiple protein patterns are obtained by repeated spin-coating, alignment, writing, and rinsing steps.

Horseradish peroxidase (HRP) was used as a model protein to investigate the effect of protein concentration, polymer concentration on the obtained patterns, and also to determine the optimal area dose (~energy) required to obtain the HRP-**poly(SET)** patterns (Figure 5-2a, 5-3, 5-4). The signal-to-noise ratio (S/N) for each condition was determined using the images of patterns stained with a fluorescent anti-HRP antibody. When the protein concentration was varied at a constant **poly(SET)** concentration, the immunostained patterns showed very high fluorescence signal (S/N>150) for concentrations at or above 10 mM. The observed increase in

S/N values could be related to higher protein concentration in the resulting gel, which also would increase the stability of the protein³⁶. The polymer concentrations were then investigated. By studying the S/N values, it was determined that the optimal polymer concentration was 0.5 wt %. At lower concentration (0.3 wt %) a weak antibody signal was observed likely because the polymer concentration was not high enough to protect the protein. Based on these results, 0.5 wt % polymer concentration and 10 μ M final protein concentration were chosen for further experiments. Importantly, the data indicate that during crosslinking of **poly(SET)**, the protein was immobilized onto the surface, likely either by grafting as a result of hydrogen abstraction or by entrapment within the network. The data also suggest that the antibody binding site of HRP was not significantly influenced by the EBL process.

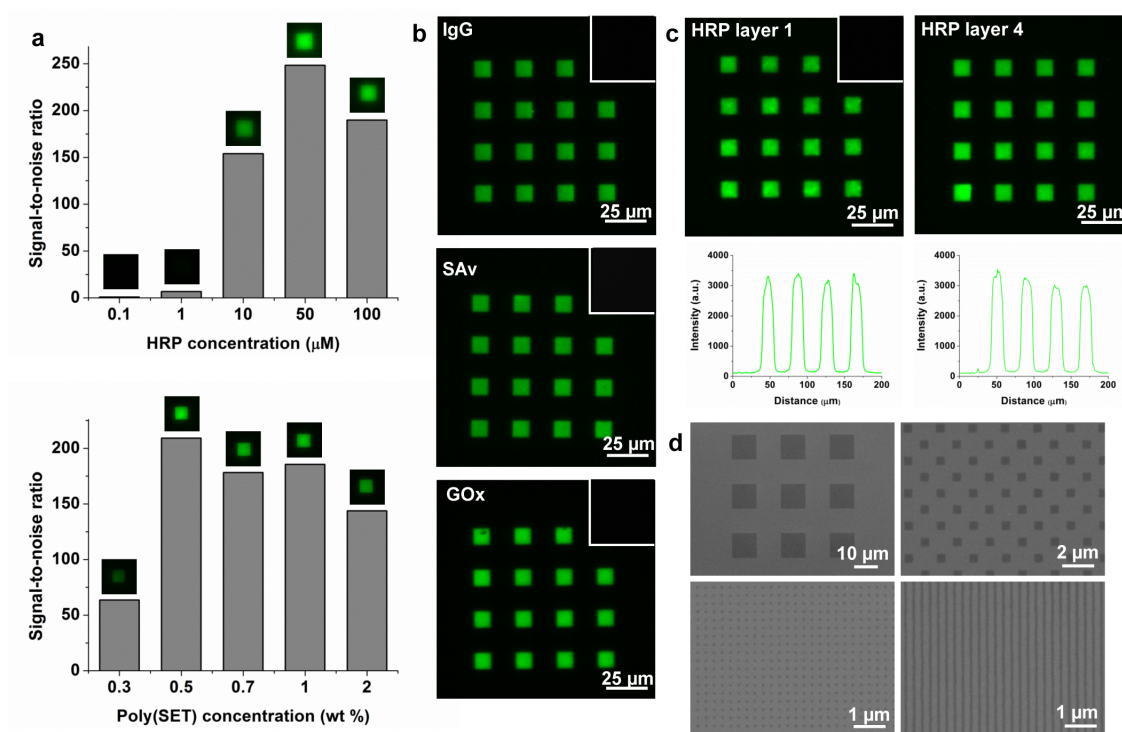


Figure 5-2. HRP, immunoglobulin G (IgG), streptavidin (SAv), and glucose oxidase (GOx) can be patterned by direct e-beam writing using trehalose glycopolymers as resists. (a) Effect of HRP and **poly(SET)** concentration on signal-to-noise ratio of **poly(SET)**-HRP patterns. Top: **poly(SET)** concentration was set to 0.5 wt% and bottom: HRP concentration was 10 mM; in each case S/N calculated for optimal dose is shown. (b) Fluorescence micrographs of fluorescent antibody stained **poly(SET)**-IgG, **poly(SET)**-SAv, **poly(SET)**-GOx, and (c) **poly(SET)**-HRP patterns (first and fourth layer written on the same substrate are shown) and fluorescence intensity profiles drawn along second row of squares from the top. Insets in (b) and (c) show that fluorescent antibodies do not bind non-specifically to **poly(SET)** patterns that did not contain respective proteins. (d) SEM micrographs showing micro- and nanopatterns of **poly(SET)**-HRP.

