Regioselective Synthesis of Glycosaminoglycan Analogs

Chengzhe Gao

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Chemistry

Kevin J. Edgar, Chair

Sam R. Turner

Alan R. Esker

Charles E. Frazier

February 18th, 2020

Blacksburg, VA

Keywords: Glycosaminoglycans (GAGs), Polysaccharides, Polysaccharide derivatives, Sulfation, Post-modification, Regioselective

Regioselective Synthesis of Glycosaminoglycan Analogs

Chengzhe Gao

Abstract

Glycosaminoglycans (GAGs), a large family of complex, unbranched polysaccharides, display a variety of essential physiological functions. The structural complexity of GAGs greatly impedes their availability, thus making it difficult to understand the biological roles of GAGs and structure-property relationships. A method that can access GAGs and their analogs with defined structure at relatively large scales will facilitate our understandings of GAG biological roles and biosynthesis modulation.

Cellulose is an abundant and renewable natural polymer. Applications of cellulose and cellulose derivatives have drawn increasing attention in recent decades. Chemical modification is an efficient method to append new functionalities to the cellulose backbones. This dissertation describes chemical modification of cellulose and cellulose derivatives to prepare unsulfated and sulfated GAG analogs. Through these studies, we have also discovered novel chemical reactions to modify cellulose. Systematic study of these novel chemistries is also included in this dissertation.

We first demonstrated our preparation of two unsulfated GAG analogs by chemical modification of a commercially available cellulose ester. Cellulose acetate was first brominated, followed by azide displacement to introduce azides as the GAG amine precursors. The resulting 6-N_3 cellulose acetate was then saponified to liberate 6-OH groups, followed by subsequent (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) oxidation of the liberated primary hydroxyl groups to carboxyl groups. Finally, the azides were reduced to amines using a novel reducing

reagent, dithiothreitol (DTT). Alternatively, another process utilized thioacetic acid to reduce azides to a mixture of amine and acetamido groups.

Through pursuing these GAG analogs, we applied novel azide reductions by DTT and thioacetic acid that are new to polysaccharide chemistry. We systematically investigated the scope of DTT and thioacetic acid azide reduction chemistry under different conditions, substrates, and functional group tolerance. Selective chlorination is another interesting reaction we discovered in functionalization of cellulose esters. We applied this chlorination reaction to hydroxyethyl cellulose (HEC). We then utilized the chlorinated HEC as a substrate for displacement reactions with different types of model nucleophiles to demonstrate the scope of its utility.

Overall, we have designed a novel synthetic route to two unsulfated GAG analogs by chemical modification of cellulose acetate. Through exploration of GAG analogs synthesis, we discovered novel methods to modify polysaccharide and polysaccharide derivatives, including azide reduction chemistry and selective chlorination reactions. Successful synthesis of various types of GAG analogs will have great potential biomedical applications and facilitate structure-activity relationship studies.

Regioselective Synthesis of Glycosaminoglycan Analogs

Chengzhe Gao

General Audience Abstract

Polysaccharides are long chains of natural sugars. Glycosaminoglycans (GAGs) are an important class of polysaccharides which have complicated chemical structures and play critical roles in many biological processes, including regulation of cell growth, promotion of cell adhesion, anticoagulation, and wound repair. Current methods to obtain these GAGs and GAG analogs are expensive, lengthy, and limited in capability. Novel methods to access these GAGs and their analogs would be promising and would facilitate understanding of biological activities of GAGs.

Cellulose is an abundant polymer on earth and provides structural reinforcement in plant cell walls. Cellulose can be further chemically modified to tailor its physiochemical properties. Cellulose and cellulose derivatives have been widely used in many industries for various applications, such as textiles, plastic films, automotive coatings, and drug formulation.

This dissertation focuses on modifying inexpensive, abundant cellulose and its derivatives to GAGs and GAG analogs. We start from the simple plant polysaccharide cellulose and obtain structurally complicated analogs of animal-sourced GAGs and GAG analogs. We reached our goal by designing a carefully crafted synthetic route, finally successfully obtaining two types of novel GAG analogs. During this process, we discovered two useful chemical reactions. We systematically investigated these chemical reactions and demonstrated their utility for polysaccharide chemical modification. These successful chemical syntheses of GAGs and their analogs will accelerate our understanding of their natural functions and have potential biomedical

applications. The novel chemical methods we discovered will be helpful in chemical modification of polysaccharides

Acknowledgements

First and foremost, I would like to thank my PhD advisor, Prof. Kevin J. Edgar, for his continued guidance and support for the past five years. Prof. Edgar is not only a research advisor to me, but also a mentor in life. I could never possibly finish my doctoral degree without his guidance and encouragement in research and life. Through his knowledge and experience in research and his optimistic attitude towards life, I grew into a more confident and mature scientist and more importantly, a better person. The opportunity to work in his group, to work with him, and to learn from him in the past five years will be an unforgettable and precious experience in my life.

I also sincerely appreciate my committee members – Prof. S. Richard Turner, Prof. Alan R. Esker, and Prof. Charles E. Frazier. Thank you all for being on my committee since my master time. Thank you all for your valuable suggestions and discussions that have helped me to gain insights and solve problems in my research.

I am fortunate to work with every member in the Edgar group. They are hard-working and brilliant colleagues and are always willing to offer useful help and discussions in research. I would like to especially thank Dr. Yifan Dong, who shared so much hands-on experience in the lab and provided valuable suggestions to my research projects and career path. Thanks to Dr. Daiqiang Xu, Dr. Xueyan Zheng, Dr. Joyann Marks, Dr. Ruoran Zhang, Dr. Xiangtao Meng, Dr. Hale Cigdem Arca, and Dr. Shu Liu. They offered tremendous help since I joined Dr. Edgar lab. I would also thank my close friend Dr. Junyi Chen. Thanks for the time we spent together in the lab, discussing research projects and sharing ideas of papers. Thanks to my lab members: Brittany Nichols, Diana

Novo, Brady Hall, Yang Zhou, Stella Petrova, Kerani Davidson, and our visiting scholar Dr. Huihui Zhang. We shared so many joyful moments and unforgettable memories.

The past years at Virginia Tech are invaluable assets for me. At Virginia Tech, I met my beloved one of the rest of my life, my girlfriend Dr. Xiuli Li. She is my angel in the morning, afternoon, and night. She shares my happiness and provides tremendous support and accompanies during my dark time. Meanwhile, she is also an honorable scientist and learning example to me. She taught me so much and her hard-working and conscientious spirit always inspires me. At Virginia Tech, I also have so many close friends: Dr. Xiaoyang Liu, Juntao Wang, Dr. Sumin Shen, and Dr. Mingjun Zhou. Many thanks for the joyful moments that we shared in the past years at Virginia Tech. In Blacksburg, I also found my first pet, my dog Toby, who generously gives me endless comfort.

Finally, I sincerely thank my parents, my mother Liping Chen and my father Fenglian Gao. No words can describe my deepest appreciation and love to them. I deeply appreciate their love, their support, and their encouragement through my life. Pursing a PhD degree was my father's dream in his 20s and he will be proud of me to complete it.

Attribution

Prof. Kevin J. Edgar was the principal investigator, advisor, and primary contributor to all research projects, manuscripts, and chapters described in this dissertation.

Stella Petrova, currently a member of the Edgar group, has contributed to the synthetic work and draft edit of Chapter 5. She is also a co-author of this manuscript in preparation.

Zachary B. Fisher, currently an undergraduate student from Department of Biochemistry at Virginia Tech, was an undergraduate researcher in the Edgar lab from August 2018 to May 2019 and has contributed to the synthesis work of Chapters 4. He is also a co-author of this manuscript.

Dr. Mingjun Zhou, currently a postdoctoral researcher at Department of Chemical Engineering at University of Massachusetts Amherst, has performed the Kaiser test in Chapter 3 and helped with lyophilizing through Chapter 3 to Chapter 5.

Dr. Xiangtao Meng, currently a principal chemist at Evonik and formerly an Edgar group member, has provided insightful synthetic discussions in Chapter 3.

Table of Contents

Chapter 1: Dissertation Overview

Glycosaminoglycans (GAGs) are a large family of complex, linear polysaccharides that display many biological functions. Except hyaluronic acid, GAGs are highly sulfated. Sulfate group are revealed to be critical to the biological functions of GAGs. However, the structural complexity of GAGs greatly impedes their availability, thus rendering difficulties to understand structure-property relationship. Current methods, including extraction, total synthesis, and chemoenzymatic synthesis have their own advantages and disadvantages. A new method that can access GAGs and their analogs with defined structure at relatively large scales will facilitate our understandings of GAG biological roles and biosynthesis modulation.

Cellulose is an abundant and renewable natural polymer. Applications of cellulose and cellulose derivatives has drawn increasing attention in recent decades. Chemical modification has been demonstrated to be an efficient method to append new functionalities to cellulose backbones. It would be advantageous to use inexpensive, abundant cellulose or its commercially available derivatives to synthesize structural complicated GAGs and their analogs.

My doctoral research in this dissertation presents our novel strategy to access GAGs and GAG analogs by chemical modification of cellulose and cellulose derivatives, which has never been described before due to the structural complexity of GAGs. We successfully designed a synthetic route to prepare two GAG analogs. Through our course to pursuing these GAG analogs, we discovered novel methods for chlorination and azide reduction, which have been demonstrated to be useful in polysaccharide modification.

Chapter 2 gives a comprehensive literature review on sulfation of polysaccharides. The sulfation method (chemical sulfation and chemoenzymatic sulfation), regioselectivity and chemoselectivity are thoroughly discussed.

Chapter 3 presents our strategy to access our designed unsulfated GAG analogs from a commercial cellulose ester. The designed GAG analogs are polysaccharides that bear carboxyl and amine, or carboxy, amine, and acetamide groups. The synthesis of such designed complicated GAG analogs is accomplished by a sequence of reactions: bromination, azide displacement, saponification, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) oxidation, and azide reduction. In the last azide reduction step, we applied novel reducing reagents, dithiothreitol (DTT) and thioacetic acid, which are new to polysaccharide chemistry.

During our GAG synthesis, we employed two azide reduction reagents, DTT and thioacetic acid, which are new to polysaccharide chemistry. **Chapter 4** describes our systematic study of the DTT and thioacetic acid azide reduction reactions in polysaccharide chemistry. Different azido polysaccharide substrates, reaction conditions, catalyst, chemoselectivity, and reaction mechanisms are systematically investigated. All of the results are satisfying. DTT can effectively reduce azido polysaccharides to the corresponding amino polysaccharides, while thioacetic acid can reduce azide to acetamide in organic solvents.

In the Edgar lab, we have demonstrated that methanesulfonyl chloride can regioselectively chlorinate cellulose esters and this chlorination is applied in our GAG synthesis. **Chapter 5** reports our attempt to apply this chlorination reaction to cellulose ether as a new strategy to functionalize cellulose ethers. Hydroxyethyl cellulose (HEC) can be selectively chlorinated at the terminal primary hydroxyl groups on the side chains. The chlorinated HEC can further undergo displacement reactions with various model nucleophiles, such as azide, amines, and thiols.

Chapter 6 summaries the research results for each chapter in this dissertation and discusses suggestions for future work.

Chapter 2: Review of Literature: Sulfation of Polysaccharides

2.1 Introduction to polysaccharides

Polysaccharides are biopolymers composed of repeating monosaccharides linked by glycosidic bonds, and they have great structural and functional diversity. Their structural diversity comes from differences in monosaccharide structure, linkage, stereochemistry, and branching. Polysaccharides are highly abundant in nature and can be obtained from many renewable sources. For example, cellulose and starch can be found in plants, while chitin and heparin can be extracted from animals. Natural polysaccharides play important biological roles, including structure reinforcement, energy storage, cellular communication, and signal identification. Due to their non-toxicity, renewability and biocompatibility, polysaccharides have great industrial applications including in textiles, food, pharmaceuticals, biofuels, and environmental protection.

2.1.1 Cellulose

First discovered by French chemist Anselme Payen in 1838, cellulose is among the most abundant polymeric raw materials with interesting properties and applications. It acts as the primary structural polymer in trees and other plants and as a storage polymer for photosynthetic products.[1] It can be obtained from wood, cotton fibers, bacteria, and certain animals (e.g. tunicates). Due to its vast abundance, low cost, biocompatibility, and biodegradability, cellulose has been the most commercially important polysaccharide with various applications.

Cellulose is a linear homopolysaccharide consisting of 1→4 linked β-D-glucopyranose monosaccharides (Figure 2.1). Each anhydroglucose unit (AGU) has three hydroxyl groups, including one primary hydroxyl group at the C6 position and two secondary hydroxyl groups at the C2 and C3 positions. These hydroxyl groups result in an extensive hydrogen bonding network,

which leads to the semi-crystalline morphology of cellulose.[2] The degree of polymerization (DP) of cellulose varies with the origin and treatment of the raw material. For instance, wood pulps have DP values in a range of 300 to 1700, while DP of cotton fibers and bacterial cellulose can vary from 800 to 10,000.

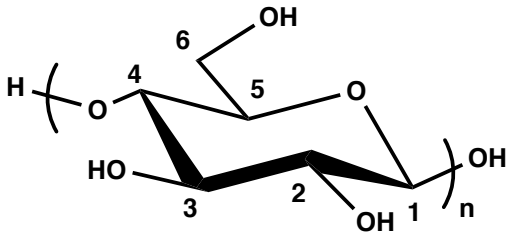


Figure 2.1 Structure of cellulose

Cellulose has desirable properties like low elasticity, biodegradability, thermal stability, and low toxicity. It has been a useful material for paper, cellulose regenerated fibers, films, coating and packaging.[3] However, wider applications are limited by its insolubility in water and common organic solvents. Furthermore, cellulose decomposes before melting and therefore it is not melt-processable. To overcome these processing issues, cellulose is derivatized to create processable derivatives with enhanced properties. Etherification and esterification are the most effective industrial strategies to introduce desired properties to cellulose derivatives.[4] Cellulose ethers and esters have been widely used in many areas including coatings, optical films, drug delivery, membranes, and composites.[5] Besides etherification and esterification, there has been a growing number of modern chemistry methods to prepare novel cellulose derivatives in the past 20 years, such as bromination/tosylation/chlorination followed by nucleophilic substitution reactions[6,7] and olefin cross metathesis.[8,9]

2.1.2 Curdlan

Curdlan is a bacterial polysaccharide that has aroused great interest in the past few decades due to its unique rheological properties and promising biological activities. It was first discovered in 1966 by Harada and coworkers[10] and the structure of curdlan was elucidated as early as 1968.[11] Curdlan consists of D-glucopyranose monosaccharides linked by β-1→3 linkages, without any branching (Figure 2.2). It is an extracellular capsular polysaccharide with DP up to 12,000, and can be produced by non-pathogenic bacteria such as *Agrobacterium*.[12]

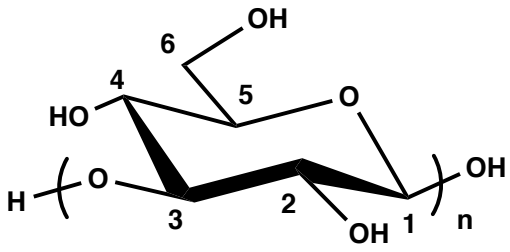


Figure 2.2 Structure of curdlan

Curdlan shows enhanced solubility versus other linear polysaccharides, such as cellulose. Curdlan is soluble in dilute alkali (0.25 M NaOH), formic acid, and some polar aprotic solvents including dimethyl sulfoxide (DMSO), *N*-methylmorpholine *N*-oxide (NMMO), and *N,N*-dimethylacetamide (DMAc) containing lithium chloride (LiCl), while like many common polysaccharides, curdlan is insoluble in water and most other organic solvents. Curdlan exhibits unique gelation properties. Heating curdlan aqueous suspension will generate two types of gels, depending on the maximum temperature used. A high-set, thermally irreversible gel can be formed when a curdlan aqueous suspension is heated to 80 °C, and a low-set, thermally reversible gel can be obtained by heating the suspension to 55 °C.[13] Gelation has been attributed to curdlan's helical structure change at the elevated temperatures. At room temperature, curdlan has a mixture of single

helix and loose triple helix structure, while at higher temperatures curdlan has a more condensed triple helix structure.

Curdlan has been used widely in the food industry and in pharmaceutics due to its gelation property, low toxicity, and biocompatibility. It was approved as a food additive in the United States in 1996 by the Food and Drug Administration (FDA). It is frequently used as a thickening agent or fat-mimic substitute in the food industry. Curdlan gel has been demonstrated to be effective as a sustained release drug delivery system. Curdlan and its derivatives display a variety of biological activities themselves, including antitumor, anti-infective, anti-inflammatory, and anticoagulant activities.[14]

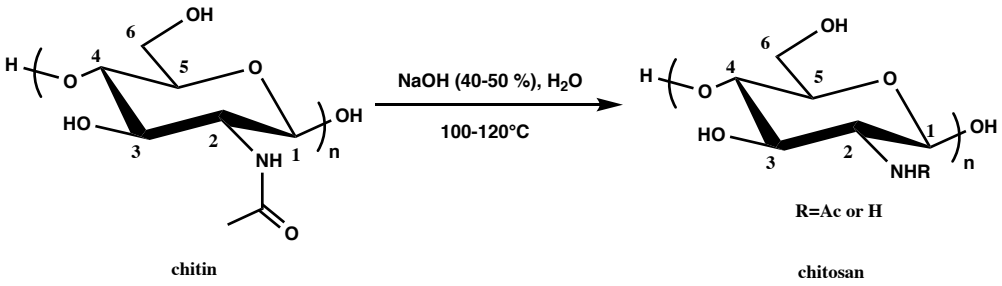
2.1.3 Chitosan and chitin

Chitin, a highly abundant amino polysaccharide, is composed of 2-acetamido-2-deoxy-β-D-glucose linked by β (1→4) glycosidic bonds. Chitin is similar to cellulose in many aspects including chemical structure, biological functions, and physicochemical properties. It serves as a structural component in many organisms, reinforcing natural matrix composites. Chitin can be extracted from many sources, including mollusks, crustaceans, insects, fungi, and algae. Chitin, as a sustainable, biocompatible, and biodegradable material, shows great potential including as a chelating agent,[15] drug carrier,[16] and in membranes.[17] However, its vast potential for useful applications is impeded by poor solubility and lack of thermal processability. Chitin is insoluble in common organic solvents and water, due to its extensive semicrystalline structure caused by hydrogen bonding, and it decomposes before melting.[18]

Chitosan, prepared by deacetylation of chitin, is a random copolymer of 2-amino-2-deoxy-β-D-glucose and 2-acetamido-2-deoxy-β-D-glucose, linked by β (1→4) linkages (Scheme 2.1). The degree of deacylation in chitosan varies from 40 % to 98 %, depending on starting material

chitin and the deacylation methods. Chitosan is soluble in dilute acidic solutions below pH 6.0, as a result of the strong basicity of the free amine groups (pK_a 6.3).[18] At low pH, the amine groups are protonated and become positively charged, making chitosan a water-soluble cationic polyelectrolyte. The most commonly used solvent system for dissolving chitosan is 1% acetic acid at pH around 4.0.[19] The significant improvement in water solubility versus chitin enhances the processability of chitosan, making film formation, coating, precipitation, and freeze-drying feasible.

Chitin and chitosan, owing to their sustainability, biocompatibility, non-toxicity, and biodegradability (even *in vivo*), have many applications in water purification, wound healing, production of paper, packing, and cosmetics.[20] Furthermore, chitin and chitosan can be readily processed into gels,[21] membranes,[22] fibers,[23] nanoparticles,[24] and sponges,[25] enhancing their biomedical applications in antibacterial materials, tissue engineering, drug delivery, and gene delivery.



Scheme 2.1 Synthesis of chitosan by alkaline hydrolysis of chitin

2.1.4 Glycosaminoglycans (GAGs)

GAGs, one of the most important macromolecule families that affect cellular functions, are a large family of long-chain, unbranched, biologically active polysaccharides, based on disaccharide repeat units. The disaccharide repeat unit usually contains a uronic acid (glucuronic

acid or iduronic acid) or galactose (in the case of keratan sulfate), along with an amino sugar (glucosamine, *N*-acetylglucosamine or *N*-acetylgalactosamine).[26] GAGs include heparin (HP), heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronic acid (HA) (Figure 2.3). Except for hyaluronic acid, GAGs are usually sulfated to various degrees and in different patterns.[27] Sulfate groups are crucial to GAG natural function because recognition and modulation of most proteins are commonly induced by the *O*-sulfate or *N*-sulfate groups on the GAG chains. GAGs exist covalently linked to proteins as part of proteoglycans, except for HA, which has high non-covalent affinity for certain proteins. All natural GAGs are negatively charged, due to carboxyl and sulfate groups on the polymer. GAGs play important roles in many essential biological processes, including blood coagulation, growth, cancer, inflammatory response, immune response, and pathogen infection.[28–30]

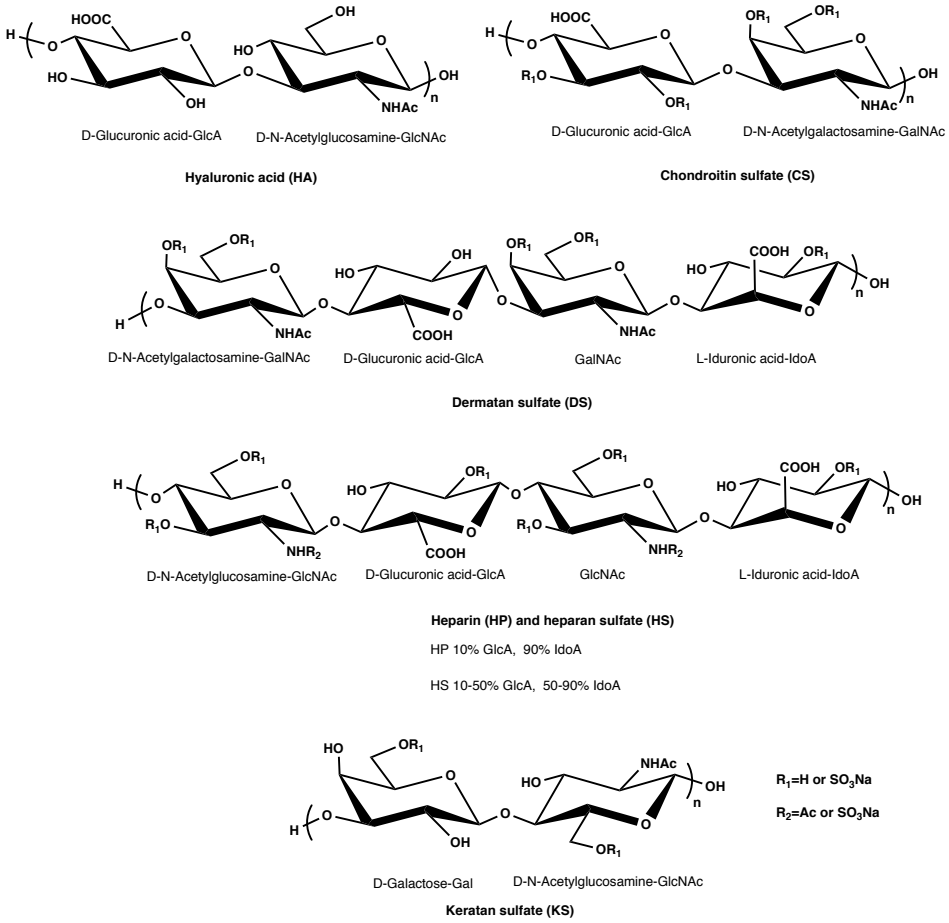


Figure 2.3 Structures of HA, CS, DS, KS, HP, and HS

2.2 Chemical sulfation of polysaccharides

Polysaccharide sulfates are polysaccharides bearing sulfuric acid half ester moieties. They are widely distributed in nature with a variety of important biological activities and biomedical applications. The most important natural polysaccharide sulfates are GAGs extracted from animals, which play key roles in physiological and pathological processes. Inspired by the biological importance of natural polysaccharide sulfates, researchers have employed chemical sulfation of natural unsulfated polysaccharides, such as cellulose, curdlan, and chitosan, to prepare novel polysaccharide sulfates. Because the half ester moiety in its protonated form ($-OSO_3H$) is so acidic

that it can lead to chain degradation, semi-synthetic polysaccharide sulfates often are isolated in salt form. Similar to natural polysaccharide sulfates, semi-synthetic polysaccharide sulfates also exhibit a large number of biological activities.[31] Furthermore, polysaccharides can be sulfated chemically at large scale, affording products with defined structural features and purity, and at low cost.

Traditional sulfation reagents like sulfuric acid, sulfuryl chloride, and other reagents including 1-piperidinesulfonic acid, chlorosulfonic acid, and sulfamic acid were first employed for sulfating polysaccharides. However, significant depolymerization was always observed, due to the breakage of glycosidic bonds under strongly acidic conditions.[32] Moreover, these sulfation reagents are hazardous and should be handled carefully. Sulfur trioxide complexes with amides (DMF) or amines (including pyridine and trimethylamine) in polar aprotic solvents provide an alternative method to sulfate polysaccharides, because of the mild reaction conditions, stability and mild acidity of the sulfation reagents, and ease of handling of those reagents. In several cases, depolymerization and cleavage of acid labile functional groups was still observed. It has been reported that adding an acid scavenger, e.g. 2-methyl-2-butene, can minimize side reactions caused by acidic conditions to the greatest extent.[33] All these sulfation reagents show preference for reaction with primary hydroxyl groups, but none of these sulfation reagents are entirely regioselective. With increased understanding of the importance of sulfate distribution patterns in the biological activities of polysaccharide sulfates, regioselective sulfation of polysaccharides is becoming a more important goal. Protecting group strategies have proved useful for regioselective synthesis of polysaccharide derivatives. Combined with sulfation reactions, they allow more regioselective sulfation of polysaccharides. A detailed discussion of sulfation of cellulose, curdlan, chitin, and chitosan is presented here.

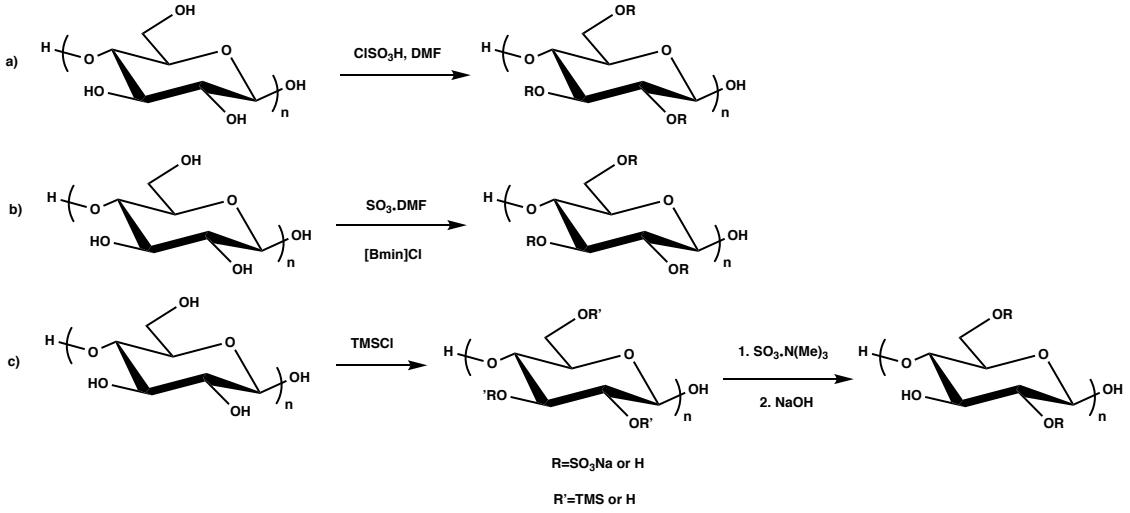
2.2.1 Sulfation of cellulose

Cellulose sulfates have been of growing interest in the past few decades, due to their potential applications in films,[34] anionic polyelectrolytes,[35,36] and their biological activities, such as anticoagulant,[37] antiviral,[38] and anti-human immunodeficiency virus (HIV) activities.[39] Owing to the abundance of cellulose, its sulfation has been extensively studied. Cellulose sulfates can be obtained under both heterogeneous and homogeneous conditions. Due to the limited solubility of cellulose, heterogeneous sulfation is a straightforward way to synthesize cellulose sulfates. For example, cellulose can be sulfated heterogeneously with H_2SO_4 in ethanol or *n*-propanol.[40,41] This reaction is usually performed below 0 °C to minimize acid-catalyzed chain cleavage. To minimize chain degradation, an alternative method utilizes SO_3•pyridine or SO_3•DMF as the sulfation reagent, H_2SO_4 as the catalyst, and dimethylformamide (DMF) as the solvent.[42] Like many other heterogeneous reactions on cellulose, the problem for heterogeneous sulfation is non-uniform sulfate group distribution. The amorphous part of the cellulose can be sulfated while the crystalline part remains unreacted. Cellulose can also be homogeneously sulfated in a variety of solvent systems that can be used to dissolve cellulose, such as N_2O_4/DMF,[43] DMAc/LiCl[44], and ionic liquids.[45–47] Compared to heterogeneous sulfation, homogeneous sulfation usually can be carried out under milder reaction conditions. As a result, usually less chain degradation is observed. Furthermore, under homogeneous conditions, the cellulose sulfates obtained are presumed to have even sulfate distributions along and between chains, and exhibit good solubility. Both heterogeneous and homogeneous sulfation show similar regioselectivity: C6 > C2 > C3.[4] Sulfation at C6 is always preferred, as a result of the wider approach angles of the primary hydroxyl groups. Hydroxyl groups at C2 are found to be more reactive than at C3 positions for sulfation. In some cases, cellulose-6-sulfate ($DS_{sulfate}$ = 0.55)[48] and cellulose-2, 6-disulfate ($DS_{sulfate}$ = 2.0)[49] can be

synthesized with almost complete regioselectivity by careful choice of sulfation reagents, solvents, and reaction conditions.

Cellulose derivatives can also be used as substrates for sulfation reactions, benefitting from their improved organic solubility versus cellulose. Such strategies are known as quasi-homogeneous sulfation. Cellulose acetate, cellulose nitrate, and trimethylsilyl cellulose are frequently used in cellulose sulfate synthesis. Cellulose acetate or cellulose acetate generated *in situ* have been studied extensively in the synthesis of cellulose sulfates. This process usually involves two steps: acetosulfation and subsequent deacylation at room temperature to avoid possible desulfation reactions.[32] In 1995, Philipp et al. reported synthesis of cellulose sulfates using $ClSO_3H/Ac_2O$/DMF at 50° C, followed by hydrolysis of ester groups under alkaline conditions.[48] In 2011, Zhang et al. employed this method to synthesize cellulose sulfates with degree of substitution of sulfate groups ($DS_{sulfate}$) ranging from 0.21 to 1.71, varying the reaction temperature, sulfation reagents, acylation reagent, and solvents.[50] In many cases, this method shows similar regioselectivity trends as described above (C6 > C2 >> C3). Cellulose silyl ethers are also useful intermediates for sulfating cellulose. For example, it was reported that trimethylsilyl cellulose (DS_{TMS} = 1.55) can be sulfated with $ClSO_3H$ in DMF, followed by treatment with NaOH in ethanol to cleave the residual trimethylsilyl groups (residual trimethylsilyl groups come from TMS groups that are not hydrolyzed under acidic conditions or substituted by sulfation reagents).[51] It is worth noting that an almost completely regioselectively substituted cellulose 6-*O*-sulfate was obtained, with $DS_{sulfate}$ at the C6 0.95 (determined by ^{13}C NMR). In 2003, Richter and Klemm reported sulfation of nearly fully substituted trimethylsilyl cellulose (DS_{TMS} = 2.9) with different SO_3 complexes.[52] When using SO_3•DMF, sulfation occurred preferentially at C6. The $DS_{sulfate}$ at the C6 position can be as high as 0.95 while $DS_{sulfate}$ at C2 is about 0.51. When SO_3•triethylamine was

employed as the sulfation reagent, a preference for sulfation at the C2 position was observed. ^{13}C NMR analysis revealed that the $DS_{sulfate}$ at C2 was 0.42 and $DS_{sulfate}$ at C6 position was 0.21. The result revealed that both the total degree of sulfation and regioselectivity depend on the sulfation reagents used. Sulfation of cellulose silyl ethers occurs at C2 and C6 and sulfation at C3 is not detected by ^{13}C NMR. Thus, this approach could be employed to synthesize cellulose-2,6-*O*-disulfate derivatives. Cellulose nitrate, typically generated *in situ* by treating cellulose with N_2O_4/DMF, can be sulfated by various sulfation reagents.[43,48] The regioselectivity of sulfation of cellulose nitrate is similar to sulfation of cellulose silyl ether derivatives. Only sulfation at C6 and C2 is observed, and the preference of C6 and C2 is dependent on sulfation reagents and reaction temperature. Investigation has shown that when using SO_3 as the sulfation reagent, sulfation at C2 dominates at low temperature (-20° C), while sulfation at C6 is preferred at room temperature (20° C).[43]

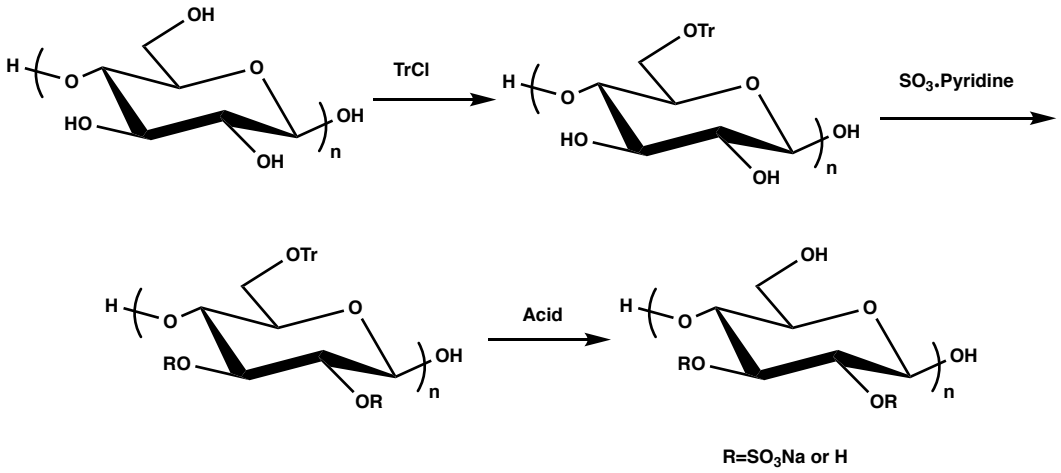


Scheme 2.2 Examples of sulfating cellulose: a) heterogeneous sulfation, b) homogeneous sulfation, c) quasi-homogeneous sulfation

Both cellulose sulfates and many other polysaccharide sulfates synthesized are typically analyzed by FT-IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis. To accurately determine the position of substitution is very challenging. Most of the publications on sulfation of polysaccharides use quantitative ^{13}C NMR spectra as the only evidence to determine $DS_{sulfate}$ at every position, despite the inaccuracy and imprecise nature of quantitative ^{13}C NMR spectra and possible errors generated in peak integration due to peak overlaps. The Mischnick group has developed a method to determine the substitution pattern in cellulose sulfates quantitatively.[53,54] Cellulose sulfates are initially permethylated by methyl iodide (MeI) in DMSO, followed by replacement of sulfate groups by acetate groups (Ac_2O) at high reaction temperature for a long time (14 h, in order to complete desulfation and acetylation), and replacement of acetate groups by deuteromethyl ethers ($NaOH/CD_3I$). Finally, the permethylated cellulose samples are hydrolyzed to monosaccharides (enzymatic or chemical approach). In the monosaccharides, the *O*-methyl-d_3 groups represent the positions of the original sulfate groups, while the *O*-methyl groups represent the positions of the unreacted hydroxyl groups. The monomer compositions were quantitatively determined by GC-MS. This method is extremely useful in assigning the substitution position. However, it is surprising that this method has only been applied in a few cases. A simple and accurate method to measure the sulfate distribution would be promising and facilitate the synthesis of both cellulose sulfates and many other polysaccharide sulfates with complete regioselectivity.

Regioselective synthesis of cellulose sulfates is challenging, due to the low reactivity and the small reactivity differences between cellulosic hydroxyl groups. Until now, the most effective strategy for regioselective synthesis of cellulose sulfates has been employing protecting groups. Triphenylmethyl group (trityl group), 4-methyloxy-triphenylmethyl group (MMTr), and 4,4'-

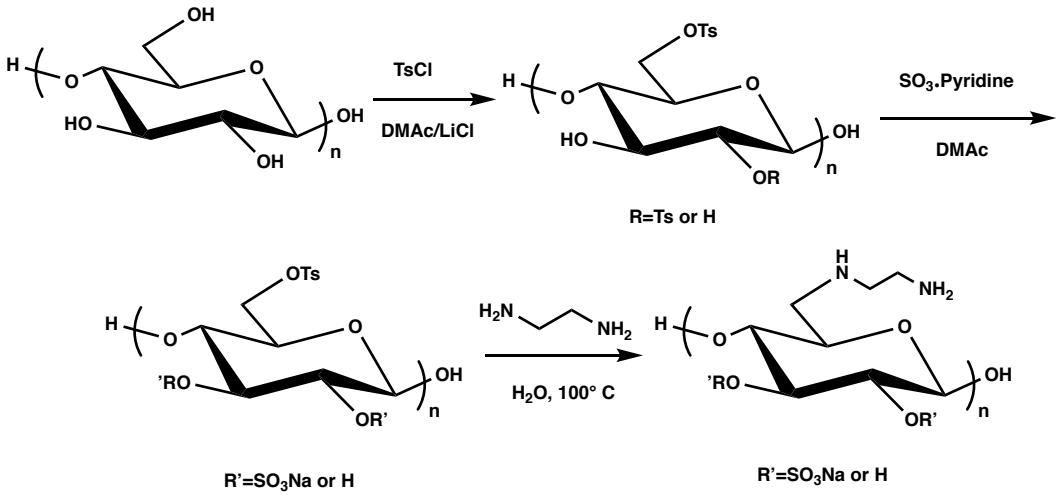
dimethoxy-triphenylmethyl group (DMTr) have been used widely to protect primary hydroxyl groups of polysaccharides. Owing to its steric demands, triphenylchloromethane will react preferentially with primary hydroxyl groups and the secondary hydroxyl groups remain largely unreacted. Moreover, the trityl or methoxytrityl groups can be easily removed under acidic conditions at room temperature. In 2000, Heinze and co-workers reported regioselective synthesis of cellulose 2,3-disulfate through three step reactions: first, introduction of trityl groups to cellulose at C6, then sulfation of secondary hydroxyl groups at C2 and C3 using SO_3• pyridine, and finally removal of trityl groups in aqueous acid (Scheme 2.3).[44] Similar strategies have also been applied to regioselective synthesis of many other polysaccharide sulfates. By similar methods, a glucogalactomannan from *Artemisia sphaerocephala*,[55] guar gum,[56] and unsulfated porphyran[57] have been regioselectively sulfated to yield the corresponding polysaccharide sulfates with sulfate groups distributed only at former secondary hydroxyl positions.



Scheme 2.3 Regioselective synthesis of cellulose-2,3-disulfate

Synthesis of cellulose sulfates bearing other substituents, such as methylated cellulose sulfate,[53] carboxyl cellulose sulfate,[58] oxyethylated cellulose sulfate,[59] and amino cellulose

sulfate,[60] has been further explored. For instance, in 2012 Heinze and co-workers reported regioselective synthesis of amino cellulose sulfates as polyzwitterions (Scheme 2.4).[60] Cellulose in DMAc/LiCl was reacted with *p*-toluenesulfonyl chloride (tosyl chloride) in the presence of a base. The tosyl group was mainly introduced to the C6 position due to lower steric hindrance there, despite some tosylation at C2 and C3. Then tosyl cellulose was sulfated with SO_3•pyridine at the C2 and C3 position in DMAc, affording a water-soluble tosyl cellulose sulfate. Subsequent treatment with NaOH aqueous solution transformed sulfonic acid half esters into sodium salts. In the final S_N2 reaction, tosyl groups were replaced by the nucleophile, 1,2-diaminoethane, to yield amino cellulose sulfates. The water solubility of the amino cellulose sulfates depended on the pH value due to the existence of both sulfate and amine groups. Amino cellulose sulfate (DS_{amine} = 0.32, $DS_{sulfate}$ = 1.21) is completely water-soluble at pH 11.5. At pH around 9, colloids were observed and a precipitate formed at pH < 9. It was proposed that when pH > 11.5 all amine groups are deprotonated and the polymer is soluble because of the sulfate groups. The precipitation formed at pH < 9 was proposed to be caused by protonation of amine groups and formation of an insoluble zwitterionic polymer, with the decrease of pH.



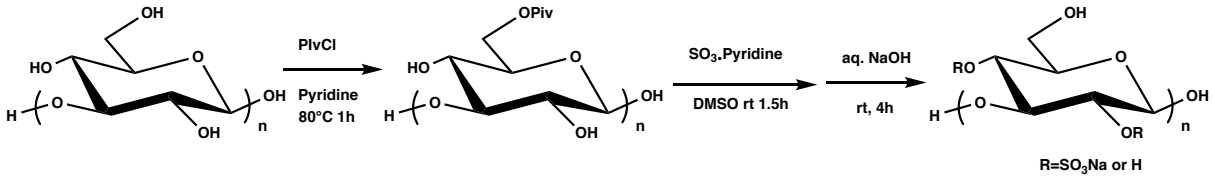
Scheme 2.4 Synthesis of amino cellulose sulfate

2.2.2 Sulfation of curdlan

Curdlan sulfates have been of great interest, owing to their high anti-HIV activity. In 1990, Yoshida and coworkers first reported homogeneous sulfation of curdlan by piperidine-*N*-sulfonic acid (PSA) in DMSO.[61] Curdlan sulfates with $DS_{sulfate}$ from 0.35 to 1.6 were synthesized by varying the reaction time, temperature, and molar ratios of sulfation reagents. Regioselectivity studies revealed that the relative hydroxyl group reactivity was O-6 > O-2 >> O-4, as revealed by ^{1}H, ^{13}C, and relayed-COSY NMR spectra. Sulfation occurred mostly at C6 at lower DS, and then at C2 as well with increasing DS. Even at $DS_{sulfate}$ as high as 1.6, very few sulfate groups at C4 were observed. In 1993, Osawa et al. studied the impact of sulfation conditions on anti-HIV activity by treating curdlan with SO_3• pyridine complex in DMF under homogeneous or heterogeneous conditions.[62] Analysis of the curdlan sulfates synthesized by homogeneous sulfation indicated that the regioselectivity was C6 > C2 > C4. However, under heterogeneous conditions, there was little difference between the reactivity of the two secondary hydroxyl groups O-2 and O-4, while sulfation of the primary hydroxyl groups O-6 was preferred. Curdlan sulfates obtained by heterogeneous sulfation exhibited unwanted cytotoxicity, while curdlan sulfates prepared by homogeneous sulfation displayed low cytotoxicity, illustrating the importance of sulfation reagents and sulfation conditions used.

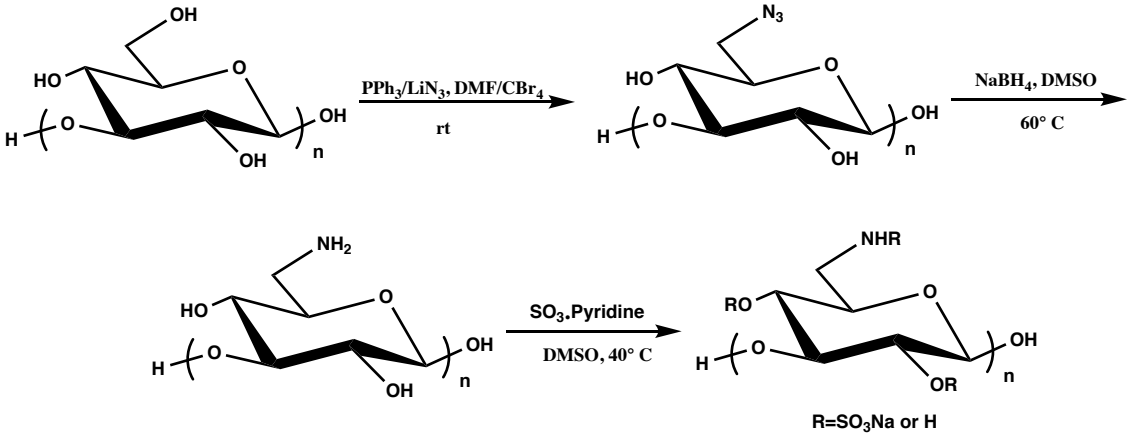
Regioselective sulfation of curdlan has also been achieved by protecting group strategies. In 1997, Gao et al. reported regioselective synthesis of curdlan-2,4-disulfate using pivaloyl (trimethylacetyl) groups to selectively protect the primary hydroxyl groups (O-6) (Scheme 2.5).[63] Pivaloyl groups are extremely sterically demanding, and thus introduction to the less hindered primary hydroxyl groups at C6 is favored. Curdlan was first treated with pivaloyl chloride in pyridine at 80° C to protect the primary hydroxyl groups. Then the protected curdlan was sulfated

by SO_3• pyridine in DMSO, followed by alkaline hydrolysis of the pivaloyl groups. The curdlan sulfate obtained had $DS_{sulfate}$ = 0.39 at C2 and $DS_{sulfate}$ = 1.00 at C4, while no sulfation at C6 was detected, as measured by ^{13}C NMR spectra. The regioselectively synthesized curdlan sulfates exhibited similar anti-HIV activity and the authors concluded that the anti-HIV activities of curdlan sulfates depended upon DS rather than upon sulfation positions.



Scheme 2.5 Synthesis of curdlan 2,4-disulfate

In order to further explore the relationship between structure and anti-HIV activity, sulfation of curdlan derivatives has also been reported. For example, Borjihan et al. reported the sulfation of aminated curdlan in 2003 (Scheme 2.6).[64] 6-Amino-6-deoxy-curdlan was prepared by one-pot bromination and azide displacement, followed by azide reduction. Subsequent sulfation of the amino and hydroxyl groups was carried out by SO_3• pyridine in DMSO at 40° C. Sulfated amino curdlan showed high anti-HIV activity and low cytotoxicity.

