Synthesis and application of specifically sulfated carbohydrates containing methacrylate functionality

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Leila Nikkhouy Albers

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: William B. Gleason

December 2008
Acknowledgments

I would like to extend my deepest appreciation to my advisor: Professor William Gleason, for the support, guidance, encouragement, and mentoring he has provided. He gave me the opportunity to learn and grow as a scientist and take control of the direction of the research.

I would like to thank Professor Carol Wells (Department of Lab Medicine and Pathology, University of Minnesota) and members of his research for their advice and assistance with biological studies of our sulfated polymers.

I would also like to thank Dr. Letitia Yao (Department of Chemistry, University of Minnesota) for her friendship, support, and guidance toward the NMR experiments.

I am also grateful to members of Professor Wang; Noelle Palumbo, for her assistance with GPC analysis and members of Professor Tranquillo; Sandy Johnson, for her friendship and assistance with UV analysis.

Finally, I would like to thank all the faculty and staff that have made my experience in the chemistry department so enjoyable and educational.
Dedication

It is my true honor to dedicate my thesis to my wonderful husband, James. I am so grateful to you for your love, support, patience, and encouragement throughout these years as I strived to succeed in my graduate studies. It is because of your sacrifice that I have been able to pursue my dreams. I am so thankful to have you as my life partner. I am also so grateful to my children; Cameron and Keon, with whom I am blessed with their love and laughter. You are my inspiration and joy every day. I couldn’t have done this without all of you and I love you all with all my heart.

Additionally, to the rest of my family especially to my parents who unconditionally supported me throughout all my educational pursuits. I am especially grateful to my brother; Massoud, and my uncle; Dr. Jamshidi, who supported me for my educational abroad. I would not be in the place I am now without them.
Abstract

Polymeric materials containing sugar moieties have proven to be important biomaterials. Hydrophobic polymers based on methyl methacrylate as well as hydrophilic polymers based on polysaccharides have been used as matrix-forming materials for tablets. A number of procedures for preparation of polysulfated carbohydrate derivatives have been published in literature\textsuperscript{1-3}. However, there are no reports of mono- or disulfated methyl methacrylate derivatives of carbohydrates.

The major focus of this thesis is synthesis and characterization of novel polymerizable methacrylate derivatives of mono- and disulfated carbohydrates. The position of sulfate group(s) as well as methyl methacrylate functionality is varied among these monomers such that upon polymerization, well defined sulfated carbohydrate containing polymers are produced. This will facilitate advances in the development of structure-activity relationships between sulfated carbohydrate polymers and their diverse biological activities.

A second area of this thesis is investigation of the potential for these novel sulfated polymers to act as biomimics of natural sulfated polysaccharides (e.g. heparan sulfate). We have studied the bacterial internalization of \textit{Staphylococcus aureus} with a number of sulfated polymers. We have also investigated the effect of the degree of sulfation of these polymers upon their interactions with bacteria such as \textit{Staphylococcus aureus}.
Computer modeling has been recognized as a sophisticated tool to investigate the interactions of ligands and proteins. This led us to another direction taken by this research project to use computational techniques to determine possible interactions of our final sulfated polymers with heparin binding proteins (HBPs) such as fibroblast growth factor (FGF). We used molecular docking as well as molecular dynamic simulation to predict binding ability of our final polymers with HBPs and provide a detailed description of these systems at the molecular level.

In an endeavor to improve the chemical and biological properties of our final polymers, we used a number of techniques such as surface grafting. Surface polymerization of our reactive carbohydrate monomers on some chemically activated surfaces such as polystyrene and glass was accomplished to enhance the hydrophilic interactions including attachment of hydrogels, immobilization of enzymes, or attraction of certain types of bacteria.

Thus, multi-faceted synthetic, computational, and biological studies that are proposed in this thesis should find wide utility for development of novel glycopolymers for biomedical applications including drug delivery.

Chapter 1. Synthesis and Characterization of Reactive Methacrylate Monomers Containing Sulfated Carbohydrates

1.1. Introduction

1.2. Background & Significance

1.2.1. Carbohydrate chemistry

1.2.2. Carbohydrates Stereochemistry

1.2.3. Sulfated sugars

1.2.4. Methacrylate-based materials

1.3. Experimental Section

1.3.1. Materials

1.3.2. Sulfation methodologies

1.3.3. Characterization methods

1.3.4. Monomer synthesis

1.3.4.1. General procedure for the methacrylation of carbohydrate derivatives
1.3.4.2. General procedure for the selective deprotection of terminal isopropylidene group……………………………………………12