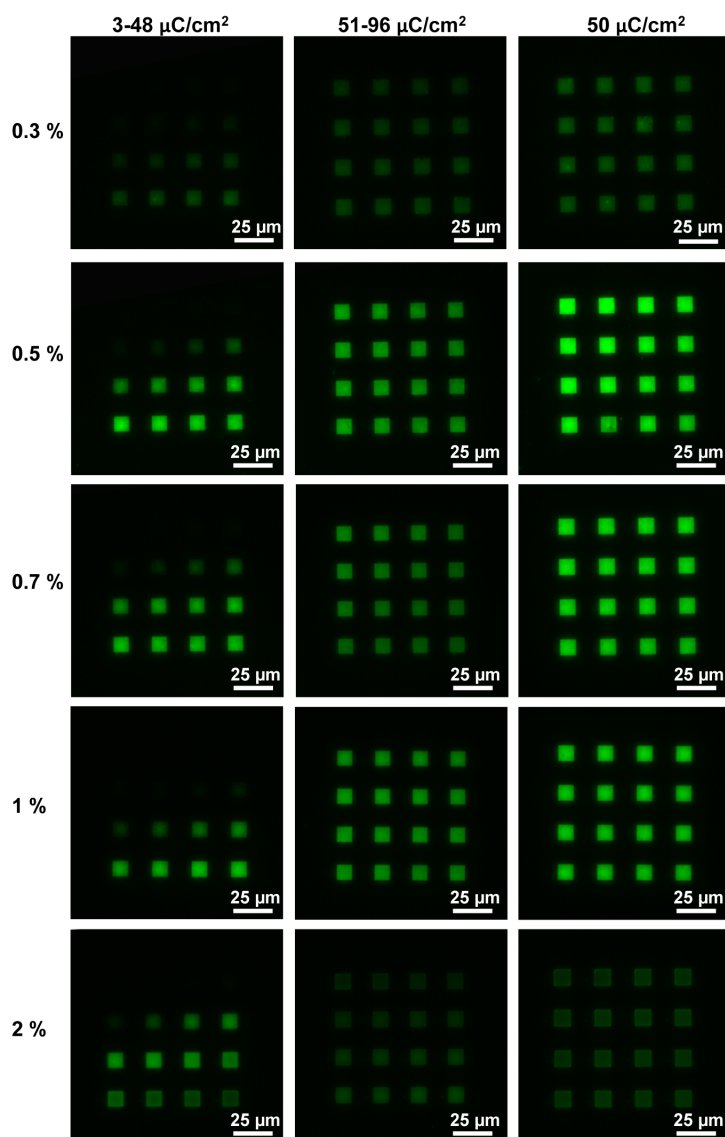


Figure 5-3. Fluorescence micrographs showing the effect of **poly(SET)** concentration on **poly(SET)**-HRP patterns. In the first two columns each square pattern was written at a different dose ranging from 3-96 $\mu\text{C}/\text{cm}^2$, therefore the patterns are visible only above a threshold dose. In the third column each square pattern was written at a constant dose of 50 $\mu\text{C}/\text{cm}^2$. HRP concentration in spin-coating solutions was 10 mM for all cases. The patterns have been stained with AlexaFluor[®] 488 goat anti-HRP antibody.

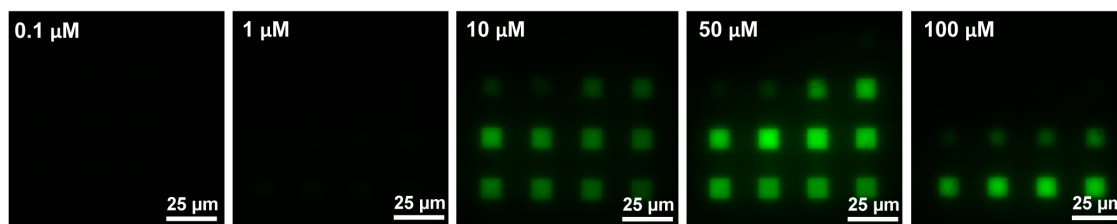


Figure 5-4. Fluorescence micrographs showing the effect of HRP concentration on **poly(SET)**-HRP patterns. Each square pattern on a single chip was written at a different dose ranging from 5-80 $\mu\text{C}/\text{cm}^2$, therefore the patterns are visible only above a threshold dose. A 0.5 wt % of **poly(SET)** solution was used for all cases. The patterns have been stained with AlexaFluor[®] 488 goat anti-HRP antibody.

To test the multiplicity of the technique, we chose HRP, glucose oxidase (GOx), immunoglobulin G (IgG), and streptavidin (SAv) because these proteins are utilized in a wide range of bionanotechnological applications such as diagnostic assays and biosensors, and also are readily available. First, dose tests for the different proteins were performed (Figure 5-5, first column). The highest S/N was obtained at an area dose of 45 mC/cm^2 for HRP, GOx, and SAv, while for IgG the optimal dose was 25 mC/cm^2 . After staining the protein- **poly(SET)** patterns that had been written at their optimal writing doses, high fluorescent signals ($\text{S/N} > 120$ for all the proteins used) were observed (Figure 5-2b). To rule out non-specific binding of antibodies to **poly(SET)**, we incubated **poly(SET)** patterns (written without protein) with all the fluorescently labeled antibodies that have been used in this work. In all of the cases no pattern was visible by fluorescence microscopy (Figure 5-2b and 5-2c insets). Therefore, the fluorescence signals seen in Figure 5-2b are due to specific antigen-antibody interactions.

Importantly, successful staining of IgG shows that their recognition property is preserved. Therefore, direct writing of IgGs with trehalose glycopolymers is a promising method of

generating antibody arrays for diagnostics and proteomics studies. Moreover, when four successive HRP patternings were applied on the same substrate, the fluorescence intensity of the first layer (exposed to four EBL cycles) was comparable to the fourth layer (Figure 5-2c). This suggests that exposure of the formed **poly(SET)**-protein patterns to repeated vacuum-vent and rinsing cycles did not have an adverse effect on the protein structure, a requirement for multicomponent patterning by EBL. The **poly(SET)**-HRP patterns generated by EBL were also visualized by scanning electron microscopy. Figure 5-2d shows square patterns with 10 μm and 500 nm side lengths as well as sub-100 nm dots and lines of **poly(SET)**-HRP.