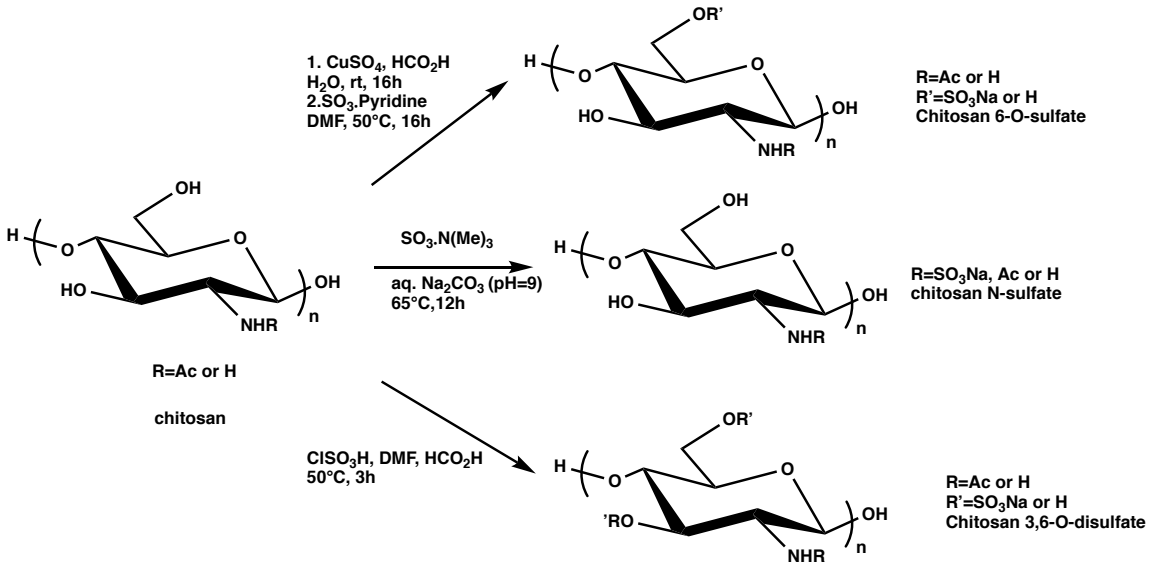


Scheme 2.6 Sulfation of 6-amino-6-deoxycurdlan

2.2.3 Sulfation of chitin and chitosan

Chitin and chitosan sulfates are important derivatives because of their structural similarity to GAGs, leading to potential applications including as blood anticoagulants, antimicrobial agents, metal ion absorbents, and anti-HIV agents. Sulfation of chitin only occurs at the hydroxyl groups (O-3 and O-6) since the acetamide groups are unreactive towards sulfation conditions. Sulfation of chitin has been reported using various sulfating reagents under homogeneous[65] or heterogeneous conditions.[66,67] The regioselectivity studies showed trends similar to those observed with other polysaccharides: sulfation at primary hydroxyl groups (O-6) is highly favored rather than at secondary hydroxyl groups at O-3. For example, selective sulfation at C6 can be achieved by homogeneous sulfation of chitin in DMAc/LiCl at room temperature by using a carefully controlled number of equivalents of SO_3• pyridine.[68] With increased temperature (45 - 75° C) and equivalents of SO_3• pyridine employed, sulfation at C3 is observed as well, with $DS_{sulfate}$ at C3 ranging from 0.48 to 0.91. Sulfation of chitosan occurs not only at the hydroxyl groups (O-3 and O-6) but also at the amine groups because of the high nucleophilicity of the amine nitrogen. Chemo- and regioselective sulfation at hydroxyl and amine groups are accessible by using different solvent systems (Scheme 2.7). Chelation of the amine groups at C2 and the hydroxyl groups at C3 with Cu(II) ions enables regioselective synthesis of chitin 6-*O*-sulfate by subsequent sulfation using SO_3• pyridine in DMF.[69] Sulfation at both O-6 and O-3 is feasible when chitosan is dissolved in dilute acid, since the nucleophilic amine groups get protonated and become no longer nucleophilic. For instance, in the chitosan formic acid-DMF mixture solution, free hydroxyl groups of chitosan react with formic acid to generate formate groups *in situ*, which subsequently react with chlorosulfonic acid to yield chitosan 3,6-*O*-disulfate.[70] The chitosan sulfates obtained showed $DS_{sulfate}$ in the range of 0.65 to 1.0 at O-6 and $DS_{sulfate}$ from 0.29 to 0.61 at O-3, as measured by

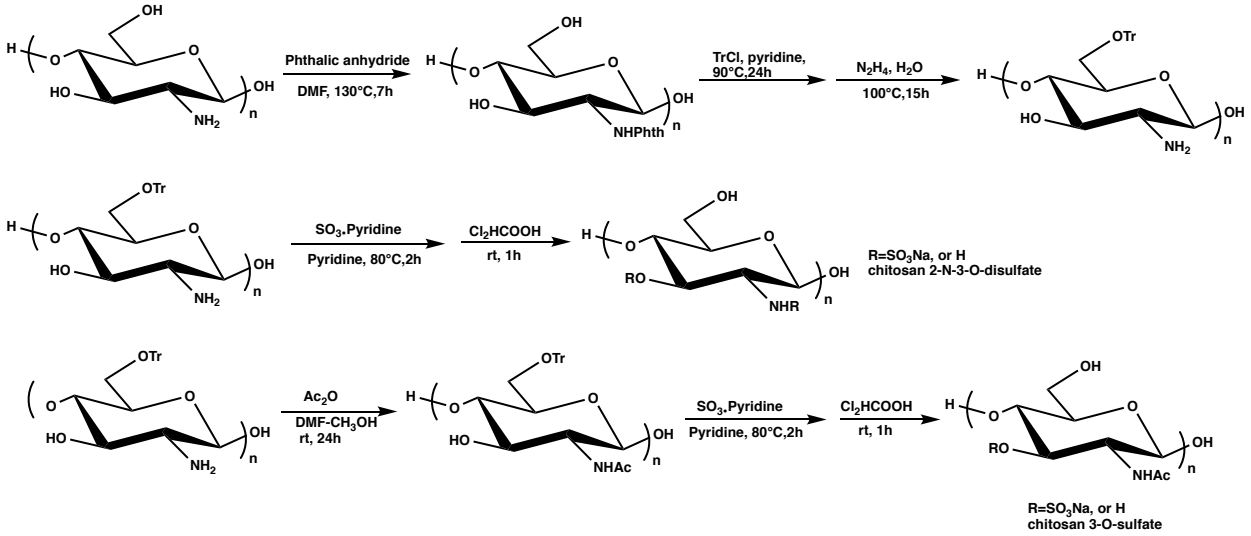
^{13}C NMR and elemental analysis. Only the free amine signal was detected in the ^{15}N NMR spectrum, indicating that there was no *N*-sulfation. In 2001, Baumann and Faust reported selective sulfation of amine groups by treatment of chitosan with a rather unreactive sulfating reagent SO_3• trimethylamine complex (its low reactivity is because trimethylamine is a stronger base than pyridine or DMF, thus trimethylamine complex with SO_3 is more stable and less reactive) in alkaline aqueous solution at 65° C.[69] This selective sulfation of amines could be attributed to the higher nucleophilicity of free amine groups than hydroxyl groups in water.



Scheme 2.7 Selective sulfation of chitosan

Protecting group strategies are extremely powerful for regioselective sulfation of chitosan. The free amine groups can be selectively protected by phthalic anhydride to afford unreactive imides, which can be deprotected by hydrazinolysis. Combined with protection of primary hydroxyls as trityl ethers, chitosan sulfates with various sulfate distributions and complete regioselectivity can be prepared. For example, Nishimura et al. reported synthesis of 3-*O*-sulfated chitosan and 2-*N*-3-*O* sulfated chitosan (Scheme 2.8).[71] First, amine groups were protected by

reacting chitosan with phthalic anhydride in DMF, followed by reaction with trityl chloride to protect O-6 and deprotection of the imides to liberate amines, affording 6-*O*-trityl chitosan as the key intermediate. The amine groups at C2 and the hydroxyl group at C3 of 6-*O*-trityl chitosan can be sulfated, with subsequent removal of trityl groups in aqueous acid, yielding 2-*N*-3-*O* sulfated chitosan. ^{13}C NMR indicated that sulfation of amines at C2 is nearly quantitative and $DS_{sulfate}$ at O-3 was measured to be 0.69, while no sulfation at O-6 was detected. 3-*O*-Sulfated chitin was synthesized by a sequence of reactions from 6-*O*-trityl chitosan, including chemoselective acylation of the amine groups, sulfation at O-3, and then removal of trityl groups. ^{13}C NMR confirmed that sulfation occurred exclusively at O-3 with $DS_{sulfate}$ 0.44. A similar strategy (phthaloylation of amines, sulfation of hydroxyl groups and deprotection of phthalimide by hydrazinolysis) has also been reported to prepare chitosan 3,6-*O*-disulfate with $DS_{sulfate}$ = 0.97 and 0.79 at C3 and C6 positions, respectively.[69]

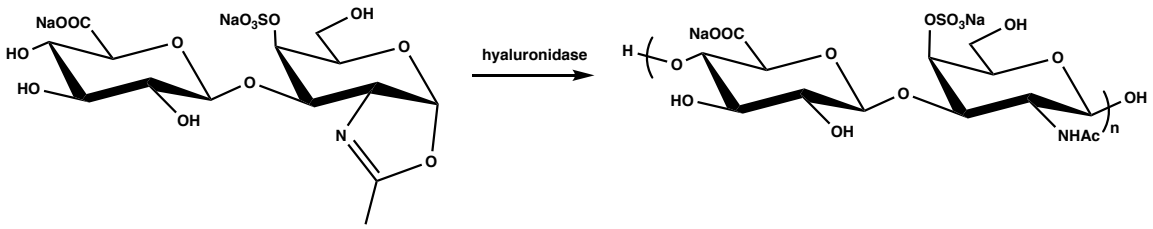


Scheme 2.8 Regioselective synthesis of chitosan 3-*O*-sulfate and chitosan 2-*N*-3-*O*-disulfate

2.3 Chemoenzymatic sulfation of polysaccharides

Many sulfated GAGs are extracted from animal tissues and purified. However, commercial preparation methods usually result in sulfated GAGs with variable molecular weight and sulfation distribution patterns, due to their polydisperse, polycomponent nature. Furthermore, as a result of the structural similarity between GAGs and their affinity for proteins, contamination also limits the biomedical applications of GAGs. In 2008, oversulfated chondroitin sulfate contaminated heparin and resulted in the death of nearly 100 patients in the US.[72–74] Thus there is a need to produce sulfated GAGs synthetically. Total synthesis has been proved useful in the synthesis of Arixtra, a pentasaccharide mimic for heparin. However, the synthesis of this pentasaccharide requires 50 steps with only 0.1% yield. Chemoenzymatic synthesis of polysaccharide sulfates has merged as a promising alternative to purely chemical synthesis.[75] Furthermore, chemoenzymatic synthesis enables production of GAG libraries which are promising tools for exploring GAG structure activity relationships, and polysaccharide-protein interactions. There are two main synthetic strategies for chemoenzymatic synthesis of polysaccharide sulfates: 1) building polysaccharide chains from disaccharides, and 2) semi-synthesis of polysaccharide sulfates from polysaccharides extracted from organisms.[76]

Employing degradation enzymes and transition state intermediates has been proved to be an effective way for building polysaccharide chains. In 2003, a disaccharide was polymerized with hyaluronidase to prepare a well-defined chondroitin-4-sulfate (Scheme 2.9).[77] The synthesized chondroitin sulfate is perfectly sulfated at C4 position in all GalNAc units, a structure which is not found in nature. The yield of the enzymatic synthesis is 79% with M_n = 12,700. Interestingly, 6-*O*-sulfate GalNAc oxazoline cannot be polymerized to give the corresponding chondroitin sulfate, which is attributed to the low reactivity of the substrate.



Scheme 2.9 Enzymatic synthesis of chondroitin-4-sulfate

Heparan sulfates with different sulfation patterns can be obtained through chemoenzymatic synthesis.[78–82] Bioengineered synthesis of heparan sulfate usually starts with heparosan, with subsequent enzymatic modifications. Heparosan, which is similar to an unsulfated and unepimerized heparin in structure, comprises a [(→4) β-D-glucuronic acid (GlcA) (1→4) *N*-acetyl-α-D-glucosamine (GlcNAc) (1→)] disaccharide repeating unit.[83,84] Chemoenzymatic synthesis of heparin and its analogues has been reported for several examples. Heparin can be synthesized by chemoenzymatic synthesis starting from *N*-acetylheparosan.[85] *N*-Sulfated heparosan can be prepared by deacylation of *N*-acetylheparosan with alkali, and then *N*-sulfation by SO_3•triethylamine or *N*-sulfotransferase. C5-epimerase coverts GlcA residues to IdoA residues. Subsequent treatment with 2-*O*-sulfotransferase sulfates the O-2 of IdoA. 6-*O*-Sulfotransferase and 3-*O*-sulfotransferase can then introduce sulfate groups to the C6 and C3 position of the GlcNAc or GlcNS units. The sulfate group distribution is carefully controlled by the ratio of substrates to enzymes. By using different combinations of synthetic enzymes, a library of heparan sulfate derivatives can be synthesized for discovering new heparan sulfates with unique biological functions.[81] For example, heparan sulfates obtained without iduronic acid residues showed the same promising anticoagulation activity as heparin.[86]

2.4. Conclusion

In summary, polysaccharide sulfate derivatives have shown great potential in different applications. Chemical sulfation of polysaccharides is a powerful method to obtain polysaccharide sulfates. Regioselective synthesis can be also accomplished by employing carefully chosen solvent systems and sulfation reagents, or by protecting group strategies. Chemoenzymatic synthesis provides an alternative method to obtain polysaccharide sulfates with very well-defined sulfation distribution, beyond what can be achieved by chemical sulfation of polysaccharides. Despite significant success in sulfation of polysaccharides, convincing analytical methods such as degradation to monomers, and analysis of the monomers have not been applied widely to the sulfated products. Moreover, regioselective synthesis of glycosaminoglycans and their analogues still remains challenging due to their structural complexity. In the future, it will be promising to completely regioselectively synthesize polysaccharide sulfates and develop a comprehensive characterization strategy. Synthesis of GAGs and their analogues with defined sulfate group distributions will also have great potential for biomedical applications.

Reference

(1) Klemm, D.; Heublein, B.; Fink, H.-P. H.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angew. Chemie Int. Ed.* **2005**, *44* (22), 3358–3393.

(2) Lavoine, N.; Desloges, I.; Dufresne, A.; Bras, J. Microfibrillated Cellulose – Its Barrier Properties and Applications in Cellulosic Materials: A Review. *Carbohydr. Polym.* **2012**, *90* (2), 735–764.

(3) Siró, I.; Plackett, D. Microfibrillated Cellulose and New Nanocomposite Materials: A Review. *Cellulose* **2010**, *17* (3), 459–494.

(4) Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J. Regioselective Esterification and Etherification of Cellulose: A Review. *Biomacromolecules* **2011**, *12* (6), 1956–1972.

(5) Edgar, K. J.; Buchanan, C. M.; Debenham, J. S.; Rundquist, P. A.; Seiler, B. D.; Shelton, M. C.; Tindall, D. Advances in Cellulose Ester Performance and Application. *Prog. Polym. Sci.* **2001**, *26* (9), 1605–1688.

(6) Petzold-Welcke, K.; Michaelis, N.; Heinze, T. Unconventional Cellulose Products Through Nucleophilic Displacement Reactions. *Macromol. Symp.* **2009**, *280* (1), 72–85.

(7) Heinze, T.; Liebert, T. Unconventional Methods in Cellulose Functionalization. *Prog. Polym. Sci.* **2001**, *26* (9), 1689–1762.

(8) Meng, X.; Matson, J. B.; Edgar, K. J. Olefin Cross-Metathesis as a Source of Polysaccharide Derivatives: Cellulose ω-Carboxyalkanoates. *Biomacromolecules* **2013**, *15* (1), 177–187.

(9) Dong, Y.; Edgar, K. J. Imparting Functional Variety to Cellulose Ethers via Olefin Cross-Metathesis. *Polym. Chem.* **2015**, *6* (20), 3816–3827.

(10) Harada, T.; Masada, M.; Hidaka, H.; Takada, M. Production of Firm, Resilient Gel-Forming Polysaccharide in Natural Medium by a Mutant of Alcaligenes Faecalis Var. Myxogenes 10C3. *J. Ferment. Technol.* **1966**, *44* (1), 20–24.

(11) Harada, T.; Misaki, A.; Saito, H. Curdlan: A Bacterial Gel-Forming β-1, 3-Glucan. *Arch. Biochem. Biophys.* **1968**, *124*, 292–298.

(12) Zhan, X.-B.; Lin, C.-C.; Zhang, H.-T. Recent Advances in Curdlan Biosynthesis, Biotechnological Production, and Applications. *Appl. Microbiol. Biotechnol.* **2012**, *93* (2), 525–531.

(13) McIntosh, M.; Stone, B. A.; Stanisich, V. A. Curdlan and Other Bacterial (1→ 3)-β-D-Glucans. *Appl. Microbiol. Biotechnol.* **2005**, *68* (2), 163–173.

(14) Zhang, R.; Edgar, K. J. Properties, Chemistry, and Applications of the Bioactive Polysaccharide Curdlan. *Biomacromolecules* **2014**, *15* (4), 1079–1096.

(15) Liu, D.; Li, Z.; Zhu, Y.; Li, Z.; Kumar, R. Recycled Chitosan Nanofibril as an Effective Cu(II), Pb(II) and Cd(II) Ionic Chelating Agent: Adsorption and Desorption Performance. *Carbohydr. Polym.* **2014**, *111*, 469–476.

(16) Berthold, A.; Cremer, K.; Kreuter, J. Preparation and Characterization of Chitosan Microspheres as Drug Carrier for Prednisolone Sodium Phosphate as Model for Anti-Inflammatory Drugs. *J. Control. Release* **1996**, *39* (1), 17–25.

(17) Mi, F.-L.; Shyu, S.-S.; Wu, Y.-B.; Lee, S.-T.; Shyong, J.-Y.; Huang, R.-N. Fabrication and Characterization of a Sponge-like Asymmetric Chitosan Membrane as a Wound Dressing. *Biomaterials* **2001**, *22* (2), 165–173.

(18) Pillai, C. K. S.; Paul, W.; Sharma, C. P. Chitin and Chitosan Polymers: Chemistry, Solubility and Fiber Formation. *Prog. Polym. Sci.* **2009**, *34* (7), 641–678.

(19) Zargar, V.; Asghari, M.; Dashti, A. A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. *ChemBioEng Rev.* **2015**, *2* (3), 204–226.

(20) Kumar, M. N. V. R. A Review of Chitin and Chitosan Applications. *React. Funct. Polym.* **2000**, *46* (1), 1–27.

(21) Nagahama, H.; Nwe, N.; Jayakumar, R.; Koiwa, S.; Furuike, T.; Tamura, H. Novel Biodegradable Chitin Membranes for Tissue Engineering Applications. *Carbohydr. Polym.* **2008**, *73* (2), 295–302.

(22) Jayakumar, R.; Rajkumar, M.; Freitas, H.; Selvamurugan, N.; Nair, S. V.; Furuike, T.; Tamura, H. Preparation, Characterization, Bioactive and Metal Uptake Studies of Alginate/Phosphorylated Chitin Blend Films. *Int. J. Biol. Macromol.* **2009**, *44* (1), 107–111.

(23) Shalumon, K. T.; Binulal, N. S.; Selvamurugan, N.; Nair, S. V.; Menon, D.; Furuike, T.; Tamura, H.; Jayakumar, R. Electrospinning of Carboxymethyl Chitin/Poly(Vinyl Alcohol) Nanofibrous Scaffolds for Tissue Engineering Applications. *Carbohydr. Polym.* **2009**, *77* (4), 863–869.

(24) Anitha, A.; Divya Rani, V. V.; Krishna, R.; Sreeja, V.; Selvamurugan, N.; Nair, S. V.; Tamura, H.; Jayakumar, R. Synthesis, Characterization, Cytotoxicity and Antibacterial Studies of Chitosan, O-Carboxymethyl and N,O-Carboxymethyl Chitosan Nanoparticles. *Carbohydr. Polym.* **2009**, *78* (4), 672–677.

(25) Muramatsu, K.; Masuda, S.; Yoshihara, Y.; Fujisawa, A. In Vitro Degradation Behavior of Freeze-Dried Carboxymethyl-Chitin Sponges Processed by Vacuum-Heating and Gamma Irradiation. *Polym. Degrad. Stab.* **2003**, *81* (2), 327–332.

(26) Gandhi, N. S.; Mancera, R. L. The Structure of Glycosaminoglycans and Their Interactions with Proteins. *Chem. Biol. Drug Des.* **2008**, *72* (6), 455–482.

(27) Prydz , K.; Kristian. Determinants of Glycosaminoglycan (GAG) Structure. *Biomolecules* **2015**, *5* (3), 2003–2022.

(28) Köwitsch, A.; Zhou, G.; Groth, T. Medical Application of Glycosaminoglycans: A Review. *J. Tissue Eng. Regen. Med.* **2018**, *12* (1), e23–e41.

(29) Kamhi, E.; Joo, E. J.; Dordick, J. S.; Linhardt, R. J. Glycosaminoglycans in Infectious Disease. *Biol. Rev.* **2013**, *88* (4), 928–943.

(30) Karst, N. A.; Linhardt, R. J. Recent Chemical and Enzymatic Approaches to the Synthesis of Glycosaminoglycan Oligosaccharides. *Curr. Med. Chem.* **2003**, *10* (19), 1993–2031.

(31) Mestechkina, N. M.; Shcherbukhin, V. D. Sulfated Polysaccharides and Their Anticoagulant Activity: A Review. *Appl. Biochem. Microbiol.* **2010**, *46* (3), 267–273.

(32) Bedini, E.; Laezza, A.; Parrilli, M.; Iadonisi, A. A Review of Chemical Methods for the Selective Sulfation and Desulfation of Polysaccharides. *Carbohydr. Polym.* **2017**, *174*, 1224–1239.

(33) Papy-Garcia, D.; Barbier-Chassefière, V.; Rouet, V.; Kerros, M.-E.; Klochendler, C.; Tournaire, M.-C.; Barritault, D.; Caruelle, J.-P.; Petit, E. Nondegradative Sulfation of Polysaccharides. Synthesis and Structure Characterization of Biologically Active Heparan Sulfate Mimetics. *Macromolecules* **2005**, *38* (11), 4647–4654.

(34) Chen, G.; Zhang, B.; Zhao, J.; Chen, H. Development and Characterization of Food Packaging Film from Cellulose Sulfate. *Food Hydrocoll.* **2014**, *35*, 476–483.

(35) Bhujbal, S. V; de Vos, P.; Niclou, S. P. Drug and Cell Encapsulation: Alternative Delivery Options for the Treatment of Malignant Brain Tumors. *Adv. Drug Deliv. Rev.* **2014**, *67*, 142–153.

(36) De Villiers, M. M.; Otto, D. P.; Strydom, S. J.; Lvov, Y. M. Introduction to Nanocoatings Produced by Layer-by-Layer (LbL) Self-Assembly. *Adv. Drug Deliv. Rev.* **2011**, *63* (9), 701–715.

(37) Groth, T.; Wagenknecht, W. Anticoagulant Potential of Regioselective Derivatized Cellulose. *Biomaterials* **2001**, *22* (20), 2719–2729.

(38) McCarthy, B. Antivirals—an Increasingly Healthy Investment. *Nat. Biotechnol.* **2007**, *25* (12), 1390.

(39) Stone, A. Microbicides: A New Approach to Preventing HIV and Other Sexually Transmitted Infections. *Nat. Rev. Drug Discov.* **2002**, *1* (12), 977.

(40) Yao, S. An Improved Process for the Preparation of Sodium Cellulose Sulphate. *Chem. Eng. J.* **2000**, *78* (2–3), 199–204.

(41) Bhatt, N.; Gupta, P. K.; Naithani, S. Preparation of Cellulose Sulfate from A-cellulose Isolated from Lantana Camara by the Direct Esterification Method. *J. Appl. Polym. Sci.* **2008**, *108* (5), 2895–2901.

(42) Zhu, L.; Qin, J.; Yin, X.; Ji, L.; Lin, Q.; Qin, Z. Direct Sulfation of Bacterial Cellulose with a ClSO3H/DMF Complex and Structure Characterization of the Sulfates. *Polym. Adv. Technol.* **2014**, *25* (2), 168–172.

(43) Wagenknecht, W.; Nehls, I.; Philipp, B. Studies on the Regioselectivity of Cellulose Sulfation in an N2O4-N, N-Dimethylformamide-Cellulose System. *Carbohydr. Res.* **1993**, *240*, 245–252.

(44) Heinze, T.; Vieira, M.; Heinze, L. New Polymers Based on Cellulose. *Lenzinger Berichte* **2000**, *79*, 39–44.

(45) Gericke, M.; Liebert, T.; Heinze, T. Interaction of Ionic Liquids with Polysaccharides, 8– Synthesis of Cellulose Sulfates Suitable for Polyelectrolyte Complex Formation. *Macromol. Biosci.* **2009**, *9* (4), 343–353.

(46) Gericke, M.; Liebert, T.; Heinze, T. Polyelectrolyte Synthesis and in Situ Complex Formation in Ionic Liquids. *J. Am. Chem. Soc.* **2009**, *131* (37), 13220–13221.

(47) Wang, Z.-M.; Li, L.; Xiao, K.-J.; Wu, J.-Y. Homogeneous Sulfation of Bagasse Cellulose in an Ionic Liquid and Anticoagulation Activity. *Bioresour. Technol.* **2009**, *100* (4), 1687–1690.

(48) Philipp, B.; Wagenknecht, W.; Wagenknecht, M.; Nehls, I.; Klemm, D.; Stein, A.; Heinze, T.; Heinze, U.; Helbig, K.; Camacho, J. Regioselektive Veresterung Und Veretherung von Cellulose Und Cellulosederivaten. Teil 2. Synthese Regioselektiv Substituierter Celluloseester. *Papier* **1995**, *49*, 58–64.

(49) Baumann, H.; Richter, A.; Klemm, D.; Faust, V. Concepts for Preparation of Novel Regioselective Modified Cellulose Derivatives Sulfated, Aminated, Carboxylated and Acetylated for Hemocompatible Ultrathin Coatings on Biomaterials. *Macromol. Chem. Phys.* **2000**, *201* (15), 1950–1962.

(50) Zhang, K.; Brendler, E.; Geissler, A.; Fischer, S. Synthesis and Spectroscopic Analysis of Cellulose Sulfates with Regulable Total Degrees of Substitution and Sulfation Patterns via 13C NMR and FT Raman Spectroscopy. *Polymer (Guildf).* **2011**, *52* (1), 26–32.

(51) Wagenknecht, W.; Nehls, I.; Stein, A.; Klemm, D.; Philipp, B. Synthesis and Substituent Distribution of Na-cellulose Sulphates via O-trimethylsilyl Cellulose as Intermediate. *Acta Polym.* **1992**, *43* (5), 266–269.

(52) Richter, A.; Klemm, D. Regioselective Sulfation of Trimethylsilyl Cellulose Using Different SO3-Complexes. *Cellulose* **2003**, *10* (2), 133–138.

(53) Gohdes, M.; Mischnick, P.; Wagenknecht, W. Methylation Analysis of Cellulose Sulphates. *Carbohydr. Polym.* **1997**, *33* (2–3), 163–168.

(54) Gohdes, M.; Mischnick, P. Determination of the Substitution Pattern in the Polymer Chain of Cellulose Sulfates. *Carbohydr. Res.* **1998**, *309* (1), 109–115.

(55) Wang, J.; Yang, W.; Wang, J.; Wang, X.; Wu, F.; Yao, J.; Zhang, J.; Lei, Z. Regioselective Sulfation of Artemisia Sphaerocephala Polysaccharide: Characterization of Chemical Structure. *Carbohydr. Polym.* **2015**, *133*, 320–327.

(56) Wang, J.; Niu, S.; Zhao, B.; Wang, X.; Yao, J.; Zhang, J.; Zhao, W.; Zhao, Y. Regioselective Synthesis of Sulfated Guar Gum: Comparative Studies of Structure and Antioxidant Activities. *Int. J. Biol. Macromol.* **2013**, *62*, 734–740.

(57) Zhang, Z.; Zhang, Q.; Wang, J.; Song, H.; Zhang, H.; Niu, X. Regioselective Syntheses of Sulfated Porphyrans from Porphyra Haitanensis and Their Antioxidant and Anticoagulant Activities in Vitro. *Carbohydr. Polym.* **2010**, *79* (4), 1124–1129.

(58) Zhang, K.; Peschel, D.; Klinger, T.; Gebauer, K.; Groth, T.; Fischer, S. Synthesis of Carboxyl Cellulose Sulfate with Various Contents of Regioselectively Introduced Sulfate and Carboxyl Groups. *Carbohydr. Polym.* **2010**, *82* (1), 92–99.

(59) Udoratina, E. V; Torlopov, M. A.; Drozd, N. N.; Makarov, V. A. Oxyethylated Cellulose Sulfates. *Polym. Sci. Ser. B* **2012**, *54* (3–4), 175–182.

(60) Heinze, T.; Genco, T.; Petzold-Welcke, K.; Wondraczek, H. Synthesis and Characterization of Aminocellulose Sulfates as Novel Ampholytic Polymers. *Cellulose* **2012**, *19* (4), 1305–1313.

(61) Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Suzuki, E.; Miyano, H.; Mimura, T.; Yoshida, O.; Yamamoto, N. Synthesis and Structural Analysis of Curdlan Sulfate with a Potent Inhibitory Effect in Vitro of AIDS Virus Infection. *Macromolecules* **1990**, *23* (16), 3717–3722.

(62) Osawa, Z.; Morota, T.; Hatanaka, K.; Akaike, T.; Matsuzaki, K.; Nakashima, H.; Yamamoto, N.; Suzuki, E.; Miyano, H.; Mimura, T. Synthesis of Sulfated Derivatives of Curdlan and Their Anti-HIV Activity. *Carbohydr. Polym.* **1993**, *21* (4), 283–288.

(63) Gao, Y.; Fukuda, A.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Nakashima, H.; Uryu, T. Synthesis of Regioselective Substituted Curdlan Sulfates with Medium Molecular

Weights and Their Specific Anti-HIV-1 Activities. *Macromolecules* **1997**, *30* (11), 3224–3228.

(64) Borjihan, G.; Zhong, G.; Baigude, H.; Nakashima, H.; Uryu, T. Synthesis and Anti-HIV Activity of 6-amino-6-deoxy-(1→ 3)-β-D-curdlan Sulfate. *Polym. Adv. Technol.* **2003**, *14* (3-5), 326–329.

(65) and, Y. Z.; Khor*, E. Preparation of C-6 Substituted Chitin Derivatives under Homogeneous Conditions. *Biomacromolecules* **2004**, *6* (1), 80–87.

(66) Cushing, I. B.; Davis, R. V.; Kratovil, E. J.; MacCorquodale, D. W. The Sulfation of Chitin in Chlorosulfonic Acid and Dichloroethane. *J. Am. Chem. Soc.* **1954**, *76* (18), 4590–4591.

(67) Tokura, S.; Itoyama, K.; Nishi, N.; Nishimura, S.-I.; Saiki, I.; Nishimura, I. A. Selective Sulfation of Chitin Derivatives for Biomedical Functions. *J. Macromol. Sci. Part A* **1994**, *31* (11), 1701–1718.

(68) Zou, Y.; Khor, E. Preparation of Sulfated-Chitins under Homogeneous Conditions. *Carbohydr. Polym.* **2009**, *77* (3), 516–525.

(69) Baumann, H.; Faust, V. Concepts for Improved Regioselective Placement of O-Sulfo, N-Sulfo, N-Acetyl, and N-Carboxymethyl Groups in Chitosan Derivatives. *Carbohydr. Res.* **2001**, *331* (1), 43–57.

(70) Zhang, K.; Helm, J.; Peschel, D.; Gruner, M.; Groth, T.; Fischer, S. NMR and FT Raman Characterisation of Regioselectively Sulfated Chitosan Regarding the Distribution of Sulfate Groups and the Degree of Substitution. *Polymer (Guildf).* **2010**, *51* (21), 4698–4705.

(71) Nishimura, S.-I.; Kai, H.; Shinada, K.; Yoshida, T.; Tokura, S.; Kurita, K.; Nakashima, H.; Yamamoto, N.; Uryu, T. Regioselective Syntheses of Sulfated Polysaccharides: Specific Anti-HIV-1 Activity of Novel Chitin Sulfates. *Carbohydr. Res.* **1998**, *306* (3), 427–433.

(72) Liu, H.; Zhang, Z.; Linhardt, R. J. Lessons Learned from the Contamination of Heparin. *Nat. Prod. Rep.* **2009**, *26* (3), 313–321.

(73) Szajek, A. Y.; Chess, E.; Johansen, K.; Gratzl, G.; Gray, E.; Keire, D.; Linhardt, R. J.; Liu, J.; Morris, T.; Mulloy, B.; et al. The US Regulatory and Pharmacopeia Response to the Global Heparin Contamination Crisis. *Nat. Biotechnol.* **2016**, *34* (6), 625–630.

(74) Guerrini, M.; Beccati, D.; Shriver, Z.; Naggi, A.; Viswanathan, K.; Bisio, A.; Capila, I.; Lansing, J. C.; Guglieri, S.; Fraser, B.; et al. Oversulfated Chondroitin Sulfate Is a Contaminant in Heparin Associated with Adverse Clinical Events. *Nat. Biotechnol.* **2008**, *26* (6), 669–675.

(75) Muthana, S.; Cao, H.; Chen, X. Recent Progress in Chemical and Chemoenzymatic Synthesis of Carbohydrates. *Curr. Opin. Chem. Biol.* **2009**, *13* (5–6), 573–581.

(76) DeAngelis, P. L.; Liu, J.; Linhardt, R. J. Chemoenzymatic Synthesis of Glycosaminoglycans: Re-Creating, Re-Modeling and Re-Designing Nature's Longest or Most Complex Carbohydrate Chains. *Glycobiology* **2013**, *23* (7), 764–777.

(77) Fujikawa, S.; Ohmae, M.; Kobayashi, S. Enzymatic Synthesis of Chondroitin 4-Sulfate with Well-Defined Structure. *Biomacromolecules* **2005**, *6* (6), 2935–2942.

(78) Chappell, E. P.; Liu, J. Use of Biosynthetic Enzymes in Heparin and Heparan Sulfate Synthesis. *Bioorg. Med. Chem.* **2013**, *21* (16), 4786–4792.

(79) Bhaskar, U.; Sterner, E.; Hickey, A. M.; Onishi, A.; Zhang, F.; Dordick, J. S.; Linhardt, R. J. Engineering of Routes to Heparin and Related Polysaccharides. *Appl. Microbiol. Biotechnol.* **2012**, *93* (1), 1–16.

(80) Xu, Y.; Masuko, S.; Takieddin, M.; Xu, H.; Liu, R.; Jing, J.; Mousa, S. A.; Linhardt, R. J.; Liu, J. Chemoenzymatic Synthesis of Homogeneous Ultralow Molecular Weight Heparins. *Science* **2011**, *334* (6055), 498–501.

(81) Liu, J.; Linhardt, R. J. Chemoenzymatic Synthesis of Heparan Sulfate and Heparin. *Nat. Prod. Rep.* **2014**, *31* (12), 1676–1685.

(82) Zhang, X.; Pagadala, V.; Jester, H. M.; Lim, A. M.; Pham, T. Q.; Goulas, A. M. P.; Liu, J.; Linhardt, R. J. Chemoenzymatic Synthesis of Heparan Sulfate and Heparin Oligosaccharides and NMR Analysis: Paving the Way to a Diverse Library for Glycobiologists. *Chem. Sci.* **2017**, *8* (12), 7932–7940.

(83) Ly, M.; Wang, Z.; Laremore, T. N.; Zhang, F.; Zhong, W.; Pu, D.; Zagorevski, D. V.; Dordick, J. S.; Linhardt, R. J. Analysis of E. Coli K5 Capsular Polysaccharide Heparosan. *Anal. Bioanal. Chem.* **2011**, *399* (2), 737–745.

(84) Wang, Z.; Ly, M.; Zhang, F.; Zhong, W.; Suen, A.; Hickey, A. M.; Dordick, J. S.; Linhardt, R. J. E. Coli K5 Fermentation and the Preparation of Heparosan, a Bioengineered Heparin Precursor. *Biotechnol. Bioeng.* **2010**, *107* (6), 964–973.

(85) Bhaskar, U.; Li, G.; Fu, L.; Onishi, A.; Suflita, M.; Dordick, J. S.; Linhardt, R. J. Combinatorial One-Pot Chemoenzymatic Synthesis of Heparin. *Carbohydr. Polym.* **2015**, *122*, 399–407.

(86) Chen, J.; Jones, C. L.; Liu, J. Using an Enzymatic Combinatorial Approach to Identify Anticoagulant Heparan Sulfate Structures. *Chem. Biol.* **2007**, *14* (9), 986–993.

Chapter 3: Efficient Synthesis of Glycosaminoglycan Analogs

(Published in *Biomacromolecules* **2019**, *20* (2), 608–617)

Chengzhe Gao[a,c] and Kevin J. Edgar[b,c*]

[a] Department of Chemistry, Virginia Tech, Blacksburg, VA, 24061

[b] Department of Sustainable Biomaterials, Virginia Tech, Blacksburg, VA, 24061

[c] Macromolecules Innovation Institute, Virginia Tech, Blacksburg, VA, 24061

Abstract

Glycosaminoglycans (GAGs) are among the most complex, biologically active polysaccharides in nature. The complexity of GAGs greatly impedes their synthesis, thus complicating the structure-property studies that are so necessary for us to understand the roles of GAGs in natural processes, in pathogen invasion, and to understand how to develop effective interventions, e.g. to prevent undesired GAG hijacking by pathogens. Total synthesis of GAG oligomers from monosaccharide building blocks is useful, but incredibly labor-intensive, expensive, and inefficient. In this study, we report a regiospecific synthetic route to two types of designed GAG analogs by chemical modification of commercially available, inexpensive cellulose acetate. Cellulose acetate was first brominated, followed by azide displacement to introduce azides as the GAG amine precursors. The resulting 6-N_3 cellulose acetate was then saponified to liberate 6-OH groups. Subsequent oxidation of the liberated primary hydroxyl groups to carboxyl groups was smoothly effected by a (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)-catalyzed process. Finally, the azides were reduced to amines using an aqueous process, new to polysaccharide chemistry, employing reduction by dithiothreitol (DTT). Alternatively, another process new to polysaccharide chemistry could be employed to convert most of the azides to acetamido groups

(mimicking those present, for example, in native hyaluronic acid) by reduction with thioacetic acid. All the intermediates and products were characterized by ^{1}H NMR, ^{13}C NMR, and FT-IR spectroscopy. This synthetic route provides access to GAG analogs that will be of great interest for exploring structure-property relationships in various biomedical applications.

3.1 Introduction

Glycosaminoglycans (GAGs) are among the most important families of biomacromolecules, with enormous influence on physiological functions including prevention of blood clotting,[1] development of the central nervous system,[2] adhesion of cells to one another,[3] signaling and recognition,[4] joint lubrication,[5] and innumerable other critical functions. They are abundant, for example in the pericellular and extracellular matrices that surround cells.[6] Important GAG families include hyaluronic acid (HA, (→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→)), heparin and heparan sulfate (HS) (both based on the backbone →4)-β-D-Glc*p*N-(1→4)-β-D-Glc*p*A-(1→, but with extensive *N*- and *O*- sulfation, as well as epimerization of GlcA to IdoA), chondroitin sulfate (CS) and the closely related dermatan sulfate (DS), and keratan sulfate (KS).[7] GAGs (with the exception of HA) are usually sulfated to various degrees and in different patterns, and are covalently linked to proteins as part of proteoglycans.[8] Heparin is an exemplary bioactive sulfated GAG that has been used clinically as an anti-coagulant drug since 1935;[9] that is to say, it was used as an anti-coagulant before its structure was known![10] The enormous value of its anti-coagulant properties (e.g. prevention of thromboses during and post-surgery, prevention of clot formation during dialysis), and the need to separate beneficial anti-coagulant activity from undesired promotion of extended bleeding have led researchers to synthesize analogs by laborious total-synthesis methods.[11] The resulting more selective heparin oligomer analogs are useful in the

clinic but extremely expensive due to the inefficiency of the total synthesis approach. This inefficiency has made it very difficult and expensive to carry out detailed, wide-ranging structure-property studies.

GAGs are structurally interesting as they begin biosynthetically as simple AB perfectly alternating copolymers, where (except in the case of KS) "A" is a 2-acetamido-2-deoxy sugar (based upon glucose (Glc) or galactose (Gal)), and "B" is glucuronic acid (GlcA). Nature introduces complexity by a combination of some or all of three post-polymerization modifications; N-deacetylation, C-5 epimerization (changing the spatial orientation of the uronic acid 6-carboxyl group to form iduronic acid (IdoA)), and sulfation of specific hydroxyls and/or amines. These modifications give a sequence of monosaccharides that contains, potentially, an enormous amount of information. As an example, a molecule of the closely related GAGs CS/DS of DP 48 (a fairly low DP for these GAGs) would be 24 dyads long; CS and DS together contain 8 common dyads (see Figure S3.1 for identities). Therefore a CS/DS molecule of this DP could have 8^{24}, or approximately 5 X 10^{21} different sequences! This complexity contributes to the difficulty of synthesizing a range of natural sequences for structure-activity studies.

Current methods to obtain GAGs and their analogs include: extraction from natural sources, total synthesis of GAG oligosaccharides, *de novo* synthesis of GAGs, and chemical and/or enzymatic modification of polysaccharides.[12] Isolation and purification from animal sources is the most widely employed strategy to obtain GAGs used in medicine.[13] However, due to the presence in most tissues of structurally similar GAGs, and the affinity of GAGs for proteins, isolated GAGs may be contaminated by proteins and other polysaccharide sulfate impurities. GAG contamination is of great concern with regard to undesired toxicity and/or eliciting of immune responses by contaminants; contamination of heparin with semi-synthetic, over-sulfated chondroitin sulfate

caused more than a hundred deaths in late 2007 and early 2008.[14] Total synthesis of oligosaccharides is another approach to GAGs with defined structure.[15] The multistep nature of total synthesis makes it nearly impossible to synthesize GAGs with native DP (ca. 50-250); low DP analogs have proven useful in the clinic as anti-coagulants with much less propensity to promote bleeding than heparin itself.[11] Chemical or enzymatic modification of polysaccharides is a useful method to obtain GAGs and their analogs with high DP. For example, starting from heparosan, the Linhardt group prepared heparin and heparan sulfates with various sulfate distributions and molecular weights by a combination of chemical synthesis, use of an epimerase, and of a series of sulfotransferases.[16–19] The Bedini group has prepared a CS and fucosylated CS library from chondroitin by a sequence of chemical reactions.[20–22] These modifications begin with polysaccharides such as heparosan[23] and unsulfated chondroitin,[24] which are produced in certain natural or bioengineered bacteria and therefore may be expensive to obtain.

GAG analogs can be also obtained by modification of common polysaccharides, including cellulose, chitin, chitosan, and curdlan. These GAG analogs are typically synthesized by sulfation of polysaccharides (e.g. chitin[25] and chitosan[26,27]) or their derivatives that bear carboxyl (e.g. cellulose carboxylate[28]) or amine/amide groups (e.g. amino cellulose[29] and amino curdlan[30]). As a result, they typically contain either carboxyl or amine/amide groups, along with sulfate groups, while natural GAGs bear both carboxyl and amine/amide groups with sulfate groups. Even not as complicated as natural GAGs in structure, the sulfated GAG analogs still display intriguing biological activities. However, GAG analogs that contain both amine and carboxyl groups are much more difficult to synthesize, hence there are very few reports on preparing them. In 2014, Tamaru et al. reported synthesis of a curdlan derivative that had both amine and carboxyl groups

at C6.[31] To our knowledge, this is the first and only report of synthesis of GAG analogs that have both carboxyl and amine groups.

Cellulose is one of the most abundant natural polymers, and its chemical modification has been investigated extensively.[32] It would be advantageous to use inexpensive, abundant cellulose or its commercially available derivatives to synthesize GAGs and their analogs. This would provide a powerful method to obtain GAGs and their analogs in relatively large amounts and enable informative structure-biological activity relationship studies. Thus, we hypothesize that we can design GAG analogs, and synthesize those analogs by chemical modification of cellulose or its derivatives.

We report herein our initial studies in this area, involving design and synthesis of two random copolymeric GAG analogs, poly(6-amino-6-deoxy-Glc-*ran*-GlcA), which we abbreviate Glc6N/GlcA, and poly(6-amino-6-deoxy-Glc-*ran*-GlcA-*ran*-6-acetamido-6-deoxy-Glc), abbreviated as Glc6NAc/GlcA/Glc6N (Figure 3.1b), each of which has entirely β-1-4 linkages, as in the →4)-β-D-Glc*p*N-(1→4)-β-D-Glc*p*A-(1→ biosynthetic precursor to heparin. The monosaccharide components are therefore identical to those of HA (Figure 3.1a) in the case of the uronic acid, and a positional isomer to the amino sugar of HA (and heparin/HS) in the case of the glucosamine (6-amino vs. natural 2-amino), where our amino sugar can be *N*-acetylated as in HA, or not. We target roughly equal DS (0.5) of amine/amide and carboxyl groups, as in natural GAGs. Our target polymers would also differ from natural GAGs in that they would be random rather than alternating copolymers, and in this initial study would not be sulfated. We hypothesize that a range of such GAG analogs can be synthesized from properly chosen cellulose acetates by careful synthetic design using a combination of highly regio- and chemoselective reactions, including Furuhata bromination, azide displacement, TEMPO oxidation, and azide reduction.

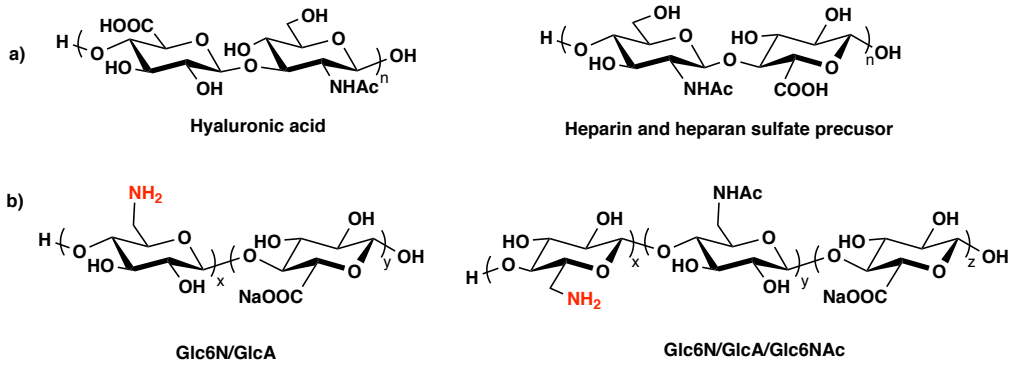


Figure 3.1 a) Hyaluronic acid (alternating copolymer, alternating β-1→3 and β-1→4 linkages) and heparin and heparan sulfate precursor (alternating copolymer, all β-1→4 linkages); b) designed GAG analogs (random copolymers, all β-1→4 linkages)

Although introduction of amines,[29,33,34] amides,[35,36] and carboxyl groups[37–39] to cellulose and other polysaccharides has been reported, synthesis of designed GAG analogs bearing amine groups, amide groups, and carboxyl groups is still very challenging. These challenges arise from solubility limitations of cellulose and its derivatives, and the need for chemo- and regioselectivity. We find, and discuss herein, that exquisitely careful synthetic design is necessary to achieve these goals and overcome these obstacles. Herein, we report such a careful synthetic design, and our exploitation of the design to attempt preparation of well-defined, structurally intriguing GAG analogs.

3.2 Experimental

3.2.1 Materials

Cellulose acetate (CA320S, DS(Ac) 1.75, DS(6-OH) 0.49 (calculated from perpropionylated samples by ^{1}H NMR), Mw = 47.6 kDa (measured by SEC), Eastman Chemical Company) was dried under vacuum at 50 °C overnight prior to use (see Tables S3.1 and Figure

S3.2 in Supplementary Information for characterization details). Pyridine, 4-dimethylaminopyridine, propionic anhydride, triphenylphosphine, N-bromosuccinimide, sodium azide, (2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO), sodium bromide, sodium hypochlorite aqueous solution (6-14% active chlorine basis), thioacetic acid, and 1,4-dithiothreitol (DTT) were from Sigma-Aldrich and used as received. Acetone and ethanol were from Fisher Scientific and used as received. Dimethylacetamide (DMAc, Fisher) and dimethyl sulfoxide (DMSO, Fisher) were kept over 4 Å molecular sieves and stored under dry N_2 until use. Regenerated cellulose dialysis tubing (Fisher, 3500 g/mol MWCO) was used as received.

3.2.2 Measurements

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in DMSO-*d6* or D_2O at room temperature with 64 scans and 15,000 scans, respectively. The quantitative ^{13}C spectrum was obtained on a Bruker Avance II 500MHz spectrometer with 30° pulse length, 10,000 scans and a relaxation delay of 5 s. Chemical shifts are reported relative to the solvent peaks. FT-IR spectra were acquired on a Nicolet 8700 instrument with 128 scans and 4 cm^{-1} resolution. All yields are reported on a molar basis, based on the average product repeat unit molecular weight as determined based on spectroscopic results.