1.3.4.3. General procedure for sulfation of carbohydrate derivatives….12

1.3.4.4. Synthesis of 6M-IGlc (10)………………………………………..13

1.3.4.5. Synthesis of 6M-DIGal (3)………………………………………..14

1.3.4.6. Synthesis of DIAll (2a)…………………………………………….14

1.3.4.7. Synthesis of 3Ac-DIGlc (4)………………………………………..15

1.3.4.8. Synthesis of 3M-IXyl (11)………………………………………..16

1.3.4.9. Synthetic approaches toward synthesis of 5M-IXyl (14)……….17

1.3.4.9.1. Synthesis of compound (14) following route I……………..18

1.3.4.9.2. Synthesis of compound (14) following route II…………….18

1.3.4.10. Synthesis of 3Am-DIAll (24)…………………………………...19

1.3.4.11. Alternative routes toward synthesis of 3M-6S-IGlc (22)………20

1.3.4.11.1. Synthesis of compound (22) using protecting group chemistry, route I………………………………………………….20

1.3.4.11.2. Synthesis of compound (22) without using protecting group chemistry, route II………………………………………..23

1.3.4.12. Synthesis of 3M-5S-IGlc (25)………………………………………..24

1.3.4.13. Alternative synthetic steps toward preparation of 6M-3,5-DS-Glc (26)………………………………………………………..26

1.3.4.13.1. Synthesis of compound (26) using protecting group chemistry, route I…………………………………………………..26
1.3.4.13.2. Synthesis of compound (26) without protecting group chemistry, route II...........................................29

1.3.4.14. Synthesis of 3M-5,6-DS-IGlc (17).............................................29

1.3.4.15. Synthesis of 3M-5,6-DS-IAll (18)..............................................30

1.3.4.16. Synthesis of 6M-3,4-DS-IGal (19)..............................................30

1.3.4.17. Synthesis of 3M-5S-IXyl (20).....................................................31

1.3.4.18. Synthesis of 5M-3S-IXyl (21).....................................................31

1.3.4.19. Attempt for preparation of 6M-3AmS-IAll (28).........................32

1.3.4.20. Deprotection of 1,2-O-isopropylidene group............................32

1.3.4.21. Temperature control NMR experiment to separate α and β anomers........................................33

1.4. Results (1.4.1-1.4.42)......................................................................34-48

1.4.43. Deprotection of 1,2-O-isopropylidene group..............................49

1.4.44. Temperature control experiment to separate α and β anomers........51

1.5. Discussion.......................................................................................54

1.6. References......................................................................................58

1.7. Appendix.........................................................................................61-90

Chapter 2. Sulfated Carbohydrate Derivatives of Methacrylate Polymers: Synthesis, Characterization, and Biological studies.......................................................91

2.1. Introduction.......................................................................................92

2.2. Background......................................................................................94

2.2.1. Staphylococcus aureus..................................................................94

2.2.2. Caco-2 and HT-29 enterocytes......................................................94
2.2.3. Sulfated carbohydrate polymers ................................................. 95
2.2.4. Methacrylate-based polysaccharides ........................................... 96
2.2.5. Pegylated heparin ................................................................. 97

2.3. Experimental Section

2.3.1. Materials ................................................................. 99
2.3.2. Characterization methods ...................................................... 99
2.3.3. Polymer synthesis ............................................................ 100
   2.3.3.1. Water soluble radical initiator for polymerization of sulfated monomers ................................................................. 100
   2.3.3.2. General procedure for polymerization of sulfated monomers ................................................................. 100
   2.3.3.3. Deprotection of 1,2-isopropylidene group from sulfated polymers ................................................................. 101
2.3.4. Methods of biological studies ............................................. 102
2.3.5. General procedures for pegylation of heparin disaccharides .......... 103
2.3.6. Interaction of heparin disaccharide PEG-NH₂ with bacteria .......... 103

2.4. Results ................................................................. 105
2.4.1. Characterization results for sulfated polymers ......................... 105
2.4.2. Effect of sulfated polymers on the internalization of Staphylococcus aureus 6390 by HT-29 and Caco-2 enterocytes ......................... 106
2.4.3. Effect of heparin disaccharides (HP DS) on internalization of S. aureus by HT-29 enterocytes pre-incubated in calcium-free medium .......... 108
2.4.4. Effect of polyethylene glycol (PEG) on internalization of E. coli by Caco-2 and HT-29 enterocytes ......................................................... 108
2.4.5. Effect of pegylated heparin on the internalization of *E-coli* by HT-29 and Caco-2 enterocytes