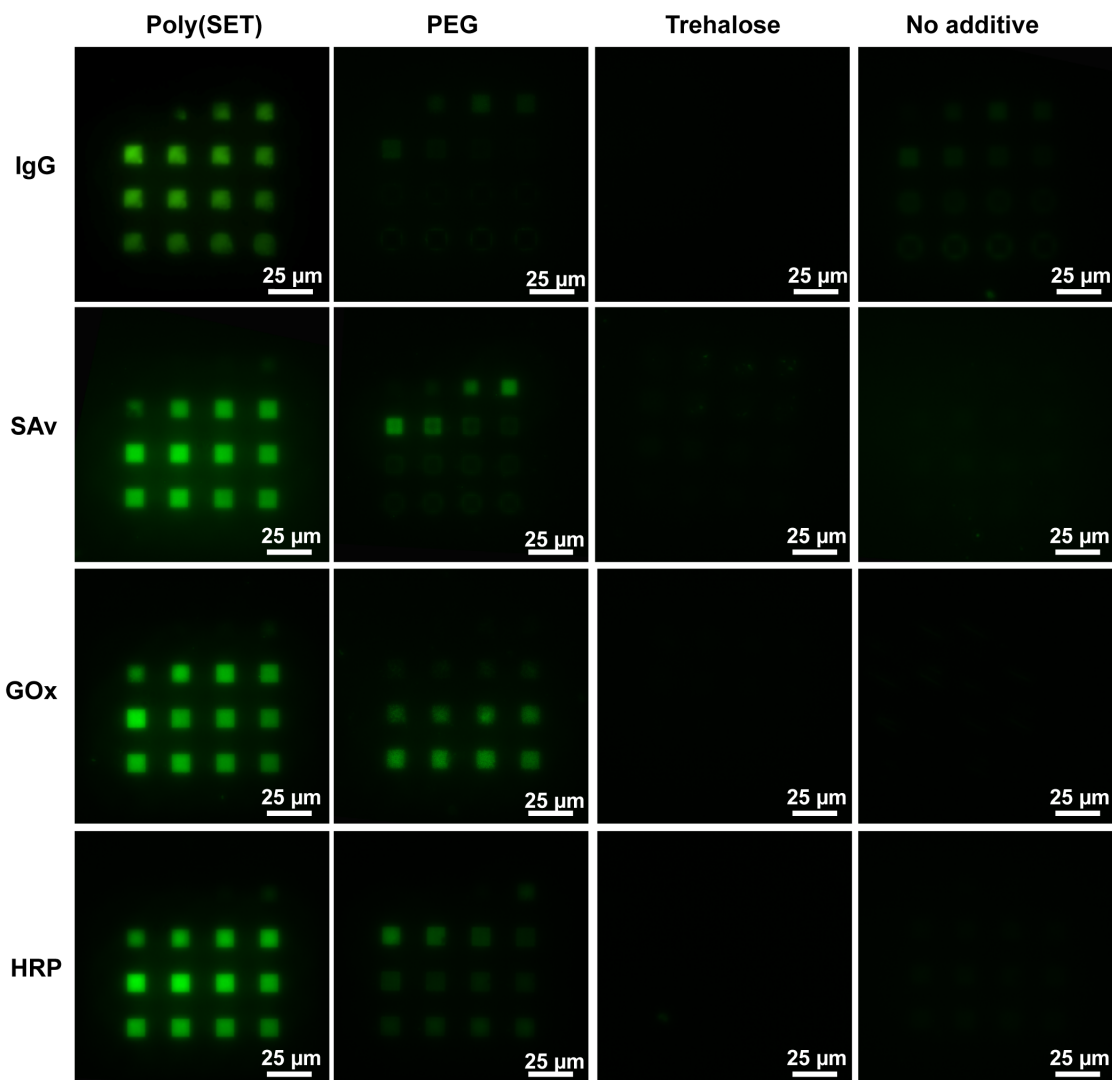


Figure 5-5. Fluorescence micrographs showing the effect of area dose for four different proteins (IgG, SAV, GOx, and HRP) written with four different excipients (**poly(SET)**, PEG, trehalose, and without excipient). Each square pattern on a single chip was written at a different dose ranging from 5-80 $\mu\text{C}/\text{cm}^2$, therefore the patterns are visible only above a threshold dose. The patterns have been stained with respective fluorescently labelled antibody for each protein. No pattern could be observed when **poly(SET)** or PEG patterns that did not contain any protein (controls) were stained with antibodies.

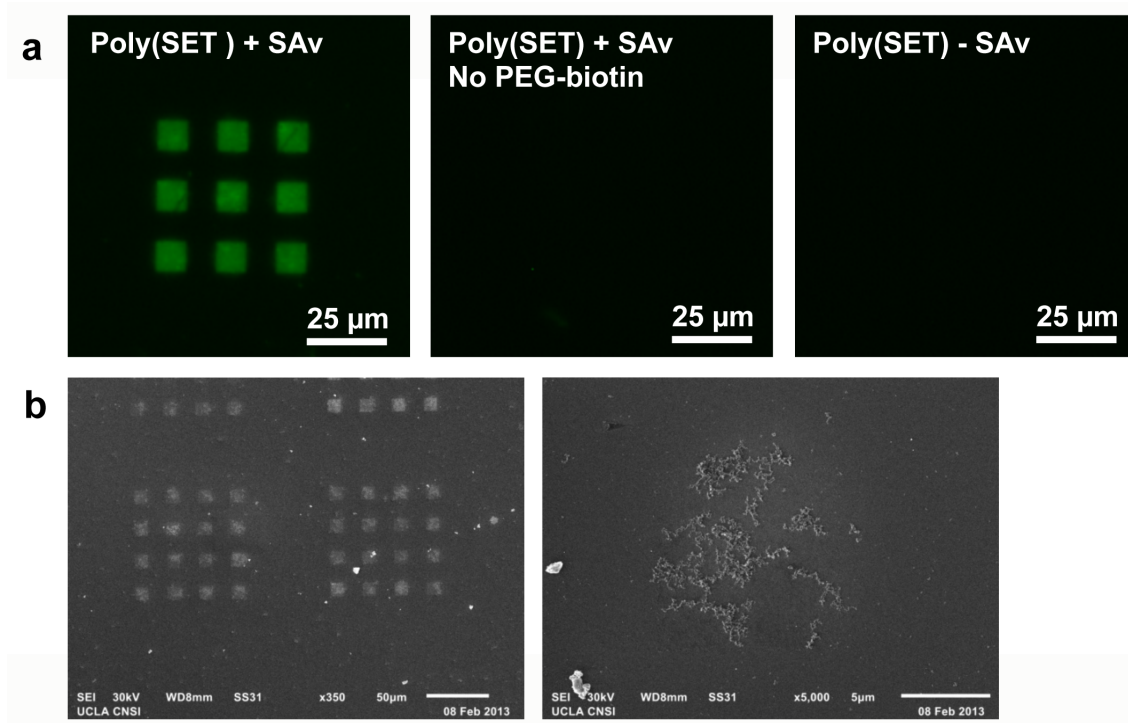


Figure 5-6. SAV and GOX patterns generated by EBL showed protein activity as assessed by biotin binding and gold nanoparticle formation respectively. (a) Fluorescence micrographs showing **poly(SET)**-SAV patterns after incubation with 8-arm PEG-biotin (0.1 % in PBS for 1h) followed by incubation with AF488 conjugated SAV (10 mg/mL in PBS for 1h). No pattern was visible when PEG-biotin incubation step was skipped or only **poly(SET)** without SAV was patterned by EBL. (b) SEM micrographs showing gold nanoparticles formed on an array of square **poly(SET)**-GOx patterns (left) and a magnified image of a single pattern (right) as a result of reduction of gold ions (3 mM) by glucose oxidase in the presence of glucose (12 mM in PBS, pH 7.4 for 8 h).