3.2.3 Regioselective bromination of CA320S

Bromination of CA320S was adapted from a previously reported method.[40] In a 250 mL three-necked round-bottom flask, CA320S (2.0 g, 8.44 mmol) was dissolved in 80 mL of DMAc. Solutions of PPh_3 (3 equiv per AGU, 25.32 mmol, 6.64 g) and NBS (3 equiv per AGU, 25.32 mmol, 4.50 g) were prepared separately by dissolving each in 20 mL of DMAc. The PPh_3 solution was added dropwise to the CA320S solution by a liquid addition funnel, followed by dropwise addition of the NBS solution. The reaction solution was then heated to 70 °C and stirred for 1 h under

nitrogen. The cooled reaction solution was then slowly poured into 2 L of a 50:50 mixture of deionized water and methanol, followed by filtration to recover the precipitate. The isolated crude product was twice re-dissolved in DMSO, then precipitated in ethanol, before vacuum drying at 40°C, affording (6-bromo-6-deoxy)-co-(6-O-acetyl)-CA320S (6-BrCA320S). ^{13}C NMR (500 MHz, DMSO-*d6*): 20.56 (O-(C=O)-<u>C</u>H$_3$), 33.06 (<u>C-6</u>-Br), 62.23 (<u>C-6'</u>-O-Ac), 71.39-79.93 (C2, C3, C4 and C5), 99.14 (C1), 102.73 (C1'), 168.92-170.24 (O-(<u>C</u>=O)-CH$_3$). Yield: 1.89 g (81.0%).

3.2.4 Synthesis of (6-azido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-N$_3$CA320S)

The azide displacement method was adapted from previous studies.[40,41] 6-BrCA320S (1.5 g, 5.582 mmol) was dissolved in 40 mL of DMSO in a 100 mL round bottom flask. Sodium azide (3 equiv per AGU, 16.74 mmol, 1.09 g) was added to the solution. The solution was stirred at 80 °C under nitrogen for 24 h. The solution was cooled to room temperature and poured into 300 mL of deionized water. The product was recovered by filtration and dried under vacuum at 40 °C to yield (6-azido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-N$_3$CA320S). ^{13}C NMR (500 MHz, DMSO-*d6*): 20.51 (O-(C=O)-<u>C</u>H$_3$), 50.27 (<u>C-6</u>-N$_3$), 62.25 (<u>C-6'</u>-O-Ac), 71.39-80.05 (C2, C3, C4 and C5), 99.21 (C1), 102.62 (C1'), 168.90-170.23 (O-(<u>C</u>=O)-CH$_3$). Yield: 1.22 g (87.5%).

3.2.5 One pot synthesis of poly(6-azido-6-deoxy-Glc-*ran*-GlcA) (Glc6N$_3$/GlcA)

6-N$_3$CA320S (1.0 g, 4.00 mmol) was added into 50 mL of 0.5 M NaOH solution in a three-necked round bottom flask. The heterogeneous reaction mixture was stirred vigorously for 12 h. The reaction solution was then cooled to 0-4 °C with an ice bath and the pH was adjusted to 10.0 by adding 0.5 M HCl. TEMPO (20 mg, 0.13 mmol) and NaBr (200 mg, 1.9 mmol) were added to the solution. NaClO solution (2 equiv per AGU, 2.8 mL) was then added dropwise by an addition funnel, then pH was adjusted to 10.0 using 0.5 M HCl. Reaction mixture pH was maintained at 10.0 by 0.5 M NaOH addition. After 6 h, the reaction was quenched by adding 2 mL of MeOH

and pH of the mixture was brought to 7.0. The product was isolated by dialysis against water for 3 days, followed by freeze-drying for 2 days, to afford poly(6-azido-6-deoxy-Glc-*ran*-GlcA) (Glc6N_3/GlcA). ^{13}C NMR (500 MHz, D_2O): 49.50 (C-6-N_3), 72.12-80.11 (C2, C3, C4 and C5), 101.60 (C1), 174.24 (-(C=O)-OH). Yield: 0.44 g (53.6%).

3.2.6 Synthesis of poly(6-amino-6-deoxy-Glc-*ran*-GlcA) (Glc6N/GlcA)

Glc6N_3/GlcA (200 mg, 0.98 mmol) was dissolved in pH 7.4 buffer (10 mL) in a 50 mL round bottom flask. Then DTT (3 equiv per AGU, 0.45 g) was slowly added to the flask and dissolved. The solution was stirred at 25 °C for 12 h under N_2. The solution was then transferred to 3500 g/mol MWCO dialysis tubing. After 3 days of dialysis against water, the solution was freeze-dried to yield poly(6-amino-6-deoxy-Glc-*ran*-GlcA) (Glc6N/GlcA). ^{13}C NMR (500 MHz, D_2O): 39.46 (C-6-NH_2), 68.96-80.15 (C2, C3, C4 and C5), 101.66 (C1), 174.24 (-(C=O)-OH). Yield: 0.15 g (86.0 %).

3.2.7 Synthesis of poly(6-amino-6-deoxy-Glc-*ran*-GlcA-*ran*-6-acetamido-6-deoxy-Glc) (Glc6NAc/GlcA/Glc6N)

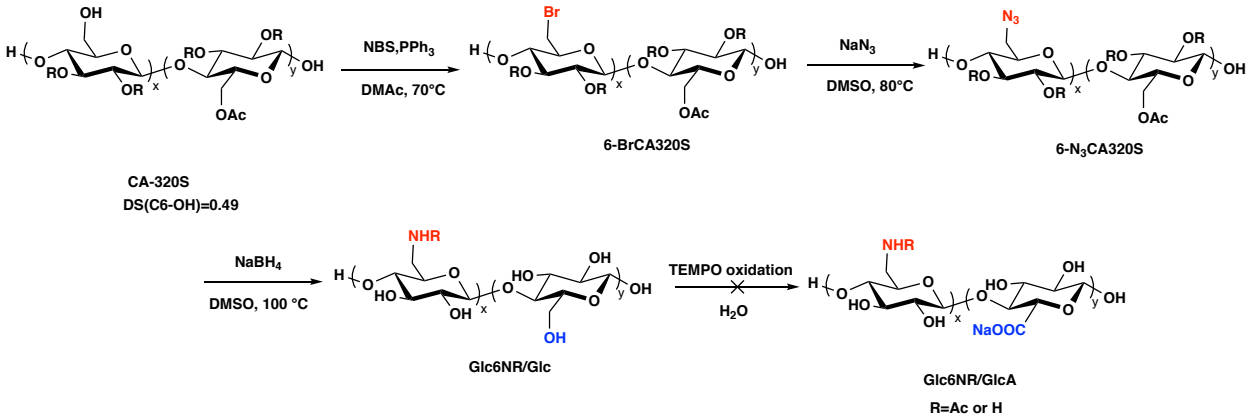
Glc6N_3/GlcA (200 mg, 0.98 mmol) was dissolved in pH 7.4 buffer (10 mL) in a 50 mL round bottom flask. Then thioacetic acid (10 equiv per AGU, 0.70 mL) was slowly added to the flask. The solution was heated to 60 °C and kept at this temperature for 48 h under N_2. The solution was then transferred to 3500 g/mol MWCO dialysis tubing. After 3 days of dialysis against ethanol, and then against water for 2 days, the solution was freeze-dried to yield poly(6-amino-6-deoxy-Glc-*ran*-GlcA-*ran*-6-acetamido-6-deoxy-Glc) (Glc6NAc/GlcA/Glc6N). ^{13}C NMR (500 MHz, D_2O): 21.86 (NH-(C=O)-CH_3), 39.55 (C-6-NH_2), 46.58 (C-6-NHAc), 71.50-80.58 (C2, C3, C4 and C5), 102.16 (C1), 174.23-174.54 (-(C=O)-OH and NH-(C=O)-CH_3). Yield: 0.14 g (63.9 %).

3.3 Results and Discussion

A commercially available random copolymer, cellulose acetate (CA320S, DS (Ac) 1.75) is an ideal starting material for our purpose. The 6-OAc can serve effectively to block roughly half of the 6-OH groups during transformation of the others into amine groups. Subsequently, those previously blocked 6-OAc positions can then be converted to carboxyl groups. By ^{1}H NMR analysis of a perpropionylated CA320S sample (Table S3.1 and Figure S3.2), DS (6-Ac) was revealed to be 0.51 and DS (6-OH) 0.49, consistent with our previous report.[40,41] This fortunate balance of DS (6-OH) and DS (6-Ac) enables synthesis of analogs with DS (NH_2) and DS (COONa) near the natural GAG targets of 0.5 each.

3.3.1 First synthetic route

Our first synthetic route to GAG analogs was by initial introduction of azide as precursor to amines or amides, by azide reduction, and then TEMPO oxidation of the remaining, saponified 6-OH groups (Scheme 3.1). Although it has been reported that chitin can undergo TEMPO oxidation without breaking amide bonds,[42,43] we were still concerned about unwanted oxidation of amine or amide groups. We began this approach by regiospecific Furuhata bromination[44] of CA320S at C6, followed by quantitative azide displacement of the 6-Br groups. The structure of 6-N_3CA320S was confirmed by FT-IR and ^{13}C NMR spectroscopy. The FT-IR spectrum (Figure 3.2) displayed a strong absorption at 2110 cm^{-1}, characteristic of azide groups. In the ^{13}C spectrum (Figure S3.4), a new peak appeared at 49 ppm, which we assigned to azide-bearing C6,[35,41] while the former C6-Br ^{13}C resonance (Figure S3.3) was absent. Failure to detect any bromide content by elemental analysis in the previous study indicated that azide displacement was quantitative and complete. DS (N_3) was 0.49, determined by elemental analysis,[40] demonstrating that the original C6-OH of CA320S had been quantitatively converted to C6-N_3.



Scheme 3.1 First proposed synthetic route to GAG analogs

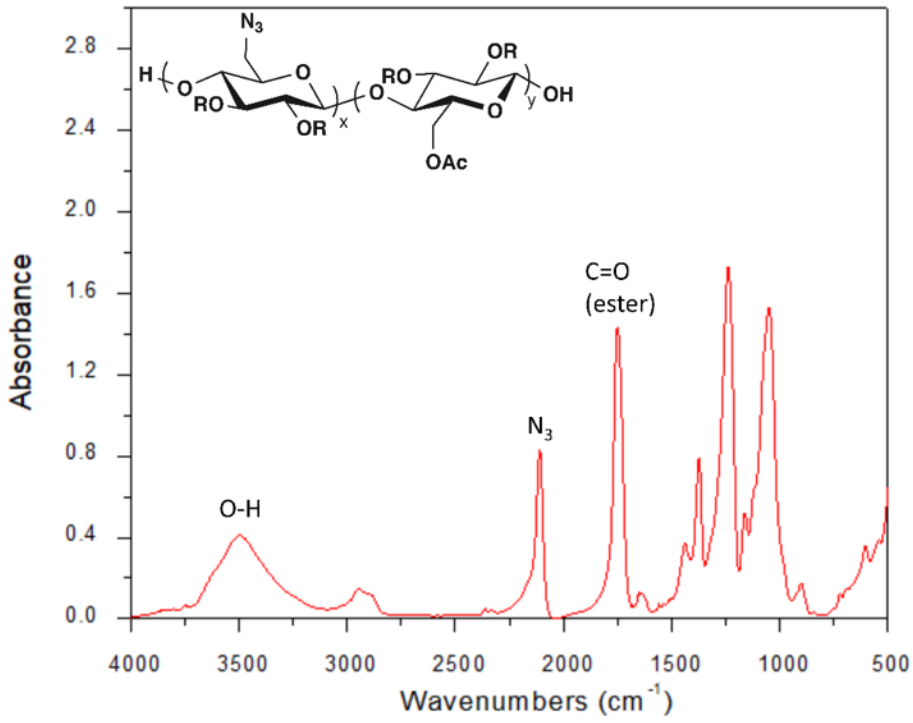


Figure 3.2 FT-IR spectrum of 6-N_3CA320S

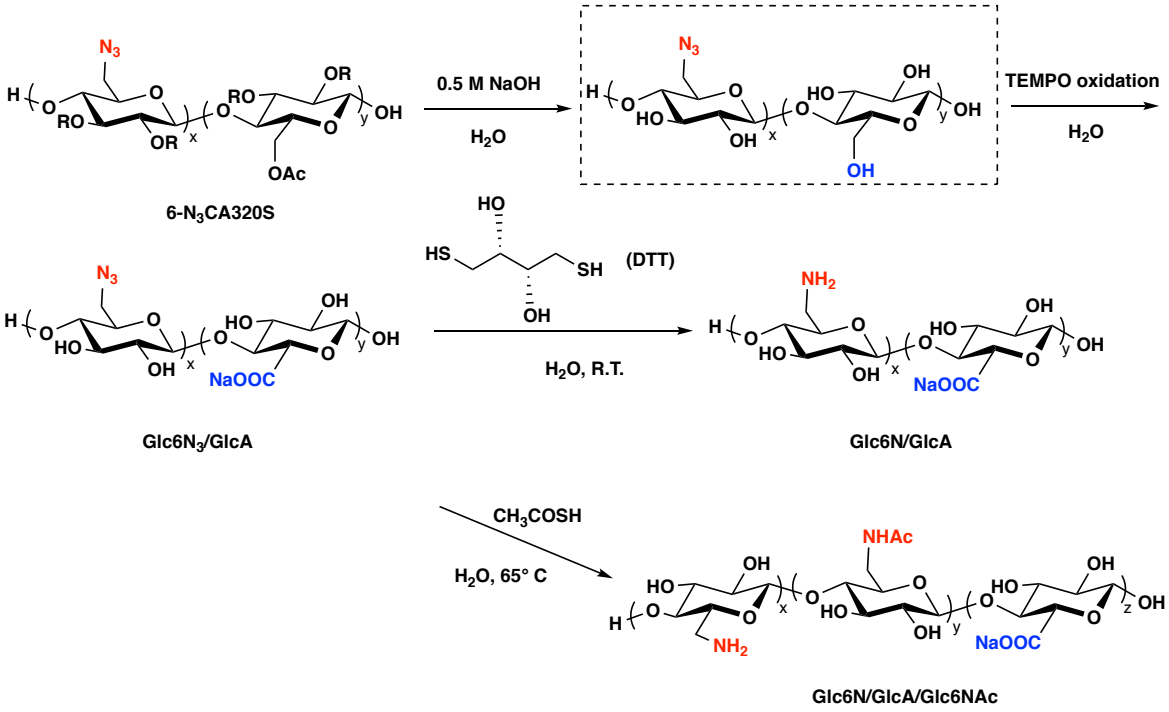
The azide groups of 6-N_3CA320S were then reduced to amines by $NaBH_4$ in DMSO. In this step, the amines generated *in situ* further reacted with the ester groups to afford amides, owing to the high nucleophilicity of amines and improved stability of amides versus esters. Such O to N acyl group transfer has been observed previously during reduction of azidopolysaccharides also

bearing ester groups[35,45,46] as well as in small molecule chemistry.[47–49] Under the strong reduction conditions (excess reagent $NaBH_4$, high temperature, long reaction time) needed for our cellulosic substrate, all or most esters were also reduced to hydroxyl groups. This borohydride reduction step accomplished three purposes; 1) azide reduction to amines; 2) acyl transfer to amides which are stable to TEMPO oxidation conditions; 3) ester reduction exposes primary 6-OH groups (O-6), enabling their subsequent TEMPO oxidation to carboxyls (uronic acids). The structure of the borohydride reduction product was confirmed by its FT-IR spectrum (Figure S3.5), where the azide absorption around 2100 cm^{-1} was absent, and the new amide absorption appeared at 1660 cm^{-1}. The significantly decreased ester absorption (1735 - 1750 cm^{-1}) and broadened OH/NH absorptions (3300 -3500 cm^{-1}) also supported the proposed structure. In the last step, the primary hydroxyl groups liberated from previous step were oxidized to carboxyls using TEMPO in water. However, although the ^{13}C spectrum of the TEMPO oxidation product (Figure S3.6), revealed the expected carboxyl carbonyl at 175 ppm, no C6-amide, C6-amine, or N-acetyl peak was observed. Even with shorter reaction time or fewer equivalents of oxidants used, these expected ^{13}C signals were absent. The apparent decomposition of the acetamido groups under the alkaline, oxidative conditions is not fully understood, but clearly it dooms this approach to GAG analogs.

3.3.2 Second, successful synthetic route to GAG analogs

To circumvent this issue, we elected to attempt to introduce carboxyl groups first, then reduce azides to amines or amides (Scheme 3.2). We will briefly describe the strategy, then detail the steps and results. Beginning with 6-N_3CA320S synthesized by the same method in section 3.1, we planned to saponify to expose all the hydroxyl groups, including the previously acetyl-protected C-6 hydroxyl groups. Then, TEMPO-mediated oxidation of the newly exposed primary 6-OH groups should afford the uronic acid-containing copolymer, Glc6N_3/GlcA. Finally, azide

reduction to amines or amines/amides mixture would afford the target GAG analogs, Glc6N/GlcA and Glc6NAc/GlcA/Glc6N.



Scheme 3.2 Successful synthesis of GAG analogs

3.3.2.1 One-pot saponification and TEMPO-mediated oxidation.

TEMPO mediated oxidation is highly selective for oxidation of primary hydroxyl groups of polysaccharides to carboxyl groups.[50] To generate the needed primary hydroxyl groups from 6-N_3CA320S, with its DS(6-N_3) of ca. 0.5 and DS(6-OAc) of ca. 0.5, the acetates were saponified under mild conditions (0.5 M NaOH aqueous solution, room temperature, 12 h). There was no need to isolate and purify the saponified product; rather, its free primary hydroxyl groups (6-OH) generated *in situ* were oxidized in the same vessel by TEMPO catalyst and NaBr/NaOCl reoxidants at 4 °C for 6 h, affording a water-soluble polymer. The FT-IR spectrum (Figure 3.3) displayed a strong carboxylate absorption at 1650 cm^{-1}, confirming successful TEMPO oxidation. The ester carbonyl at 1750 cm^{-1} disappeared, and intensity of the OH stretch from 3000 cm^{-1} to 3500 cm^{-1}

increased, supporting the conclusion that all ester groups had been saponified. The strong azide absorption at 2100 cm^{-1} supported the hypothesis that the azides were stable towards the saponification and TEMPO oxidation reaction conditions employed. In the ^{13}C NMR spectrum (Figure 3.4a), a new resonance at 174 ppm was assigned to the carboxylate carbons, consistent with previously reported chemical shifts of uronic acid carboxylates.[51,52] In addition, a resonance at 49 ppm was consistent with the continued presence of C6 bearing azide groups, while the absence of acetate resonances (methyl groups at 20 ppm and ester carbonyl at 170 ppm) supported full saponification. Failure to detect a hydroxyl-bearing C6 resonance in the range of 55-65 ppm provided further support to the hypothesis that all free 6-OH groups were oxidized to carboxyls. The DS(carboxyl) was 0.49 by quantitative ^{13}C NMR, confirming virtually complete TEMPO oxidization (> 96%) of available 6-OH groups, since DS (6-OH) of the 6-N_3CA320S deacylation product was 0.51. We were also able to obtain a partially oxidized product by shorter TEMPO oxidation reaction time. The partially oxidized product showed FT-IR and ^{13}C NMR spectra similar to those of the fully oxidized product, except for the new ^{13}C NMR resonance assigned to C6 bearing a hydroxyl substituent at 59 ppm (Figure 3.4b). Ester saponification and introduction of the charged carboxylate groups significantly altered polymer solubility. While 6-N_3CA320S is soluble in polar aprotic solvents, such as DMSO, DMF, and DMAc, but insoluble in water, Glc6N_3/GlcA is soluble in water and insoluble in any organic solvent tested. This complicated the subsequent azide reduction, since such reductions are typically carried out in organic solvents.

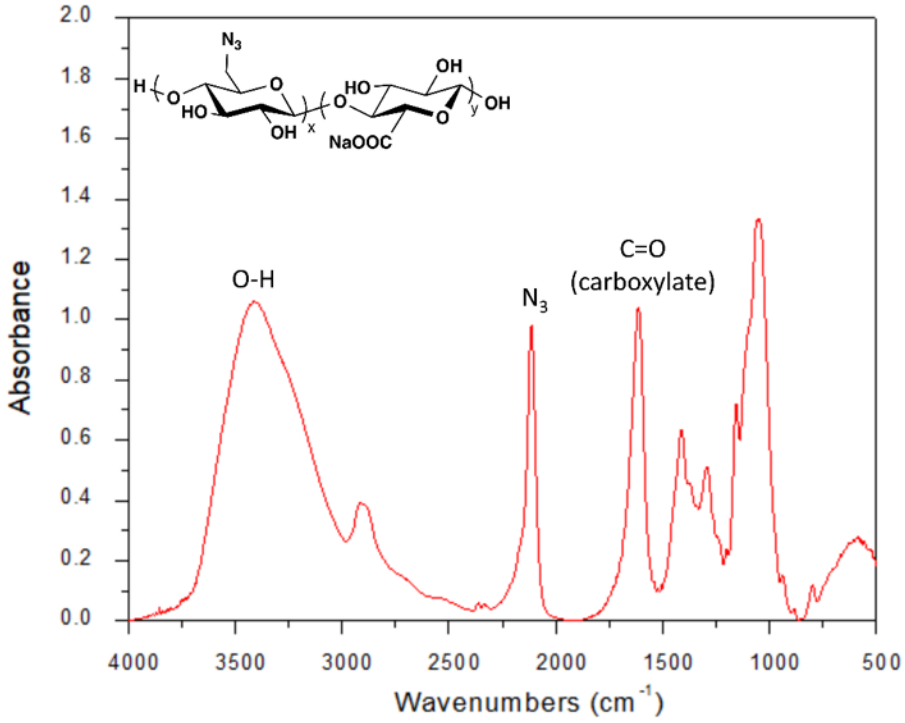


Figure 3.3 FT-IR spectrum of TEMPO oxidation product Glc6N_3/GlcA

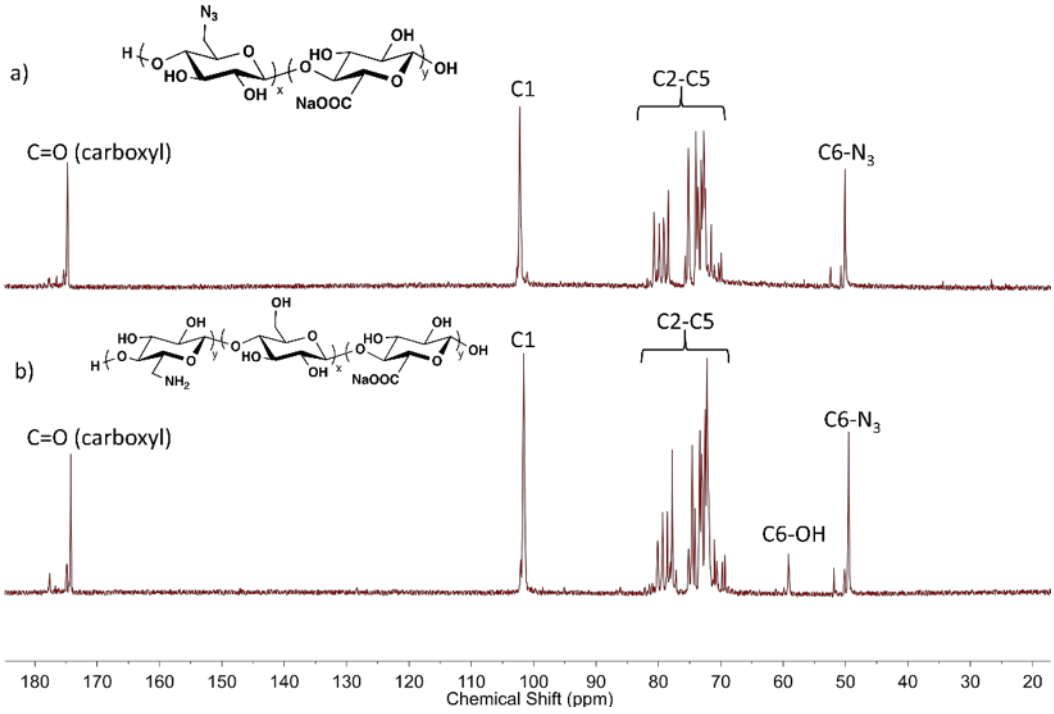


Figure 3.4 ^{13}C NMR spectrum of a) fully TEMPO oxidized product Glc6N_3/GlcA b) partially TEMPO oxidized product Glc6N_3/GlcA/Glc

3.3.2.2 Azide reduction by DTT

After successful ester saponification and oxidation of the resulting 6-OH groups to carboxylate in one pot, the only remaining step was to selectively reduce azides to amines and/or amides. Effective methods for reduction of azides attached to polysaccharides have included sodium borohydride ($NaBH_4$),[34,45] lithium aluminum hydride ($LiAlH_4$),[29] or Staudinger (PPh_3/H_2O) reductions[33,35]. These reagents usually work best in organic solvents, which can't be used in our case because Glc6N_3/GlcA is soluble only in water, and not in any of the (polar) organic solvents investigated (acetone, THF, chloroform, DMSO, DMAc, DMF, NMP). The chemoselectivity required by the presence of carboxylate groups is another important consideration. $NaBH_4$ and $LiAlH_4$ react with water at room temperature or above, so are not good candidates to reduce a polymer soluble only in water. Therefore we first investigated the approach of reducing the azides by Staudinger reaction in mixed water/cosolvent systems, including THF, DMAc and ethanol/DMAc as cosolvents. However, none of these water/cosolvent systems can dissolve both the water-soluble polymer and organic-soluble triphenylphosphine. We therefore sought a method to reduce azide to amine in water chemoselectively, without reducing carboxylate groups. It is well-known that thiols are useful reagents for reduction of azides to amines in small molecule organic chemistry.[53] In 1989, Daly and Lee reported reduction of 6-azido-6-deoxy cellulose esters to the corresponding amines by 1,3-propanedithiol.[54] Although these investigators only achieved a low conversion of azides to amines, these results inspired us to find a water-soluble thiol reagent as an alternative for our GAG analog azide reduction. 1,4-Dithiothreitol (DTT) is a water-soluble reagent that is frequently used in small molecule chemistry to reduce disulfide bonds[55,56] and azides[57,58]. However, application of DTT to reduce azide-bearing polysaccharides has not been reported previously.

We therefore treated Glc6N_3/GlcA with DTT in water, using mild conditions to maximize chemoselectivity (room temperature, 12 h). We were excited to find that ^{13}C NMR and FT-IR confirmed successful synthesis of our first designed GAG analog, Glc6N/GlcA. By ^{13}C NMR (Figure 3.5), the resonance for C6 bearing N_3 (49 ppm) was absent, while a new resonance at 39 ppm was assigned as the amine-bearing C6, indicating successful reduction of azide to amine. Retention of the carboxylate peak at 174 ppm supported the notion that no carboxylate groups had been reduced. By FT-IR (Figure 3.6), the strong azide peak formerly at 2100 cm^{-1} was absent, further supporting complete azide reduction. Positive confirmation of the presence of amines was important, since this reaction had not previously been applied to polysaccharides, and we wished to supplement standard spectroscopic techniques. We obtained positive confirmation by applying the Kaiser test, which is sensitive for primary amines.[59,60] In the Kaiser test, the color changed from light yellow to blue after heating (Figure S3.7a), supporting the presence of the expected primary 6-amino groups. Our preliminary kinetic study revealed that the DTT reduction was surprisingly fast, being complete within 2 h, as indicated by ^{13}C NMR and FT-IR. DTT provides many advantages over current polysaccharide azide reduction methods, owing to its high chemoselectivity, mild reaction conditions, and ease of operation. Reducing reagents $NaBH_4$ and $LiAlH_4$ usually require high temperatures and long reaction times. They are also known to reduce various functional groups, including esters.[61] Staudinger reaction is a mild and selective method to reduce azide to amine or amide, but the resulting aminated polysaccharide derivatives are frequently contaminated by residual triphenylphosphine and its oxide, which are extremely difficult to remove quantitatively from polysaccharides.

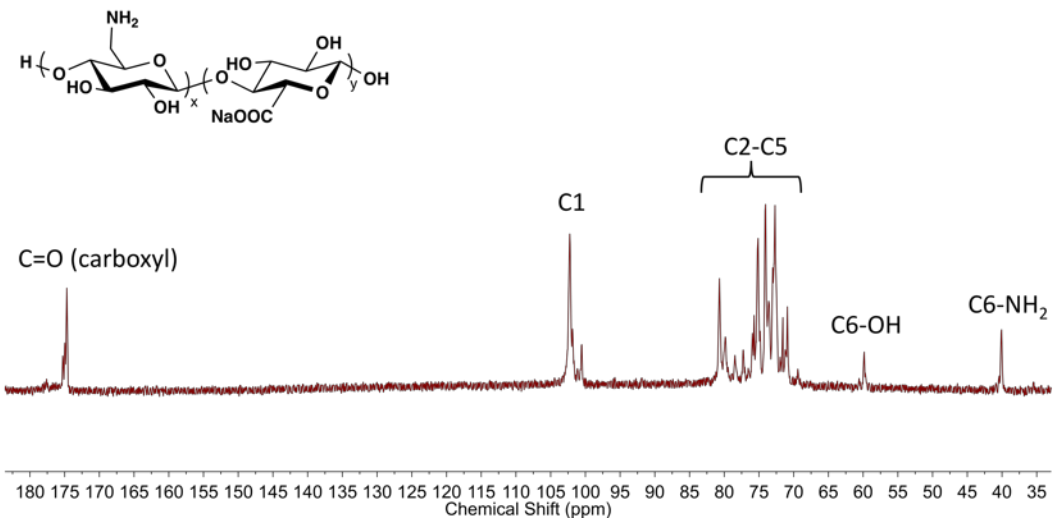


Figure 3.5 ^{13}C NMR spectrum of Glc6N/GlcA

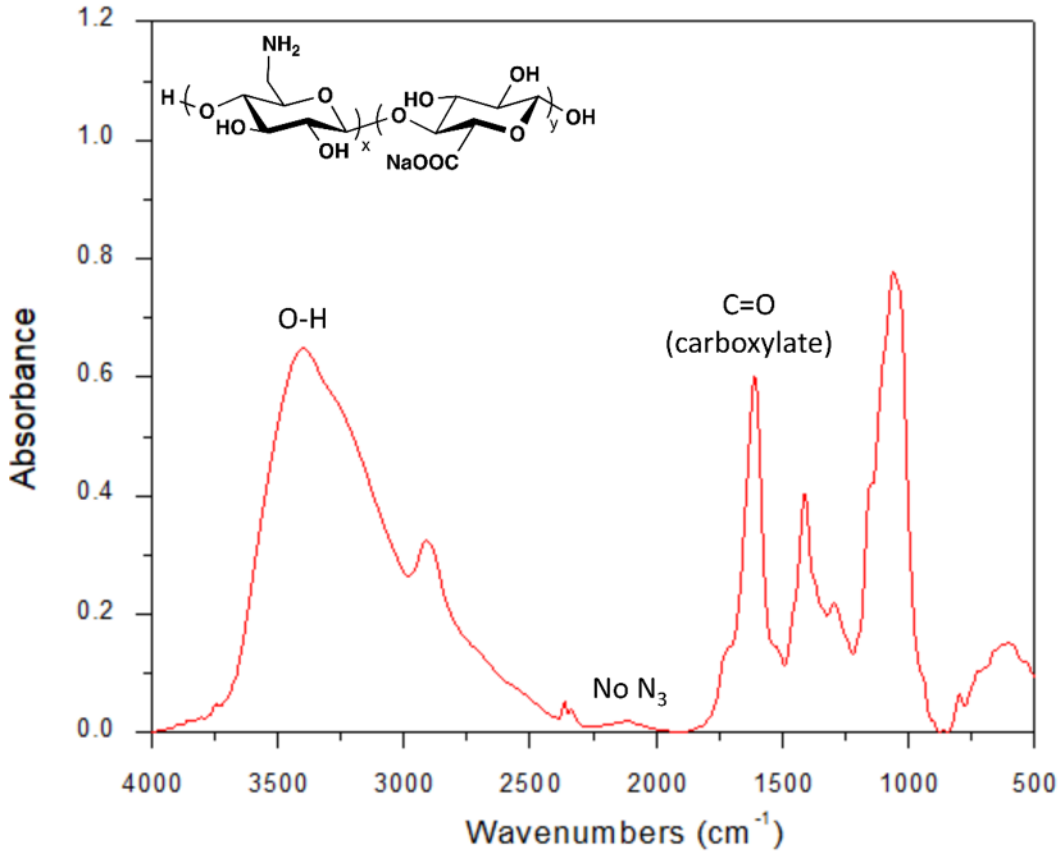


Figure 3.6 FT-IR spectrum of Glc6N/GlcA

3.3.2.3 Azide reduction by thioacetic acid

While heparin contains 2-amino-2-deoxyglucose monosaccharides (many of which are N-sulfated), most other GAGs have 2-acetamido-2-deoxy moieties. Thus, the ability to be able to reduce our azido moieties directly to amides would afford GAG analogs of significant interest. Although amides can be obtained by Staudinger reaction of polysaccharide azides in the presence of carboxylic anhydrides[35,36,46], or likely by selective acylation of the 6-amino groups of Glc6N/GlcA, these methods would be difficult to apply in the present case due to the solvent insolubility of Glc6N_3/GlcA. It has been shown in small molecule organic[62] and in carbohydrate chemistry[63] that thioacetic acid can selectively reduce azide to acetamido, without forming amine as the intermediate.[64,65] We therefore hypothesized that thioacetic acid would selectively reduce azide to acetamido in polysaccharides (though frequently small molecule reactions don't work well for recalcitrant polysaccharides).

We thus explored treatment of Glc6N_3/GlcA with thioacetic acid, again choosing relatively mild conditions to promote chemoselectivity (pH 7.4 buffer, 65 °C, 48 h). We were surprised to find that the azides were reduced directly to an amine/amide mixture, rather than only to amides. A new ^{13}C NMR resonance (Figure 3.7) appeared at 46 ppm, assigned to the acetamido-substituted C-6, while the former C6-N_3 resonance at 50 ppm was absent. A new ^{13}C NMR resonance appeared at 22 ppm, assigned as the *N*-acetyl methyl groups, further confirming successful reduction to acetamido groups. Surprisingly, amine-substituted C-6 was also observed at 40 ppm. We were happy to observe the absence of acetate carbonyl signals at around 170 ppm, eliminating the possibility that *O*-acylation by thioacetic acid may have occurred,[66] perhaps due to the low reactivity of hydroxyl groups in aqueous conditions. The presence of amine in the product was further confirmed by the color change from light yellow to blue after heating in the Kaiser test

(Figure S3.7b). In the FT-IR spectrum (Figure S3.8), no azide absorption at 2100 cm^{-1} was observed, indicating complete azide reduction. A new ^{1}H NMR (Figure S3.9) resonance appeared in the range of 1.9-2.0 ppm, assigned to acetamido methyl groups. DS (NHAc) was 0.35 by ^{1}H NMR integration. Considering that DS (N_3) was 0.49 in the starting material, DS (NH_2) was calculated to be 0.14 by difference; thus conversion to amides was ca. 71%, and conversion to amines was ca. 29%. The observed partial conversion to amine is particularly interesting, since the thioacetic acid reduction mechanism has been well-established for small molecules and does not involve formation of amine as the intermediate.[64,65] Typically, the reaction involves a [3+2] cycloaddition to form a thiatriazoline (five membered ring) intermediate, followed by a retro [3+2] cycloaddition to afford a hydroxyimine (Scheme 3.3). The hydroxyimine then tautomerizes to give an amide as the final product. We propose the possible mechanistic explanation that in our case, the imines also undergo competitive hydrolysis to form amines under aqueous condition. This proposed explanation is in good agreement with our observation that formation of amides dominates over formation of amines, since one would expect tautomerization to be faster than bimolecular imine hydrolysis. It is, however, unproven, and will require additional experimentation to reveal the true mechanism for obtaining an amide/amine mixture. The novel conversion of azide to a mixture of amines and amides by thioacetic acid provides a very interesting GAG analog, which bears amine, amide, and carboxyl groups and has structural similarity to unepimerized and unsulfated heparin.

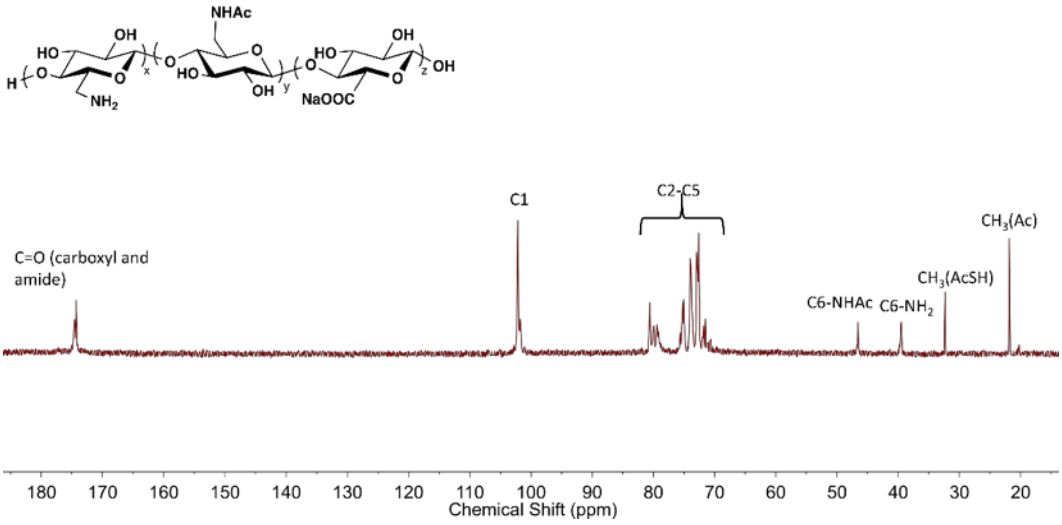
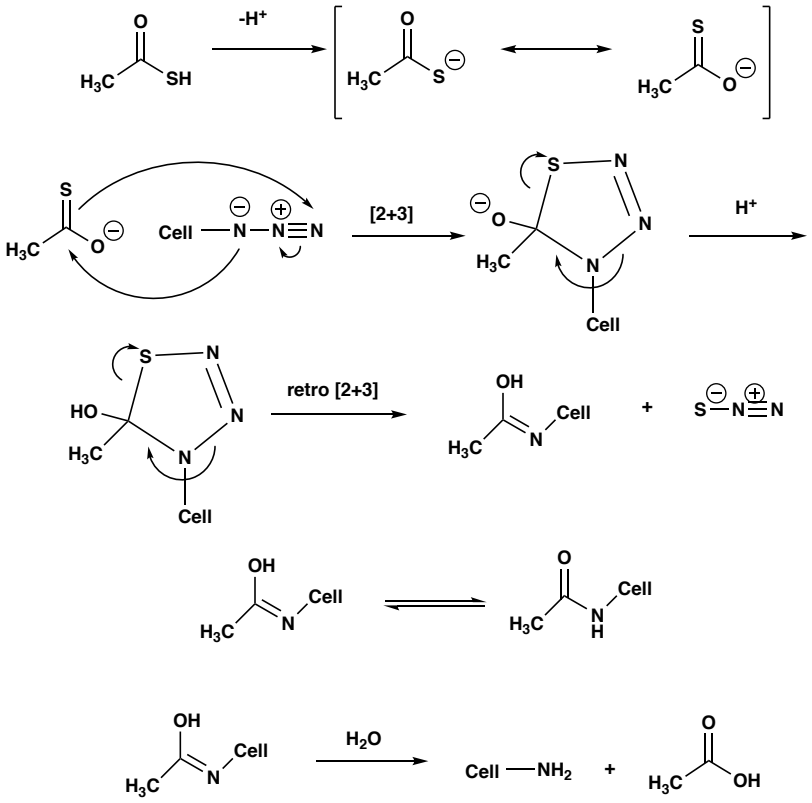


Figure 3.7 ^{13}C NMR spectrum of Glc6NAc/GlcA/Glc6N



Scheme 3.3 Proposed mechanism of thioacetic acid reduction

Sample	DP	DS	Solubility[a]
CA320S	131.2[b]	DS (Ac) = 1.75[c]	DMSO, DMF, DMAc
6-BrCA320S		DS (Br) = 0.49[d]	DMSO, DMF, DMAc
6-N_3CA320S	131.0[b]	DS (N_3) = 0.49[d]	DMSO, DMF, DMAc
Glc6N_3/GlcA	NA[e]	DS (COONa) = 0.49[f]	H_2O
Glc6N/GlcA	NA[e]		H_2O
Glc6NAc/GlcA/Glc6N	NA[e]	DS (NHAc) = 0.35[c]	H_2O

Table 3.1 Summary of starting material, all intermediates, and final products

[a] Solubility was tested at the concentration of 5 mg/ mL. [b] Measured by SEC in DMAc [c] Measured by ^{1}H NMR spectroscopy. [d] Measured by elemental analysis. [e] We were unable to get a useful MW by aqueous SEC due to strong aggregation observed in that solvent. We also peracylated these water-soluble polymers to enhance organic solvent solubility, in order to permit measurement of molecular weight by solvent SEC. However, strong aggregation of the peracetylated derivatives was also observed, preventing us from getting useful data by SEC in a DMAc system. [f] Measured by quantitative ^{13}C NMR spectroscopy

3.4 Conclusions

In this work, we describe an unsuccessful attempt, and then a successful design of a synthetic route to GAG analogs, which bear both amine and carboxyl groups, or a combination of

amines/amides and carboxyl groups. These analogs have DS ca. 0.5 of both amine/amides and carboxyl groups, as in natural GAGs, as a result of starting with the readily available, relatively inexpensive commercial cellulose acetate, CA320S, which has DS(Ac) at C6 of almost exactly 0.5. The successful route involved regiospecific C6 bromination and azide displacement, installing the amine/amide precursor at C6. Saponification of the acetyl moieties exposed the remaining C6 oxygen moieties as primary hydroxyls susceptible to TEMPO oxidation, which was carried out with very high chemoselectivity, without the need to isolate or purify the saponification product, to afford a water soluble polymer. Finally, we carried out azide reduction by DTT in water, applied herein for the first time to polysaccharides, to accomplish smooth reduction of azides to amines, and afford a GAG analog that mimicks the heparin backbone. Separately, we found that thioacetic acid reduction, again being applied for the first time in polysaccharide chemistry, reduced azide to the corresponding acetamide, but with a significant side "reduction" to amine (though mechanistically likely an imine hydrolysis), thus producing an interesting, new GAG analog that contains both amines and amides. Our original hypothesis that we could synthesize GAG analogs from cellulose acetate by regioselective modifications was proved correct, and was even extended by the reduction chemistry we discovered. It is also noteworthy that every step from the azide onward was carried out in aqueous media, providing a relatively green, selective, efficient pathway from a cellulose ester to these analogs.

We are exploring the biological activities of these synthesized analogs, and will report structure-activity insights in future publications. The amine-containing materials also have potential for formation of hydrogels that would have promise in tissue engineering, controlled release, and many other applications. We are also interested in exploring subsequent, regioselective sulfation of these GAG analogs, which should permit synthesis of a library of GAG

analogs (e.g. *N*-sulfated, *O*-sulfated, and/or *N*,*O*-sulfated) that even more closely resemble bioactive GAGs like HS, CS, and DS, allowing us to study GAG analog structure activity relationships systematically. Caution is necessary in exploring biological effects and possible toxicity of these semi-synthetic GAG analogs, since certain unnatural GAG analogs (e.g. oversulfated chondroitin sulfate) have been shown to be highly toxic.[14] It will also be of interest to further explore and delineate the potential of the reducing reagents DTT and thioacetic acid for azide reduction in polysaccharide chemistry.

Supporting information

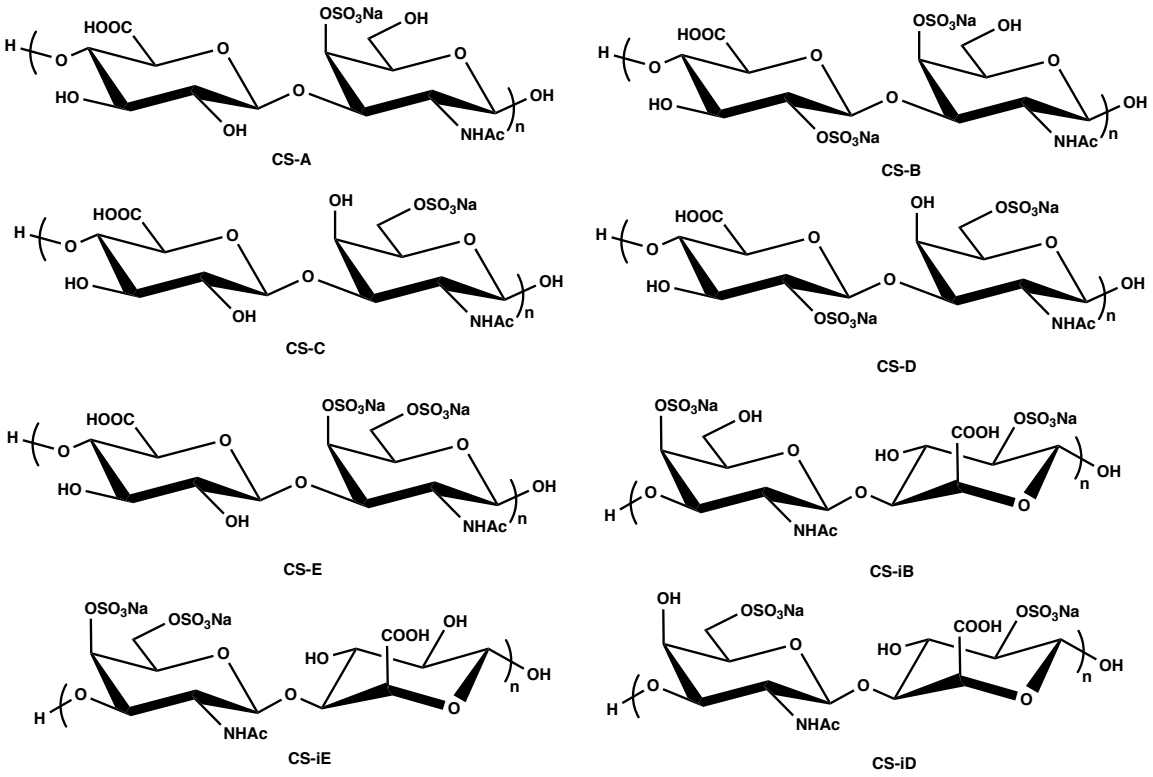


Figure S3.1. Eight common CS/DS dyads

Perpropionylation of CA320S

Cellulose acetate (CA320S) was perpropionylated using methods adapted from previous studies. In a 50 mL round-bottom flask, CA320S (0.2 g) was dissolved in 4 mL of pyridine at 80 ˚C under nitrogen. 4-Dimethylaminopyridine (20 mg) and propionic anhydride (4 mL) were slowly added to the solution and stirred at 80 ˚C for 24 h. The reaction mixture was then cooled to room temperature and slowly poured into ethanol (200 mL). The precipitate was isolated by filtration, then re-dissolved in acetone (10 mL) and re-precipitated in ethanol (100 mL). The sample was collected by filtration and dried in a vacuum oven at 40 °C to yield perpropionylated CA320S.

Table S3.1 Positional Degrees of Substitution (DS) of Perpropionylated CA320S.