2.5. Discussion

2.6. References

2.7. Appendix

Chapter 3. Sulfated Carbohydrate Derivatives of Methacrylate Polymers: Computational Studies: Molecular Docking and Molecular Dynamic Calculations

3.1. Introduction

3.2. Background

3.2.1. Molecular docking

3.2.2. Molecular dynamic

3.3. Calculation Section

3.3.1. Computational methodologies

3.3.1.1. AUTODOCK 3

3.3.1.2. GROMACS

3.3.2. AutoDock procedure

3.3.2.1. Preparation of the macromolecule file

3.3.2.2. Preparation of the ligand file

3.3.2.3. Preparation of the grid parameters

3.3.2.4. Preparation of the docking parameters

3.3.2.5. Starting the Autogrid and Autodock

3.3.2.6. General procedures for docking sulfated polymers into 2AXM

3.3.3. Ligands (Sulfated carbohydrate derivatives of methyl methacrylate
3.4. Results.............................................................................................................137
    3.4.1. Interpretation of docking results.........................................................137
    3.4.2. General process for interpretation of docking sulfated polymers into
            2AXM......................................................................................................137
    3.4.3. Docking results of sulfated polymers into 2AXM binding pocket
            (3.4.3.1 - 3.4.3.17)..................................................................................138-150
3.5. Discussion......................................................................................................151
    3.5.1. Docking the sulfated polymers into 2AXM binding pocket.................151
    3.5.2. Dynamic simulation of sulfated polymers into 2AXM binding pocket...153
3.6. References....................................................................................................154

Chapter 4. Surface Polymerization of Methacrylate Carbohydrate Derivatives......156
4.1. Introduction...................................................................................................157
4.2. Background and Significance......................................................................159
    4.2.1. Surface modification of polymers......................................................159
    4.2.2. Surface grafting..................................................................................160
        4.2.2.1. Technique of “grafting to”.........................................................160
        4.2.2.2. Technique of “grafting from”.....................................................160
    4.2.3. Surface Polymerization on polystyrene..............................................161
    4.2.4. Polymerization on surface of glass....................................................163
    4.2.5. Reducing sugars................................................................................164
4.3. Experimental Section...................................................................................166
4.3.1. Materials ..............................................................................................................166

4.3.2. Methods of analysis ............................................................................................166
    4.3.2.1. Reducing sugar determination .......................................................................167
        4.3.2.1.1. Reducing sugar by copper bicinechoninate ...........................................167
        4.3.2.1.2. Reducing sugar by Park-Johnson’s method .........................................168

4.3.3. General procedure for obtaining standard curves ............................................169

4.3.4. Surface polymerization on polystyrene surface by Bamford’s method ...169
    4.3.4.1. General procedure for grafting 3M-DIGlc or 6M-DIGal on surface
        of polystyrene 24-well plates ............................................................................170
    4.3.4.2. General procedure for grafting 3M-DIGlc or 6M-DIGal on surface
        of glass ..............................................................................................................171

4.4. Results ....................................................................................................................172
    4.4.1. Standard curve for glucose and deblocked methacrylated glucose polymer
        using copper bicinechoninate assay ..............................................................172
    4.4.2. Standard curve for glucose and deblocked methacrylated glucose polymer
        using Park-Johnson’s assay ..........................................................................173
    4.4.3. Calibration curve for galactose and deblocked methacrylated galactose
        polymer using copper bicinechoninate assay ..............................................174
    4.4.4. Calibration curve for galactose and deblocked methacrylated galactose
        polymer using Park-Johnson’s assay ...........................................................175
    4.4.5. Surface grafting of glucose monomer onto polystyrene ................................176
    4.4.6. Surface grafting of galactose monomer onto polystyrene ............................177
    4.4.7. Surface grafting of glucose and galactose monomer onto glass plate ......178
4.5. Discussion ........................................................................................................179
4.6. References .......................................................................................................181
Bibliography ..........................................................................................................183
List of Figures