Observing protein activity is a critical component for protein arrays. The IgG exhibited activity; it still bound to antibodies as described above. The activity of SAV patterns was assessed by a biotin binding assay (Figure 5-6a). After generating **poly(SET)**-SAV patterns, the

substrates were incubated with an 8-arm PEG-biotin. Subsequent incubation with AF488 labeled streptavidin was used to detect the surface bound PEG-biotin in a sandwich assay format. The **poly(SET)**-SAv patterns showed strong fluorescence signal after incubation with PEG-biotin and fluorescent streptavidin indicating that the written SAv molecules retained their biotin binding properties and thus the biological activity. No signal was observed when only **poly(SET)** was patterned or when the PEG-biotin incubation step was skipped. These control experiments confirmed that the observed fluorescence signal for **poly(SET)**-SAv patterns was indeed due to specific interaction of streptavidin with biotin. The activity of **poly(SET)**-HRP patterns was assessed using a tetramethylbenzidine (TMB) assay. The observed activity of **poly(SET)**-HRP patterned substrates was higher (0.037 ± 0.009 a.u., $p < 0.035$) than that measured for control substrates that had been spin-coated with **poly(SET)**-HRP and charged into SEM chamber but not exposed to e-beams and washed (0.019 ± 0.005 a.u.). The signal observed for control substrates is likely due to non-specifically adsorbed protein that was not removed during the washing step. Importantly, **poly(SET)** was not a substrate for TMB and did not influence the measurements. Finally, the activity of **poly(SET)**-GOx patterns were visualized by formation of gold nanoparticles on the generated patterns as a result of reduction of gold ions by glucose oxidase (Figure 5-6b).³⁷ The data together demonstrate that the direct write of these proteins with **poly(SET)** resulted in bioactive protein patterns.

Single Protein Patterning with Different Excipients

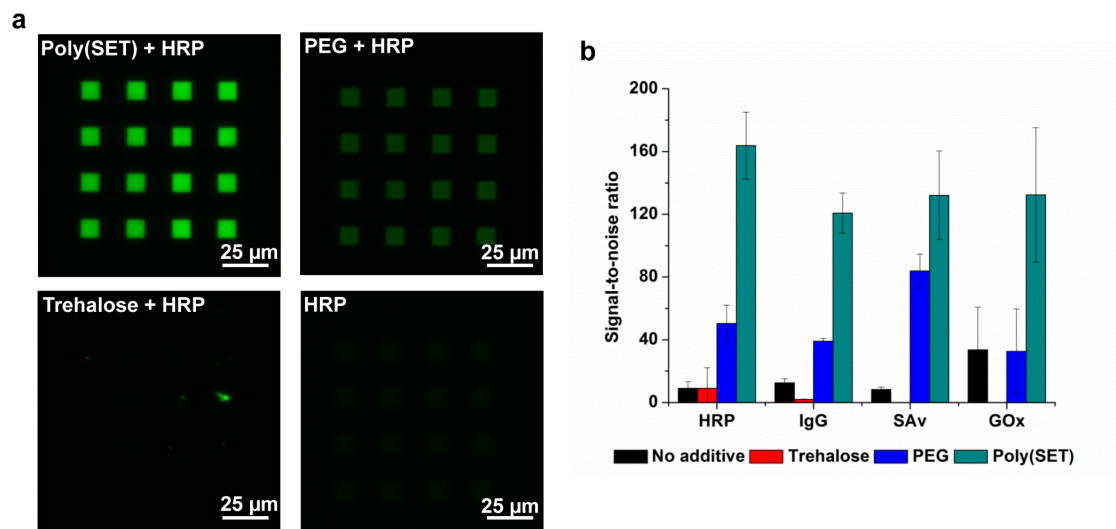


Figure 5-7. Effect of different excipients on patterning of HRP, IgG, SAV, and GOx. (a) Fluorescence micrographs of HRP patterned with **poly(SET)**, PEG, trehalose, or without additive after staining with AlexaFluor® 488 goat anti-HRP. (b) Signal-to-noise ratios calculated for different proteins patterned with different excipients. S/N values were significantly higher for **poly(SET)** patterns compared to other excipients ($p < 5.3 \times 10^{-5}$, $< 6.6 \times 10^{-5}$, < 0.019 , < 0.038 for HRP, IgG, SAV, GOx, respectively)