	DS(Ac)	DS(Pr)
6-	0.53	0.49
2,3-	0.84	1.19

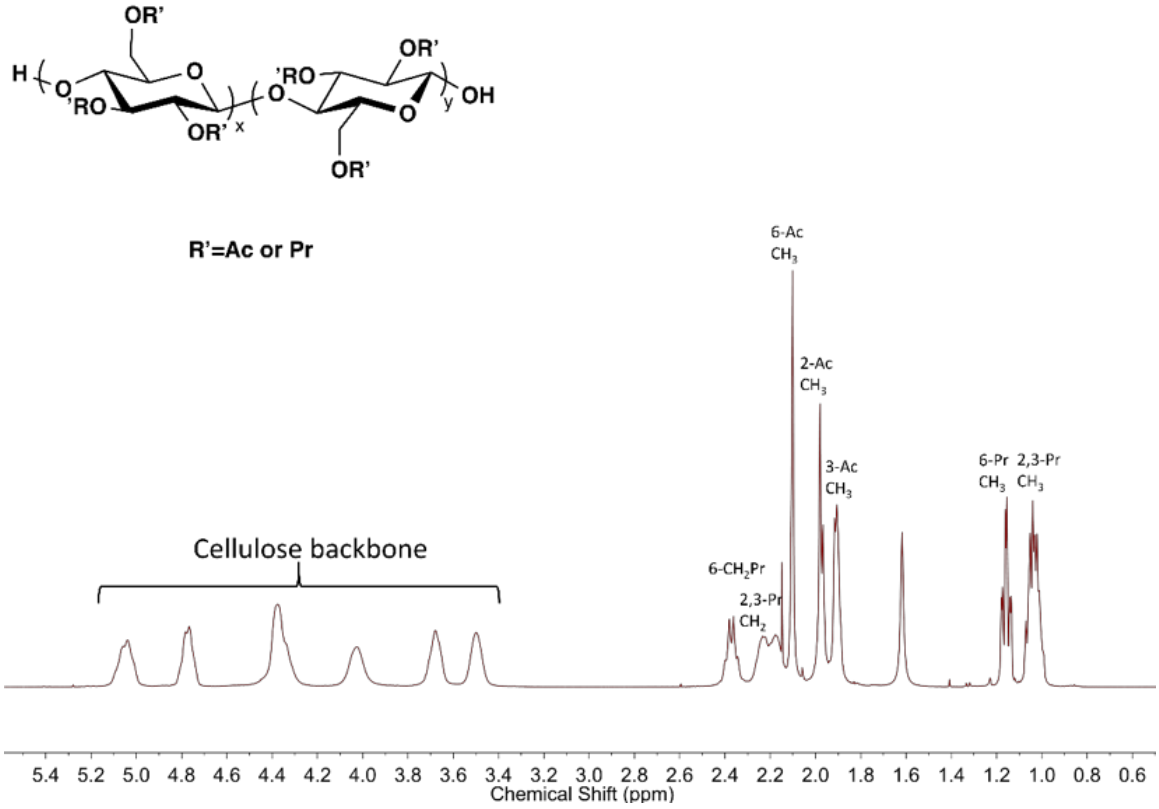


Figure S3.2. ^{1}H NMR spectrum of perpropionylated CA320S

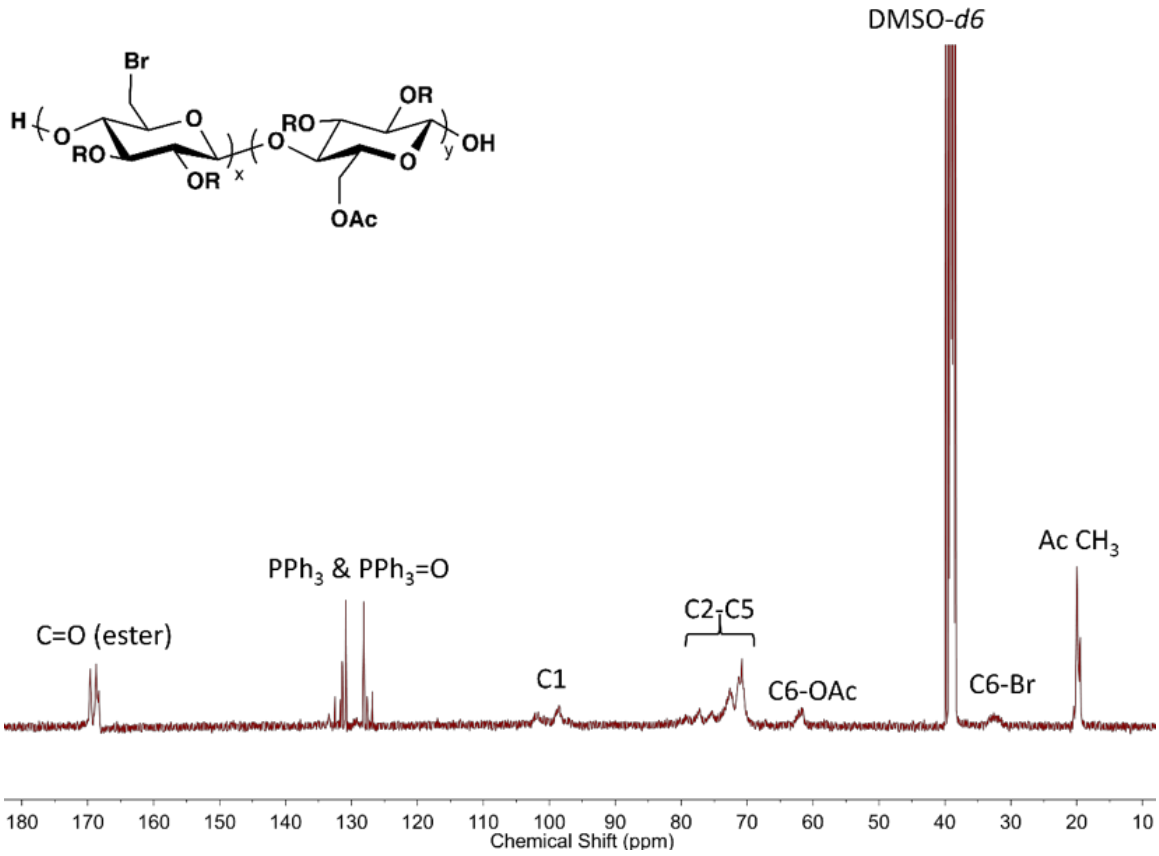


Figure S3.3. ^{13}C NMR spectrum of 6-BrCA320S

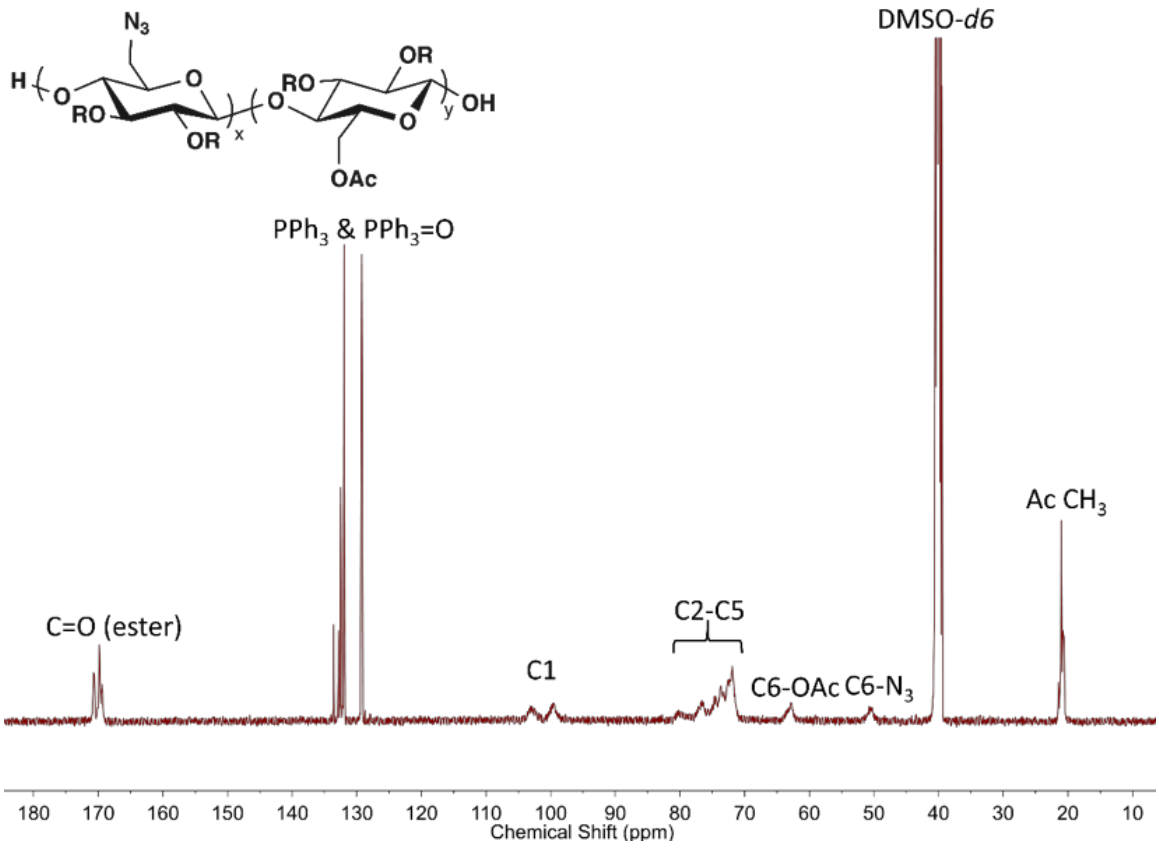


Figure S3.4. ^{13}C NMR spectrum of 6-N_3CA320S

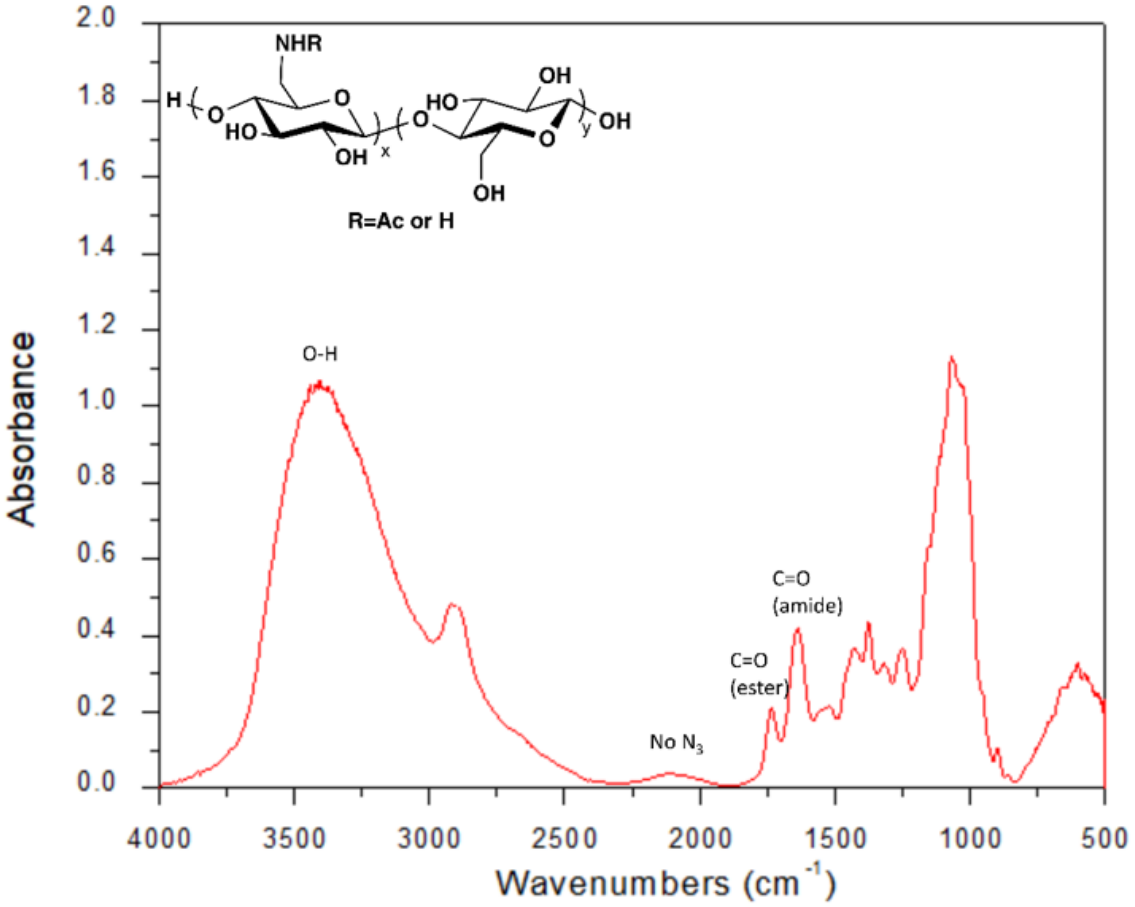


Figure S3.5. FT-IR spectrum of $NaBH_4$ reduction product obtained by synthetic route 1

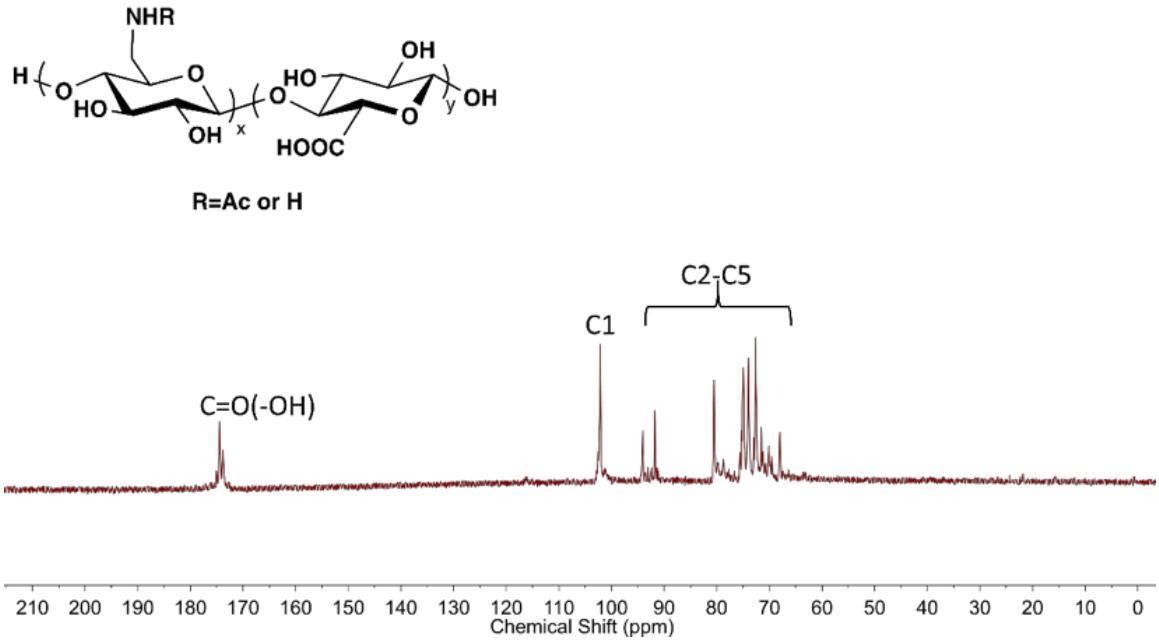
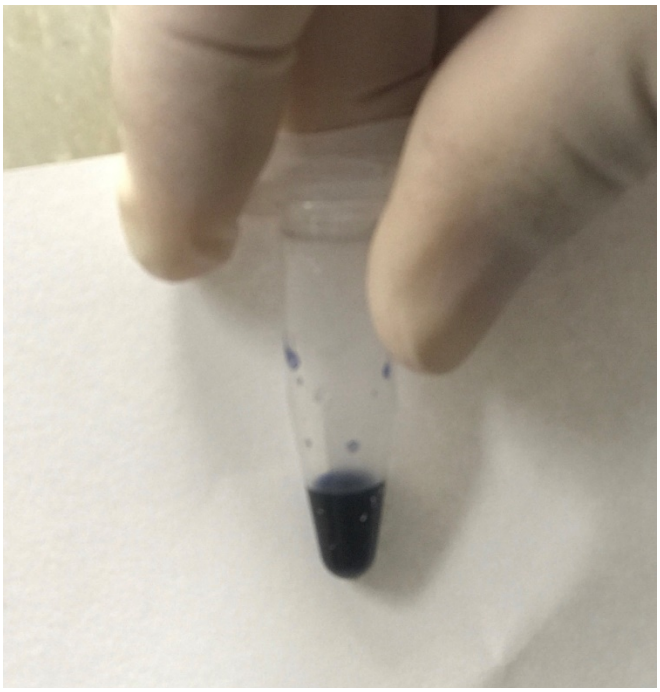


Figure S3.6. ^{13}C NMR spectrum of TEMPO oxidation product obtained by synthetic route 1

a)



b)

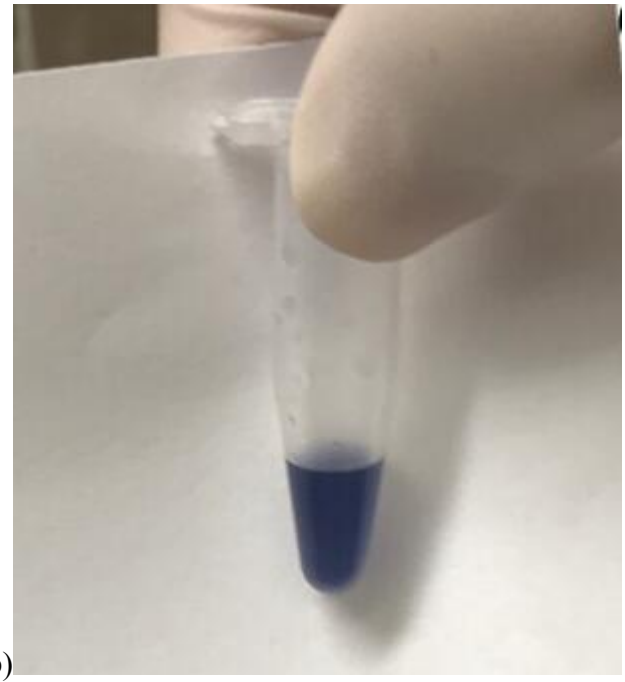


Figure S3.7. Kaiser test result of a) Glc6N/GlcA b) Glc6NAc/GlcA/Glc6N after heating

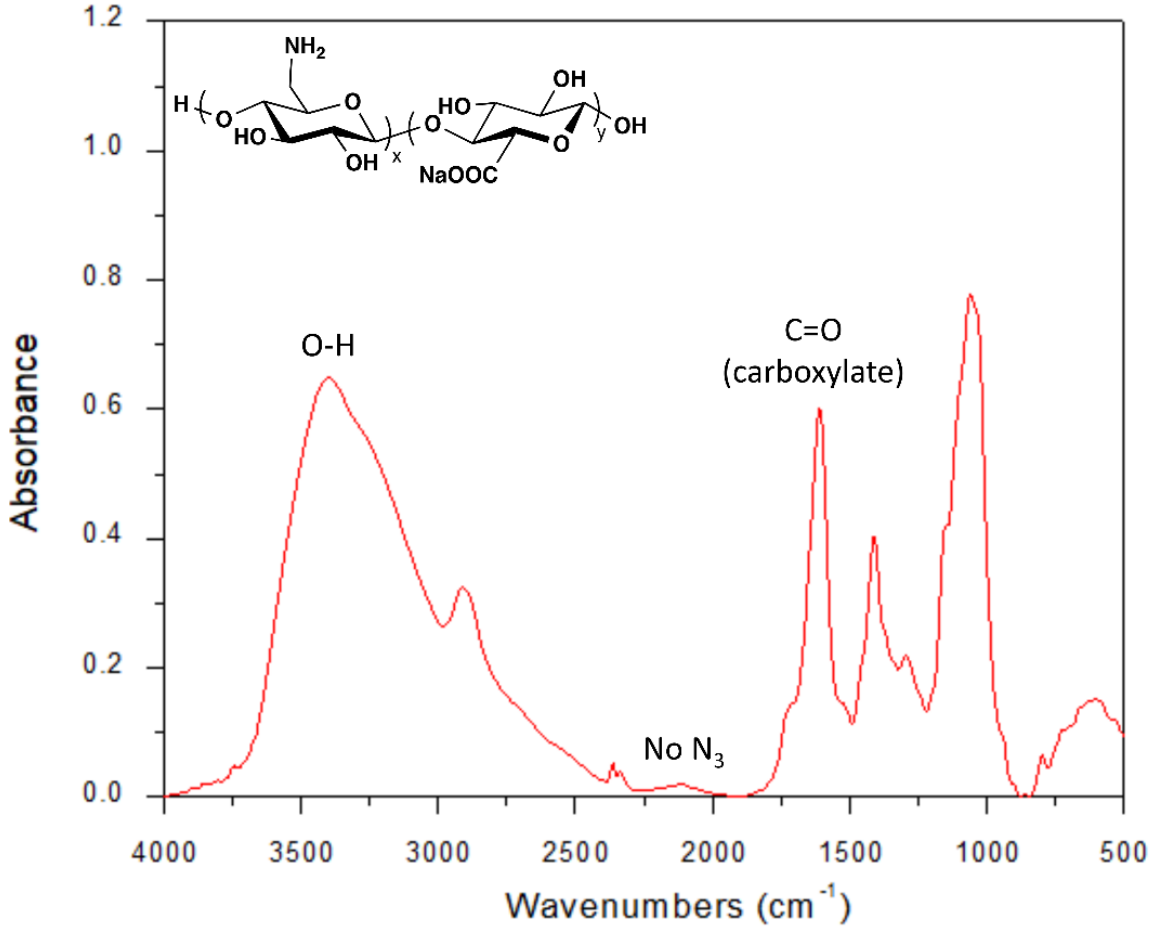


Figure S3.8. FT-IR spectrum of Glc6NAc/GlcA/Glc6N

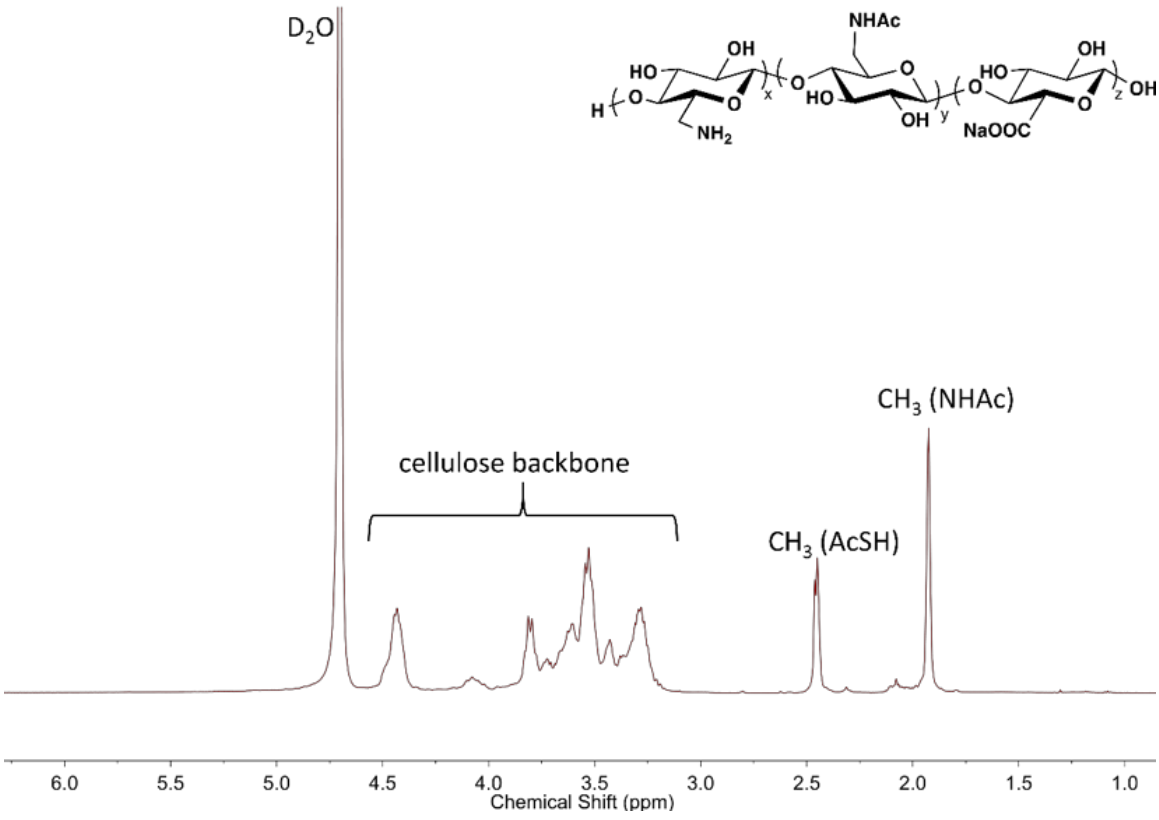


Figure S3.9. [1]H NMR spectrum of Glc6NAc/GlcA/Glc6N

Reference

(1) Teien, A. N.; Abildgaard, U.; Höök, M. The Anticoagulant Effect of Heparan Sulfate and Dermatan Sulfate. Thromb. Res. 1976, 8 (6), 859–867.

(2) Pulsipher, A.; Griffin, M. E.; Stone, S. E.; Brown, J. M.; Hsieh-Wilson, L. C. Directing Neuronal Signaling through Cell-Surface Glycan Engineering. J. Am. Chem. Soc. 2014, 136 (19), 6794–6797.

(3) Gopal, S.; Bober, A.; Whiteford, J. R.; Multhaupt, H. A. B.; Yoneda, A.; Couchman, J. R. Heparan Sulfate Chain Valency Controls Syndecan-4 Function in Cell Adhesion. J. Biol. Chem. 2010, 285 (19), 14247–14258.

(4) Bucior, I.; Burger, M. M. Carbohydrate–Carbohydrate Interactions in Cell Recognition. Curr. Opin. Struct. Biol. 2004, 14 (5), 631–637.

(5) Zhang, Z.; Christopher, G. F. The Nonlinear Viscoelasticity of Hyaluronic Acid and Its Role in Joint Lubrication. Soft Matter 2015, 11 (13), 2596–2603.

(6) Zhang, F.; Zhang, Z.; Linhardt, R. J. Glycosaminoglycans. In Handbook of Glycomics; Elsevier, 2010; pp 59–80.

(7) Gandhi, N. S.; Mancera, R. L. The Structure of Glycosaminoglycans and Their Interactions with Proteins. Chem. Biol. Drug Des. 2008, 72 (6), 455–482.

(8) Soares da Costa, D.; Reis, R. L.; Pashkuleva, I. Sulfation of Glycosaminoglycans and Its Implications in Human Health and Disorders. Annu. Rev. Biomed. Eng. 2017, 19 (1), 1–26.

(9) Mousavi, S.; Moradi, M.; Khorshidahmad, T.; Motamedi, M. Anti-Inflammatory Effects of Heparin and Its Derivatives: A Systematic Review. Adv. Pharmacol. Sci. 2015, 2015, 507151.

(10) Durant, G. J.; Hendrickson, H. R.; Montgomery, R. Studies on the Structure of Heparin. Arch. Biochem. Biophys. 1962, 99 (3), 418–425.

(11) Chang, C.-H.; Lico, L. S.; Huang, T.-Y.; Lin, S.-Y.; Chang, C.-L.; Arco, S. D.; Hung, S.-C. Synthesis of the Heparin-Based Anticoagulant Drug Fondaparinux. Angew. Chemie Int. Ed. 2014, 53 (37), 9876–9879.

(12) Fu, L.; Suflita, M.; Linhardt, R. J. Bioengineered Heparins and Heparan Sulfates ☆. Adv. Drug Deliv. Rev. 2016, 97, 237–249.

(13) Datta, P.; Linhardt, R. J.; Sharfstein, S. T. Industrial Production of Glycosaminoglycans. In Reference Module in Life Sciences; Elsevier, 2017.

(14) Guerrini, M.; Beccati, D.; Shriver, Z.; Naggi, A.; Viswanathan, K.; Bisio, A.; Capila, I.; Lansing, J. C.; Guglieri, S.; Fraser, B.; et al. Oversulfated Chondroitin Sulfate Is a Contaminant in Heparin Associated with Adverse Clinical Events. Nat Biotechnol 2008, 26 (6), 669–675.

(15) Mende, M.; Bednarek, C.; Wawryszyn, M.; Sauter, P.; Biskup, M. B.; Schepers, U.; Bräse, S. Chemical Synthesis of Glycosaminoglycans. Chem. Rev. 2016, 116 (14), 8193–8255.

(16) DeAngelis, P. L.; Liu, J.; Linhardt, R. J. Chemoenzymatic Synthesis of Glycosaminoglycans: Re-Creating, Re-Modeling and Re-Designing Nature's Longest or Most Complex Carbohydrate Chains. Glycobiology 2013, 23 (7), 764–777.

(17) Xu, Y.; Masuko, S.; Takieddin, M.; Xu, H.; Liu, R.; Jing, J.; Mousa, S. A.; Linhardt, R. J.; Liu, J. Chemoenzymatic Synthesis of Homogeneous Ultralow Molecular Weight Heparins. Science 2011, 334 (6055), 498–501.

(18) Xu, Y.; Cai, C.; Chandarajoti, K.; Hsieh, P.-H.; Li, L.; Pham, T. Q.; Sparkenbaugh, E. M.; Sheng, J.; Key, N. S.; Pawlinski, R.; et al. Homogeneous Low-Molecular-Weight Heparins with Reversible Anticoagulant Activity. Nat. Chem. Biol. 2014, 10 (4), 248–250.

(19) Zhang, X.; Pagadala, V.; Jester, H. M.; Lim, A. M.; Pham, T. Q.; Goulas, A. M. P.; Liu, J.; Linhardt, R. J. Chemoenzymatic Synthesis of Heparan Sulfate and Heparin Oligosaccharides and NMR Analysis: Paving the Way to a Diverse Library for Glycobiologists. Chem. Sci. 2017, 8 (12), 7932–7940.

(20) Bedini, E.; De Castro, C.; De Rosa, M.; Di Nola, A.; Iadonisi, A.; Restaino, O. F.; Schiraldi, C.; Parrilli, M. A Microbiological-Chemical Strategy to Produce Chondroitin Sulfate A,C. Angew. Chemie Int. Ed. 2011, 50 (27), 6160–6163.

(21) Bedini, E.; De Castro, C.; De Rosa, M.; Di Nola, A.; Restaino, O. F.; Schiraldi, C.; Parrilli, M. Semi-Synthesis of Unusual Chondroitin Sulfate Polysaccharides Containing GlcA(3-O-Sulfate) or GlcA(2,3-Di-O-Sulfate) Units. Chem. - A Eur. J. 2012, 18 (7), 2123–2130.

(22) Laezza, A.; Iadonisi, A.; Castro, C. De; De Rosa, M.; Schiraldi, C.; Parrilli, M.; Bedini, E. Chemical Fucosylation of a Polysaccharide: A Semisynthetic Access to Fucosylated Chondroitin Sulfate. Biomacromolecules 2015, 16 (7), 2237–2245.

(23) Wang, Z.; Ly, M.; Zhang, F.; Zhong, W.; Suen, A.; Hickey, A. M.; Dordick, J. S.; Linhardt, R. J. E. Coli K5 Fermentation and the Preparation of Heparosan, a Bioengineered Heparin Precursor. Biotechnol. Bioeng. 2010, 107 (6), 964–973.

(24) Cimini, D.; Restaino, O. F.; Catapano, A.; De Rosa, M.; Schiraldi, C. Production of Capsular Polysaccharide from Escherichia Coli K4 for Biotechnological Applications. Appl. Microbiol. Biotechnol. 2010, 85 (6), 1779–1787.

(25) Zou, Y.; Khor, E. Preparation of Sulfated-Chitins under Homogeneous Conditions. Carbohydr. Polym. 2009, 77 (3), 516–525.

(26) Baumann, H.; Faust, V. Concepts for Improved Regioselective Placement of O-Sulfo, N-Sulfo, N-Acetyl, and N-Carboxymethyl Groups in Chitosan Derivatives. Carbohydr. Res. 2001, 331 (1), 43–57.

(27) Zhang, K.; Helm, J.; Peschel, D.; Gruner, M.; Groth, T.; Fischer, S. NMR and FT Raman Characterisation of Regioselectively Sulfated Chitosan Regarding the Distribution of Sulfate Groups and the Degree of Substitution. Polymer (Guildf). 2010, 51 (21), 4698–4705.

(28) Zhang, K.; Peschel, D.; Klinger, T.; Gebauer, K.; Groth, T.; Fischer, S. Synthesis of Carboxyl Cellulose Sulfate with Various Contents of Regioselectively Introduced Sulfate and Carboxyl Groups. Carbohydr. Polym. 2010, 82 (1), 92–99.

(29) Liu, C.; Baumann, H. Exclusive and Complete Introduction of Amino Groups and Their N-Sulfo and N-Carboxymethyl Groups into the 6-Position of Cellulose without the Use of Protecting Groups. Carbohydr. Res. 2002, 337 (14), 1297–1307.

(30) Borjihan, G.; Zhong, G.; Baigude, H.; Nakashima, H.; Uryu, T. Synthesis and Anti-HIV Activity of 6-amino-6-deoxy-(1→ 3)-β-D-curdlan Sulfate. Polym. Adv. Technol. 2003, 14 (3-5), 326–329.

(31) Tamaru, S.; Tokunaga, D.; Hori, K.; Matsuda, S.; Shinkai, S. Giant Amino Acids Designed on the Polysaccharide Scaffold and Their Protein-like Structural Interconversion. Org. Biomol. Chem. 2014, 12 (5), 815–822.

(32) Klemm, D.; Heublein, B.; Fink, H.-P. H.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie Int. Ed. 2005, 44 (22), 3358–3393.

(33) Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H. Preparation of Amylose Derivatives Selectively Modified at C-6. 6-Amino-6-Deoxyamylose. Macromolecules 1994, 27 (11), 2917–2922.

(34) Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F. Facile Synthesis of 6-Amino-6-Deoxycellulose. Carbohydr. Res. 2005, 340 (7), 1403–1406.

(35) Fox, S. C.; Edgar, K. J. Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O -Acylated 6-Amino-6-Deoxy-Cellulose. Biomacromolecules 2012, 13 (4), 992–1001.

(36) Zhang, R.; Edgar, K. J. Synthesis of Curdlan Derivatives Regioselectively Modified at C-6: O-(N)-Acylated 6-Amino-6-Deoxycurdlan. Carbohydr. Polym. 2014, 105, 161–168.

(37) Tahiri, C.; Vignon, M. R. TEMPO-Oxidation of Cellulose: Synthesis and Characterisation of Polyglucuronans. Cellulose 2000, 7 (2), 177–188.

(38) Saito, T.; Isogai, A. TEMPO-Mediated Oxidation of Native Cellulose. The Effect of Oxidation Conditions on Chemical and Crystal Structures of the Water-Insoluble Fractions. Biomacromolecules 2004, 5 (5), 1983–1989.

(39) Gomez-Bujedo, S.; Etienne Fleury, A.; Vignon, M. R. Preparation of Cellouronic Acids and Partially Acetylated Cellouronic Acids by TEMPO/NaClO Oxidation of Water-Soluble Cellulose Acetate. Biomacromolecules 2004, 5 (2), 565–571.

(40) Liu, S.; Edgar, K. J. Water-Soluble Co-Polyelectrolytes by Selective Modification of Cellulose Esters. Carbohydr. Polym. 2017, 162, 1–9.

(41) Gao, C.; Liu, S.; Edgar, K. J. Regioselective Chlorination of Cellulose Esters by Methanesulfonyl Chloride. Carbohydr. Polym. 2018, 193, 108–118.

(42) Fan, Y.; Saito, T.; Isogai, A. Chitin Nanocrystals Prepared by TEMPO-Mediated Oxidation of α-Chitin. Biomacromolecules 2008, 9 (1), 192–198.

(43) Kato, Y.; Kaminaga, J.; Matsuo, R.; Isogai, A. TEMPO-Mediated Oxidation of Chitin, Regenerated Chitin and N-Acetylated Chitosan. Carbohydr. Polym. 2004, 58 (4), 421–426.

(44) Furuhata, K.; Koganei, K.; Chang, H.-S.; Aoki, N.; Sakamoto, M. Dissolution of Cellulose in Lithium Bromide-Organic Solvent Systems and Homogeneous Bromination of Cellulose with N-Bromosuccinimide Triphenylphosphine in Lithium Bromide - N,N-Dimethylacetamide. Carbohydr. Res. 1992, 230, 165–177.

(45) Zhang, R.; Edgar, K. J. Water-Soluble Aminocurdlan Derivatives by Chemoselective Azide Reduction Using NaBH 4. Carbohydr. Polym. 2015, 122, 84–92.

(46) Pereira, J. M.; Edgar, K. J. Regioselective Synthesis of 6-Amino- and 6-Amido-6-Deoxypullulans. Cellulose 2014, 21 (4), 2379–2396.

(47) Cao, P.; Raleigh, D. P. Ester to Amide Switch Peptides Provide a Simple Method for Preparing Monomeric Islet Amyloid Polypeptide under Physiologically Relevant Conditions and Facilitate Investigations of Amyloid Formation. J. Am. Chem. Soc. 2010, 132 (12), 4052–4053.

(48) Sohma, Y.; Hayashi, Y.; Skwarczynski, M.; Hamada, Y.; Sasaki, M.; Kimura, T.; Kiso, Y. O?N Intramolecular Acyl Migration Reaction in the Development of Prodrugs and the Synthesis of Difficult Sequence-Containing Bioactive Peptides. Biopolymers 2004, 76 (4), 344–356.

(49) Sproviero, J. F. Stereospecificity of the O→N Acyl Migration in 2,3,4,6-Tetra-O-Benzoyl-β-D-Mannopyranosylamine. Carbohydr. Res. 1973, 26 (2), 357–363.

(50) Bragd, P. L.; van Bekkum, H.; Besemer, A. C. TEMPO-Mediated Oxidation of Polysaccharides: Survey of Methods and Applications. Top. Catal. 2004, 27 (1–4), 49–66.

(51) Spatareanu, A.; Bercea, M.; Budtova, T.; Harabagiu, V.; Sacarescu, L.; Coseri, S. Synthesis, Characterization and Solution Behaviour of Oxidized Pullulan. Carbohydr. Polym. 2014, 111, 63–71.

(52) Tahiri, C.; Vignon, M. R. TEMPO-Oxidation of Cellulose: Synthesis and Characterisation of Polyglucuronans. Cellulose 2000, 7 (2), 177–188.

(53) Bräse, S.; Gil, C.; Knepper, K.; Zimmermann, V. Organic Azides: An Exploding Diversity of a Unique Class of Compounds. Angew. Chemie Int. Ed. 2005, 44 (33), 5188–5240.

(54) Daly, W. H.; Soo, L. Peptide Graft Copolymers from Soluble Aminodeoxycellulose Acetate. In American Chemical Society, Polymer Preprints, Division of Polymer Chemistry; 1989.

(55) Konigsberg, W. [13] Reduction of Disulfide Bonds in Proteins with Dithiothreitol. Methods Enzymol. 1972, 25, 185–188.

(56) Zhang, Q.; Aleksanian, S.; Noh, S. M.; Oh, J. K. Thiol-Responsive Block Copolymer Nanocarriers Exhibiting Tunable Release with Morphology Changes. Polym. Chem. 2013, 4 (2), 351–359.

(57) Staros, J. V.; Bayley, H.; Standring, D. N.; Knowles, J. R. Reduction of Aryl Azides by Thiols: Implications for the Use of Photoaffinity Reagents. Biochem. Biophys. Res. Commun. 1978, 80 (3), 568–572.

(58) Baker, N. D.; Griffin, R. J.; Irwin, W. J.; Slack, J. A. The Reduction of Aryl Azides by Dithiothreitol: A Model for Bioreduction of Aromatic Azido-Substituted Drugs. Int. J. Pharm. 1989, 52 (3), 231–238.

(59) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. Anal. Biochem. 1970, 34 (2), 595–598.

(60) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. Quantitative Monitoring of Solid-Phase Peptide Synthesis by the Ninhydrin Reaction. Anal. Biochem. 1981, 117 (1), 147–157.

(61) Brown, M. S.; Rapoport, H. The Reduction of Esters with Sodium Borohydride 1. J. Org. Chem. 1963, 28 (11), 3261–3263.

(62) Rosen, T.; Lico, I. M.; Chu, D. T. W. A Convenient and Highly Chemoselective Method for the Reductive Acetylation of Azides. J. Org. Chem. 1988, 53 (7), 1580–1582.

(63) Vogel, P.; Robina, I. Synthesis of Monosaccharides and Analogs. In Comprehensive Glycoscience; Elsevier, 2007; pp 489–582.

(64) Robert, K. V; Ning, S.; Ronald, S. R.; Lawrence, W. J. Mechanism of Thio Acid/Azide Amidation. J. Am. Chem. Soc. 2006, 128 (17), 5695–5702.

(65) Ning, S.; Sreenivas, K.; Rachel, G.; Lawrence, W. J. The Reaction of Thio Acids with Azides: A New Mechanism and New Synthetic Applications. J. Am. Chem. Soc. 2003, 125 (26), 7754–7755.

(66) Kumar, S.; Kohli, K. Chemical Modification of Wood: Reaction with Thioacetic Acid and Its Effect on Physical and Mechanical Properties and Biological Resistance. In Renewable-Resource Materials; Springer US: Boston, MA, 1986; pp 147–160.

Chapter 4: Azide Reduction by DTT or Thioacetic Acid Provides Access to Amino and Amido Polysaccharides

(Published in *Cellulose* **2019**, *26* (1), 445–462)

*Chengzhe Gao[a,c], Zachary B. Fisher[b] and Kevin J. Edgar[c,d] **

[a] Department of Chemistry, Virginia Tech, Blacksburg, VA, 24061

[b] Department of Biochemistry, Virginia Tech, Blacksburg, VA, 24061

[c] Macromolecules Innovation Institute, Virginia Tech, Blacksburg, VA, 24061

[d] Department of Sustainable Biomaterials, Virginia Tech, Blacksburg, VA, 24061

Abstract

Amino and amido polysaccharides include some of the most complex, fascinating natural polymers, due in part to their important biological activities and intriguing biomedical applications. Chemical modification strategies have recently been created in order to access semi-synthetic amino polysaccharides. They typically involve a three-step reaction sequence: introducing a leaving group, azide displacement of that leaving group, and azide reduction to amine or amide. The final azide reduction to amine or amide is a key step, and current reduction methods are limited by chemoselectivity and solubility issues. In this study, we describe an efficient approach to selectively reduce polysaccharide azides to amines, employing 1,4-dithiothreitol (DTT). Azide reduction by DTT has been demonstrated to be effective across a broad range of substrates and under mild conditions. Different polysaccharides (cellulose and curdlan) and organic solvents (DMF, DMAc, and DMSO) were explored, as was chemoselectivity in the presence of functional groups such as esters, with satisfying reduction results in all cases. We further report a new approach to reduce polysaccharide azides directly to acetamides using thioacetic acid, with high

conversion, without going through the free amine intermediate. This is new to polysaccharide chemistry and cannot be achieved by any current method. Thorough characterization revealed that the thioacetic acid reduction was chemoselective, with no side reactions observed. Mechanistic study showed that the reaction does not go through an intermediate amine, and appears to be concerted. These azide reductions by DTT and thioacetic acid provide access to amino and amido polysaccharides that will be of great interest for exploring future applications and understanding structure property relationships of amino and amido polysaccharides.

4.1 Introduction

Natural amino and amido polysaccharides include some of the most complex, information-rich, diversely bioactive polymers in nature. Glycosaminoglycans (GAGs), one important class of amino and amido polysaccharides, are essential in a wide variety of organisms and drive many physiological processes, including prevention of blood clotting, cell adhesion, cell signaling and recognition, and joint lubrication, to name only a few.[1] Chitin, among the most abundant natural amido polysaccharides, functions as a structural polymer in crustaceans, reinforcing natural matrix shell composites.[2] Chitosan, the semisynthetic, partially N-deacylated derivative of chitin, has been demonstrated to be useful in many pharmaceutical and biomedical applications. Chitosan is mildly basic (pK_A ca. 6),[3] and when protonated can form complexes with many anionic molecules due to electrostatic interaction. Chitosan can also bind with proteins of the tight junctions between gastrointestinal enterocytes, and thereby facilitate tight junction opening which enhances bioavailability of drugs (e.g. polypeptides) that could not otherwise passively permeate across membranes. Thus, chitosan has been widely used in delivery of anionic drugs, nucleic acids, and certain proteins.[4] Chitosan also has important applications in wound healing, anti-bacterial

membranes, and tissue engineering.[5] However, amino and amido polysaccharides are often isolated from natural tissues, and so are prone to contamination by proteins or other polysaccharides, which can limit their utility.

In order to explore structure activity relationships and develop novel chitosan analogs, amination and amidation of natural polysaccharides, including cellulose,[6–8] curdlan,[9,10] pullulan,[11] and amylose[12] have been explored extensively. Polysaccharide amination has typically involved three steps: conversion of a polysaccharide hydroxyl group (the primary, 6-OH) to a good leaving group, followed by azide displacement, and final azide reduction to amine, in some cases incorporating *in situ* acylation to amide.[13] The appended leaving groups are usually selected from among bromide,[14] tosyl (Ts),[15] or chloride,[16] because of ease of introduction and sufficient reactivity with nucleophiles.[17] Introduction of the leaving group in each case occurs regiospecifically (Br, Cl) or preferably (Ts) at C6 position, largely because of wider available approach angles and greater ability to participate in S_N2 reactions at that position.[18] Each of these leaving groups can be quantitatively substituted by azide because of its high nucleophilicity. These useful polysaccharide azides[19,20] are promising precursors for amino polysaccharide derivatives with DS(NH_2) up to 1, provided that the azide can be conveniently reduced.

Commonly employed methods to reduce azide to amine in polysaccharide chemistry include the Staudinger reaction and metal hydrides. The Staudinger reaction employs triphenylphosphine (PPh_3) and water to efficiently reduce azide to amine at room temperature;[13] its mild nature (ester groups for example are unscathed) and typically quantitative yields make it very attractive. However, the obtained aminated polysaccharide is typically contaminated by triphenylphosphine oxide (PPh_3=O), which is extremely difficult to separate from the polysaccharide. Insoluble PPh_3=O may also contribute to the apparent poor solubility (in both

organic solvents and water) frequently observed[7] for these Staudinger amine products. Alternatively, strong metal hydride reducing agents like sodium borohydride ($NaBH_4$) or lithium aluminum hydride ($LiAlH_4$) can be used to reduce polysaccharide azides to amines. The obtained aminated polysaccharides typically have much better solubility than Staudinger reduction products. For example, 6-amino-6-deoxy curdlan obtained by $NaBH_4$ azide reduction is soluble in DMSO as well as in water.[10] These reagents are water sensitive, and generate hydrogen gas when excess reagent is hydrolyzed. Elevated temperatures are typically necessary in order to get high or complete conversion to amine. Chemoselectivity of $NaBH_4$ or $LiAlH_4$ can be problematic, as they are broadly reactive towards many other functional groups including esters.[10] Introducing amides to the polysaccharide backbone can be even more challenging; the only practical methods have involved amine or ylide acylation. Amine acylation is attractive in theory because amines are better nucleophiles than hydroxyl groups. However, frequently amine selectivity is imperfect in polysaccharides, and in fact selective acylation of amine without hydroxyl acylation has been reported in only a few cases for natural amino polysaccharides, including chitosan,[21] chondroitin sulfate,[22] and heparan.[23,24] All examples require amino polysaccharides that are soluble in water or water/alcohol mixtures, excluding important water-insoluble polysaccharides like cellulose. Another method for appending amide groups is Staudinger reduction (PPh_3) to the iminophosphorane ylide in the presence of a carboxylic anhydride; clean imide acylation and loss of PPh_3=O converts azide to amide in many polysaccharides, including cellulose,[7] pullulan,[11] and curdlan.[9] The reaction suffers however from the issues of difficult PPh_3=O separation mentioned earlier. Lack of chemoselectivity is another important drawback, since carboxylic anhydrides will esterify the hydroxyl groups as well. In summary, the Staudinger reaction (PPh_3, H_2O) and metal hydride reductants are useful for reducing polysaccharide azides to amines, but each has

limitations, including solubility or chemoselectivity. In addition, there is a dearth of efficient methods for appending amide groups to polysaccharide backbones. This leads us to seek new methods to reduce polysaccharide azides to amines or amides with complete chemoselectivity, affording uncontaminated products in high yields, and with tolerance for a range of solvents.