Figure 1.1. Structures of two epimers, glucose and galactose..............................6
Figure 1.2. D-glucose anomers..............................................................................6
Figure 1.3. Structure of non-reducing sugar, DIGlc..............................................7
Figure 1.4. Most commonly repeated disaccharide unit of heparin.......................7
Figure 1.5. General procedure for synthesis of compounds (1) and (2)...............12
Figure 1.6. General procedure for preparation of compounds (5) and (6).............12
Figure 1.7. Synthesis of 6M-IGlc (10).................................................................13
Figure 1.8. Synthesis of 6M-DIGal (3).................................................................14
Figure 1.9. Synthesis of DIAll (2a).....................................................................15
Figure 1.10. Synthesis of 3Ac-DIGlc (4).............................................................16
Figure 1.11. Synthesis of 3M-IXyl (11)...............................................................17
Figure 1.12. Synthesis of 5M-IXyl (14) using route I.........................................18
Figure 1.13. Synthesis of 5M-IXyl (14)...............................................................19
Figure 1.14. Synthesis of 3Am-DIAll (24)...........................................................20
Scheme 1.1. Initial synthetic steps toward synthesis of monomer (22), route I........21
Figure 1.15. Synthesis of 3M-6S-IGlc (22).........................................................23
Scheme 1.2. Synthetic steps for preparation of compound (22), route II..............23
Scheme 1.3. Synthesis of 3M-5S-IGlc (25).........................................................24
Figure 1.16. Synthesis of 3M-5S-IGlc (25).........................................................25
Scheme 1.4. Initial synthetic steps toward synthesis of monomer (26)...............26
Figure 1.17. Synthesis of 3MEM-6M-IGlc (1i)...............................................28
Scheme 1.5. Synthesis of 6M-3,5-DS-IGlc (26) ............................................. 29
Scheme 1.6. Synthesis of 3M-5,6-DS-IGlc (17) .............................................. 30
Scheme 1.7. Synthesis of 3M-5,6-DS-IAll (18) ............................................. 30
Scheme 1.8. Synthesis of 6M-3,4-DS-IGal (19) ............................................. 31
Figure 1.18. Synthesis of 3M-5S-IXyl (20) .................................................. 31
Figure 1.19. Synthesis of 5M-3S-IXyl (21) .................................................. 32
Scheme 1.9. Synthetic route toward synthesis of 6M-3AmS-IAll (28) .......... 32
Figure 1.20. Deprotection of 1,2-O-isopropylidene group using %5 HCl at 60°C ........... 50
Figure 1.21. Difference between Hα and Hβ chemical shift (σ) and coupling constant (J) in 2,3-disulfated glucosamine ................................................................. 51
Figure 1.22. 1H NMR of 2,3-disulfated glucosamine at 22°C ....................... 52
Figure 1.23. Chemical shift changes of D2O in 2,3-disulfated glucosamine with Temperature and appearance of Hβ at 50°C (a) and 60°C (b) ...................... 53
Figure 1.24. Mechanism of cleavage of vicinal diol in (3a) by NaIO4 ............... 55
Figure 1.25. Possible mechanism for formation of side products in cleavage of diol in (3a) using NaIO4 ................................................................. 56
Figure 2.1. Structure of water soluble initiator (VA044) ................................ 100
Figure 2.2. Polymerization of P3M-5,6-DS-IGlc (P17) ................................. 101
Figure 2.3. Deprotection of 1,2-isopropylidene group by acid-base reagents .... 101
Figure 2.4. Deprotection of 1,2-isopropylidene group by K10 clay .................. 101
Figure 2.5. General synthetic step for preparation of heparin disaccharide (IV-H, II-H, I-S, III-S) ................................................................. 103
Figure 2.6. Effect of P17 on internalization of S. aureus 6390 by HT-29 enterocytes ... 106
Figure 2.7. Effect of P17 on internalization of *S. aureus* 6390 by Caco-2 enterocytes...106

Figure 3.1. Details of hydrogen bonding between aFGF protomers (ribbons) and heparin (ball and stick) in aFGF dimmers A and C…………………………………….120

Figure 3.2. “Lock and Key”, representing enzyme and substrate…………………….122

Figure 3.3. “Lock and Key”, representing concept of molecular docking compared to “Hand-in-Glove”………………………………………………………….122

Figure 3.4. The representation of AutoDock and AutoGrid…………………………..126

Figure 3.5. Chem 3D structure of copolymer of sulfated glucose-sulfated galactose-methyl methacrylate (2mm-sulfglu-4mm-sulfgal-2mm)………………………..134

Figure 3.6. Docking results of ms-iso-3mglu into 2AXM binding pocket……………..139

Figure 3.7. Docking results of ms-iso-6mgal into 2AXM binding pocket……………..140

Figure 3.8. Structure of carbohydrate derivatives of methyl methacrylate……………..141

Figure 3.9. Docking results of ms-iso-6mgal into 2AXM binding pocket……………..144

Figure 3.10. Docking results of ms-syn-6mgal into 2AXM binding pocket……………145

Figure 3.11. Docking results of 2mm-sulfglu-4mm-sulfgal-2mm into 2AXM binding pocket……………………………………………………………….147