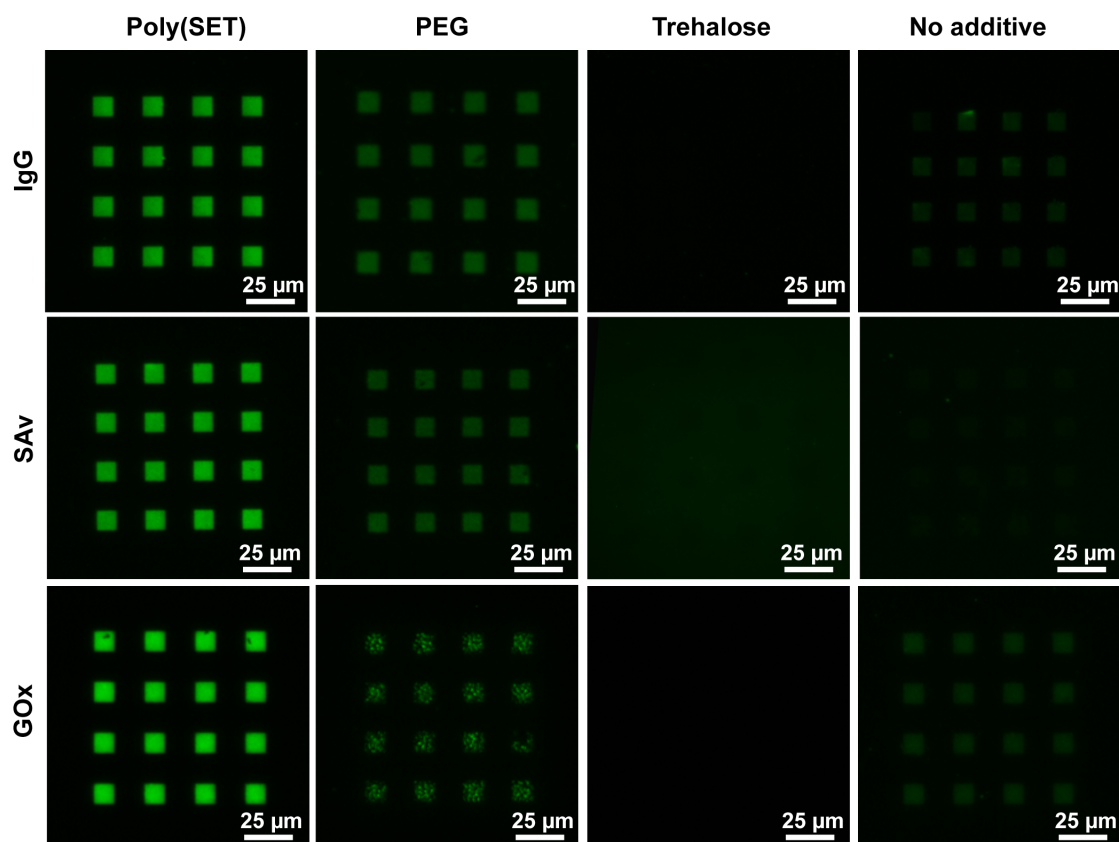


Figure 5-8. Fluorescence micrographs showing IgG, SAv, and GOx patterned with either **poly(SET)**, or PEG, or trehalose, or without any excipient. Substrates were prepared by spin-coating protein-excipient solutions.

Several compounds including amino acids,³⁸ sugars,^{39,40} and polymers⁴⁰ have been shown to improve protein stability. Therefore, the effectiveness of PEG and trehalose were compared to **poly(SET)** in the stabilization of HRP, IgG, GOx, and SAv. First, the optimal writing dose (the dose that gave the highest S/N) was determined for each excipient-protein (Figure 5-5), then the S/N values were determined for each excipient-protein pattern that had been written at the optimal dose (Figure 5-7 and 5-8). In all cases, significantly higher fluorescence signals were observed for protein patterns that had been written with **poly(SET)** compared to those that had been written with PEG, trehalose, or with no additive (Figure 5-7a and 5-8). Figure 5-7b shows

that the S/N values for **poly(SET)** (average S/N = 120-164 for different proteins) were consistently higher than PEG (average S/N = 32-84 for different proteins). Since both polymers can be crosslinked by e-beam irradiation, the lower signals observed for PEG are due to a less efficient stabilization capability or to a less efficient grafting efficiency of this polymer compared to **poly(SET)**. It has been observed that trehalose glycopolymers are superior stabilizers to PEG when exposed to other stresses (heat and lyophilization) in solution.^{33,34} In the case of trehalose and no additive the fluorescence signals were very low; this is to be expected since ablation of proteins by e-beams is known, and furthermore trehalose does not crosslink during the e-beam process.

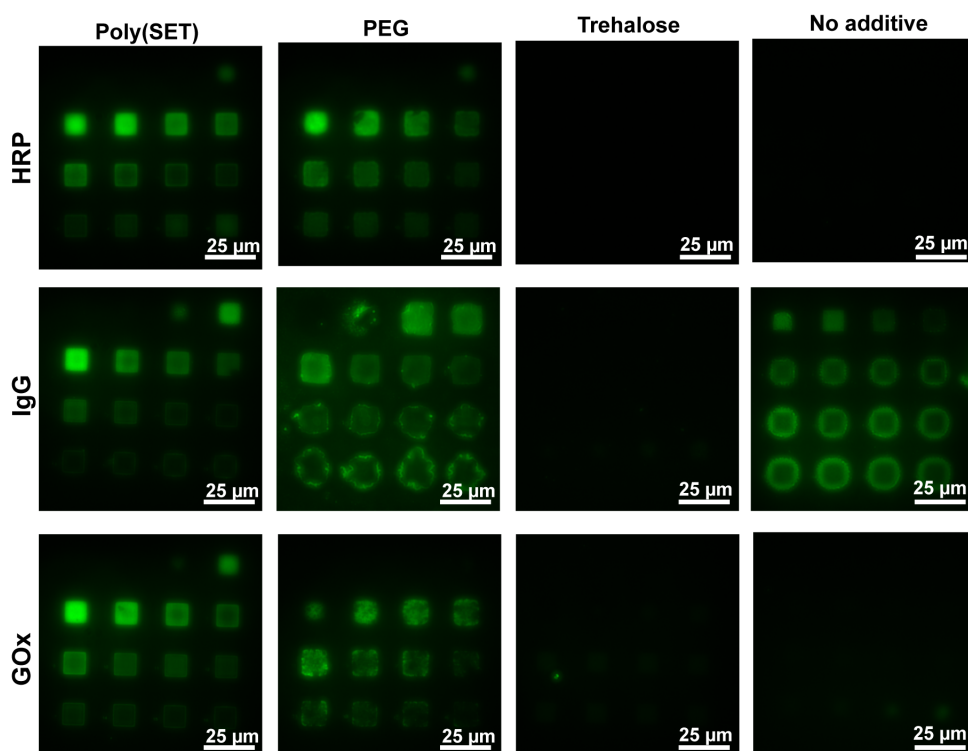


Figure 5-9. Fluorescence micrographs showing HRP, IgG, and GOx patterned with either **poly(SET)**, or PEG, or trehalose, or without any excipient. Each square pattern on a single chip was written at a different dose ranging from 5 to 80 mC/cm². Therefore, only the square patterns above a threshold dose are visible. Substrates were coated by spreading protein-excipient solutions and vacuum drying.

To rule out any differences in the amount of protein coated onto the surface of the substrates during spin-coating with different excipients, we prepared substrates by placing a 5 mL drop of protein-excipient solution onto the chips and let it spread over the surface instead of spin coating. After, drying these substrates under vacuum, patterns were generated by EBL and stained with fluorescent antibodies (Figure 5-9). When the solution was spread onto the substrates and dried, higher S/N values (average S/N ranged from 70 to 184, Figure 5-10) were obtained with PEG compared to those obtained for the spin-coated substrates (average S/N

ranged from 32 to 51). Nevertheless, the average S/N for **poly(SET)** was the highest in all cases (average S/N ranged from 197 to 240, Figure 5-10).