It has been well known in organic chemistry that thiols are effective reducing agents for conversion of small molecule azides to amines.[25] Furthermore, in 1989, Daly and Lee reported attempts using 1,3-propanedithiol to selectively reduce a 6-azido-6-deoxy cellulose ester to the corresponding amine. Their success was partial, as only a small proportion of the azides were reduced; nonetheless, this inspired us to consider reducing thiols as alternative polysaccharide azide reducing reagents. Recently, our lab reported employing 1,4-dithiothreitol (DTT) to reduce azide to amine in water during the synthesis of GAG analogs.[27] DTT has been demonstrated to be a powerful reducing agent for azides[28,29] and disulfides[30,31] in organic chemistry and biochemistry. Our previous study revealed that cellulose C-6 azides were completely reduced to amines in the presence of carboxyl groups. This aqueous azide reduction could not be achieved by Staudinger reaction or metal hydride ($NaBH_4$ or $LiAlH_4$) reduction, due to solubility issues (Staudinger), reactivity with water (metal hydrides), or the likelihood of inadequate chemoselectivity (reduction of carboxyl groups; metal hydrides).

A general, predictable method to selectively convert polysaccharide azides to amides would be of great interest, since there are many bioactive, highly functional amidopolysaccharides in nature (e.g. chitin, hyaluronic acid, chondroitin sulfate). Thioacetic acid can selectively reduce azide to acetamide in organic[32] and carbohydrate chemistry[33–35]. Mechanistic studies have indicated that this interesting small molecule reduction does not involve the amine as an intermediate.[36] While utilizing thioacetic acid to reduce azides in water as part of our previous

GAG analog synthesis, we obtained an unexpected *mixture* of acetamide and amine (amide:amine ratio 7:3). While this first demonstration of thioacetic acid reduction of azide to amide in polysaccharide chemistry was promising, formation of amine impeded acquisition of important polysaccharide derivatives bearing only amides.

Despite successful initial application of DTT and thioacetic acid to cellulose azide reduction in water, there remain many important unresolved questions. What is the scope of the DTT reduction of azide to amine, with regard to polysaccharide and ability to use commonly used organic solvents? Can we use thioacetic acid to selectively reduce polysaccharide azides to amides in organic solvents, without formation of amine? Under conditions where thioacetic acid reduction of polysaccharide azides produces both amide and amine products, can that ratio be controlled? Can we gain mechanistic insight into the fascinating thioacetic acid reduction of polysaccharide azides? Addressing these questions, we hypothesize that we can apply DTT and thioacetic acid to selectively reduce azido polysaccharides to the corresponding amines and/or amides in predictable fashion. Herein, we report our attempts to use DTT to reduce azido curdlans including those that also contain ester groups, and azido cellulose ester derivatives, to the corresponding amines. We also describe thioacetic acid reduction of azido curdlans including those that also contain ester groups, as well as azido pullulan, to the corresponsive amides, and initial experiments to probe the reaction mechanism.

4.2 Experimental

4.2.1 Materials

Cellulose acetate (CA320S, DS(Ac) 1.75 (measured by proton NMR of the perpropionylated derivative),[37–39] Mw = 47.6 kDa (measured by SEC),[16] Eastman Chemical

Company) and curdlan (degree of polymerization (DP) = 421 (measured by SEC),[40] Wako Chemicals) were dried under vacuum at 50 °C overnight prior to use. Pyridine, 4-dimethylaminopyridine (DMAP), acetic anhydride, propionic anhydride, triphenylphosphine, N-bromosuccinimide, sodium azide, thioacetic acid, 1,4-dithiothreitol (DTT), and anhydrous *N,N*-dimethylformamide (DMF) were from Sigma-Aldrich and used as received. Acetone, ethanol, and methanol were from Fisher Scientific and used as received. *N,N*-Dimethylacetamide (DMAc, Fisher) and dimethyl sulfoxide (DMSO, Fisher) were kept over 4 Å molecular sieves and stored under dry N_2 until use. Regenerated cellulose dialysis tubing (Fisher, 3500 g/mol MWCO) was used as received.

4.2.2 Measurements

One dimensional ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in DMSO-*d6* or D_2O or DMF-*d7* at room temperature with 64 scans and 15,000 scans, respectively. 2D-Heteronuclear Multiple Bond Correlation (HMBC) spectra were acquired on a Bruker Avance II 500MHz spectrometer in DMF-*d7* with 256 t1 increasement and 128 scans. Chemical shifts are reported relative to solvent peaks. FT-IR spectra were acquired on a Nicolet 8700 instrument with 128 scans and 4 cm^{-1} resolution.

4.2.3 Syntheses of 6-azido-6-deoxy-curdlan (6-N3Curdlan), 6-azido-6-deoxy-2,4-di-O-acetylcurdlan (6-N3CurdlanAc), 6-azido-6-deoxy-2,4-di-O-propionyl-curdlan (6-N3CurdlanPr), (6-azido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-N3CA320S), and 6-azido-6-deoxy-pullulan (6-N3Pullulan) – see Supplementary Information for details.

4.2.4 Reaction of 6-azido-6-deoxy-curdlan with 1,4-dithiothreitol (DTT)

In a 50 mL round bottom flask, 6-azido-6-deoxy-curdlan (200 mg, 1.07 mmol) was dissolved in 10 mL of anhydrous DMSO. DTT (0.82 g, 5 eq per anhydroglucose unit (AGU)) was

added slowly to the solution. The solution was stirred at room temperature (RT) for 12 h. Dialysis against water for 3 days and then freeze-drying afforded 6-amino-6-deoxy-curdlan (6-NH_2Curdlan). ^{13}C NMR (500 MHz, D_2O): 40.22 (C6-NH_2), 69.19 (C4), 71.08 (C2), 73.35 (C5), 81.61 (C3), 101.76 (C1). ^{1}H NMR (500 MHz, D_2O): 3.03 – 4.76 (curdlan backbone). IR (KBr): 3367 (O-H), 2916 (C-H), and 1575 (N-H). Yield: 135 mg (78.4%).

4.2.5 Reaction of 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan with DTT

6-Azido-6-deoxy-2,4-di-*O*-acetylcurdlan (200 mg, 0.73 mmol) was dissolved in 10 mL of anhydrous DMSO in a 50 mL round bottom flask, then DTT (0.57 g, 5 eq per AGU) was added to the flask. After stirring 12 h at RT, the solution was transferred at 3500 g/mol MWCO dialysis tubing and dialyzed against water for 3 days. The product was collected by filtration to afford 6-acetamido-6-deoxy-*O*-acetylcurdlan (6-NHAcCurdlanAc). ^{13}C NMR (500 MHz, DMF-*d7*): 21.06 (O-(C=O)-$\underline{C}H_3$), 22.46 (NH-(C=O)-$\underline{C}H_3$), 40.53 (C6-NHAc), 70.59 (C4), 73.49 (C2), 75.46 (C5), 80.67 (C3), 100.72 (C1), 170.02 (O-($\underline{C}$=O)-CH_3), 171.81 (NH-($\underline{C}$=O)-CH_3). ^{1}H NMR (500 MHz, DMF-*d7*): 1.80 – 2.32 (O-(C=O)-C$\underline{H}_3$ and NH-($\underline{C}$=O)-C$\underline{H}_3$), 3.01 – 6.14 (curdlan backbone). IR (KBr): 3396 (O-H), 2931 (C-H), 1746 (C=O, ester), 1655 (C=O, amide), and 1554 (N-H). Yield: 122 mg (67.3%).

4.2.6 Reaction of (6-azido-6-deoxy)-co-(6-*O*-acetyl)-CA320S with DTT

In a 50 mL round bottom flask, 6-N_3CA320S (200 mg, 0.80 mmol) was dissolved in 10 mL of anhydrous DMSO. DTT (0.61 g, 5 eq per AGU) was then added to the solution. The mixture was stirred at RT for 24 h. The product was isolated by dialysis against water for 3 days and then freeze-dried to give (6-acetamido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-NHAcCA320S). ^{13}C NMR (500 MHz, D_2O): 18.80-19.93 (O-(C=O)-$\underline{C}H_3$ and NH-(C=O)-$\underline{C}H_3$), 39.44 ($\underline{C6}$-NHAc), 62.07 ($\underline{C\text{-}6}$'-O-Ac), 70.08-78.52 (C2, C3, C4 and C5), 99.31 (C1), 102.06 (C1'), 172.17-172.98

(O-(C=O)-CH_3 and NH-(C=O)-CH_3). IR (KBr): 3434 (O-H), 2921 (C-H), 1744 (C=O, ester), 1641 (C=O, amide), and 1563 (N-H). Yield: 118 mg (62.3%).

4.2.7 Reaction of 6-azido-6-deoxy-curdlan with thioacetic acid (AcSH)

In a 50 mL round bottom flask, 6-azido-6-deoxy-curdlan (200 mg, 1.07 mmol) was dissolved in 10 mL of anhydrous DMF. Thioacetic acid (0.76 mL, 10 eq per AGU) and 2,6-lutidine (1.23 mL, 10 eq per AGU) were added separately to the solution. The reaction solution was heated to 65 °C and stirred for 24 h. The cooled solution was then dialyzed against ethanol for 3 days and against water for 3 days, followed by freeze-drying to yield 6-acetamido-6-deoxy-curdlan (6-NHAcCurdlan). ^{13}C NMR (500 MHz, DMF-*d7*): 22.00 (NH-(C=O)-CH_3), 39.94 (C6-NHAc), 69.49 (C4), 73.55 (C2), 74.81 (C5), 84.65 (C3), 103.22 (C1), 171.81 (NH-(C=O)-CH_3). ^{1}H NMR (500 MHz, DMF-*d7*): 1.75 - 2.04 (NH-(C=O)-CH_3), 2.95 – 6.39 (curdlan backbone). IR (KBr): 3418 (O-H), 2916 (C-H), 1652 (C=O, amide), and 1557 (N-H). Yield: 154 mg (70.9%).

4.2.8 Reaction of 6-azido-6-deoxy-2,4-di-*O*-propionylcurdlan with thioacetic acid (AcSH)

6-Azido-6-deoxy-2,4-di-*O*-propionylcurdlan (200 mg, 0.67 mmol) was dissolved in 10 mL of anhydrous DMF in a 50 mL round bottom flask. Thioacetic acid (0.48 mL, 10 eq per AGU) and 2,6-lutidine (0.77 mL, 10 eq per AGU) were added separately to the solution. The reaction solution was stirred at 65 °C for 24 h. The solution was dialyzed against ethanol for 3 days, then against water for 3 days, followed by freeze-drying to yield 6-acetamido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-NHAcCurdlanPr). ^{13}C NMR (500 MHz, DMF-*d7*): 8.85 (O-(C=O)-CH_2CH_3), 22.31 (NH-(C=O)-CH_3), 27.24 (O-(C=O)-CH_2CH_3), 40.38 (C6-NHAc), 69.73 (C4), 72.67 (C2), 73.24 (C5), 78.32 (C3), 100.77 (C1), 169.96 (NH-(C=O)-CH_3), 173.19 (O-(C=O)-CH_2CH_3). ^{1}H NMR (500 MHz, DMF-*d7*): 0.93 – 1.28 (O-(C=O)-CH_2CH_3), 1.83 - 1.99 (NH-(C=O)-CH_3), 2.23

– 2.67 (O-(C=O)-C$\underline{H}_2$CH$_3$), 3.10 – 5.20 (curdlan backbone). IR (KBr): 3394 (O-H), 2983 and 2945 (C-H), 1750 (C=O, ester), 1662 (C=O, amide), and 1546 (N-H). Yield: 138 mg (65.5%).

4.2.9 Reaction of 6-azido-6-deoxy-pullulan with thioacetic acid (AcSH)

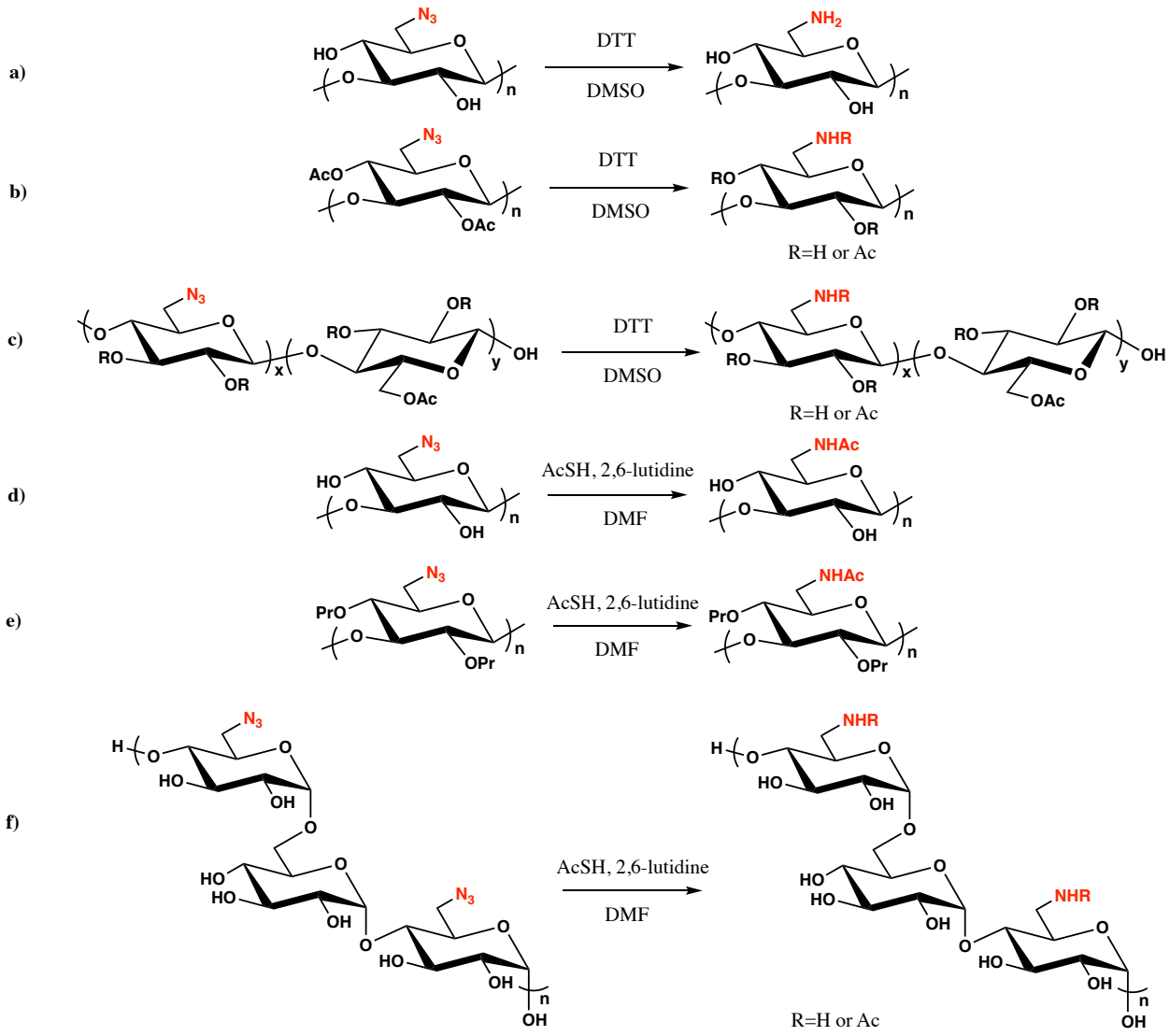
6-Azido-6-deoxy-pullulan (80 mg, 0.45 mmol) was dissolved in 5 mL of water : DMF mixed solvent (v : v = 19 : 1) in a 25 mL round bottom flask. Thioacetic acid (0.32 mL, 10 eq per AGU) was added dropwise to the solution, followed by slow addition of 2,6-lutidine (0.48 mL, 10 eq per AGU). The reaction solution was stirred at 65 °C for 24 h. The solution was dialyzed against ethanol for 3 days, then against water for 3 days, followed by freeze-drying to yield 6-acetamido-6-deoxy-pullulan (6-NHAcPullulan). ^{13}C NMR (500 MHz, D$_2$O-DMF-*d7* mixture): 20.96, 21.08 (NH-(C=O)-$\underline{C}$H$_3$), 39.28, 40.00 (C6-NHAc), 46.22 46.80 (C6-NH$_2$), 59.61 (C6'), 65.82-72.70 (C2, C3, C5), 80.35 (C4), 96.53 (C1), 99.72 (C1'), 172.89, 173.03 (NH-($\underline{C}$=O)-CH$_3$). ^{1}H NMR (500 MHz, D$_2$O-DMF-*d7* mixture): 1.79 - 2.02 (NH-(C=O)-C$\underline{H}_3$), 3.00 – 5.50 (pullulan backbone). IR (KBr): 3379 (O-H), 2928 (C-H), 1645 (C=O, amide), and 1558 (N-H). Yield: 71 mg (82.6%).

4.3 Results and discussion

4.3.1 Synthesis of azido curdlan, azido curdlan esters, and azido cellulose esters

Azido polysaccharide derivatives are important intermediates for this study. Regioselective syntheses of 6-N$_3$Curdlan,[9] 6-N$_3$CurdlanAc,[9] 6-N$_3$CurdlanPr,[9] 6-N$_3$CA320S,[16] and 6-N$_3$Pullulan [11] were carried out as previously reported. The primary hydroxyl group was first replaced by a good leaving group, bromide or chloride in our case, by regiospecific Furuhata bromination (NBS, PPh$_3$) or regioselective chlorination using methanesulfonyl chloride.[16] Then the leaving group at C6 was substituted by the highly nucleophilic azide, affording 6-azido-6-deoxy-curdlan, 6-azido CA320S, and 6-azido-6-deoxy-pullulan. 6-Azido-6-deoxy-curdlan was further esterified in

pyridine/anhydride mixture (acetic anhydride or propionic anhydride) with DMAP as the catalyst to give the corresponding 6-azido curdlan acetate or propionate. Structures of 6-N_3Curdlan, 6-N_3CurdlanAc, 6-N_3CurdlanPr, 6-N_3CA320S, and 6-N_3Pullulan were confirmed by FT-IR, ^{1}H NMR, and ^{13}C NMR. Successful azide incorporation was confirmed by its characteristic FTIR absorption at around 2100 cm^{-1} (Figure 4.1a) and the ^{13}C NMR peak around 51 ppm (C6-N_3) (Figure 4.2a). Subsequent esterification of azido curdlan was also supported by the ester carbonyl absorption around 1750 cm^{-1} (FT-IR, Figure 4.3a) and the carbonyl resonance at 170 ppm in the ^{13}C NMR spectrum.



Scheme 4.1 Reactions of a) 6-N_3Curdlan, b) 6-N_3CurdlanAc, and c) 6-N_3CA320S with DTT and reaction of d) 6-N_3Curdlan, e) 6-N_3CurdlanPr, and f) 6-N_3Pullulan with thioacetic acid.

4.3.2 Azide reduction by 1,4-dithiothreitol (DTT)

We began by extending our recent first application of DTT, also known as Cleland's reagent, to polysaccharide azide reduction, demonstrated in selective reduction of a GAG analog azide to amine in water.[27] We chose 6-azido-6-deoxy-curdlan (6-N_3Curdlan) as model substrate, beginning with reaction conditions similar to those used for DTT reductions of azide to amine in small molecule chemistry. 6-N_3Curdlan was treated with DTT (10 eq) and triethylamine (5 eq) in DMSO at RT for 12 h to yield water-soluble 6-amino-6-deoxy-curdlan (6-NH_2Curdlan). In the product FT-IR spectrum (Figure 4.1b), the azide peak at 2108 cm^{-1} had completely disappeared, indicating complete azide reduction. A new absorption at 1575 cm^{-1} was assigned as N-H bending. The ^{13}C spectrum (Figure 4.2b) provides conclusive evidence of the product structure: a new resonance appeared at 40 ppm, assigned to the amine-bearing C6, while the former C6-N_3 resonance (51 ppm) was absent. After successful DTT reduction of 6-N_3Curdlan to 6-NH_2Curdlan, we further explored the effect of bases and solvents. Base is believed to facilitate reduction by deprotonation of the thiol groups from DTT to form more nucleophilic thiol anions. Different molar ratios (5 eq or 2 eq per AGU) of triethylamine and *N,N*-diisopropylethylamine were explored. Each reaction condition gave complete azide reduction; to our surprise, even in the absence of a base! The impact of reaction solvent was also investigated. Often polar aprotic solvents, such as DMF, DMSO, and DMAc, are suitable for dissolution of polysaccharides and their derivatives. Frequently only one polar aprotic solvent type works well for a particular polysaccharide. For example, pullulan is only soluble in DMF and insoluble in DMSO and DMAc, while dextran is

only soluble in DMSO and insoluble in DMF and DMAc. We were pleased to find that complete conversion of 6-N_3Curdlan to amine was observed in DMSO, DMF, and DMAc. Including previous reports on its utility in water, DTT reduction of polysaccharide azides is clearly effective in a usefully wide range of solvents. All amino curdlans thus prepared are fully soluble in water, similar to amino curdlans obtained by $NaBH_4$ reduction,[10] while the amino curdlan obtained by Staudinger reaction (PPh_3, H_2O) is not soluble in common solvents.[9]

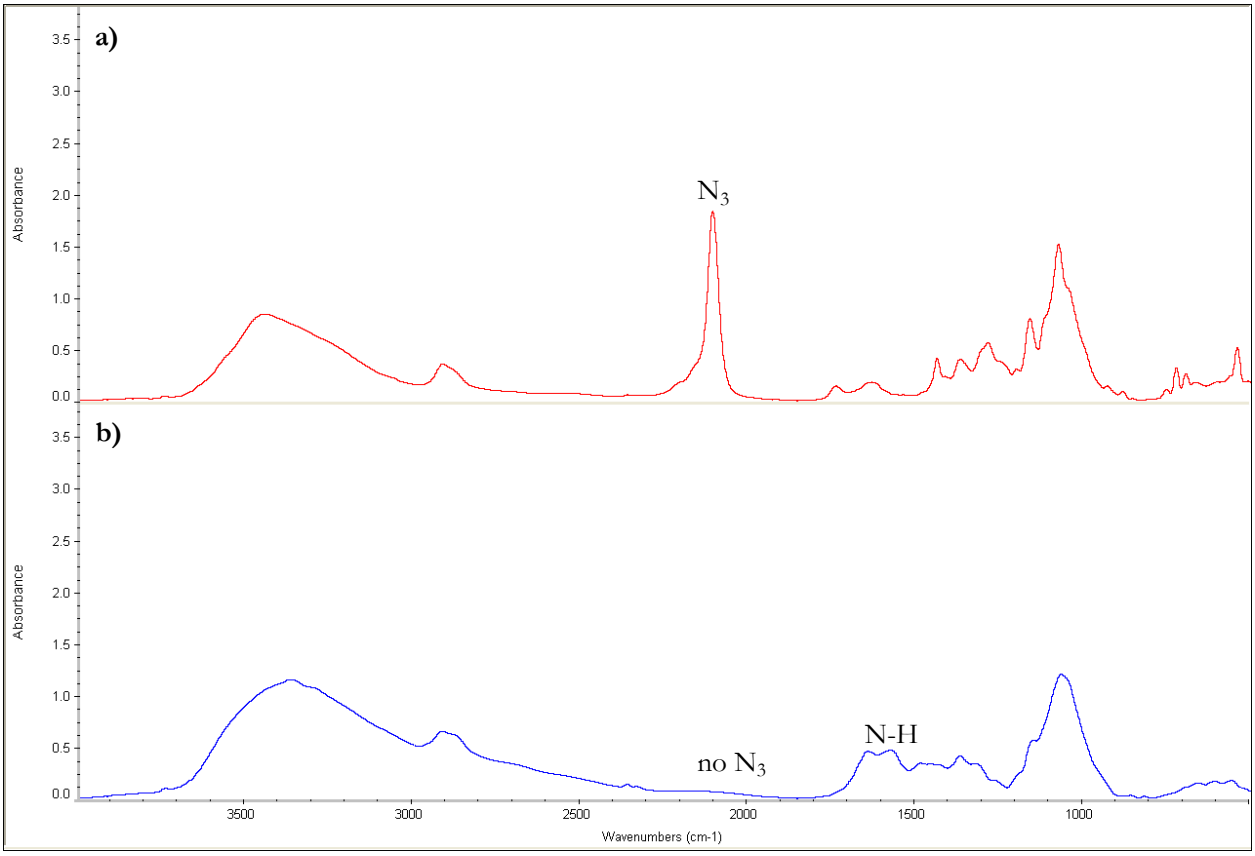


Figure 4.1 FT-IR spectra of (a) 6-azido-6-deoxy-curdlan (6-N_3Curdlan) and (b) 6-amino-6-deoxy curdlan (6-NH_2Curdlan).

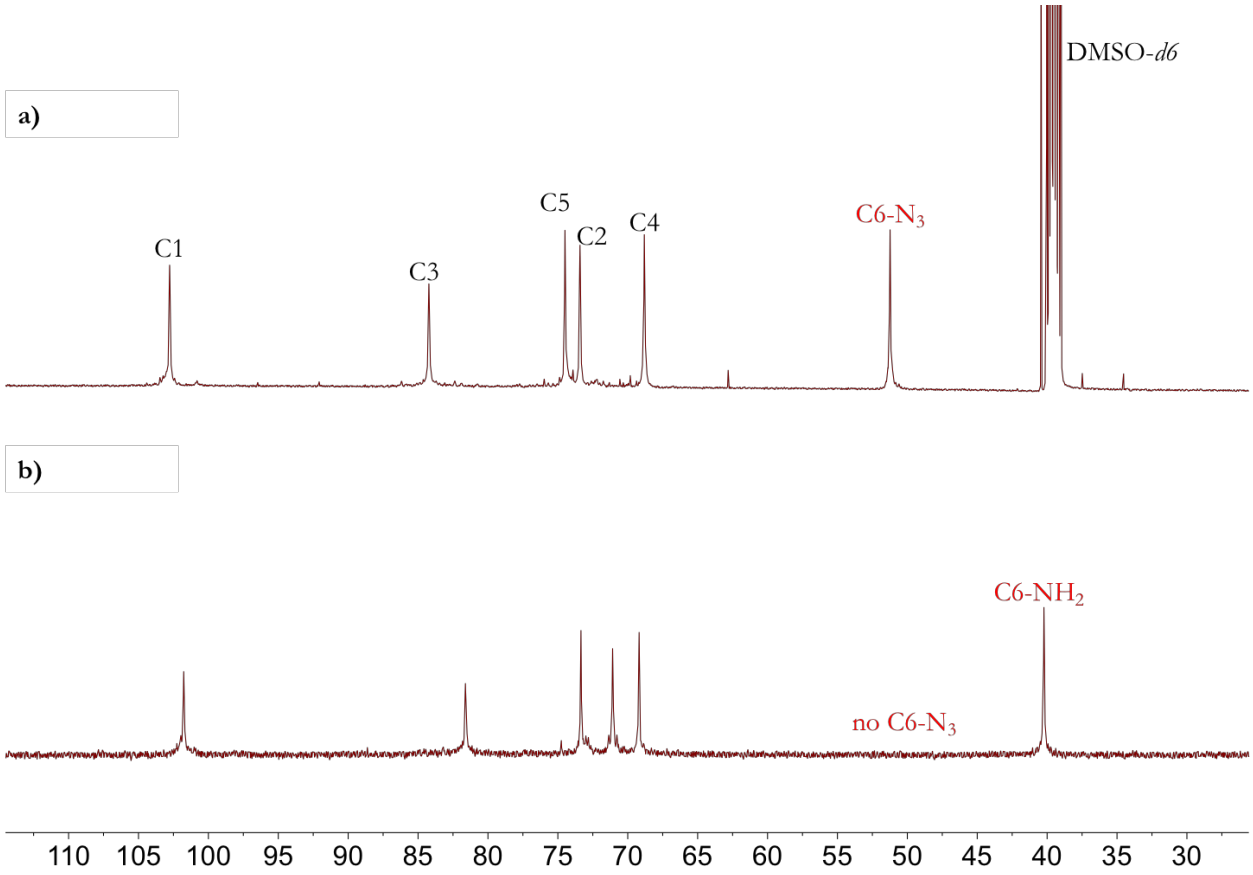


Figure 4.2 ^{13}C NMR spectra of (a) 6-azido-6-deoxy-curdlan (6-N_3Curdlan) and (b) 6-amino-6-deoxy curdlan (6-NH_2Curdlan).

We briefly explored other sulfur-containing reagents to reduce azide to amine. Hydrogen sulfide, sodium hydrosulfide, and sodium sulfide have been used as reducing reagents as early as the 1800s, and it has been reported that H_2S[41] and sodium sulfide[42] can reduce azide to amine. A mechanistic study indicated that SH^- was an important intermediate in the reduction.[43] For this reason, we chose sodium hydrosulfide (NaHS) to attempt reduction of 6-N_3Curdlan to 6-NH_2Curdlan. During the reaction, bubbles were generated and the solution color turned from yellow to green. Azide-related peaks disappeared in both FT-IR and ^{13}C NMR spectra. However, no resonance for C6 bearing amine (40-50 ppm) was observed in the ^{13}C NMR spectrum; in fact,

no signals were observed in the typical C6 region of the ^{13}C NMR spectrum. We do not fully understand the unusual disappearance of C6 signals. The previous literature[44] indicated that very high purity of sodium hydrosulfide was essential for chemical reactions and impurity will have a significant negative effect on reactions. It is possible that the failure of this reaction was due to impurities in purchased NaSH.

After demonstrating that DTT cleanly reduces 6-N_3Curdlan to 6-NH_2Curdlan, we wished to further explore the chemoselectivity of this reduction. Can DTT selectively reduce azide without reacting with other functional groups? Esters are important polysaccharide functional groups, for example in cellulose where they enhance solubility, extrudability, and other properties of the resulting commercially important derivatives.[45] To test whether ester reduction would compete, we treated 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan (6-N_3CurdlanAc) with DTT (5 eq) in DMSO at RT for 12 h. Instead of an amino curdlan ester, an amido curdlan ester, 6-acetamido-6-deoxy-*O*-acetylcurdlan (6-NHAcCurdlanAc), was obtained as the product. Its structure was confirmed by FT-IR and ^{13}C NMR spectroscopy. In the FT-IR spectrum (Figure 4.3b), the azide absorption at 2104 cm^{-1} was absent, while a new amide carbonyl stretch (1655 cm^{-1}) and a new amide N-H bending absorbance (1554 cm^{-1}) appeared, consistent with complete conversion of azide to amide. In addition, the O-H stretch (3396 cm^{-1}) was enhanced, demonstrating loss of some ester groups. A strong ester C=O stretch remained at 1746 cm^{-1}, supporting the hypothesis that DTT does not reduce ester groups. Formation of acetamide groups was further confirmed by ^{13}C NMR (Figure 4.4). New ^{13}C NMR resonances appeared at 40 ppm, assigned to the amide-bearing C6, and at 172 ppm and 22 ppm, assigned as the resonances of the carbonyl and methyl groups, respectively, from the acetamido moiety, while the azide-bearing C6 resonance at 50 ppm disappeared. The continuing presence of some esters is further confirmed by acetyl carbonyl and methyl resonances

at 170 ppm and 21 ppm, respectively. The data are fully consistent with the hypothesis that the nucleophilic amine formed *in situ* by DTT reduction reacts intra- or intermolecularly with an ester group to form a more stable amide as the final product, with no direct ester reduction. Such acyl transfer reactions have been previously observed in polysaccharide chemistry[10,46] as well as in other areas of organic chemistry. Two distinct methyl groups appeared in the ^{1}H NMR spectrum (Figure S4.3) at 1.85 and 2.07 ppm, assigned as the resonance of methyl groups from acetamido and acetate groups. The sum of DS(NHAc) and DS(OAc) was measured to be 1.89 by ^{1}H NMR integration (DS(OAc) ca. 0.98, DS(NHAc) ca. 0.91, some peak overlap). Considering that DS (OAc) was ca. 2.0 in the starting 6-N_3CurdlanAc, this confirms that the acetamides come from acetate acyl migration. It is difficult to more positively confirm the absence of any small amount of amine by ^{13}C NMR, due to C6-amide and C6-amine signal overlap at ca. 40 ppm.

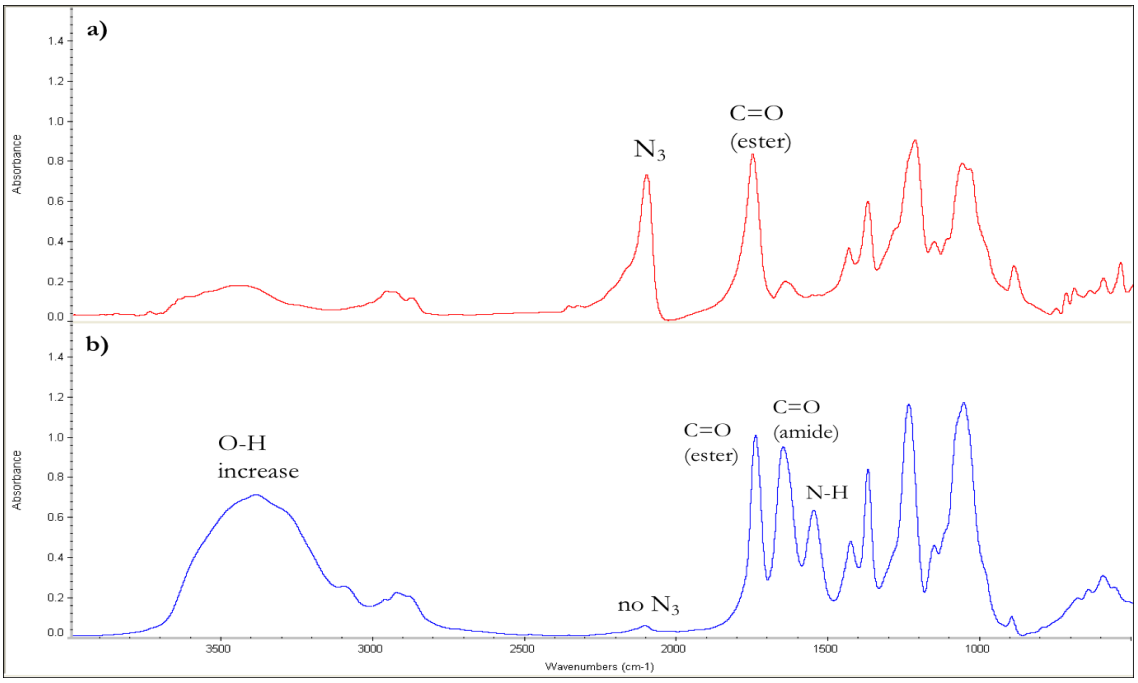


Figure 4.3 FT-IR spectra of (a) 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan (6-N_3CurdlanAc) and (b) 6-acetamido-6-deoxy-*O*-acetylcurdlan (6-N_3CurdlanAc).

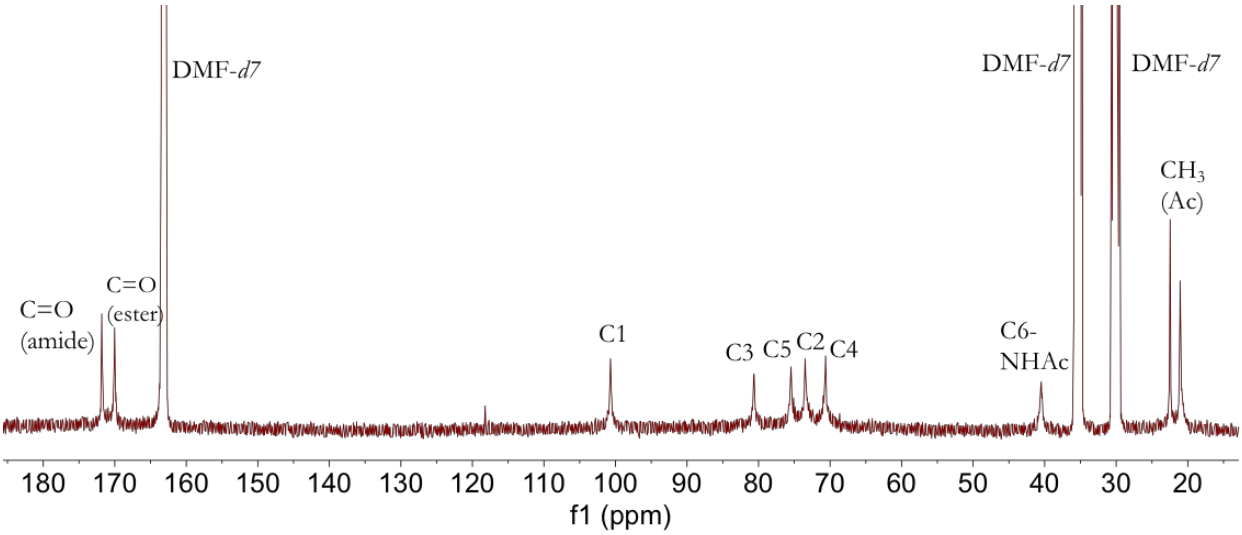


Figure 4.4 ^{13}C NMR spectrum of 6-acetamido-6-deoxy-*O*-acetylcurdlan (6-NHAcCurdlanAc).

We further employed DTT to reduce an azido cellulose ester, (6-azido-6-deoxy)-co-(6-*O*-acetyl)-CA320S (6-N_3CA320S), to yield (6-acetamido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-NHAcCA320S) under reaction conditions similar to those for the curdlan derivatives. FT-IR (Figure S4.1) and ^{13}C NMR (Figure S4.2) spectra with features akin to those noted in the 6-NHAcCurdlanAc spectra were obtained, further exemplifying clean transformation of azide to amine, followed by acyl transfer, with no ester reduction.

4.3.3 Azide reduction by thioacetic acid

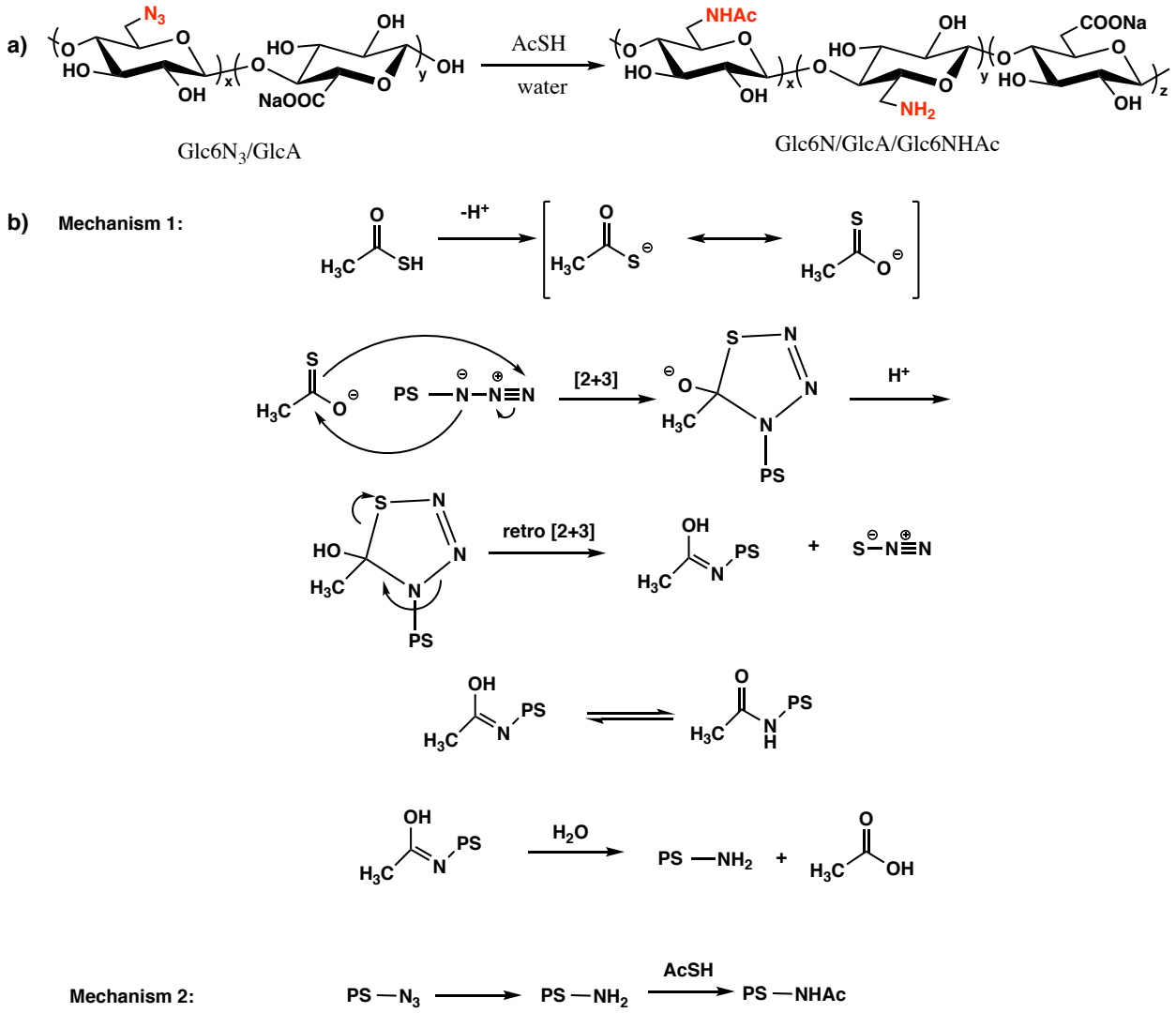
In our previous studies, we applied thioacetic acid (AcSH) to reduce a GAG analog azide in water, obtaining a mixture of the amide and, surprisingly, the amine (Scheme 4.2a). Based on published mechanistic studies on small molecules,[36] we proposed a similar mechanism that attributed amine formation to hydrolysis of a hydroxyimine intermediate (Scheme 4.2b, mechanism 1), in which case the amine is not an intermediate on the way to the amide. Another possible mechanism would be initial reduction of azide to amine, then acylation of the amine by thioacetic acid to yield the amide. This mechanism (Scheme 4.2b, mechanism 2) would be

consistent with observation of amine products (Scheme 4.2b, mechanism 2). We wished to identify conditions in organic solvents that minimize the formation of amine and afford only amides. Possible esterification is another chemoselectivity concern about thioacetic acid reduction in organic solvents, as it has been reported that thioacetic acid can esterify cellulose from wood [47]. In our previous study, we observed no esterification of free GAG analog hydroxyls by thioacetic acid, but that reduction was carried out in water, in which net acylation by thioacetic acid would be less likely. We further explored this reduction, watching for possible acylation of curdlan hydroxyl groups in organic media.

We first treated 6-azido-6-deoxy curdlan (6-N_3Curdlan) with AcSH (20 eq) in DMF at 65 °C for 48 h. In the product FT-IR spectrum (Figure 4.5b), a new amide carbonyl signal appeared at 1652 cm^{-1} and a new N-H bending absorbance appeared at 1557 cm^{-1}, supporting amide formation. However, a residual azide signal at 2108 cm^{-1} demonstrated that azide reduction was incomplete, further confirmed by the presence of the azide-bearing C6 at 50 ppm in ^{13}C NMR spectrum. Even with longer reaction time (72 h), elevated reaction temperature (80 °C), or increased equivalents of thioacetic acid (30 eq or 40 eq), residual azide was always observed; the reaction was slow and incomplete. In the small molecule AcSH reduction literature, investigators reported that this reaction can be accelerated by 2,6-lutidine base.[32] We therefore treated 6-N_3Curdlan with AcSH (20 eq) in the presence of 2,6-lutidine (10 eq) in DMF at 65 °C for 48 h. Successful reduction to amide was confirmed by the amide carbonyl at 1652 cm^{-1} and N-H bend at 1557 cm^{-1}, and its completion was demonstrated by disappearance of the azide absorption at 2108 cm^{-1} (Figure 4.5c).Further confirmation was offered by the ^{13}C NMR spectrum (Figure 4.6b), where the former C6 bearing azide signal at 50 ppm disappeared, and a new peak appeared at 40 ppm, assigned as the amide-bearing C6. The presence of the acetamide group is further confirmed

by the amide carbonyl resonance at 172 ppm and the methyl at 22 ppm. All chemical shifts are consistent with those of the acetamide moiety of 6-NHAcCurdlanAc. It is satisfying that even with fewer equivalents of AcSH (10 eq) and shorter reaction time (24 h), the reaction was complete. We also attempted to carry out the AcSH reduction in DMSO, but observed the disappearance of liquid DMSO and thioacetic acid, and a large quantity of yellow precipitate was formed. The unusual phenomena can be explained by reaction between DMSO and thioacetic acid; Whiting and Walton reported that DMSO reacted with AcSH at room temperature to form elemental sulfur (S_8).[48] It was important to confirm chemoselectivity of the AcSH reduction, particularly with respect to formation of amine and/or esterification. We can get at DS(NH_2) indirectly by measuring DS (NHAc) through integration of the acetamide methyl in the ^{1}H NMR spectrum (Figure S4.4), which in this case was measured as 0.93. As the starting DS (N_3) was ca. 1.0, this supports the hypothesis that AcSH cleanly reduced azide to amide, with no or negligible formation of amine. With regard to the possible reaction of thioacetic acid with hydroxy groups, in the FT-IR spectrum (Figure 4.5c) no ester carbonyl stretches are observed in the vicinity of 1750 cm^{-1}. By ^{13}C NMR (Figure 4.6b) no ester carbonyl or methyl resonances are observed; by ^{1}H NMR (Figure S4.4), only the single acetamide methyl peak was observed, all supporting absence of *O*-esterification. As a control experiment, we treated curdlan with AcSH (20 eq) in DMAc/LiCl at 80 °C for 48 h. No acetate groups were evident in the product (^{1}H NMR, Figure S4.5). We also used a mixture of H_2O/DMF (v : v = 1 : 9) to conduct the reaction, testing whether amine would be generated by hydroxyimine hydrolysis by the water cosolvent. However, DS (NHAc) measured by ^{1}H NMR was 0.93, identical to that of the product obtained in pure DMF, suggesting that adding water to the organic solvent will not lead to formation of a substantial amount of amine. The result seems to be reasonable since the tautomerization of hydroxyimine is unimolecular and in theory should

be much faster than the biomolecular hydrolysis reaction proposed to lead to amine formation. Clearly, the observed formation of amine in water is an interesting and not fully understood reaction.



Scheme 4.2 a) Aqueous thioacetic acid reduction in our previous report and b) two postulated mechanisms

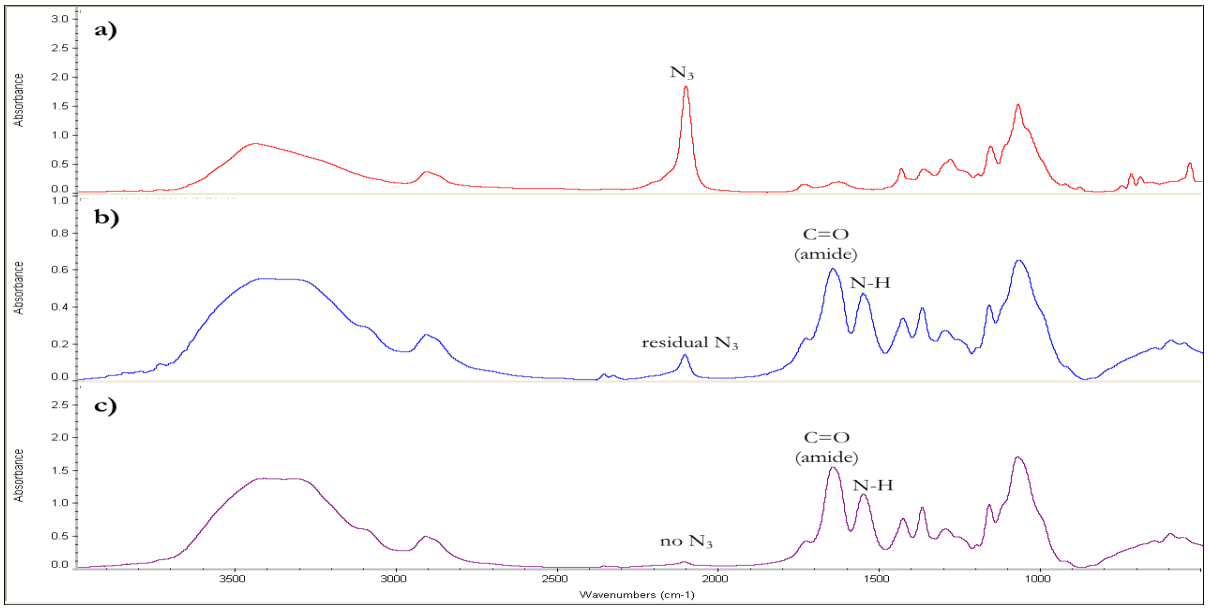


Figure 4.5 FT-IR spectra of a) 6-azido-6-deoxy-curdlan (6-N_3Curdlan), b) incomplete thioacetic acid azide reduction product, and c) complete azide reduction product: 6-acetamido-6-deoxy-curdlan (6-NHAcCurdlan).

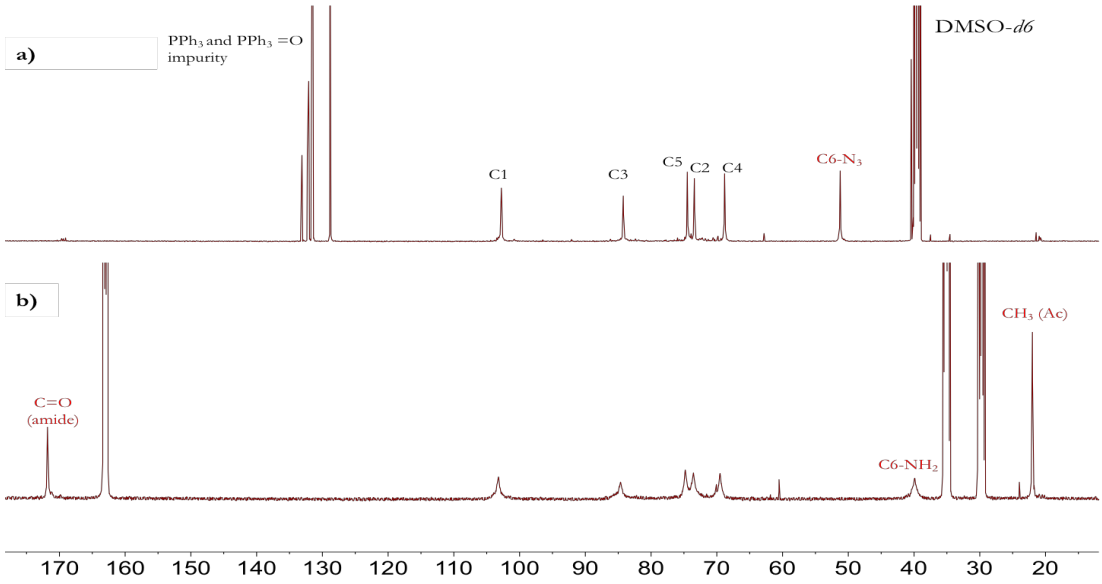


Figure 4.6 ^{13}C NMR spectra of a) 6-azido-6-deoxy-curdlan (6-N_3Curdlan) and b) 6-acetamido-6-deoxy-curdlan (6-NHAcCurdlan).

In order to further understand the mechanism and scope of the AcSH reduction, we designed an experiment that we thought would provide important insight, treating 6-azido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-N_3CurdlanPr) with AcSH. If the mechanism is concerted (mechanism 1, Scheme 4.2b) and does not involve an amine intermediate, azides will be converted only to acetamide groups. If instead azide is first reduced to amine and then converted to amide (mechanism 2, Scheme 4.2b), the free amine should react by propionyl transfer, forming a **propionamide,** competitive with reaction with thioacetic acid and formation of acetamide. Thus a mixture of acetamide and propionamide would be expected if amine is an intermediate in conversion to amide. The product of this reaction showed no azide absorption at 2101 cm^{-1}, but an amide carbonyl signal at 1662 cm^{-1} and N-H bending at 1546 cm^{-1} by FT-IR appeared (Figure 4.7b), confirming successful azide reduction to amide. By ^{13}C NMR (Figure S4.5), two new resonances appeared at 170 ppm and 22 ppm, assigned as the carbonyl and methyl groups from the acetamide substituent. An HMBC spectrum (Figure 4.8) confirms product structure. The new formed amide carbonyl at 170 ppm correlates only with the methyl protons (1.82 – 2.02 ppm) from acetyl groups, while the methyl protons (0.95 – 1.25 ppm) from propionyl groups were detected to correlate only with the ester carbonyl at 173 ppm, illustrating that acetamide is formed exclusively, with no evidence of propionamide formation, indicating no O to N propionyl group transfer. DS (Ac) and DS (Pr) were calculated to be 0.93 and 1.87 respectively, by ^{1}H NMR integration (Figure S4.6), confirming the lack of acyl transfer. All evidence strongly supports the concerted mechanism (1 in Scheme 4.2b), and the absence of intermediate amine. The result also indicates minimal to zero DS(amine) in the final product, since any residual amine (not only in an intermediate, but in the product as well) would lead to detectable propionamide by O, N acyl transfer**.**

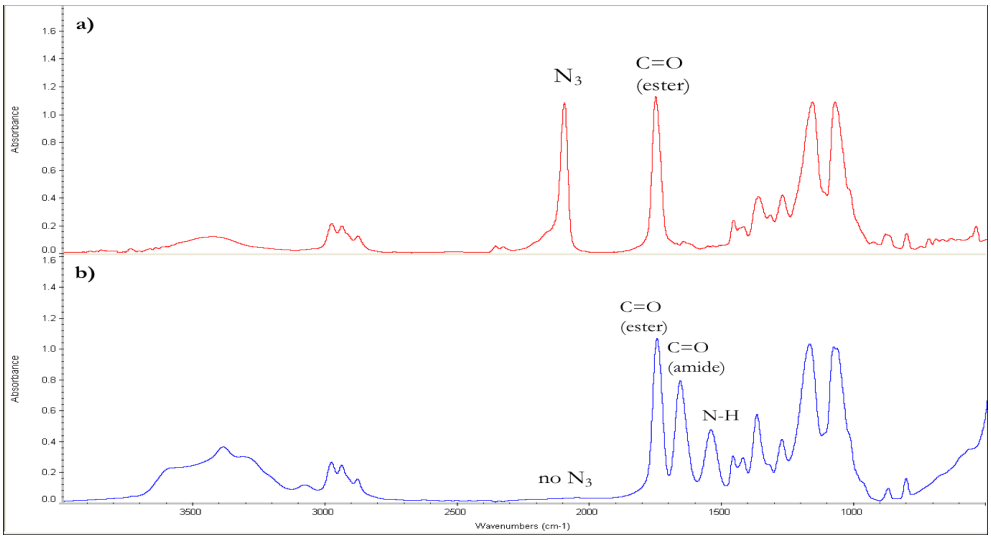


Figure 4.7 FT-IR spectra of a) 6-azido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-N_3CurdlanPr) and b) 6-acetamido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-NHAcCurdlanPr).

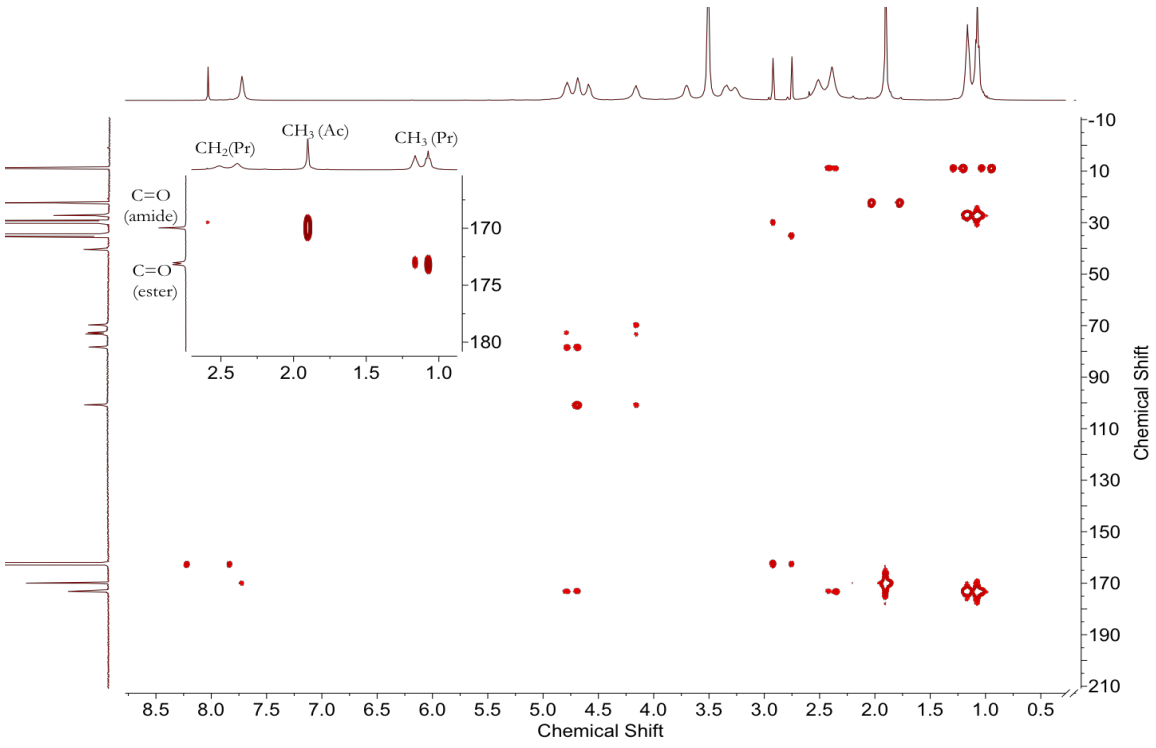


Figure 4.8 HMBC spectrum of 6-acetamido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-NHAcCurdlanPr).

We were interested in exploring the AcSH azide reduction in water, in order to observe whether water concentration influenced amine vs. amide formation (remembering that a mixture was observed in our earlier GAG analog work). 6-Azido-6-deoxy-pullulan (6-N_3Pullulan) was treated with AcSH and 2,6-lutidine in water : DMF (19 : 1 v : v) (the small amount of DMF was used to help dissolve 6-N_3Pullulan) at 65 °C for 18 h. Conversion of azide to acetamide was confirmed by FT-IR (Figure S4.7) and ^{13}C NMR (Figure 4.9) by similar logic as described in earlier examples. However, a small ^{13}C resonance appeared at 46 ppm, assigned to the amine-bearing C6. The small peak was consistent with a low proportion of amine groups. This result supports our hypothesis that amine formation can result from interception of the hydroxyimine intermediate by reaction with water. We wished to explore whether added 2,6-lutidine would impact amide/amine ratio, and so treated $GlcN_3$/GlcA with AcSH and 2,6-lutidine in water at 65 °C (Scheme 4.2a, but with added 2,6-lutidine). The fast generation of bubbles and rapid color change (from yellow to orange in < 4 h) suggested more rapid reaction. In fact, in the presence of 2,6-lutidine the reaction was complete in < 12 h, compared with 48 h in the absence of 2,6-lutidine. By ^{13}C NMR (Figure S4.8) the product contained both amine and amide bearing C6 resonances, and DS(NHAc) was 0.37 by ^{1}H NMR, almost identical to the result without 2,6-lutidine (DS(NHAc) 0.35). Thus 2,6-lutidine accelerates the reaction rate but does not significantly change the proportion of amine/amide products, so appears not to relatively favor or disfavor hydroxyimine hydrolysis. It is interesting to note that AcSH reduction in water affords different results from reduction in DMF, being surprisingly faster and affording amines as minor products along with the amide, which is the major product in water but exclusive product in DMF. It has been illustrated by Hackenberger and co-workers in 2014 that product ratio (amide, thioamide, and

amine) from aqueous thioacetic acid azide reduction can be affected by adjusting pH.[49] We are continuing to explore potential methods to fine-tune the amine/amide product ratio, since this is highly relevant to effective synthesis of the important GAG analogs, for one example.

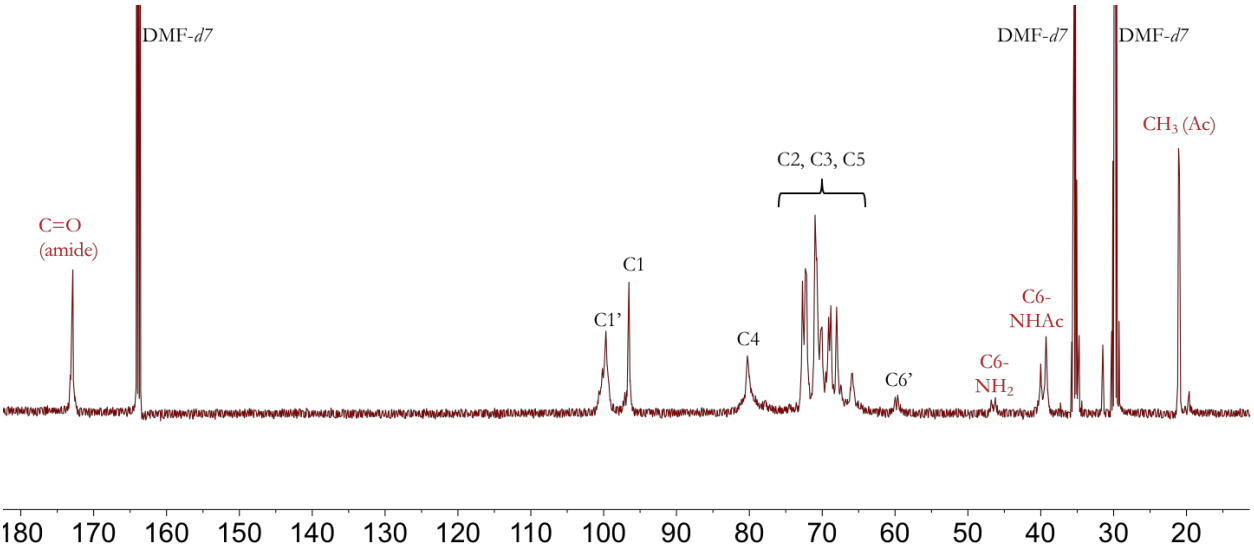


Figure 4.9 ^{13}C NMR spectrum of 6-acetamido-6-deoxy-pullulan (6-NHAcPull).

4.4 Conclusions

We describe herein an efficient strategy to reduce azide moieties on polysaccharides to amines and amides using sulfur-containing reagents, DTT and thioacetic acid respectively. We demonstrate that DTT is effective in reducing azide to amine in polysaccharides of various structures (cellulose and curdlan) and in several polar aprotic solvents (DMSO, DMAc, and DMF). We demonstrate chemoselectivity towards reduction of the sensitive ester moiety, although transacylation from ester to amine to yield more stable amides is a likely subsequent reaction. This inevitable acyl migration, coupled with the selectivity of DTT towards azide rather than ester reduction, affords an interesting route to polysaccharide C-6 amides with now-free hydroxyl groups at other positions, that could be subsequently derivatized in order to achieve target properties. Conversion of azide to acetamide, which is even more challenging and has not been

described previously in polysaccharide chemistry, can be achieved by reaction with thioacetic acid in DMF. By employing 2,6-lutidine as base, we show that reasonable reaction rates and complete conversion of azide to acetamide can be achieved. We present evidence that strongly supports a concerted mechanism leading to a hydroxyimine product, that can tautomerize to the amide or be hydrolyzed to amine, depending on reaction conditions. We rule out hydroxyl acylation and direct reduction to amine as possible side reactions. We show that thioacetic acid reduces an azido pullulan in water; in that solvent the amide is the primary product, but a small amount of amine was also formed. This indicates that under aqueous conditions chemoselectivity of thioacetic acid reduction is not complete for amide formation as it is in organic solvents.

The DTT reduction to amine has many advantages over current azide reduction methods, because of its mild and efficient nature, good solubility (and purity) of the product amines, and tolerance towards reduction-sensitive functional groups like ester and carboxyl. Importantly, as demonstrated in our previous work, water is a good solvent for DTT reduction, unlike conventional Staudinger and metal hydride reductions. Thioacetic acid reduction of azide to acetamide in DMF is new to polysaccharide chemistry, accomplishing a transformation for which there is no other one-step alternative, and provides a new pathway to amido polysaccharide derivatives. Acetamides are important functional groups in many polysaccharides, such as chitin and all of the GAGs. Thioacetic acid reduction in water is less chemoselective, affording amides but also amines as minor products. These mixed amine/amide products could in fact be useful in some cases, e.g. in GAG analog synthesis.

We have partially confirmed our starting hypothesis, achieving deeper understanding of both DTT and AcSH reductions so as to expand their scope and obtain polysaccharide amines and amides from polysaccharide azides in relatively predictable, convenient fashion. Our mechanistic

understanding and ability to fully manipulate these chemistries to achieve desired results are still incomplete, however. In future work we will seek to understand how to manipulate reaction conditions so as to be able to eliminate amine formation in water, or conversely to control the amine/amide ratio to a desired target. Deeper understanding of this interesting reaction mechanism will help us approach these goals.

Supporting information

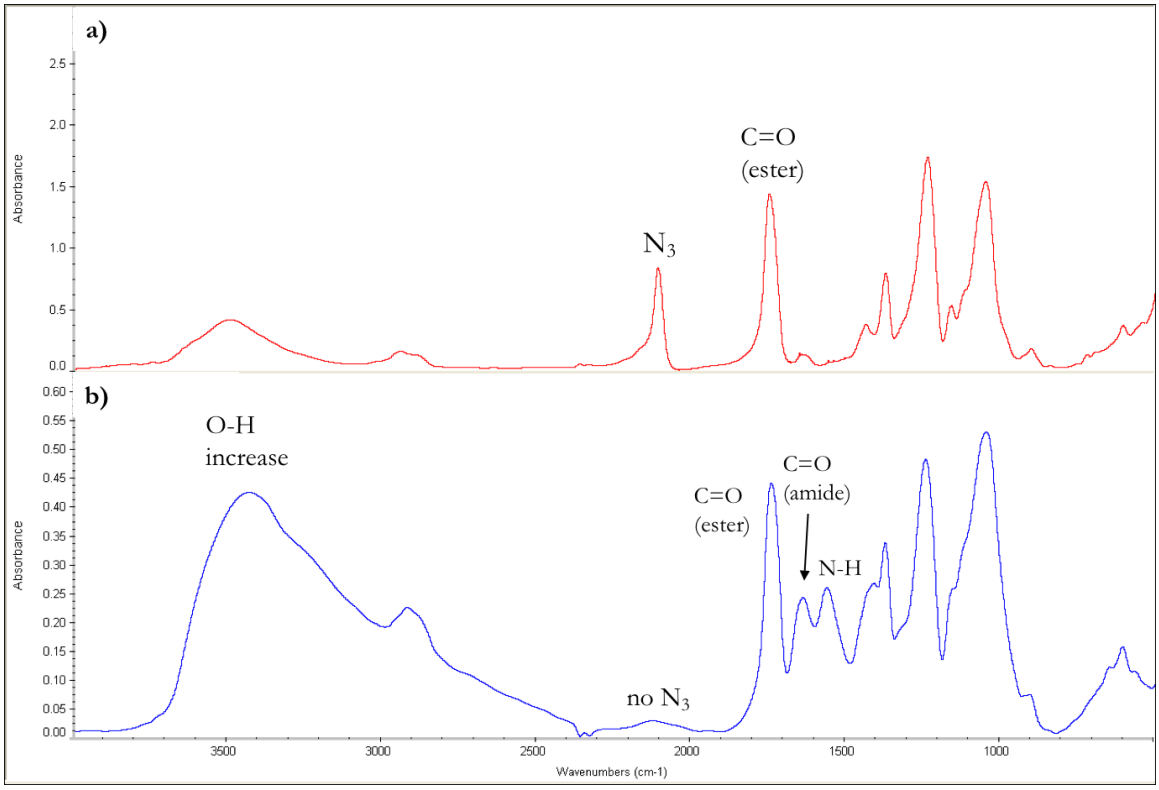


Figure S4.1 FT-IR spectra of (6-azido-6-deoxy)-co-(6-*O*-acetyl)-CA320S (6-N_3CA320S) and b) (6-acetamido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-NHAcCA320S).

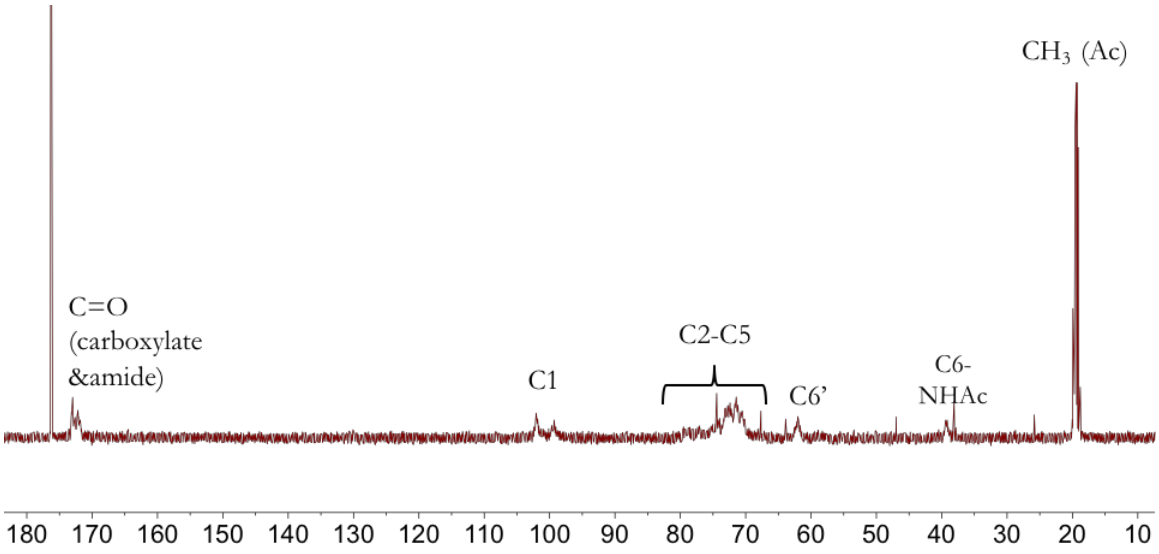


Figure S4.2 ^{13}C NMR spectrum of (6-acetamido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-NHAcCA320S).

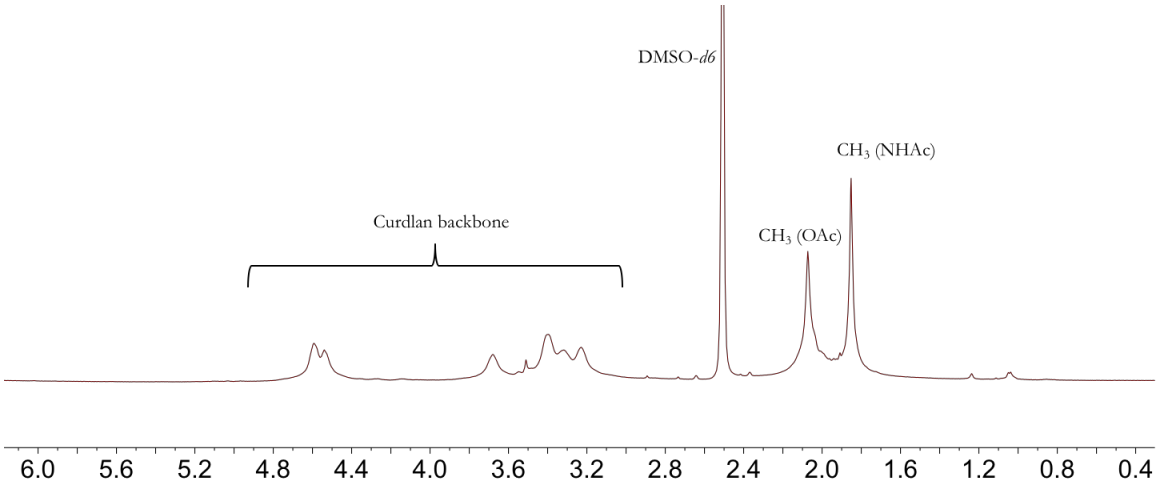


Figure S4.3 ^{1}H NMR spectrum of 6-acetamido-6-deoxy-*O*-acetyl-curdlan (6-NHAcCurdlanAc).

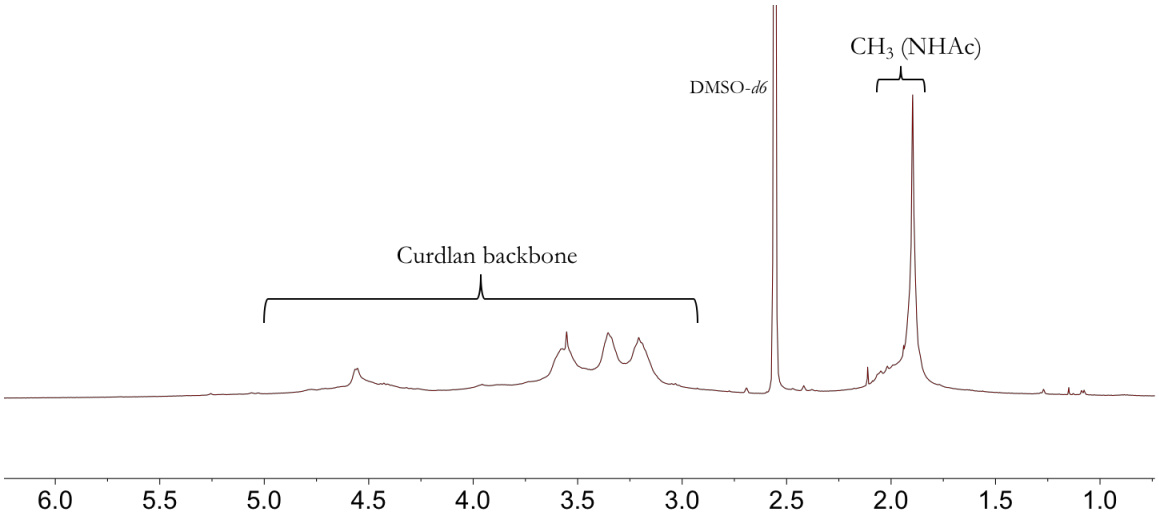


Figure S4.4 ^{1}H NMR spectrum of 6-acetamido-6-deoxy-curdlan (6-NHAcCurdlan).

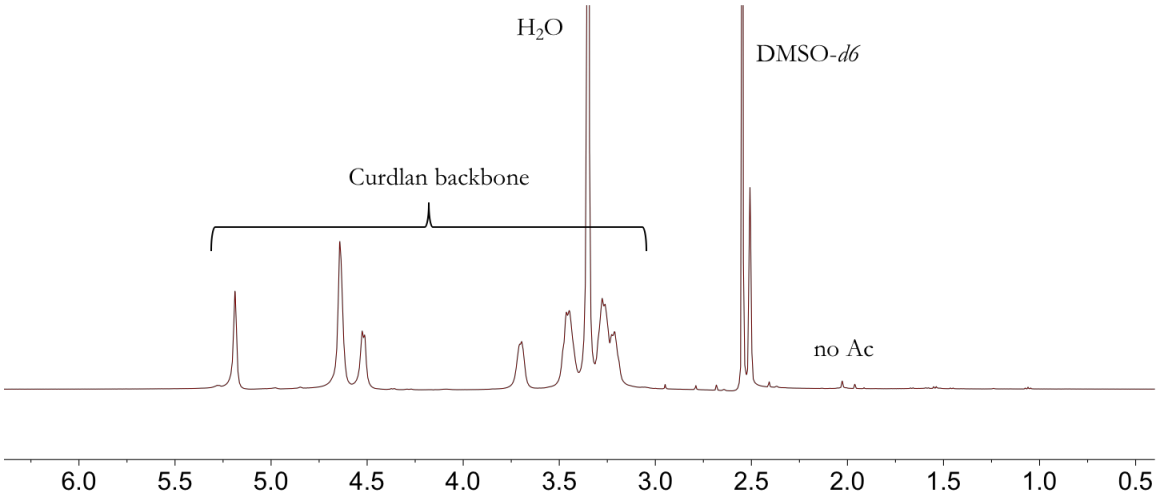


Figure S4.5 ^{1}H NMR spectrum of product obtained by treating curdlan with thioacetic acid

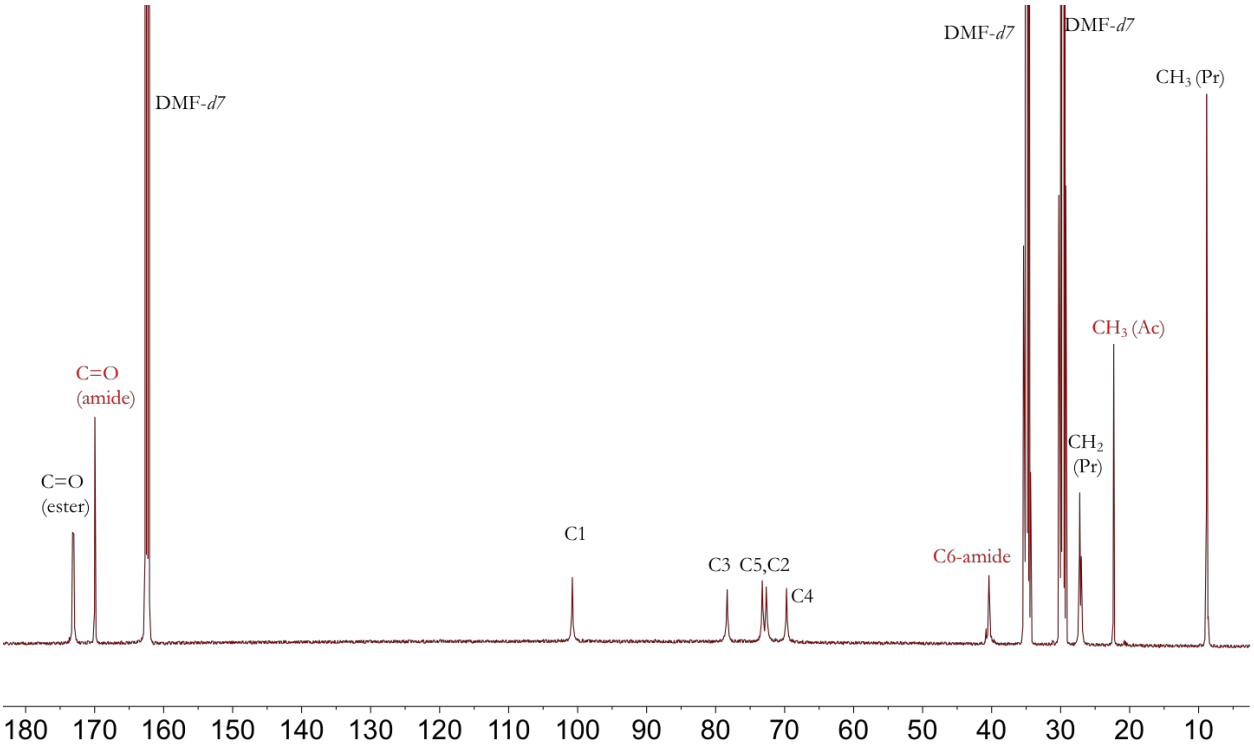


Figure S4.6 ^{13}C NMR spectrum of 6-acetamido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-NHAcCurdlanPr)

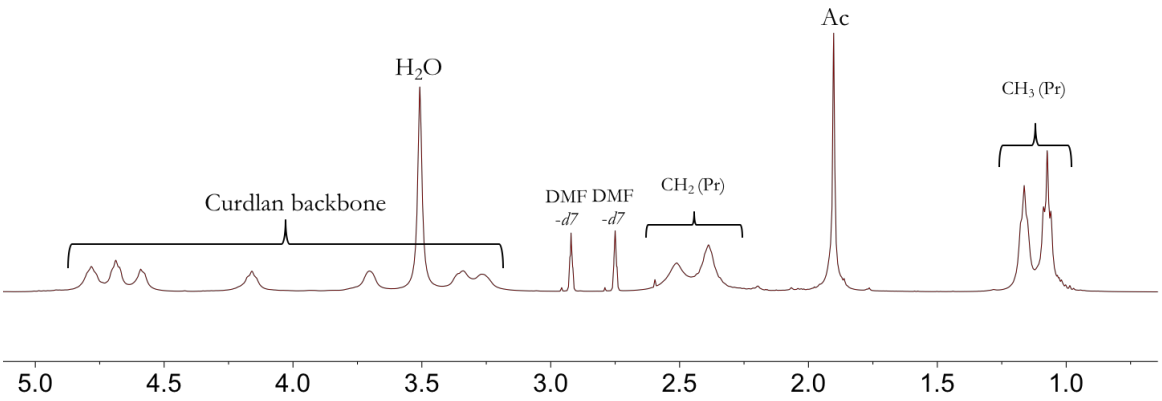


Figure S4.7 ^{1}H NMR spectrum of 6-acetamido-6-deoxy-2,4-di-*O*-propionylcurdlan (6-NHAcCurdlanPr).

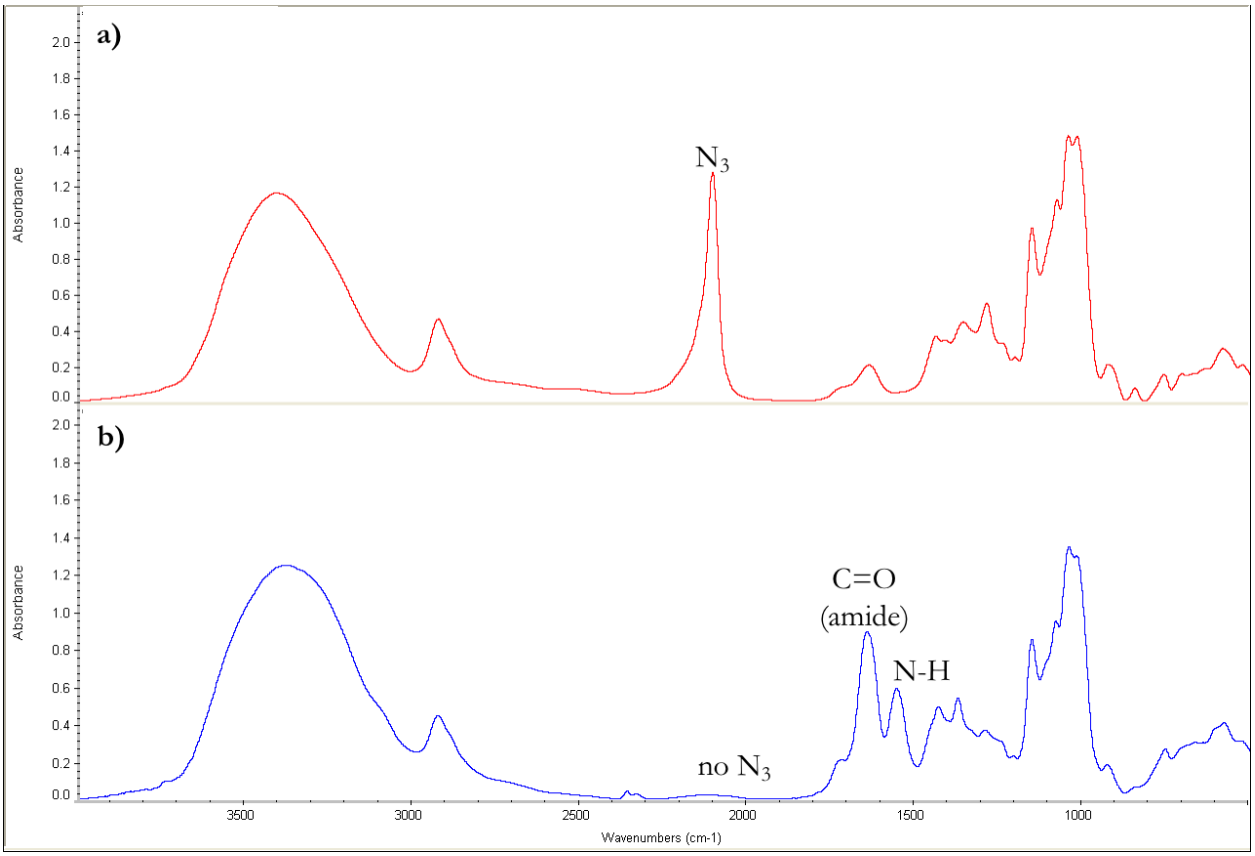


Figure S4.8 FT-IR spectra of a) 6-azido-6-deoxy-pullulan (6-N_3Pullulan) and b) 6-acetamido-6-deoxy-pullulan (6-NHAcPullulan)

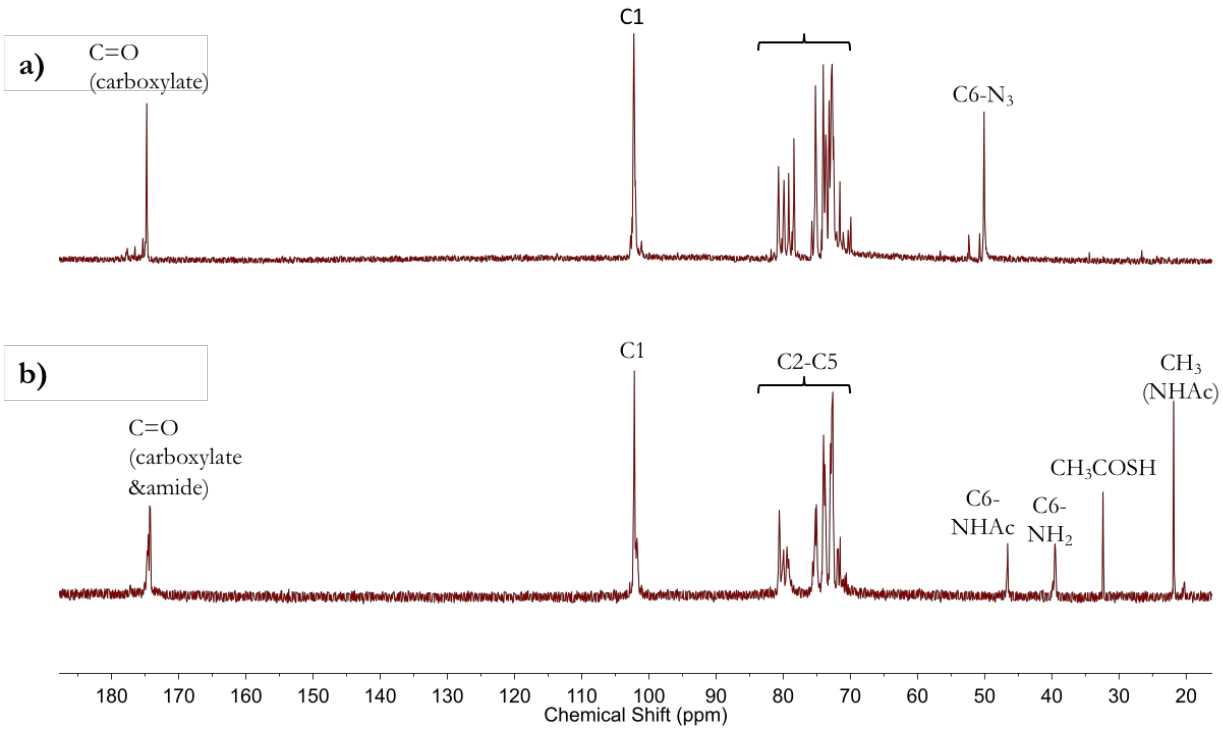


Figure S4.9 ^{13}C NMR spectra of products by thioacetic acid aqueous reduction of a) $Glc6N_3/GlcA$; b) obtained Glc6N/GlcA/Glc6NHAc under catalysis by 2,6-lutidine

Syntheses of 6-azido-6-deoxy-curdlan (6-N_3Curd), 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan (6-N_3CurdAc), 6-azido-6-deoxy-2,4-di-*O*-propionyl-curdlan (6-N_3CurdPr), (6-azido-6-deoxy)-co-(6-O-acetyl)-CA320S (6-N_3CA320S), and 6-azido-6-deoxy-pullulan (6-N_3Pull)

Syntheses of 6-N_3Curdlan, 6-N_3CurdlanAc, 6-N_3CurdlanPr, 6-N_3CA320S, and 6-N_3Pullulan were adapted from previous reports.

Curdlan was dissolved in DMAc/LiBr by a procedure reported in previous studies. A mixture of curdlan (4.0 g) and DMAc (120 mL) was heated to 165 °C and kept at that temperature for 30 minutes with vigorous stirring. LiBr (36.00 g) was added to the mixture, and the mixture was stirred at 165 °C for 10 min. The slurry was then slowly cooled to room temperature with stirring. A transparent solution was obtained after 2 h. PPh_3 (25.96 g, 4 eq per AGU) and NBS (17.58, 4 eq per AGU) were separately dissolved in 40 mL of DMAc. To a solution of curdlan (4.0 g) in DMAc/LiBr, the PPh_3 solution was added dropwise by an addition funnel, followed by dropwise addition of NBS solution. The reaction mixture was heated to 70 °C for 1 h under nitrogen. The solution was then cooled and slowly poured into 2 L of a 50:50 mixture of deionized water and methanol. The crude product was collected by filtration, then re-dissolved in DMSO and re-precipitated in ethanol. The product was dried in a vacuum oven overnight at 40 °C to afford 6-bromo-6-deoxycurdlan (6-BrCurd).

Dry 6-bromo-6-deoxycurdlan (2.5 g, 11.1 mmol) was dissolved in 50 mL of DMSO in a 100 mL round bottom flask. NaN_3 (3.6 g, 5 eq per AGU) was added to the flask. The solution was stirred at 80 °C for 24 h. The solution was then poured into 500 mL of deionized water, followed by filtration to collect the product. The product was dried under vacuum at 40 °C overnight to yield 6-azido-6-deoxycurdlan (6-N_3Curd).

6-Azido-6-deoxycurdlan (0.5 g, 2.67 mmol), DMAP (20 mg), pyridine (2.2 mL, 10 eq per AGU), and 10 eq of carhoxylic anhydride (acetic or propionic anhydride) were added to a 50 mL round-bottom flask. The mixture was stirred at 80 °C for 24 h. The solution was then poured into 50 mL of deionized water, followed by filtration to recover the precipitate. The crude product was re-dissolved in 5 mL of DMSO and then re-precipitated into 50 mL of methanol. The product was washed extensively with ethanol and deionized water before vacuum drying at 40 °C overnight to yield 6-azido-6-deoxycurdlan acetate (6-N_3CurdAc) and 6-azido-6-deoxycurdlan propionate (6-N_3CurdPr).

6-N_3CA320S was prepared in the same method with different amounts of material and reagents. CA320S (1.0 g) was dissolved in 40 mL of DMAc in a three-neck flask. PPh_3 (3 equiv per AGU, 3.32 g) and NBS (3 equiv per AGU, 2.25 g) solution were added dropwise to the flask. The identical procedure was followed to yield (6-bromo-6-deoxy)-co-(6-*O*-acetyl)-CA320S (6-BrCA320S). 6-BrCA320S (0.5 g, 1.86 mmol) was dissolved in 20 mL of anhydrous DMSO. NaN_3 (0.36g, 3 eq per AGU) was added to the flask. Then a similar procedure was followed to give (6-azido-6-deoxy)-co-(6-*O*-acetyl)-CA320S (6-N_3CA320S).

Pullulan (2.0 g) and LiBr (2.34 g) were dissolved in DMF (80 mL) at 70 °C until a homogeneous solution formed. PPh_3 (12.88 g, 4 eq per AGU) and NBS (8.76 g, 4 eq per AGU) were separately dissolved in 15 mL of DMF, followed by slow addition to pullulan solution by an addition funnel. The solution was heated to 70 °C for 3 h under nitrogen. The solution was then slowly poured into 1 L of ethanol. The product was collected by filtration, yielding 6-bromo-6-deoxy-pullulan (6-BrPull). 6-BrPulluan (1.0 g) was dissolved in 40 mL of DMF at 80 °C. Then NaN_3 (5 eq per AGU) was added to the flask. The solution was kept at 80 °C for 24 h. The solution

was then dialysis against water for 3 days, followed by freeze-drying to yield 6-azido-6-deoxy-pullulan (6-N_3Pull).

Reference

(1) Köwitsch, A.; Zhou, G.; Groth, T. Medical Application of Glycosaminoglycans: A Review. *J. Tissue Eng. Regen. Med.* **2018**, *12* (1), e23–e41.

(2) Kumar, M. N. V. R. A Review of Chitin and Chitosan Applications. *React. Funct. Polym.* **2000**, *46* (1), 1–27.

(3) Rinaudo, M.; Pavlov, G.; Desbrières, J. Influence of Acetic Acid Concentration on the Solubilization of Chitosan. *Polymer (Guildf).* **1999**, *40* (25), 7029–7032.

(4) Bernkop-Schnürch, A.; Dünnhaupt, S. Chitosan-Based Drug Delivery Systems. *Eur. J. Pharm. Biopharm.* **2012**, *81* (3), 463–469.

(5) Zargar, V.; Asghari, M.; Dashti, A. A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications. *ChemBioEng Rev.* **2015**, *2* (3), 204–226.

(6) Liu, C.; Baumann, H. Exclusive and Complete Introduction of Amino Groups and Their N-Sulfo and N-Carboxymethyl Groups into the 6-Position of Cellulose without the Use of Protecting Groups. *Carbohydr. Res.* **2002**, *337* (14), 1297–1307.

(7) Fox, S. C.; Edgar, K. J. Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively *O* -Acylated 6-Amino-6-Deoxy-Cellulose. *Biomacromolecules* **2012**, *13* (4), 992–1001.

(8) Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F. Facile Synthesis of 6-Amino-6-Deoxycellulose. *Carbohydr. Res.* **2005**, *340* (7), 1403–1406.

(9) Zhang, R.; Edgar, K. J. Synthesis of Curdlan Derivatives Regioselectively Modified at C-6: O-(N)-Acylated 6-Amino-6-Deoxycurdlan. *Carbohydr. Polym.* **2014**, *105*, 161–168.

(10) Zhang, R.; Edgar, K. J. Water-Soluble Aminocurdlan Derivatives by Chemoselective Azide Reduction Using NaBH 4. *Carbohydr. Polym.* **2015**, *122*, 84–92.

(11) Pereira, J. M.; Edgar, K. J. Regioselective Synthesis of 6-Amino-and 6-Amido-6-Deoxypullulans. *Cellulose* **2014**, *21* (4), 2379–2396.

(12) Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H. Preparation of Amylose Derivatives Selectively Modified at C-6. 6-Amino-6-Deoxyamylose. *Macromolecules* **1994**, *27* (11), 2917–2922.

(13) Liu, S.; Edgar, K. J. Staudinger Reactions for Selective Functionalization of Polysaccharides: A Review. *Biomacromolecules* **2015**, *16* (9), 2556–2571.

(14) Furuhata, K.; Koganei, K.; Chang, H.-S.; Aoki, N.; Sakamoto, M. Dissolution of Cellulose in Lithium Bromide-Organic Solvent Systems and Homogeneous Bromination of Cellulose with N-Bromosuccinimide-Triphenylphosphine in Lithium Bromide-N,N-Dimethylacetamide. *Carbohydr. Res.* **1992**, *230* (1), 165–177.

(15) Klemm, D.; Heinze, T.; Philipp, B.; Wagenknecht, W. New Approaches to Advanced Polymers by Selective Cellulose Functionalization. *Acta Polym.* **1997**, *48* (8), 277–297.

(16) Gao, C.; Liu, S.; Edgar, K. J. Regioselective Chlorination of Cellulose Esters by Methanesulfonyl Chloride. *Carbohydr. Polym.* **2018**, *193*, 108–118.

(17) Petzold-Welcke, K.; Michaelis, N.; Heinze, T.; Petzold-Welcke, K.; Michaelis, N.; Heinze, T.; Petzold-Welcke, K.; Michaelis, N.; Heinze, T. Unconventional Cellulose Products through Nucleophilic Displacement Reactions. *Macromol. Symp.* **2009**, *280* (1), 72–85.