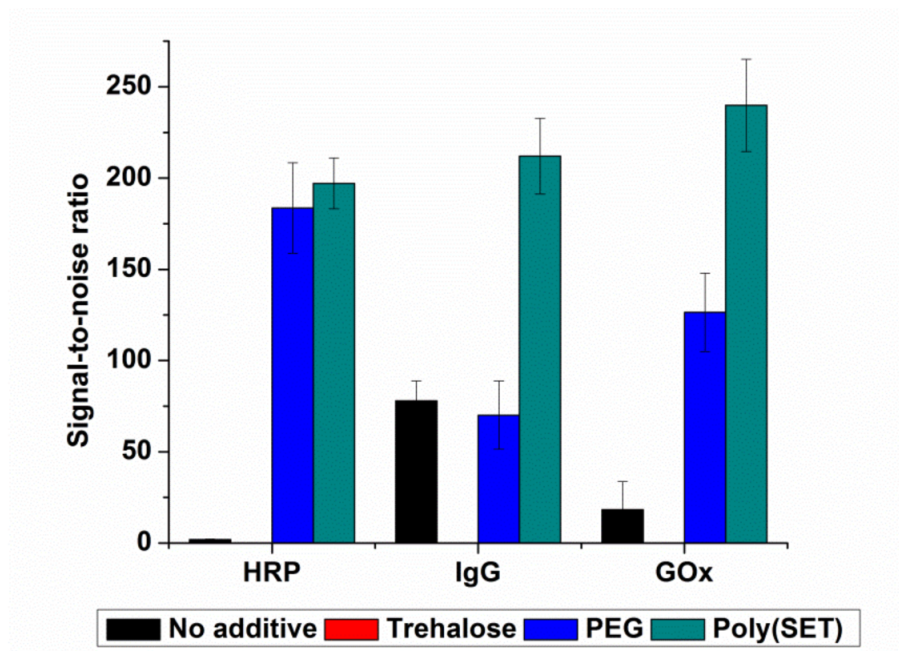


Figure 5-10. Maximum signal-to-noise ratios for HRP, IgG, and GOx patterned with either **poly(SET)**, or PEG, or trehalose, or without any excipient. Substrates were coated by spreading protein-excipient solutions and vacuum drying.

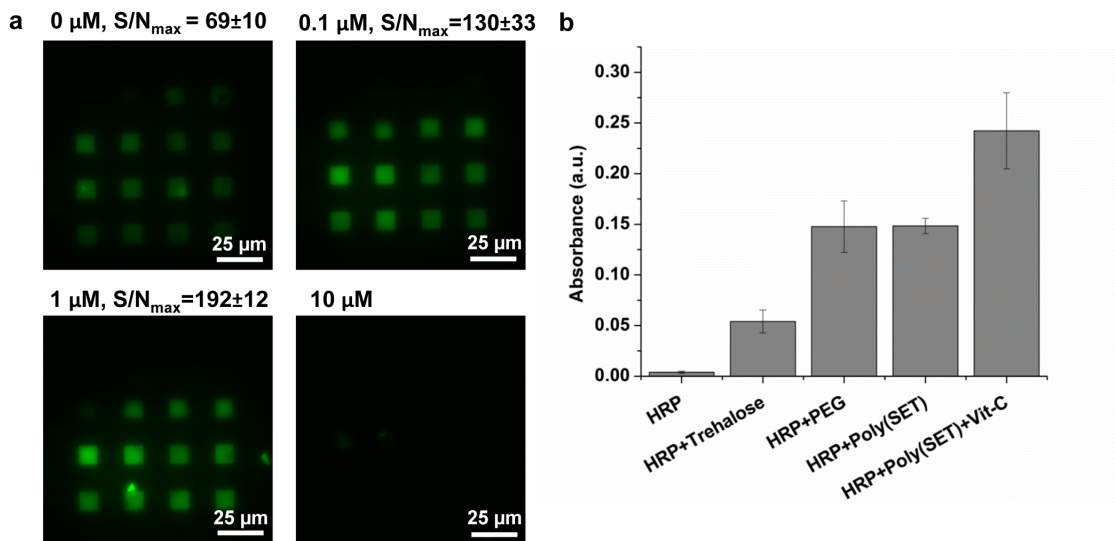


Figure 5-11. (a) Fluorescence micrographs showing patterns of **poly(SET)**-HRP containing different amounts of ascorbic acid (Vitamin C) and written at a dose range of 5 to 80 mC/cm². (b) Activity of HRP after incubation under high vacuum conditions for 4h with different excipients.

In an effort to understand the mechanisms of protein stabilization by **poly(SET)** during EBL process, we investigated the effect of radicals formed upon e-beam irradiation and the effect of high vacuum conditions (Figure 5-11). When **poly(SET)**-HRP coated substrates having different concentrations of ascorbic acid (Vitamin C) as a radical scavenger were exposed to electron beams, an increase in the fluorescence intensity was observed with increasing ascorbic acid concentrations (Figure 5-11a). The calculated S/N values increased from 69 ± 10 to 192 ± 12 when the ascorbic acid concentration was increased from 0 mM to 1 mM. This suggests that likely the ascorbic acid molecules reduce the amount of inter- and intra-molecular crosslinking reactions by scavenging the formed radicals and help in preservation of protein structure. As expected, the minimum dose required for crosslinking also increased with increasing

concentration of ascorbic acid and eventually a concentration of 10 mM prevented crosslinking reactions and thus pattern formation. The S/N value for no ascorbic acid was lower than shown in Figure 5-2 because in order to prevent over-exposure of the ascorbic acid samples, the samples without additive were underexposed. We also investigated the activity of HRP with different excipients after being exposed to high vacuum conditions for 4h. HRP loses activity just upon exposure to the vacuum, but in the presence of trehalose, PEG, or **poly(SET)** the activity of HRP is much higher (Figure 5-11a). PEG and **poly(SET)** were equally as good at stabilizing the protein to vacuum. Thus, the results observed when patterning by electron beam lithography must be a result of **poly(SET)**'s superior ability to stabilize against exposure to electron beam irradiation. Interestingly, in the presence of both **poly(SET)** and ascorbic acid, the highest activity for HRP was observed, indicating that ascorbic acid helps stabilization of HRP during desiccation.

Micro- and Nanopatterning of Multiple Proteins

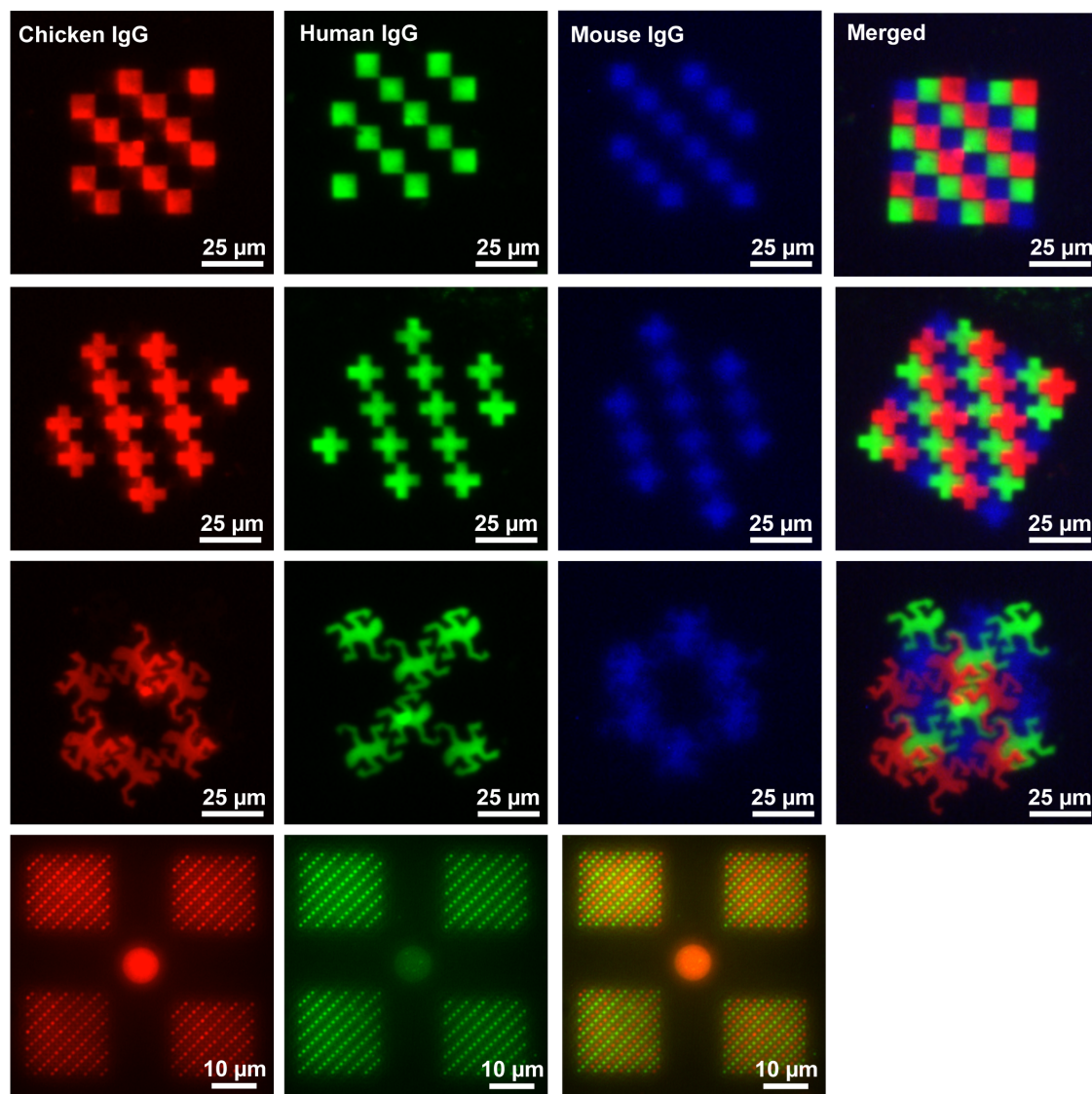


Figure 5-12. Multiple protein micro- and nanopatterns generated by sequentially writing human, chicken, and mouse IgGs. Fluorescence micrographs showing fluorescent antibody stained individual IgG patterns written with **poly(SET)**; chicken IgG (red channel), human IgG (green channel), mouse IgG (blue channel). The merged images show all the patterned IgGs forming a tricolor checkerboard, a cross-array, a replica of M.C. Escher's work "Reptiles", and a hexagonal array of 500 nm square patterns.

To demonstrate the applicability of the direct EBL technique in generating multiplexed antibody arrays in close proximity, contiguous micropatterns of multiple proteins were generated using human, chicken, and mouse immunoglobulins (Figure 5-12). First, **poly(SET)**-human IgG patterns were written to create a checkerboard patterns or arrays of crosses. After removing the noncrosslinked polymer and protein from the surface by washing and spin-coating with the **poly(SET)**-chicken IgG, a complementary pattern was written. Then, a third layer was written with mouse IgG. Sequential staining of the patterns with respective labelled antibodies allowed visualization of tricolor checkerboard or cross-array patterns. It can be seen that fluorescent antibodies are mainly confined to the areas where respective IgGs had been written without significant cross-reactivity.

With electron beam lithography, it is possible to make patterns based on user-created designs (so called arbitrary patterns). To illustrate that complex multicomponent patterns can be obtained with the direct protein patterning technique, a replica of M.C. Escher's work "Reptiles" was generated with the three IgGs in the same manner as described above. The resulting patterns replicate the design of the painting and demonstrate the complexity of patterns possible, as well as the ability to pattern different proteins in close proximity. Such patterns could be very useful in generating bioactive surfaces for investigating fundamental questions about protein-protein interactions, as well as studying the effect of geometry on cell-behavior or the effect of multivalency in cell-behavior.

Multicomponent patterning is also important for generating high density, high sensitivity arrays that could be very useful in fields such as proteomics and diagnostics. To investigate this, hexagonal nanoarrays of human and chicken IgGs were written. Each pattern in these arrays is $500 \times 500 \text{ nm}^2$. The center-to-center distance between human and chicken IgGs were set to 1000

nm for better visualization of individual elements in the arrays. The data show that indeed nanoarrays are possible.

5.4 Conclusion

In conclusion, the use of the trehalose glycopolymer has been shown to allow direct patterning of proteins by providing stabilization to high energy radiation and vacuum conditions during electron beam lithography. The versatility of this direct patterning approach has been demonstrated by generating complex, multicomponent micro- and nanopatterns of proteins. Although protein patterns were demonstrated, it is readily envisioned that this approach can be extended to other biomolecules such as DNA, carbohydrates etc. Thus, we expect that this biomolecule patterning technique will find broad application in different disciplines and will be important in designing bioanalytical assays, biosensors, microreactors, and bioactive interfaces for cell culture.