(18) Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J. Regioselective Esterification and Etherification of

Cellulose: A Review. *Biomacromolecules* **2011**, *12* (6), 1956–1972.

(19) Liebert, T.; Hänsch, C.; Heinze, T. Click Chemistry with Polysaccharides. *Macromol. Rapid Commun.* **2006**, *27* (3), 208–213.

(20) Meng, X.; Edgar, K. J. "Click" Reactions in Polysaccharide Modification. *Prog. Polym. Sci.* **2016**, *53*, 52–85.

(21) Nishimura, S.-I.; Kai, H.; Shinada, K.; Yoshida, T.; Tokura, S.; Kurita, K.; Nakashima, H.; Yamamoto, N.; Uryu, T. Regioselective Syntheses of Sulfated Polysaccharides: Specific Anti-HIV-1 Activity of Novel Chitin Sulfates. *Carbohydr. Res.* **1998**, *306* (3), 427–433.

(22) Madhunapantula, S. V.; Achur, R. N.; Bhavanandan, V. P.; Gowda, D. C. The Effect of Substitution of the N-Acetyl Groups of N-Acetylgalactosamine Residues in Chondroitin Sulfate on Its Degradation by Chondroitinase ABC. *Glycoconj. J.* **2007**, *24* (8), 465–473.

(23) Huang, L.; Kerns, R. J. Diversity-Oriented Chemical Modification of Heparin: Identification of Charge-Reduced N-Acyl Heparin Derivatives Having Increased Selectivity for Heparin-Binding Proteins. *Bioorg. Med. Chem.* **2006**, *14* (7), 2300–2313.

(24) Hirano, S.; Ohashi, W. N-Acylation of N-Desulfate Heparin. *Agric. Biol. Chem.* **1976**, *40* (12), 2501–2502.

(25) Bräse, S.; Gil, C.; Knepper, K.; Zimmermann, V. Organic Azides: An Exploding Diversity of a Unique Class of Compounds. *Angew. Chemie Int. Ed.* **2005**, *44* (33), 5188–5240.

(26) Daly, W. H.; Soo, L. Peptide Graft Copolymers from Soluble Aminodeoxycellulose Acetate. In *American Chemical Society, Polymer Preprints, Division of Polymer Chemistry*; 1989.

(27) Gao, C.; Edgar, K. J. Efficient Synthesis of Glycosaminoglycan Analogs. *Biomacromolecules* **2018**.

(28) Staros, J. V.; Bayley, H.; Standring, D. N.; Knowles, J. R. Reduction of Aryl Azides by

Thiols: Implications for the Use of Photoaffinity Reagents. *Biochem. Biophys. Res. Commun.* **1978**, *80* (3), 568–572.

(29) Baker, N. D.; Griffin, R. J.; Irwin, W. J.; Slack, J. A. The Reduction of Aryl Azides by Dithiothreitol: A Model for Bioreduction of Aromatic Azido-Substituted Drugs. *Int. J. Pharm.* **1989**, *52* (3), 231–238.

(30) Konigsberg, W. [13] Reduction of Disulfide Bonds in Proteins with Dithiothreitol. *Methods Enzymol.* **1972**, *25*, 185–188.

(31) Jacobs, S.; Cuatrecasas, P. Disulfide Reduction Converts the Insulin Receptor of Human Placenta to a Low Affinity Form. *J. Clin. Invest.* **1980**, *66* (6), 1424–1427.

(32) Ning, S.; Sreenivas, K.; Rachel, G.; Lawrence, W. J. The Reaction of Thio Acids with Azides: A New Mechanism and New Synthetic Applications. *J. Am. Chem. Soc.* **2003**, *125* (26), 7754–7755.

(33) Yang, Y. Y.; Ficht, S.; Brik, A.; Wong, C. H. Sugar-Assisted Ligation in Glycoprotein Synthesis. *J. Am. Chem. Soc.* **2007**, *129* (24), 7690–7701.

(34) Ochiai, H.; Huang, W.; Wang, L.-X. Expeditious Chemoenzymatic Synthesis of Homogeneous N-Glycoproteins Carrying Defined Oligosaccharide Ligands. *J. Am. Chem. Soc.* **2008**, *130* (41), 13790–13803.

(35) Crich, D.; Yang, F. Phenylthiomethyl Glycosides: Convenient Synthons for the Formation of Azidomethyl and Glycosylmethyl Glycosides and Their Derivatives. *Angew. Chemie Int. Ed.* **2009**, *48* (47), 8896–8899.

(36) Robert, K. V; Ning, S.; Ronald, S. R.; Lawrence, W. J. Mechanism of Thio Acid/Azide Amidation. *J. Am. Chem. Soc.* **2006**, *128* (17), 5695–5702.

(37) Liebert, T.; Hussain, M. A.; Heinze, T. Structure Determination of Cellulose Esters via

Subsequent Functionalization and NMR Spectroscopy. *Macromol. Symp.* **2005**, *223* (1), 79–92.

(38) Zheng, X.; Gandour, R. D.; Edgar, K. J. Probing the Mechanism of TBAF-Catalyzed Deacylation of Cellulose Esters. *Biomacromolecules* **2013**, *14* (5), 1388–1394.

(39) Liu, S.; Edgar, K. J. Water-Soluble Co-Polyelectrolytes by Selective Modification of Cellulose Esters. *Carbohydr. Polym.* **2017**, *162*, 1–9.

(40) Liu, S.; Gao, C.; Mosquera-Giraldo, L. I.; Taylor, L. S.; Edgar, K. J. Selective Synthesis of Curdlan ω-Carboxyamides by Staudinger Ylide Nucleophilic Ring-Opening. *Carbohydr. Polym.* **2018**, *190*, 222–231.

(41) Lippert, A. R.; New, E. J.; Chang, C. J. Reaction-Based Fluorescent Probes for Selective Imaging of Hydrogen Sulfide in Living Cells. *J. Am. Chem. Soc.* **2011**, *133* (26), 10078–10080.

(42) Kazemi, F.; Kiasat, A. R.; Sayyahi, S. Chemoselective Reduction of Azides with Sodium Sulfide Hydrate under Solvent Free Conditions. *Phosphorus, Sulfur Silicon Relat. Elem.* **2004**, *179* (9), 1813–1817.

(43) Henthorn, H. A.; Pluth, M. D. Mechanistic Insights into the H_2S-Mediated Reduction of Aryl Azides Commonly Used in H_2S Detection. *J. Am. Chem. Soc.* **2015**, *137* (48), 15330–15336.

(44) Hartle, M. D.; Meininger, D. J.; Zakharov, L. N.; Tonzetich, Z. J.; Pluth, M. D. NBu_4SH Provides a Convenient Source of HS^- Soluble in Organic Solution for H_2S and Anion-Binding Research. *Dalt. Trans.* **2015**, *44* (46), 19782–19785.

(45) Edgar, K. J.; Buchanan, C. M.; Debenham, J. S.; Rundquist, P. A.; Seiler, B. D.; Shelton, M. C.; Tindall, D. Advances in Cellulose Ester Performance and Application. *Prog. Polym.*

Sci. **2001**, *26* (9), 1605–1688.

(46) Meng, X.; Roy Choudhury, S.; Edgar, K. J. Multifunctional Cellulose Esters by Olefin Cross-Metathesis and Thiol-Michael Addition. *Polym. Chem.* **2016**, *7* (23), 3848–3856.

(47) Kumar, S.; Kohli, K. Chemical Modification of Wood: Reaction with Thioacetic Acid and Its Effect on Physical and Mechanical Properties and Biological Resistance. In *Renewable-Resource Materials*; Springer US: Boston, MA, 1986; pp 147–160.

(48) Whiting, A.; Walton, S. Unexpected Exothermic Reaction between Thioacetic Acid and DMSO. *Org. Process Res. Dev.* **2006**, *10* (4), 846–846.

(49) Mühlberg, M.; Siebertz, K. D.; Schlegel, B.; Schmieder, P.; Hackenberger, C. P. R. Controlled Thioamide vs. Amide Formation in the Thioacid–Azide Reaction under Acidic Aqueous Conditions. *Chem. Commun.* **2014**, *50* (35), 4603.

Chapter 5: Functionalization of Hydroxyethylcellulose by Selective Chlorination

(Manuscript in preparation)

Chengzhe Gao,[a,c] Stella P. Petrova,[a,c] and Kevin J. Edgar[b,c,*]

[a]*Department of Chemistry, Virginia Tech, Blacksburg, VA, 24061*

[b]*Department of Sustainable Biomaterials, Virginia Tech, Blacksburg, VA, 24061*

[c]*Macromolecules Innovation Institute, Virginia Tech, Blacksburg, VA, 24061*

E-mail: kjedgar@vt.edu; Tel: +1-540-231-0674

Abstract

Selective chemical modification of cellulose and cellulose derivatives is challenging due to their generally low reactivity and poor solubility. Halogenation followed by halide displacement has been demonstrated to be a valuable strategy to append new functionalities to polysaccharides and their derivatives. In this paper, we report a simple and efficient pathway to modify commercial, inexpensive hydroxyethyl cellulose (HEC). First, methanesulfonyl chloride (MsCl) can selectively chlorinate the terminal primary hydroxyl groups from HEC (degree of substitution (DS) 1.5, molar substitution (MS) 2.5), thereby affording high chloride content (DS (Cl) 1.68). Then, the resulting chlorinated HEC can undergo nucleophilic displacement reactions with various nucleophiles including azide (NaN_3), amine (1-methylimidazole), and thiols (3-mercaptopropionic acid and 2-mercapto ethanol). All products were characterized by NMR and FT-IR spectroscopy. Through this strategy, we prepared a library of HEC derivatives, including cationic and anionic derivatives, which are of great interest in various applications such as surfactants, gas separation membranes, and amorphous solid dispersions in oral drug delivery.

5.1 Introduction

Cellulose is one of the most abundant natural polymers, playing an important role in structural reinforcement of plant cell walls.[1] It is an attractive material for a variety of applications[2] because it is renewable, its hydroxyl groups can be chemically functionalized, and has high mechanical strength. It is estimated that nature creates 10^{10} to 10^{11} tons of new cellulose annually, and native cellulose is widely used in the paper, textile, and food industries. However, cellulose is insoluble in common organic and aqueous solvent systems, and it cannot be thermally processed (decomposes before melting), hindering wider application of cellulose as a sustainable material. Chemical functionalization of the three free cellulose hydroxyl groups can modify its physiochemical properties, permitting processing and tailoring for application needs. Surprisingly, despite the vast amount of available cellulose and the practical utility of cellulose derivatives, there are only a few important classes of commercial cellulose derivatives; cellulose esters (e.g. acetate, propionate, butyrate, succinate, and phthalate), cellulose ethers (e.g. methyl, ethyl, hydroxyethyl, hydroxypropyl, and carboxymethyl ethers), and cellulose ether esters.[2–4] This narrow range of derivatives is mainly a result of the similar and low reactivity of cellulose hydroxyl groups. As a result of low reactivity and high crystallinity, harsh conditions are also typically necessary to functionalize cellulose. For example, esterification of cellulose normally requires strong acids, and etherification methods require strongly alkaline conditions, limiting the types of functional groups that may be selectively appended. The increasing societal demand for sustainable materials makes it imperative to develop novel, efficient, and effective chemistries to selectively modify cellulose and its derivatives.

Cellulose ethers have been used in a wide range of applications, e.g. as thickeners, binders, lubricants, and rheology modifiers in numerous products such as food, pharmaceuticals, personal care products, oil field chemicals, construction, paper, and adhesives.[4] They are typically water soluble and in some cases can be further elaborated by functionalizing the remaining ring hydroxyls, or newly introduced hydroxyl groups (hydroxyethyl and hydroxypropyl groups).[5] Commercially, cellulose ethers can be esterified to afford cellulose ether esters to tune the hydrophilicity of the polymer including for coatings and drug delivery applications. There are also extensive reports on chemical modifications of terminal hydroxyl groups on HEC and hydroxypropyl cellulose (HPC) by further etherification and esterification [6]. For example, cationic HEC derivatives with quaternary ammonium groups can be prepared by etherification of HEC and have demonstrated to be useful as surfactants, e.g. in cosmetics [7,8]. Recently, the Edgar group has applied various chemical methods including selective oxidation of particular hydroxyl groups of hydroxyalkyl ethers, and olefin cross-metathesis to append terminal carboxyl groups to cellulose ethers for amorphous solid dispersion (ASD) applications.[9–12] Olefin cross-metathesis typically involves three steps: etherification of hydroxyl groups to append a terminal olefin handle, cross-metathesis with acrylate derivatives, and hydrogenation or thiol-ene click reactions to eliminate the resulting α-β unsaturation to afford a stable product. Through this strategy, a wide range of cellulose ether derivatives with enhanced ASD performance were prepared, and the structure-activity relationships were thoroughly investigated, demonstrating substantial potential for cellulose ether modification.[13]

One of the most useful cellulose modification strategies is to convert the primary hydroxyl group to a good leaving group (e.g. tosylate, mesylate, -Cl, -Br, -I), followed by nucleophilic displacement.[14,15] Employing this strategy a variety of cellulose derivatives containing azides,

amines, thiols, and heterocycles has been prepared.[16–20] While this strategy is well established for modifications of native polysaccharides, only a few investigators have explored halogenation strategies for functionalizing hydroxyethyl polysaccharides. Eissa et al. applied Furuhata bromination (PPh_3 and NBS) to HEC to successfully convert the terminal hydroxyl groups on the HEC side chain to bromide, then displaced bromide with azide, creating a partner for subsequent azide alkyne click reactions.[21] Joubert et al. synthesized azide substituted HEC in similar fashion, creating a substrate for click reactions to graft poly(ionic liquid)s to the HEC backbone.[22] In both cases, Furuhata bromination was employed and azide was the only nucleophile described.[23] To our knowledge, tosylate and chloride, two convenient and relatively inexpensive leaving groups frequently used in polysaccharide chemistry, have never been introduced to functionalize HEC. Each leaving group has its own advantages and disadvantages. Tosylate is the most commonly used leaving group in cellulose chemistry due to its availability, hydrolytic stability, wide range of compatible solvents (for example complex organic solvents, NaOH/urea aqueous solution, and ionic liquids), and product solubility in common organic solvents. However, tosylation of polysaccharides is typically not entirely regioselective, and side reactions such as chlorination can compete.[24] Bromide can be introduced usually with beautiful chemo- and regioselectivity by reaction with PPh_3 and NBS; however, the by-product triphenylphosphine oxide (PPh_3=O) is very challenging to remove quantitatively from the products.[25] Inexpensive chlorination reagents are available, and recently a method has been published for regioselective chlorination of cellulose 6-OH groups with no observed by-product formation issues.[26] Chloride is however typically inferior, e.g. vs. bromide, as a leaving group. Thus, it would be of great interest to systematically investigate the modification of HEC by introducing leaving groups other than bromide, then evaluate displacement of the chloride with various nucleophiles.

Recently, the Edgar lab has discovered that methanesulfonyl chloride (MsCl) can chemo- and regioselectively chlorinate primary hydroxyl groups on cellulose esters such as low DS cellulose acetate (DS(Ac) 1.8, DS(primary OH) 0.5).[26] The 6-chloro-6-deoxy cellulose esters can then undergo nucleophilic displacement reactions with a wide variety of nucleophiles, including amines, thiols, and azide. This chlorination method displayed many advantages including ease of purification, low cost reagents, and sufficient reactivity. HEC contains a high content of primary hydroxyl groups; the sum of the unreacted C-6 OH groups and the DS(HE), since every oligo(hydroxyethyl) chain terminates with a primary OH group. Moreover, compared to the free hydroxyl groups on the cellulose backbone, the oligo(hydroxyethyl) terminal hydroxy groups of HEC should be more reactive due to their wider approach angles. However, it remains unknown whether MsCl can chlorinate primary terminal hydroxyl groups and whether the putative chlorinated HEC would be a useful substrate for further nucleophilic substitution reactions to afford HEC derivatives.

Thus, we hypothesize that methanesulfonyl chloride can selectively chlorinate the HEC terminal hydroxy groups, replacing the terminal -OH with -Cl. We further hypothesize that the introduced chloride can be displaced by different nucleophiles, creating useful HEC derivatives. If reduced to practice, this two-step sequence will be a powerful strategy for hydroxyethyl polysaccharide functionalization. Herein, we report our attempt to prove or refute these hypotheses.

5.2 Experimental

5.2.1 Materials

HEC (structure shown in Scheme 5.1, m=1.5, molar substitution (average number of substituents per anhydroglucose ring, MS) = 2.5, degree of substitution (average number of

position substituted per anhydroglucose ring, DS) = 1.5 (reported by previous publications),[27] average Mw ~ 90,000), methanesulfonyl chloride (MsCl) (99.7+%), sodium azide, 1-methylimidazole (99+%), sodium iodide, 4-dimethylaminopyridine (99%), *N, N*-diisopropylethylamine （98+）, and thioacetic acid were from Sigma Aldrich and used as received. Acetone and ethanol were from Fisher Scientific and used as received. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) from Fisher were kept over 4 Å molecular sieves and stored under dry N_2 until use. Regenerated cellulose dialysis tubing (Fisher, 3500 g/mol MWCO) was used as received.

5.2.2 Measurements

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in DMSO-*d6* or D_2O at room temperature using 64 scans and 15,000 scans, respectively. Chemical shifts are reported relative to the solvent peaks. FT-IR spectra were acquired on a Nicolet 8700 instrument with 128 scans and 4 cm^{-1} resolution. Elemental analysis (EA) was performed by Micro Analysis Inc. using a CE440 analyzer. Carbon, hydrogen and nitrogen contents were determined by flask combustion followed by ion chromatography. Chlorine and sulfur contents were measured by flask combustion followed by titration. All yields are reported on a molar basis, based on the average product repeat unit molecular weight as determined based on spectroscopic and elemental analysis results. DS values were calculated according to the following equations:

$$DS_{chloride} = \frac{132.11 \times Cl\%}{35.45 \times C\%}$$

$$DS_{azide} = \frac{132.11 \times N\%}{42.03 \times C\%}$$

$$DS_{mesylate} = \frac{132.11 \times S\%}{32.07 \times C\%}$$

5.2.3 Methods

5.2.3.1 Regioselective chlorination of hydroxyethyl cellulose

In a 100 mL round-bottom (RB) flask, HEC (1.0 g, 3.6 mmol AGU, 5.4 mmol terminal primary OH) was dissolved in 40 mL of anhydrous DMF under mechanical stirring overnight until a homogeneous solution was obtained. MsCl (4.3 mL, 10 equiv. per terminal primary OH) was added dropwise to the solution. The reaction mixture was kept at 75 °C for 4 h under N_2 as the solution turned from colorless to yellow. The solution was cooled to room temperature and slowly poured into 400 mL of deionized water. The crude product was isolated by filtration, extensively washed with water and ethanol, and vacuum dried overnight at 40 °C to yield ω-(2-chloroethyl)-hydroxyethyl cellulose (HEC-Cl). ^{13}C NMR (500 MHz, DMSO-*d6*): 43.58 (-O-CH_2- $\underline{C}H_2$-Cl), 60.22 (C-6-O-), 69.69, 70.56 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 71.43-81.44 (C2, C3, C4 and C5), 101.89 (C1), and 161.30 (O-(C=O)-H). Elemental analysis results: %C 42.47, %H 5.73, %Cl 19.24, %S 0.4, and %N not found (below the detection limit). DS calculated by EA: DS(Cl) = 1.68. Maximum possible DS(Mesyl) calculated by EA (assuming all S comes from mesyl groups): DS(Ms) = 0.03. Yield: 921 mg (83.6%).

5.2.3.2 Synthesis of ω-(2-azidoethyl)-hydroxyethyl cellulose (HEC-N_3)

HEC-Cl (0.5 g, 1.7 mmol AGU, 2.5 mmol Cl) was dissolved in 20 mL of anhydrous DMSO in a 100 mL RB flask. To the solution was added NaN_3 (813 mg, 5 equiv. per Cl). The reaction was heated to 75 °C for 24 h under N_2. The solution was poured into 200 mL of deionized water, filtered, and the solid product washed with water. The product was dried in a vacuum oven overnight at 40 °C to yield HEC-N_3. ^{13}C NMR (500 MHz, DMSO-*d6*): 50.01 (-O-CH_2- $\underline{C}H_2$-N_3), 60.22 (C-6-O-), 69.30, 69.72, 69.82 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 70.87-82.06 (C2, C3, C4 and C5), and

100.26-103.58 (C1). Elemental analysis results: %C 41.09, %H 5.67, %N 21.03, %Cl 1.25, and %S 0.4. DS calculated by EA: DS(N_3) = 1.60, DS(Cl) = 0.11. Yield: 416 mg (80.6%).

5.2.3.3 Synthesis of ω-(2-acetamidoethyl)-hydroxyethyl cellulose (HEC-NHAc)

In a 50 mL RB flask, 200 mg of HEC-N_3 (0.65 mmol AGU, 0.97 mmol N_3) was dissolved in 10 mL of anhydrous DMF. Thioacetic acid (0.68 mL, 10 equiv. per N_3) and 2,6-lutidine (1.12 mL, 10 equiv. per N_3) were added separately to the solution. The reaction solution was stirred at 65 °C for 24 h. The cooled solution was dialyzed against ethanol for 3 days and against water for another 3 days, then freeze-dried to yield HEC-NHAc. ^{1}H NMR (500 MHz, DMSO-*d6*): 1.75-1.95 (NH-(C=O)-$\underline{C}H_3$), and 2.80-5.11 (cellulose backbone and -O-C$\underline{H}_2$-C$\underline{H}_2$-O-); ^{13}C NMR (500 MHz, DMSO-*d6*): 22.58 (NH-(C=O)-$\underline{C}H_3$), 60.30 ($\underline{C}$-6-O-), 69.17, 69.59, 69.75 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 70.90-81.80 (C2, C3, C4 and C5), 100.45-104.02 (C1), and 169.35 (NH-($\underline{C}$=O)-CH_3). Yield: 124 mg (57.5 %).

5.2.3.4 Synthesis of ω-(2-(1-methyl-3-imidazolio)ethyl)-hydroxyethyl cellulose (HEC-MeIM)

HEC-Cl (200 mg, 0.67 mmol AGU, 1.00 mmol Cl) was dissolved in 10 mL of anhydrous DMSO in a 50 mL RB flask. NaI (450 mg, 3 equiv. per Cl) and 1-methylimidazole (3.2 mL, 40 equiv. per Cl) were slowly added to the flask. The solution was stirred at 80 °C for 48 h under N_2. The product was collected by dialysis of the cooled solution against ethanol for 3 days and against 0.9 % sodium chloride solution for another 3 days, then freeze-dried to yield HEC-MeIM. ^{1}H NMR (500 MHz, DMSO-*d6*): 3.01-4.58 (cellulose backbone, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-, and N-$\underline{C}H_3$), and 7.33-7.61 (N-C$\underline{H}$=CH-N-CH_3, N-CH=C$\underline{H}$-N-CH_3, and N=C$\underline{H}$-N-CH_3); ^{13}C NMR (500 MHz, DMSO-*d6*): 35.89 (N-$\underline{C}H_3$), 49.00 (-O-CH_2-$\underline{C}H_2$-N), 60.34 ($\underline{C}$-6-O-), 68.45, 69.59 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 70.95-81.50 (C2, C3, C4 and C5), 101.96 (C1), 122.56, 123.46 (N-$\underline{C}$H=CH-N-CH_3 and N-CH=$\underline{C}$H-N-CH_3), and 136.43 (N=$\underline{C}$H-N-CH_3). Yield: 105 mg (42.5 %).

5.2.3.5 Synthesis of ω-(2-thioethyl) hydroxyethyl cellulose (HEC-ME)

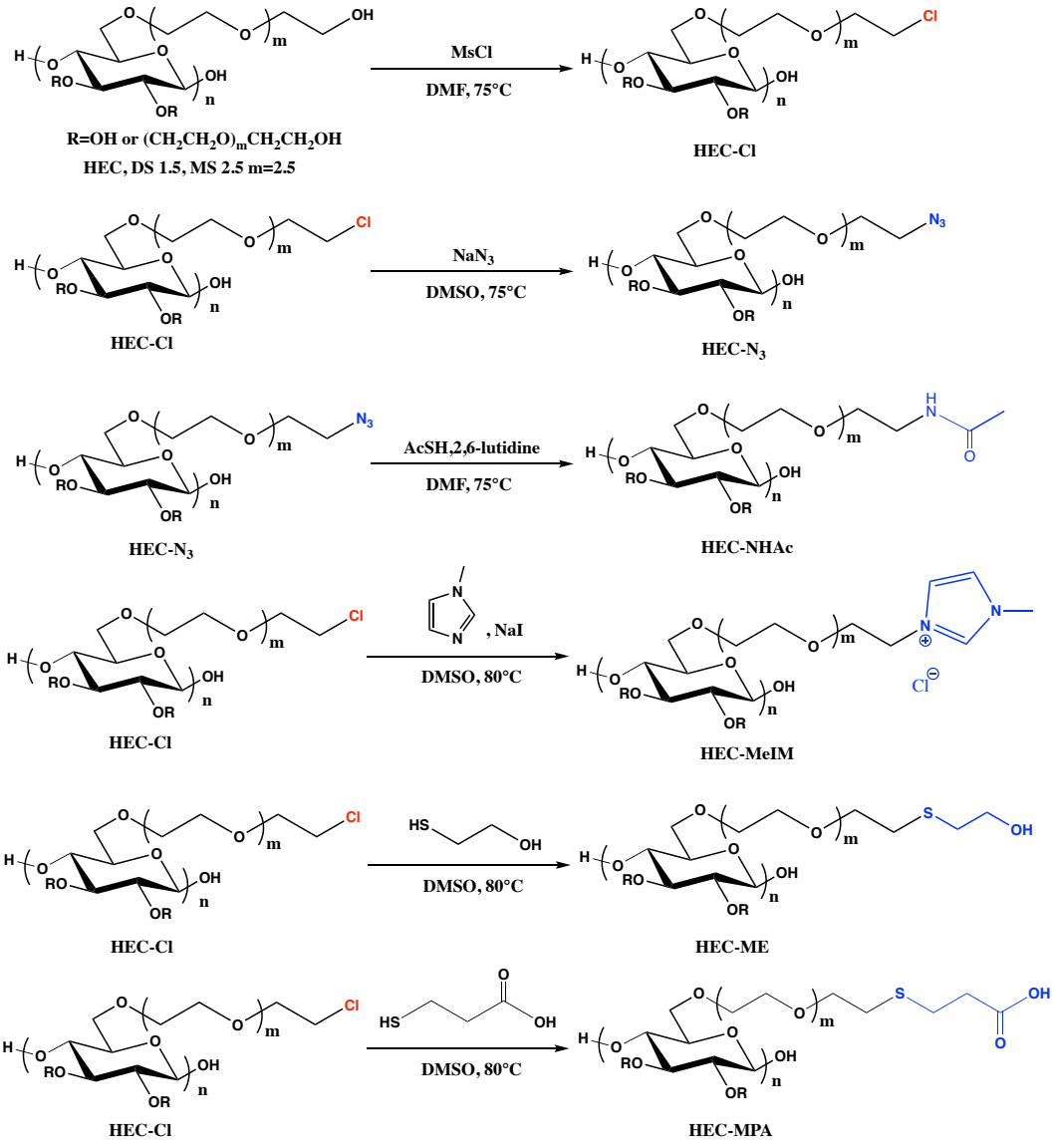
In a 50 mL RB flask, HEC-Cl (200 mg, 0.67 mmol AGU, 1.00 mmol Cl) was dissolved in 10 mL of anhydrous DMSO. NaI (450 mg, 3 equiv. per Cl), *N, N*-diisopropylethylamine (3.5 mL, 20 equiv. per Cl), and 2-mercaptoethanol (1.4 mL, 20 equiv. per Cl) were added to the flask. The solution was stirred at 80°C for 24 h under N_2. The product was collected by dialysis of the cooled solution against ethanol for 3 days and against water for another 3 days, then freeze-dried to yield HEC-ME. ^{1}H NMR (500 MHz, DMSO-*d6*): 2.58, 2.66 (S-C$\underline{H_2}$-CH_2-OH), and 2.98-4.91 (cellulose backbone, -O-C$\underline{H_2}$-C$\underline{H_2}$-O-, and -S-CH_2-C$\underline{H_2}$-OH); ^{13}C NMR (500 MHz, DMSO-*d6*): 30.94 (-S-$\underline{C}H_2$-CH_2-OH), 34.32 (-O-CH_2-$\underline{C}H_2$-S-), 43.61 (-O-CH_2-$\underline{C}H_2$-Cl), 60.25 ($\underline{C\text{-}6}$-O-), 61.03 (-S-CH_2-$\underline{C}H_2$-OH), 69.81, 70.35,70.58 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 71.77-81.65 (C2, C3, C4 and C5), and 102.32 (C1). Yield: 148 mg (61.4%).

5.2.3.6 Synthesis of ω-(2-carboxyethylthio) hydroxyethyl cellulose (HEC-MPA)

In a 50 mL RB flask, HEC-Cl (200 mg, 0.67 mmol AGU, 1.00 mmol Cl) was dissolved in 10 mL of anhydrous DMSO. NaI (450 mg, 3 equiv. per Cl), *N, N*-diisopropylethylamine (3.5 mL, 20 equiv. per Cl), and 3-mercaptopropionic acid (1.8 mL, 20 equiv. per Cl) were added to the flask. The solution was stirred at 80°C for 24 h under N_2. The reaction mixture was precipitated into 100 mL of deionized water and dialyzed against water to remove impurities. The product was collected by filtration and dried under vacuum at 40°C to yield HEC-MPA. ^{1}H NMR (500 MHz, DMSO-*d6*): 2.60, 2.71, 2.87 (S-C$\underline{H_2}$-CH_2-COOH and S-CH_2-C$\underline{H_2}$-COOH), and 3.06-4.60 (cellulose backbone and -O-C$\underline{H_2}$-C$\underline{H_2}$-O-); ^{13}C NMR (500 MHz, DMSO-*d6*): 33.15, 33.40, 33.84, 34.00 (-S-$\underline{C}H_2$-CH_2-COOH, -S-CH_2-$\underline{C}H_2$-COOH, -O-CH_2-$\underline{C}H_2$-S-), 44.03 (-O-CH_2-$\underline{C}H_2$-Cl), 64.02 ($\underline{C\text{-}6}$-O-), 68.68, 70.23,71.01 (-O-$\underline{C}H_2$-$\underline{C}H_2$-O-), 72.73-82.26 (C2, C3, C4 and C5), 102.34 (C1), and 171.66, 173.15 (-S-CH_2-CH_2-$\underline{C}$OOH). Yield: 124 mg (46.0 %).

5.3 Results and Discussion

In this work, we selected HEC with MS = 2.5 and DS = 1.5 as starting material because of its commercial availability, high content of terminal primary hydroxyl groups, and good solubility in organic solvents.



Scheme 5.1 Selective chlorination and subsequent displacement by azide, amines, and thiols

5.3.1 Selective chlorination of HEC by MsCl

Methanesulfonyl chloride has been previously shown to successfully chlorinate ring hydroxyl groups of polysaccharides, polysaccharide derivatives, and carbohydrates.[28–30] Our lab has also previously employed MsCl to regioselectively chlorinate the remaining C-6 primary hydroxyl groups of a commercial cellulose acetate (DS(Ac) 1.8).[26] We attempted to chlorinate the terminal primary hydroxyl groups of the HEC oligo(hydroxyethyl) substituents under similar conditions, that is in DMF at 75 °C using MsCl as chlorination reagent. We obtained a product in whose ^{13}C NMR spectrum (Figure 5.1) we were delighted to observe a new C-Cl resonance at 43.6 ppm, consistent with chloride-bearing primary carbons as previously reported,[26,28] indicating a successful chlorination reaction. In the ^{13}C NMR spectrum, the HEC side chain methylene groups display higher signal-to-noise ratio than cellulose backbone resonances, which is attributed to higher mobility of the flexible oligo(hydroxyethyl) side chains than the rigid cellulose backbone. It is worth-noting that the C-Cl signal in the ^{13}C NMR spectrum also possesses a high signal-to-noise ratio, similar to those of the HEC side chain methylene groups, suggesting that the C-Cl may have an environment similar to that of the HEC side chain. This is consistent with the hypothesis that most or all of the chlorination occurred on the oligo(hydroxyethyl) hydroxyl terminus. Elemental analysis for chlorine (19.24%) afforded a calculated DS (Cl) value of 1.68. Considering that the DS(HE) of the starting ether (and thus of ω-OH groups) is ca. 1.5, and that the DS(6-OH) of starting HEC must be quite low (etherification at C6 being favored),[31,32] this elemental analysis result suggests that essentially all the terminal oligo(hydroxyethyl) hydroxyls have been successfully replaced by chloride. Elemental analysis results also provide useful evidence about chlorination chemoselectivity. Sulfur content was 0.4 % and nitrogen was not found, suggesting that no more than a low DS(mesyl) (≤ 0.03) is appended to the polymer, implying selectivity of

chlorination vs. mesylation ≥ 43 : 1. This further confirms that the product contains no DMF by-products, either entrapped or appended.[24] No mesylate resonances were observed in the product ^{13}C NMR (-$OSO_2\underline{C}H_3$ around 38 ppm) or ^{1}H NMR (-$OSO_2C\underline{H}_3$ around 3-4 ppm) (Figure S5.1) spectra, confirming the low content or absence of mesylate groups. Small resonances attributed to formates were observed in the ^{13}C NMR (O-(<u>C</u>=O)-H at 163 ppm) and ^{1}H NMR (O-(C=O)-<u>H</u> at 8.1 - 8.4 ppm) spectra; small amounts of formates have been observed in many other MsCl chlorination examples and are consistent with the reaction mechanism.[24,33] The MsCl chlorination mechanism has been well described in previous publications.[24,26] Based on this mechanism, we speculate that the regioselectivity of the HEC chlorination arises from the large difference in available approach angles between the terminal hydroxyl groups on the flexible oligo(hydroxyethyl) chains, and the rigid cellulose chain 6-OH groups, particularly since the last step of the chlorination mechanism involves a S_N2 displacement by chloride. While the chlorinated HEC is no longer soluble in water, it does have good solubility in polar aprotic solvents like DMF, DMSO, and DMAc.

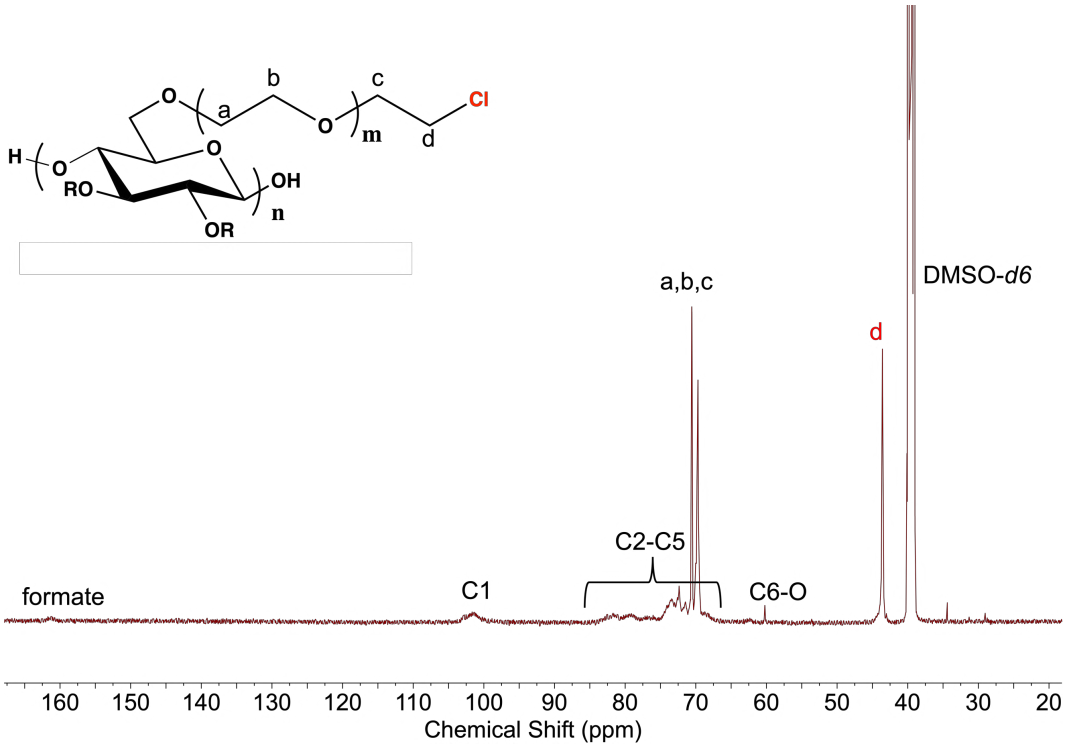


Figure 5.1 ^{13}C NMR spectrum of HEC-Cl

5.3.2 Azide displacement of chloride and subsequent azide reduction (synthesis of HEC-N_3 and HEC-NHAc)

Having successfully chlorinated HEC, we explored subsequent utilization of HEC-Cl as an intermediate to prepare various HEC derivatives by nucleophilic displacement reactions, simultaneously acquiring more evidence about the regioselectivity of this chlorination reaction. We initially chose the strong nucleophile azide; numerous reports have indicated that polysaccharides containing leaving groups such as bromide,[34] chloride,[28] and tosylate[17] can be quantitatively displaced by azide at C6. Thus, the azide will be an ideal nucleophile to test the reactivity of the chloride leaving groups we appended to HEC. Residual chloride would indicate that replacement of secondary OH groups (C2, C3) may have occurred to some extent, since S_N2 displacement of these ring secondary chlorides should be very difficult. Furthermore, azide has

been demonstrated to be a powerful functional group in polysaccharide chemistry; it can be reduced to amine,[25] amide,[35] or imine groups,[36,37] or can serve as a partner in Huisgen click reactions.[17,38] HEC-Cl was treated with sodium azide (DMSO, 80 °C, 24 h), affording HEC-N_3, as confirmed by FT-IR, ^{13}C NMR, and elemental analysis. In the FT-IR spectrum (Figure 5.2a), the characteristic azide signal appeared at 2100 cm^{-1}, indicating successful azide incorporation. By ^{13}C NMR (Figure 5.3), a new resonance at 50 ppm was assigned as the azide-bearing primary carbon, shift consistent with previous reports [39]. Meanwhile, the former chloride-bearing primary carbon signal at 44 ppm was absent, consistent with high displacement conversion. Elemental analysis again provides useful information: DS (N_3) and DS (Cl) were calculated to be 1.61 and 0.11 respectively, indicating high displacement conversion (around 95 %). The high conversion suggests that the chlorination reaction is predominantly regioselective at the terminal, primary hydroxy groups, since chlorides at C2 and C3 are unlikely to be displaced by the azide and should be detected by elemental analysis. The observed low % Cl may have been the result of incomplete azide substitution, or partial chlorination at the secondary hydroxyl groups. In our previous work with cellulose acetate chlorination by MsCl, we observed no replacement of secondary OH groups by Cl. Although azide is a strong nucleophile, high but incomplete azide displacement conversion is frequently observed when the DS of the leaving group is high. Thus, we postulate that the remaining chloride in the HEC-N_3 product arises mostly from the unreacted chloride connected to the primary carbon on the HEC side chain, rather than from C2/C3 appended chlorides.

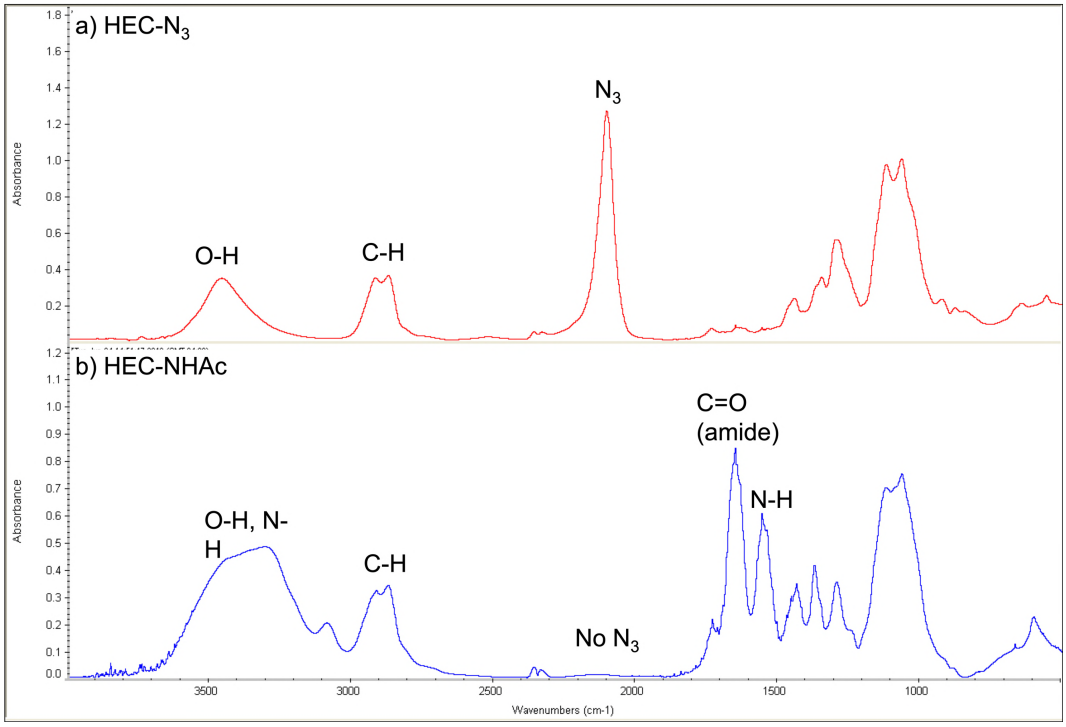


Figure 5.2 FT-IR spectra of a) HEC-N_3 and b) HEC-NHAc

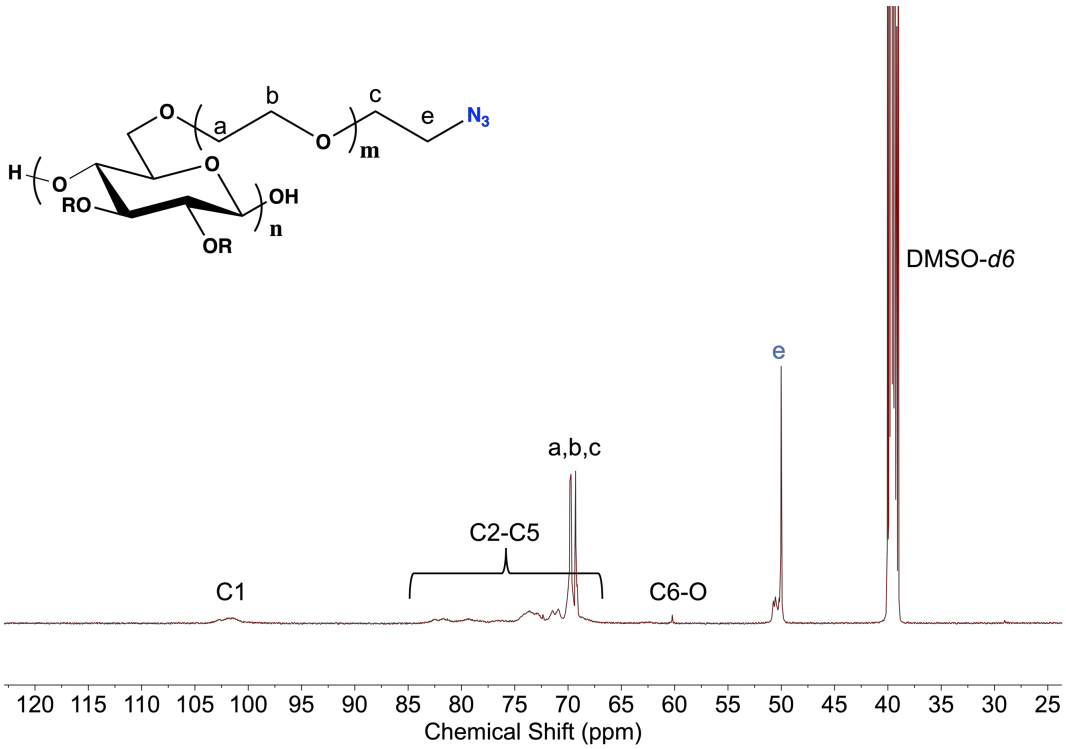


Figure 5.3 ^{13}C NMR spectrum of HEC-N_3

With HEC-N_3 in hand, we explored useful azide transformations. Since the utility of HEC-N_3 in click reactions has been demonstrated previously,[21] we decided to attempt to reduce the azides to amides. Amido and amino polysaccharides, including chitin, chitosan, and glycosaminoglycans (GAGs), are important natural polysaccharides, some of which have great complexity and biological activity, and are rich information carriers.[40,41] Very recently, our lab demonstrated that thioacetic acid (AcSH) can selectively reduce azide to acetamide in polysaccharide chemistry.[42,43] In this work, we reduced the HEC-N_3 to HEC-NHAc using thioacetic acid in conditions similar to those that were effective with GAG analogs (DMF, 65 °C, 24 h, 2,6-lutidine as catalyst). In the FT-IR spectrum (Figure 5.2b), the amide carbonyl at 1650 cm^{-1} and N-H bend at 1560 cm^{-1} demonstrated successful azide reduction to acetamide, and disappearance of the azide absorption at 2100 cm^{-1} were consistent with complete conversion. Formation of the acetamide was also confirmed by the ^{1}H and ^{13}C NMR spectra. In the ^{13}C NMR spectrum (Figure 5.4), the previous primary carbon-bearing azide signal at 50 ppm disappeared, while the acetamide group carbonyl resonance appeared at 169 ppm, and the amide methyl at 23 ppm. A new ^{1}H resonance (Figure S5.2) appeared in the range of 1.8-2.0 ppm, assigned to acetamido methyl groups. All signals are consistent with those of polysaccharide acetamide moieties described in previous publications.[25,35,42] Selective reduction to amide or amine is of course highly useful; thus we also attempted to reduce the azide to amine using dithiothreitol or sodium borohydride, as has been successful for polysaccharide 6-N_3 groups.[25,42] FT-IR of the product indicated complete disappearance of the azide group. However, the obtained product was insoluble in common solvents, making NMR characterization of the amine infeasible. We still lack the evidence to confirm or refute amine formation due to these solubility challenges.