5.5 References

- (1) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760-1763.
- (2) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101-2105.
- (3) Christman, K. L.; Enriquez-Rios, V. D.; Maynard, H. D. *Soft Matter* **2006**, *2*, 928-939.
- (4) Weinrich, D.; Jonkheijm, P.; Niemeyer, C. M.; Waldmann, H. *Angew. Chem. Int. Ed.* **2009**, *48*, 7744-7751.
- (5) Kingsmore, S. F. *Nat. Rev. Drug Discovery* **2006**, *5*, 310-320.
- (6) Mitchell, P. *Nat. Biotechnol.* **2002**, *20*, 225-229.
- (7) Kane, R. S.; Takayama, S.; Ostuni, E.; Ingber, D. E.; Whitesides, G. M. *Biomaterials* **1999**, *20*, 2363-2376.
- (8) Falconnet, D.; Csucs, G.; Grandin, H. M.; Textor, M. *Biomaterials* **2006**, *27*, 3044-3063.
- (9) Lehnert, D.; Wehrle-Haller, B.; David, C.; Weiland, U.; Ballestrem, C.; Imhof, B. A.; Bastmeyer, M. *J. Cell Sci.* **2004**, *117*, 41-52.
- (10) Arnold, M.; Hirschfeld-Warneken, V. C.; Lohmuller, T.; Heil, P.; Blummel, J.; Cavalcanti-Adam, E. A.; Lopez-Garcia, M.; Walther, P.; Kessler, H.; Geiger, B.; Spatz, J. P. *Nano Lett.* **2008**, *8*, 2063-2069.
- (11) Chen, C. S.; Mrksich, M.; Huang, S.; Whitesides, G. M.; Ingber, D. E. *Science* **1997**, *276*, 1425-1428.
- (12) Kilian, K. A.; Bugarija, B.; Lahn, B. T.; Mrksich, M. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 4872-4877.

- (13) Kim, D. H.; Lee, H.; Lee, Y. K.; Nam, J. M.; Levchenko, A. *Adv. Mater.* **2010**, *22*, 4551-4566.
- (14) Barbulovic-Nad, I.; Lucente, M.; Sun, Y.; Zhang, M. J.; Wheeler, A. R.; Bussmann, M. *Crit. Rev. Biotechnol.* **2006**, *26*, 237-259.
- (15) Bernard, A.; Delamarche, E.; Schmid, H.; Michel, B.; Bosshard, H. R.; Biebuyck, H. *Langmuir* **1998**, *14*, 2225-2229.
- (16) Delamarche, E.; Bernard, A.; Schmid, H.; Michel, B.; Biebuyck, H. *Science* **1997**, *276*, 779-781.
- (17) Christman, K. L.; Schopf, E.; Broyer, R. M.; Li, R. C.; Chen, Y.; Maynard, H. D. *J. Am. Chem. Soc.* **2009**, *131*, 521-527.
- (18) Hong, Y.; Krsko, P.; Libera, M. *Langmuir* **2004**, *20*, 11123-11126.
- (19) Derby, B. *J. Mater. Chem.* **2008**, *18*, 5717-5721.
- (20) Lee, K. B.; Lim, J. H.; Mirkin, C. A. *J. Am. Chem. Soc.* **2003**, *125*, 5588-5589.
- (21) Huo, F. W.; Zheng, Z. J.; Zheng, G. F.; Giam, L. R.; Zhang, H.; Mirkin, C. A. *Science* **2008**, *321*, 1658-1660.
- (22) Lim, J. H.; Ginger, D. S.; Lee, K. B.; Heo, J.; Nam, J. M.; Mirkin, C. A. *Angew. Chem. Int. Ed.* **2003**, *42*, 2309-2312.
- (23) Kim, S.; Marelli, B.; Brenckle, M. A.; Mitropoulos, A. N.; Gil, E. S.; Tsioris, K.; Tao, H.; Kaplan, D. L.; Omenetto, F. G. *Nat. Nanotechnol.* **2014**, DOI:10.138/NNANO.2014.47.
- (24) Saaem, I.; Tian, J. *Adv. Mater.* **2007**, *19*, 4268-4271.
- (25) Rundqvist, J.; Mendoza, B.; Werbin, J. L.; Heinz, W. F.; Lemmon, C.; Romer, L. H.; Haviland, D. B.; Hoh, J. H. *J. Am. Chem. Soc.* **2007**, *129*, 59-67.

- (26) Pesen, D.; Heinz, W. F.; Werbin, J. L.; Hoh, J. H.; Haviland, D. B. *Soft Matter* **2007**, *3*, 1280-1284.
- (27) Christman, K. L.; Vazquez-Dorbatt, V.; Schopf, E.; Kolodziej, C. M.; Li, R. C.; Broyer, R. M.; Chen, Y.; Maynard, H. D. *J. Am. Chem. Soc.* **2008**, *130*, 16585-16591.
- (28) Alpert, P. *Integr. Comp. Biol.* **2005**, *45*, 685-695.
- (29) Billi, D.; Potts, M. *Res. Microbiol.* **2002**, *153*, 7-12.
- (30) Benaroudj, N.; Lee, D. H.; Goldberg, A. L. *J. Biol. Chem.* **2001**, *276*, 24261-24267.
- (31) Yoshinaga, K.; Yoshioka, H.; Kurosaki, H.; Hirasawa, M.; Uritani, M.; Hasegawa, K. *Biosci. Biotechnol. Biochem.* **1997**, *61*, 160-161.
- (32) De Carlo, S.; Adrian, H.; Kallin, P.; Mayer, J. M.; Dubochet, J. *J. Microsc.* **1999**, *196*, 40-45.
- (33) Mancini, R. J.; Lee, J.; Maynard, H. D. *J. Am. Chem. Soc.* **2012**, *134*, 8474-8479.
- (34) Lee, J.; Lin, E. W.; Lau, U. Y.; Hedrick, J. L.; Bat, E.; Maynard, H. D. *Biomacromolecules* **2013**, *14*, 2561-2569.
- (35) Dey, R. K.; Cui, B. *Nanotechnology* **2013**, *24*, 5.
- (36) Bovaird, J. H.; Ngo, T. T.; Lenhoff, H. M. *Clin. Chem.* **1982**, *28*, 2423-2426.
- (37) de la Rica, R.; Bat, E.; Herpoldt, K. L.; Xie, H.; S., B.; Maynard, H. D.; Stevens, M. M. *Adv. Funct. Mater.* **2014**, DOI:10.1002/adfm.201304047.
- (38) Chen, B.; Bautista, R.; Yu, K.; Zapata, G. A.; Mulkerrin, M. G.; Chamow, S. M. *Pharm. Res.* **2003**, *20*, 1952-1960.
- (39) Kendrick, B. S.; Chang, B. S.; Arakawa, T.; Peterson, B.; Randolph, T. W.; Manning, M. C.; Carpenter, J. F. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 11917- 11922.
- (40) Keefe, A. J.; Jiang, S. Y. *Nat. Chem.* **2012**, *4*, 60-64.