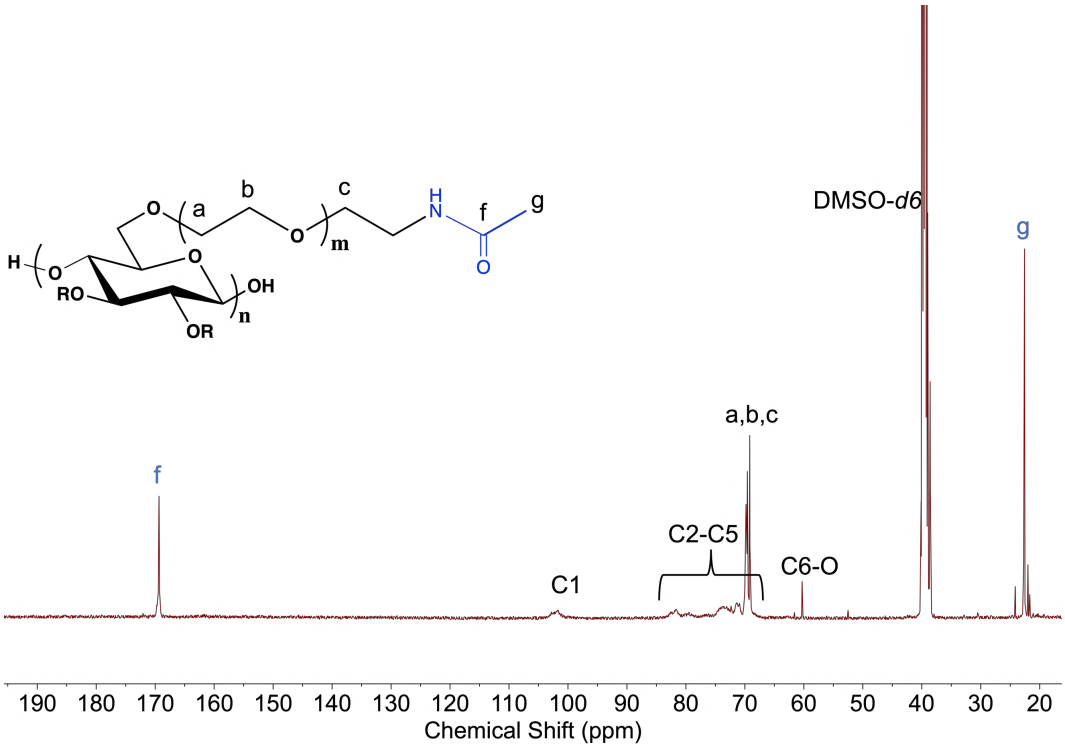


Figure 5.4 ^{13}C NMR spectrum of HEC-NHAc

5.3.3 Reaction with amines (Synthesis of HEC-MeIM)

After examining the reactivity of chloride in HEC-Cl with the strong azide nucleophile, we then explored its reaction with an amine-based nucleophile. The aromatic amine 1-methylimidazole was chosen as a gateway and proof of concept to cationic HEC derivatives, reacting with 1-methylimidazole in DMSO (80 °C, 48 h). We hypothesized that the chloride on HEC-Cl would be adequately reactive towards S_N2 displacement by imidazole, even given its inferiority to bromide as a leaving group. However, to our surprise, we obtained a water insoluble product and no 1-methylimidazolium moiety was observed in the product ^{1}H and ^{13}C NMR spectra. This indicated that the approach angle effect could not compensate for the less effective chloride leaving group. To improve reaction conversion, sodium iodide (NaI) was added to generate HEC-I *in situ* since iodide is a better leaving group than chloride.[44] In the presence of 3 equivalents of

NaI per Cl, we were encouraged to obtain a water-soluble polymer. The product showed a new resonance at 7.3-7.6 ppm (^{1}H NMR, Figure S5.3) from the aromatic protons of the 1-methylimidazolium moiety and a new sharp peak at 4.3 ppm from the *N*-methyl group, indicating successful incorporation of 1-methylimidazolium. A new ^{13}C NMR resonance (Figure 5.5) appeared at 50 ppm, assigned to the imidazole substituted cellulose C-6, and resonances at 122 and 136 ppm were assigned to the imidazolium aromatic carbons. Meanwhile, no obvious C-Cl signal was observed at 44 ppm, consistent with high displacement conversion. Cationic polysaccharide derivatives have been widely applied as surfactants,[45] gas separation membranes,[46] and as vehicles for drugs, DNA/RNA, and peptide delivery.[47] Cationic HEC-MeIM has promising potential, for example, in gas separation membranes.

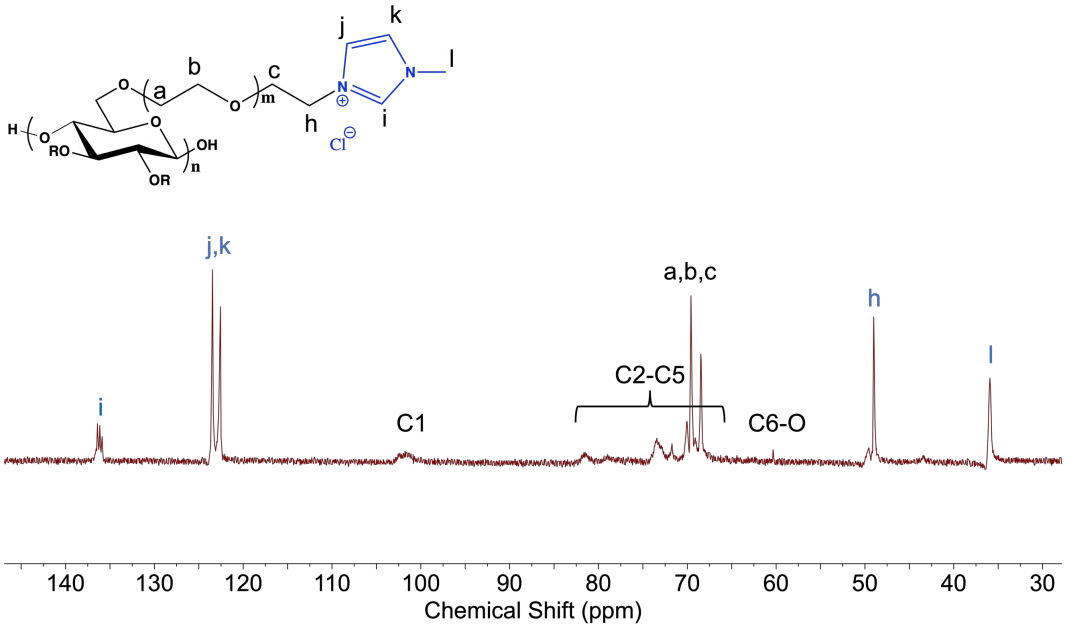


Figure 5.5 ^{13}C NMR spectrum of HEC-MeIM

5.3.4 Reaction with thiols (Synthesis of HEC-ME and HEC-MPA)

Thiols are reactive nucleophiles and displacement reactions using thiols have afforded useful cellulose derivatives, of interest for amorphous solid dispersion,[10] and for wastewater treatment applications.[48] We choose a neutral thiol, 2-mercaptoethanol, and a negatively charged thiol, 3-mercaptopropionic acid, as models to study the displacement reactions of HEC-Cl with thiols. HEC-Cl was first treated with 2-mercaptoethanol in the presence of *N*, *N*-diisopropylethylamine (DIPEA) to deprotonate the thiol (DMSO, 80 °C, 24 h). In the product ^{13}C NMR spectrum (Figure 5.6), new resonances appeared at 34 ppm, assigned to the sulfur bearing primary carbon, and at 31 ppm and 61 ppm, assigned to the two methylene groups from the thioether moiety. The C-Cl peak was still observed at 45 ppm, indicating incomplete chloride displacement. Appearance of the methylene group signal in the range of 2.6 to 2.7 ppm in the ^{1}H NMR spectrum confirmed the desired displacement. Although HEC-ME has a terminal hydroxyl group like HEC, HEC-ME is surprisingly insoluble in water. This might be the result of the incomplete chloride displacement.

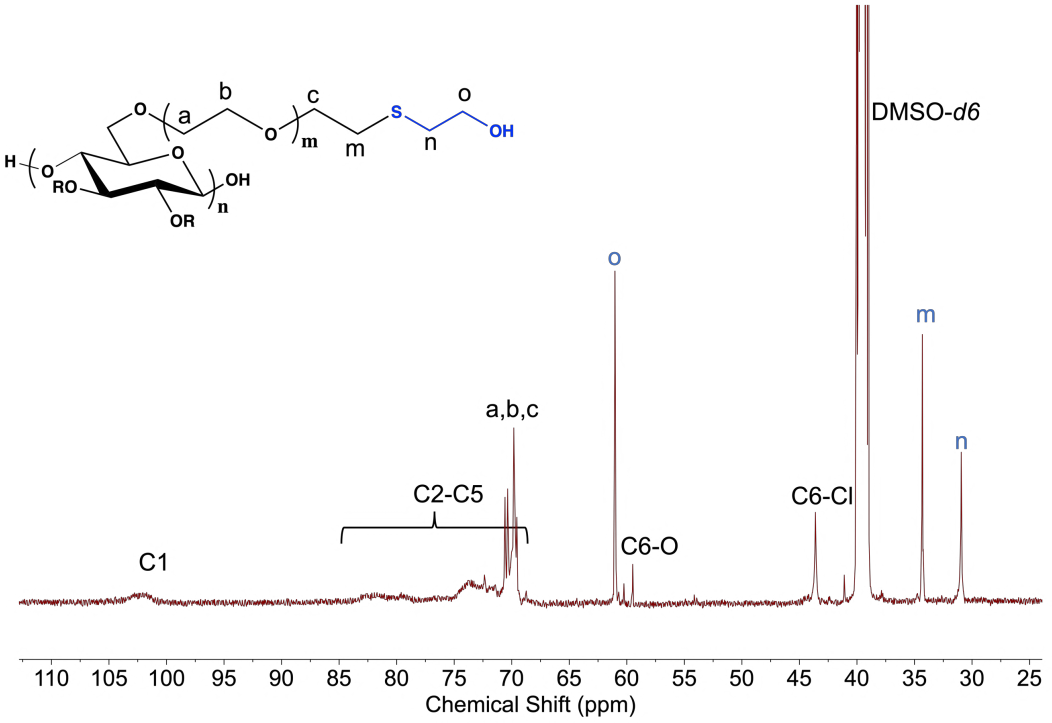


Figure 5.6 ^{13}C NMR spectrum of HEC-Me

HEC-Cl was then treated with 3-mercaptopropionic acid under similar conditions (DIPEA, NaI, DMSO, 80 °C, 24 h). Two new ^{1}H NMR resonances (Figure S5.5) between 2.6 and 2.9 ppm were assigned as the thioether methylene groups. In the product ^{13}C NMR spectrum (Figure 5.7), a new signal appeared at 33-34 ppm, assigned to the overlap of the S-bound HEC-terminal, primary carbon and two methylene groups, and at 172 and 173 ppm, assigned to carboxyl carbonyl groups. A resonance for unreacted C-Cl signal again appeared at 43 ppm, illustrating incomplete chloride displacement by the thiol even in the presence of NaI. Cellulose ether derivatives with terminal carboxyl groups possess intriguing applications in amorphous solid dispersions.[11] This example of successfully preparing a HEC derivative with a terminal carboxyl group provides an efficient and economical synthetic strategy to prepare HEC derivatives for ASD applications and enables the

systematic study of structure-property relationships in the future, perhaps competing with the olefin cross-metathesis strategy which requires a costly ruthenium complex catalyst.

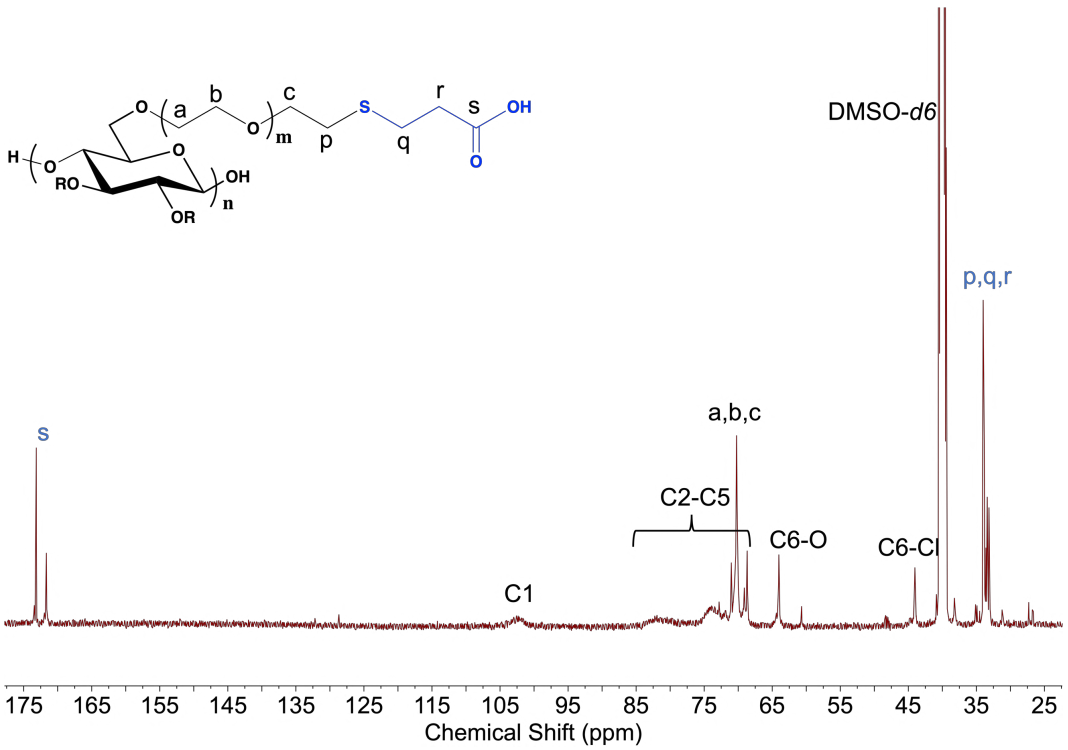


Figure 5.7 ^{13}C NMR spectrum of HEC-MPA

5.4 Conclusions

We have successfully developed a facile and efficient method to functionalize hydroxyethyl cellulose (HEC) by selective chlorination, and explored the utility of the resulting HEC-Cl in subsequent nucleophilic displacement reactions. HEC can be selectively chlorinated by MsCl at the terminal, primary hydroxy groups with negligible side reactions (mesylation, formate group formation), with high conversion (DS (Cl) 1.68). We also illustrated the utility of the resulting chlorinated HEC for appending new functional groups, synthesizing various HEC derivatives with defined structures. The chlorinated HEC can undergo displacement with strong

nucleophiles (azide) and with high conversion (95 %), affording a useful precursor for click reactions or azide reduction reactions. To demonstrate azide as an important intermediate, HEC-N_3 was reduced to HEC-NHAc using thioacetic acid (AcSH), creating intriguing amido HEC derivatives. We further functionalized the chlorinated HEC by weaker nucleophiles like amines and thiols. The chloride is less reactive with these nucleophiles, resulting in low reaction conversions under some conditions. Even the reduced steric hindrance of the HEC side chain cannot always compensate for the relatively poor leaving potential of the chloride. Therefore, we discovered that adding NaI in some cases significantly improved the reaction conversion because of the better leaving potential of the iodide versus the chloride. By adding NaI *in situ*, we prepared amine and thiol substituted HEC derivatives, including cationic (HEC-MeIM) and anionic (HEC-MPA) HEC derivatives. In all of the displacement examples (azide, amines, and thiols) we noted that the displacement conversion of HEC-Cl was lower than in our previous studies using C-6 chlorinated cellulose acetate. We postulate that HEC-Cl has a higher DS compared to the chlorinated cellulose acetate (HEC-Cl DS (Cl) 1.68 vs CA320S-Cl 0.49), presenting a higher challenge to achieve complete conversion of the leaving group. We are continuing to explore optimized reaction conditions to further improve reaction conversions.

Chlorination followed by nucleophilic displacement has been demonstrated to be an efficient route to functionalizing HEC. This strategy provides access to many HEC derivatives which are otherwise challenging to synthesize and are potentially useful in practical applications. For example, cationic HEC derivatives can be used as surfactants or gas separation membranes, while HEC anionic derivatives can be used in amorphous solid dispersions. The applications of the obtained HEC derivatives, including both gas separation and amorphous solid dispersions, are currently being investigated in our lab. Reaction of other polysaccharides with ethylene oxide also

provide hydroxyethyl derivatives (for example, hydroxyethyl starch that is an important plasma extender in traumatic injuries[49]), so that broader application of this strategy should prove useful.

Supporting information

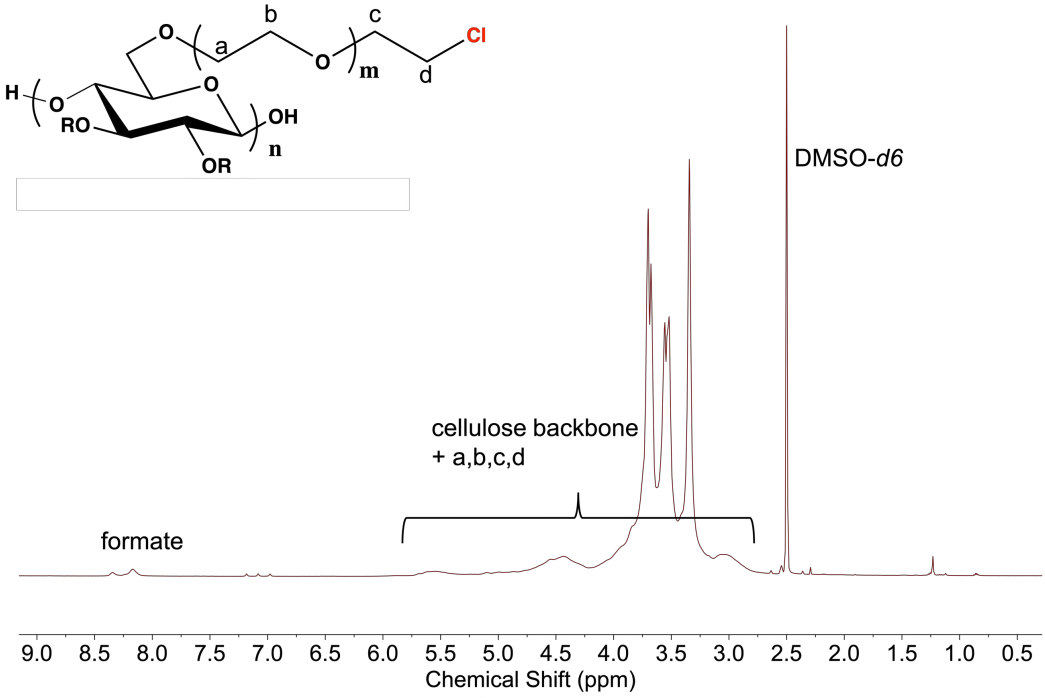


Figure S5.1 ^{1}H NMR spectrum of HEC-Cl

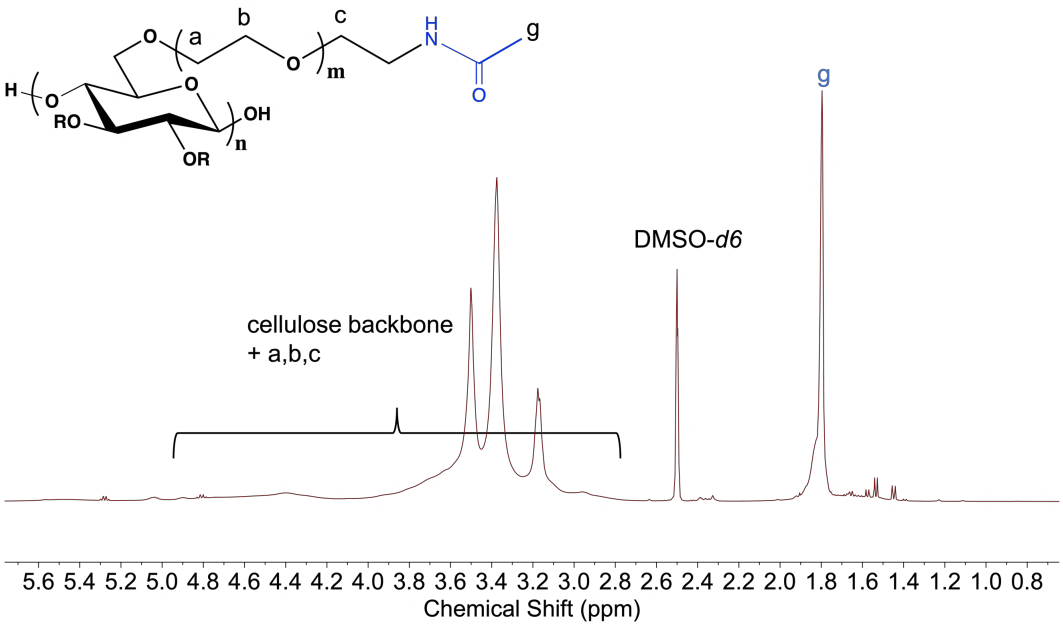


Figure S5.2 ^{1}H NMR spectrum of HEC-NHAc

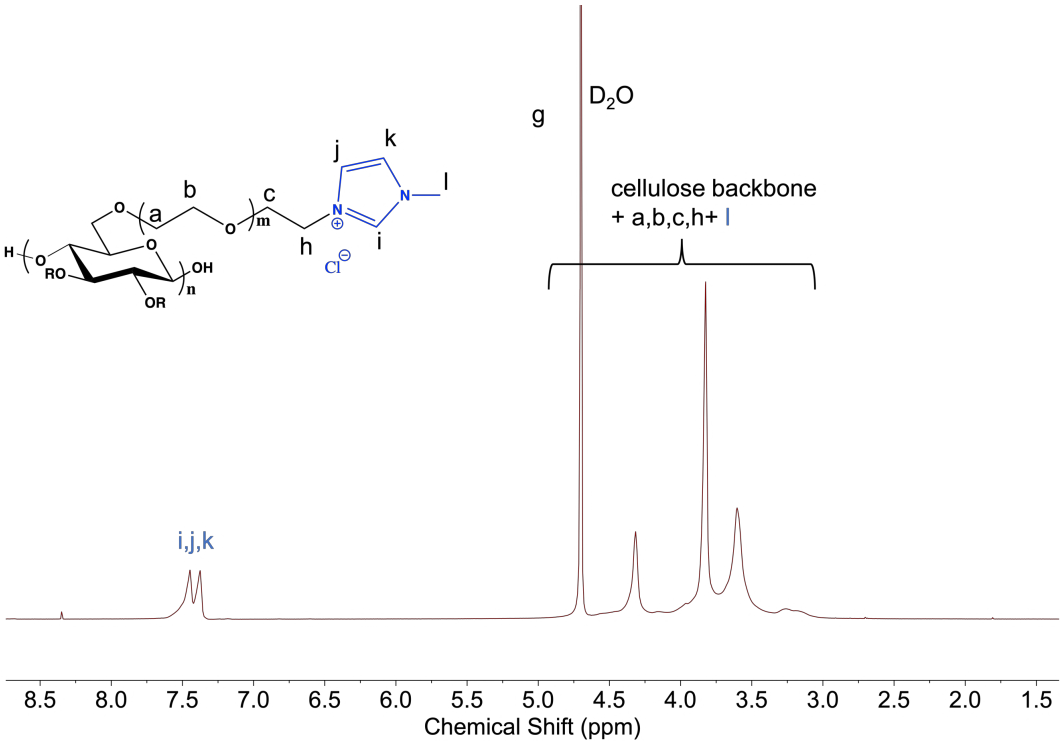


Figure S5.3 ^{1}H NMR spectrum of HEC-MeIM

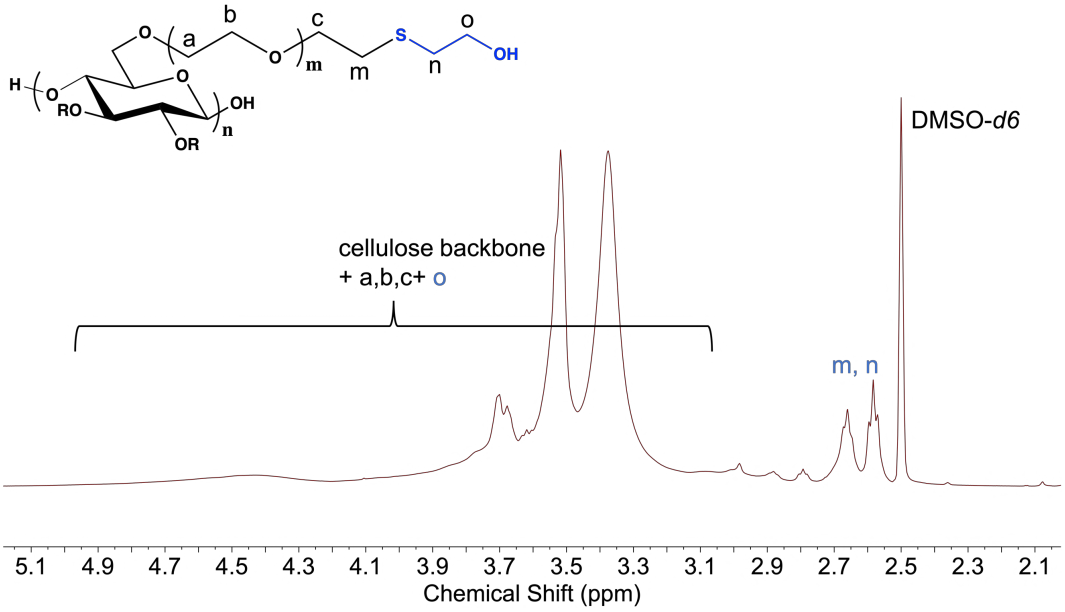


Figure S5.4 [1]H NMR spectrum of HEC-Me

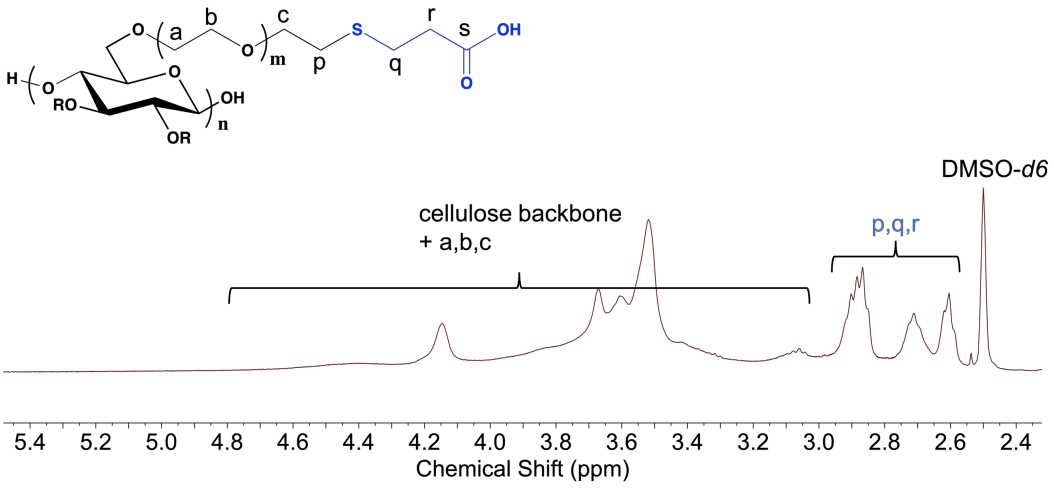


Figure S5.5 [1]H NMR spectrum of HEC-MPA

Reference

(1) Klemm, D.; Heublein, B.; Fink, H.-P. H.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie Int. Ed. 2005, 44 (22), 3358–3393.

(2) Edgar, K. J.; Buchanan, C. M.; Debenham, J. S.; Rundquist, P. A.; Seiler, B. D.; Shelton, M. C.; Tindall, D. Advances in Cellulose Ester Performance and Application. Prog. Polym. Sci. 2001, 26 (9), 1605–1688.

(3) Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J. Regioselective Esterification and Etherification of Cellulose: A Review. Biomacromolecules 2011, 12 (6), 1956–1972.

(4) Arca, H. C.; Mosquera-Giraldo, L. I.; Bi, V.; Xu, D.; Taylor, L. S.; Edgar, K. J. Pharmaceutical Applications of Cellulose Ethers and Cellulose Ether Esters. Biomacromolecules 2018, 19 (7), 2351–2376.

(5) Wang, X.; Wu, M.; Zhang, B.; Zhang, Y.; Hu, C.; Shi, L.; Lv, Y.; Ran, R. Phase-Transfer Method Synthesis Hydroxyethyl Cellulose Lauryl Ether. Colloids Surfaces A Physicochem. Eng. Asp. 2019, 562, 383–391.

(6) Abbas, K.; Amin, M.; Hussain, M. A.; Sher, M.; Abbas Bukhari, S. N.; Edgar, K. J. Design, Characterization and Pharmaceutical/Pharmacological Applications of Ibuprofen Conjugates Based on Hydroxyethylcellulose. RSC Adv. 2017, 7 (80), 50672–50679.

(7) Zhou, S.; Xu, C.; Wang, J.; Golas, P.; Batteas, J.; Kreeger, L. Phase Behavior of Cationic Hydroxyethyl Cellulose−Sodium Dodecyl Sulfate Mixtures: Effects of Molecular Weight and Ethylene Oxide Side Chain Length of Polymers. Langmuir 2004, 20 (20), 8482–8489.

(8) Svensson, A.; Sjöström, J.; Scheel, T.; Piculell, L. Phases and Structures of a Polyion-Surfactant Ion Complex Salt in Aqueous Mixtures: Cationic Hydroxyethyl Cellulose with

Dodecylsulfate Counterions. Colloids Surfaces A Physicochem. Eng. Asp. 2003, 228 (1–3), 91–106.

(9) Dong, Y.; Edgar, K. J. Imparting Functional Variety to Cellulose Ethers via Olefin Cross-Metathesis. Polym. Chem. 2015, 6 (20), 3816–3827.

(10) Dong, Y.; Mosquera-Giraldo, L. I.; Taylor, L. S.; Edgar, K. J. Tandem Modification of Amphiphilic Cellulose Ethers for Amorphous Solid Dispersion via Olefin Cross-Metathesis and Thiol-Michael Addition. Polym. Chem. 2017, 8 (20), 3129–3139.

(11) Dong, Y.; Mosquera-Giraldo, L. I.; Taylor, L. S.; Edgar, K. J. Amphiphilic Cellulose Ethers Designed for Amorphous Solid Dispersion via Olefin Cross-Metathesis. Biomacromolecules 2016, 17 (2), 454–465.

(12) Dong, Y.; Mosquera-Giraldo, L. I.; Troutman, J.; Skogstad, B.; Taylor, L. S.; Edgar, K. J. Amphiphilic Hydroxyalkyl Cellulose Derivatives for Amorphous Solid Dispersion Prepared by Olefin Cross-Metathesis. Polym. Chem. 2016, 7 (30), 4953–4963.

(13) Dong, Y.; Matson, J. B.; Edgar, K. J. Olefin Cross-Metathesis in Polymer and Polysaccharide Chemistry: A Review. Biomacromolecules 2017, 18 (6), 1661–1676.

(14) Heinze, T.; Liebert, T. Unconventional Methods in Cellulose Functionalization. Prog. Polym. Sci. 2001, 26 (9), 1689–1762.

(15) Petzold-Welcke, K.; Michaelis, N.; Heinze, T.; Petzold-Welcke, K.; Michaelis, N.; Heinze, T.; Petzold-Welcke, K.; Michaelis, N.; Heinze, T. Unconventional Cellulose Products through Nucleophilic Displacement Reactions. Macromol. Symp. 2009, 280 (1), 72–85.

(16) Liu, C.; Baumann, H. Exclusive and Complete Introduction of Amino Groups and Their N-Sulfo and N-Carboxymethyl Groups into the 6-Position of Cellulose without the Use of Protecting Groups. Carbohydr. Res. 2002, 337 (14), 1297–1307.

(17) Liebert, T.; Hänsch, C.; Heinze, T. Click Chemistry with Polysaccharides. Macromol. Rapid Commun. 2006, 27 (3), 208–213.

(18) Berlin, P.; Klemm, D.; Tiller, J.; Rieseler, R. A Novel Soluble Aminocellulose Derivative Type: Its Transparent Film-Forming Properties and Its Efficient Coupling with Enzyme Proteins for Biosensors. Macromol. Chem. Phys. 2000, 201 (15), 2070–2082.

(19) Knaus, S.; Mais, U.; Binder, W. H. Synthesis, Characterization and Properties of Methylaminocellulose. Cellulose 2003, 10 (2), 139–150.

(20) Aoki, N.; Furuhata, K.-I.; Saegusa, Y.; Nakamura, S.; Sakamoto, M. Reaction of 6-Bromo-6-Deoxycellulose with Thiols in Lithium Bromide-N,N-Dimethylacetamide. J. Appl. Polym. Sci. 1996, 61 (7), 1173–1185.

(21) Eissa, A. M.; Khosravi, E.; Cimecioglu, A. L. A Versatile Method for Functionalization and Grafting of 2-Hydroxyethyl Cellulose (HEC) via Click Chemistry. Carbohydr. Polym. 2012, 90 (2), 859–869.

(22) Joubert, F.; Yeo, R. P.; Sharples, G. J.; Musa, O. M.; Hodgson, D. R. W.; Cameron, N. R. Preparation of an Antibacterial Poly(Ionic Liquid) Graft Copolymer of Hydroxyethyl Cellulose. Biomacromolecules 2015, 16 (12), 3970–3979.

(23) Furuhata, K.; Koganei, K.; Chang, H.-S.; Aoki, N.; Sakamoto, M. Dissolution of Cellulose in Lithium Bromide-Organic Solvent Systems and Homogeneous Bromination of Cellulose with N-Bromosuccinimide-Triphenylphosphine in Lithium Bromide-N,N-Dimethylacetamide. Carbohydr. Res. 1992, 230 (1), 165–177.

(24) McCormick, C. L.; Dawsey, T. R.; Newman, J. K. Competitive Formation of Cellulose P-Toluenesulfonate and Chlorodeoxycellulose during Homogeneous Reaction of p-Toluenesulfonyl Chloride with Cellulose in N,N-Dimethylacetamide-Lithium Chloride. Carbohydr. Res. 1990, 208, 183–191.

(25) Zhang, R.; Edgar, K. J. Water-Soluble Aminocurdlan Derivatives by Chemoselective Azide Reduction Using NaBH 4. Carbohydr. Polym. 2015, 122, 84–92.

(26) Gao, C.; Liu, S.; Edgar, K. J. Regioselective Chlorination of Cellulose Esters by Methanesulfonyl Chloride. Carbohydr. Polym. 2018, 193, 108–118.

(27) Zhou, D.; Xiang, L.; Zeng, R.; Cao, F.; Zhu, X.; Wang, Y. Graft Copolymer Composed of Cationic Backbone and Bottle Brush-like Side Chains as a Physically Adsorbed Coating for Protein Separation by Capillary Electrophoresis. J. Sep. Sci. 2011, 34 (23), 3441–3450.

(28) Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H. Preparation of Amylose Derivatives Selectively Modified at C-6. 6-Amino-6-Deoxyamylose. Macromolecules 1994, 27 (11), 2917–2922.

(29) Ren, J.; Wang, P.; Dong, F.; Feng, Y.; Peng, D.; Guo, Z. Synthesis and Antifungal Properties of 6-Amino-6-Deoxyinulin, a Kind of Precursors for Facile Chemical Modifications of Inulin. Carbohydr. Polym. 2012, 87 (2), 1744–1748.

(30) Evans, M. E.; Long, L.; Parrish, F. W. Reaction of Carbohydrates with Methylsulfonyl Chloride in N,N-Dimethylformamide. Preparation of Some Methyl 6-Chloro-6-Deoxyglycosides. J. Org. Chem. 1968, 33 (3), 1074–1076.

(31) Mischnick, P.; Momcilovic, D. Chemical Structure Analysis of Starch and Cellulose Derivatives. In Advances in Carbohydrate Chemistry and Biochemistry; Academic Press Inc., 2010; Vol. 64, pp 117–210.

(32) Mischnick, P.; Niedner, W.; Adden, R. Possibilities of Mass Spectrometry and Tandem-Mass Spectrometry in the Analysis of Cellulose Ethers. Macromol. Symp. 2005, 223 (1), 67–78.

(33) Sato, T.; Koizumi, J.; Ohno, Y.; Endo, T. An Improved Procedure for the Preparation of Chlorinated Cellulose with Methanesulfonyl Chloride in a Dimethylformamide–Chloral–Pyridine Mixture. J. Polym. Sci. Part A Polym. Chem. 1990, 28 (8), 2223–2227.

(34) Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F. Facile Synthesis of 6-Amino-6-Deoxycellulose. Carbohydr. Res. 2005, 340 (7), 1403–1406.

(35) Zhang, R.; Edgar, K. J. Synthesis of Curdlan Derivatives Regioselectively Modified at C-6: O-(N)-Acylated 6-Amino-6-Deoxycurdlan. Carbohydr. Polym. 2014, 105, 161–168.

(36) Zhang, R.; Liu, S.; Edgar, K. J. Efficient Synthesis of Secondary Amines by Reductive Amination of Curdlan Staudinger Ylides. Carbohydr. Polym. 2017, 171, 1–8.

(37) Liu, S.; Gao, C.; Mosquera-Giraldo, L. I.; Taylor, L. S.; Edgar, K. J. Selective Synthesis of Curdlan ω-Carboxyamides by Staudinger Ylide Nucleophilic Ring-Opening. Carbohydr. Polym. 2018, 190, 222–231.

(38) Meng, X.; Edgar, K. J. “Click” Reactions in Polysaccharide Modification. Prog. Polym. Sci. 2016, 53, 52–85.

(39) Fox, S. C.; Edgar, K. J. Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O-Acylated 6-Amino-6-Deoxy-Cellulose. Biomacromolecules 2012, 13 (4).

(40) Kumar, M. N. V. R. A Review of Chitin and Chitosan Applications. React. Funct. Polym. 2000, 46 (1), 1–27.

(41) Afratis, N.; Gialeli, C.; Nikitovic, D.; Tsegenidis, T.; Karousou, E.; Theocharis, A. D.; Pavão, M. S.; Tzanakakis, G. N.; Karamanos, N. K. Glycosaminoglycans: Key Players in Cancer Cell Biology and Treatment. FEBS J. 2012, 279 (7), 1177–1197.

(42) Gao, C.; Fisher, Z. B.; Edgar, K. J. Azide Reduction by DTT or Thioacetic Acid Provides Access to Amino and Amido Polysaccharides. Cellulose 2019, 26 (1), 445–462.

(43) Gao, C.; Edgar, K. J. Efficient Synthesis of Glycosaminoglycan Analogs. Biomacromolecules 2019, 20 (2), 608–617.

(44) Evano, G.; Nitelet, A.; Thilmany, P.; Dewez, D. F. Metal-Mediated Halogen Exchange in Aryl and Vinyl Halides: A Review. Frontiers in Chemistry. Frontiers Media S. A April 1, 2018.

(45) Wang, K.; Ye, L. Structure and Property of Cationic Hydroxyethyl Cellulose. Polym. - Plast. Technol. Eng. 2010, 49 (8), 807–811.

(46) Li, F.; Fei, P.; Cheng, B.; Meng, J.; Liao, L. Synthesis, Characterization and Excellent Antibacterial Property of Cellulose Acetate Reverse Osmosis Membrane via a Two-Step Reaction. Carbohydr. Polym. 2019, 216, 312–321.

(47) Bernkop-Schnürch, A.; Dünnhaupt, S. Chitosan-Based Drug Delivery Systems. Eur. J. Pharm. Biopharm. 2012, 81 (3), 463–469.

(48) Aoki, N.; Fukushima, K.; Kurakata, H.; Sakamoto, M.; Furuhata, K. 6-Deoxy-6-Mercaptocellulose and Its S-Substituted Derivatives as Sorbents for Metal Ions. React. Funct. Polym. 1999, 42 (3), 223–233.

(49) Reinhart, K.; Hartog, C. S. Hydroxyethyl Starch in Patients with Trauma. Br. J. Anaesth. 2012, 108 (2), 321–322.

Chapter 6: Summary and future work

The dissertation presents successful modification of cellulose and cellulose derivatives to regioselectively synthesize glycosaminoglycans (GAGs) and their analogs. This provides a novel strategy to access GAGs and their analogs with defined structure and will facilitate understanding of GAG biological roles and biosynthesis modulation mechanism. During our GAG synthesis, we also discovered a novel chlorination reaction by methanesulfonyl chloride, and azide reductions by dithiothreitol and thioacetic acid. This dissertation also describes our systematic study of these reactions and their utilization in polysaccharide functionalization.

6.1 Efficient synthesis of unsulfated glycosaminoglycan analogs

In Chapter 3, we first described our design and synthesis of unsulfated GAG analogs. Two unsulfated GAG analogs were designed to bear both amine and carboxyl groups, or a combination of amines/amides and carboxyl groups. These analogs have DS around 0.5 of both amine/amides and carboxyl groups, identical to natural GAGs. After an unsuccessful attempt, we proposed a green synthetic route to the unsulfated GAG analogs. We were fortunate to utilize a commercial cellulose acetate, CA320S, as our starting material, which has both DS(Ac) and DS(OH) at C6 almost exactly 0.5. The successful route started with regiospecific Furuhata bromination at C6 and azide displacement, installing the amine/amide precursor at C6. Then saponification of the acetyl moieties exposed the remaining C6 oxygen moieties as primary hydroxyl groups, which can undergo (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) oxidation without the need to isolate or purify the saponification product, affording a water-soluble polymer. Finally, we carried out aqueous azide reduction by dithiothreitol (DTT), applied for the first time to polysaccharides, to accomplish azide reduction to amines. Alternatively, we employed thioacetic acid, again being applied for the first time in polysaccharide chemistry, to reduce azide to the corresponding

acetamide and amine mixture. The unsulfated GAG analogs have various potential biological activities and the amine-containing analog also has potential for formation of hydrogels that would have promise in tissue engineering, controlled release, and many other applications. The design of this synthetic route provides access to various sulfated GAG analogs.

6.2 Azide reduction by DTT or thioacetic acid provides access to amino and amido polysaccharides

In Chapters 3, DTT and thioacetic acid were employed to reduce azide to amines and amides under aqueous conditions. In Chapter 4, we systematically studied these new DTT and thioacetic acid azide reductions on polysaccharides. We demonstrated that DTT is effective in reducing azide to amine in polysaccharides of various structures (cellulose and curdlan) and in several polar aprotic solvents (DMSO, DMAc, and DMF). Conversion of azide to acetamide, which is even more challenging and has not been described previously in polysaccharide chemistry, was achieved by reaction with thioacetic acid in DMF with 2,6-lutidine as catalyst. We investigated chemoselectivity and reaction mechanism of thioacetic acid reduction as well. We ruled out hydroxyl acylation and direct reduction to amine as possible side reactions. We presented evidence that strongly supports a concerted mechanism leading to the amide formation. Other than organic solvents, we also studied water as the solvent in thioacetic acid reduction. In water, amide was the primary product, but a small amount of amine was also formed. This suggests that under aqueous conditions chemoselectivity of thioacetic acid reduction is not complete for amide formation, as it is in organic solvents.

The DTT reduction to amine has many advantages over current azide reduction methods, due to its mild and efficient nature, functional group tolerance, and successful reduction in water.

Thioacetic acid reduction of azide to acetamide in DMF is new to polysaccharide chemistry, accomplishing a transformation for which there is no other one-step alternative, and provides a new pathway to amido polysaccharide derivatives. Thioacetic acid reduction in water is less chemoselective, affording amides but also amines as minor products. These mixed amine/amide products could in fact be useful in some cases, e.g. in GAG analog synthesis.

6.3 Functionalization of hydroxyethyl cellulose by selective chlorination

Our lab has previously discovered that methanesulfonyl chloride (MsCl) can selectively chlorinate primary hydroxyl groups of cellulose esters. In Chapter 5, we report our attempt to apply this MsCl chlorination to functionalizing a cellulose ether, hydroxyethyl cellulose (HEC). We employed MsCl to selectively chlorinate terminal primary hydroxy groups on hydroxyethyl cellulose side chains. We obtained chlorinated HEC with a high content of chloride (DS (Cl) 1.68) and negligible side reactions (mesylation, formate group formation). The chlorinated HEC can undergo displacement with various model nucleophiles (azide, amines, and thiols) with moderate to high conversion. Through this strategy, we prepared many HEC derivatives which are otherwise challenging to synthesize and are potentially useful in practical applications. For example, we synthesized azido HEC as a precursor for azide reduction or as a partner for click reactions. We further utilized thioacetic acid to reduce HEC-N_3 to the corresponding acetamide. We also prepared cationic and anionic HEC derivatives, which have potential applications in surfactants, gas separation membranes, and amorphous solid dispersions. Overall, this strategy, MsCl chlorination and subsequent displacement, provides a novel approach for functionalizing commercial, inexpensive HEC for various applications.

6.4 Proposed future work

GAGs and their analogs display many significant biological roles and have been demonstrated to be useful in biomedical applications such as in hydrogels for drug delivery. The natural GAGs are alternating copolymers and they typically possess 1→4 or a combination of 1→3 and 1→4 linkages. Our GAG analogs only have 1→4 linkages from the starting material cellulose derivatives. Due to the nature of polymer post-modification, our GAG is a random copolymer, therefore different than the alternating copolymers from nature. The reactions employed in our syntheses, such as bromination, TEMPO oxidation, and DTT reduction, have been proven to be effective on a wide range of polysaccharides. Our synthetic route should also work on other polysaccharides to prepare GAG analogs. In the future, it would be advantageous to start with different polysaccharides and prepare different libraries of GAG analogs. For example, it will be of great interest to start with curdlan, a polysaccharide with 1→3 linkages, to investigate the linkage effect on biological activities. It would be also very interesting to start with some alternating polysaccharides to prepare GAG analogs with alternating structure. Our collaborator, Prof. Antoni Planas, has successfully prepared an alternating polysaccharide with repeat unit 6'-azido-6'-deoxycellobiose by enzymatic polymerization.[1] Such an alternating polysaccharide copolymer will be an ideal substrate for our purposes. The azide will be the precursor of amine and amide, and the primary hydroxyl groups can undergo TEMPO oxidation, followed by subsequent sulfation reactions, affording alternating sulfated GAG analogs.

GAGs are usually highly sulfated. For example, the DS (sulfate) of heparin is as high as 1.5. In our current sulfated GAG synthesis, the maximum DS we achieved is around 0.5. The DS of sulfate and the distribution pattern of sulfate groups are essential to GAGs biological activities.[2] Nature controls DS of sulfate groups by modulating sulfation enzyme activities. We could achieve

higher DS and further control the DS of sulfate groups by varying sulfation reaction conditions. A systematically study of sulfation of our GAG analogs by varying reaction temperature, solvents, time, and sulfation reagents would afford GAG analogs with different DS (sulfate). Thus, we can not only control the sulfation distribution pattern by varying reaction sequence but also control the extent of sulfation under different sulfation conditions. This will help us understand the impact of sulfate DS and distribution pattern on the biological activities of GAGs.

Finally, work in our group have demonstrated that cellulose ether derivatives with terminal carboxy groups have important applications in amorphous solid dispersion.[3,4] In our HEC chlorination work, the chlorinated HEC can be displaced by amine groups. Amino acids are powerful building blocks for synthetic chemistry. It is worthwhile to attempt to use the amine groups of amino acids to displace the chloride of HEC-Cl, yielding cellulose ether derivatives with terminal carboxy groups. This reaction will utilize polysaccharide derivatives and amino acid and the resulted polysaccharide peptide conjugate would be hypothesized to be biocompatible in many situations, and relatively non-toxic. Polymers used for amorphous solid dispersion usually need to have balanced hydrophobicity and hydrophilicity.[5,6] Such balance may be tailored by selecting and comparing natural amino acids bearing substituents of varying hydrophobicity, shape, and charge. This method has the potential to prepare a novel class of HEC derivatives for amorphous solid dispersion applications.

Reference

(1) Codera, V.; Edgar, K. J.; Faijes, M.; Planas, A. Functionalized Celluloses with Regular Substitution Pattern by Glycosynthase-Catalyzed Polymerization. *Biomacromolecules* **2016**, *17* (4), 1272–1279.

(2) Gandhi, N. S.; Mancera, R. L. The Structure of Glycosaminoglycans and Their Interactions with Proteins. *Chem. Biol. Drug Des.* **2008**, *72* (6), 455–482.

(3) Dong, Y.; Mosquera-Giraldo, L. I.; Taylor, L. S.; Edgar, K. J. Amphiphilic Cellulose Ethers Designed for Amorphous Solid Dispersion via Olefin Cross-Metathesis. *Biomacromolecules* **2016**, *17* (2), 454–465.

(4) Dong, Y.; Matson, J. B.; Edgar, K. J. Olefin Cross-Metathesis in Polymer and Polysaccharide Chemistry: A Review. *Biomacromolecules* **2017**, *18* (6), 1661–1676.

(5) Frank, D. S.; Matzger, A. J. Effect of Polymer Hydrophobicity on the Stability of Amorphous Solid Dispersions and Supersaturated Solutions of a Hydrophobic Pharmaceutical. *Mol. Pharm.* **2019**, *16* (2), 682–688.

(6) Chavan, R. B.; Rathi, S.; Jyothi, V. G. S. S.; Shastri, N. R. Cellulose Based Polymers in Development of Amorphous Solid Dispersions. *Asian Journal of Pharmaceutical Sciences*. Shenyang Pharmaceutical University May 1, 2019, pp 248–264.