Figure 3.12. Docking results of ms-syn-3mxyl into 2AXM binding pocket……………149

Figure 4.1. Structure of carbohydrate derivatives of methyl methacrylate……………158

Figure 4.2. Schematic illustration of “grafting to” and “grafting from” techniques…….161

Figure 4.3. Steps in thermal graft polymerization onto glass plate by azo group initiator…………………………………………………………………….163

Figure 4.4. Comparison between reducing sugar compounds with non-reducing ones.164
Figure 4.5. Oxidation of C1 of polymer (P-3M-DIGlc)………………………………..165
Figure 4.6. The structure of main component of copper bicinchoninate assay……..168
Figure 4.7. Structures of two important components of surface grafting onto glass…..171
Figure 4.8. Standard curve for glucose and glucose polymer using copper bicinchoninate assay……………………………………………………………………………172
Figure 4.9. Standard curve for glucose and glucose polymer using Park-Johnson’s method………………………………………………………………………….173
Figure 4.10. Standard curve for galactose and galactose polymer using copper bicinchoninate assay…………………………………………………………………..174
Figure 4.11. Standard curve for galactose and galactose polymer using Park-Johnson’s method………………………………………………………………………..175
List of Tables

Table 1.1. Structures of target sulfated monomers ............................................. 3
Table 2.1. Structures of synthesized sulfated carbohydrate polymers ................. 93
Table 2.2. Molecular weight determinations for sulfated polymers ..................... 105
Table 2.3. Effect of P17 on internalization of S. aureus 6390 by HT-29 and Caco-2 ... 106
Table 2.4. Internalization of S. aureus by HT-29 enterocytes pre-incubated in calcium-
   Free medium ± 25 µg/mL selected heparin disaccharides (HP DS) ................. 108
Table 2.5. Effect of HMW PEG on internalization of E. coli by Caco-2 and HT-29
   enterocytes ................................................................................................. 109
Table 3.1. Interactions between aFGF protomers and heparin ............................. 119
Table 3.2. Chem 3D structures of mono or disulfated 3-O-methacryloyl-glucose
   polymers .................................................................................................... 132
Table 3.3. Chem 3D structures of mono or disulfated 6-O-methacryloyl-galactose
   polymers .................................................................................................... 133
Table 3.4. Chem 3D structures of mono or disulfated 6-O-methacryloyl-glucose
   polymers .................................................................................................... 134
Table 3.5. Chem 3D structures of monosulfated methacrylated xylose polymers .... 135
Table 3.6. Names of docked polymers with their generated file for AutoDock ........ 136
Table 3.7. Docking results of ms-iso-3mglu into 2AXM binding pocket ............... 138
Table 3.8. Docking results of ms-syn-3mglu into 2AXM binding pocket ............... 139
Table 3.9. Docking results of ds-iso-3mglu into 2AXM binding pocket ............... 140
Table 3.10. Docking results of ds-syn-3mglu into 2AXM binding pocket ............. 141
Table 3.11. Docking results of ms-iso-6mglu into 2AXM binding pocket…………….142
Table 3.12. Docking results of ms-syn-6mglu into 2AXM binding pocket…………….142
Table 3.13. Docking results of ds-iso-6mglu into 2AXM binding pocket…………….143
Table 3.14. Docking results of ds-syn-6mglu into 2AXM binding pocket…………….143
Table 3.15. Docking results of ms-iso-6mgal into 2AXM binding pocket…………….144
Table 3.16. Docking results of ms-syn-6mgal into 2AXM binding pocket…………….145
Table 3.17. Docking results of ds-iso-6mgal into 2AXM binding pocket…………….146
Table 3.18. Docking results of ds-syn-6mgal into 2AXM binding pocket…………….146
Table 3.19. Docking results of 2mm-sulfglu-4mm-sulfgal-2mm into 2AXM binding
    pocket…………………………………………………………………………...147
Table 3.20. Docking results of ms-iso-3mxyl into 2AXM binding pocket…………….148
Table 3.21. Docking results of ms-syn-3mxyl into 2AXM binding pocket…………….148
Table 3.22. Docking results of ms-iso-5mxyl into 2AXM binding pocket…………….149
Table 3.23. Docking results of ms-syn-5mxyl into 2AXM binding pocket…………….150
Table 4.1. Polymer glucose grafted on surface of polystyrene……………………….176
Table 4.2. Polymer galactose grafted on surface of polystyrene……………………….177
Table 4.3. Polymer glucose or galactose on surface of glass……………………….178
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIGlc</td>
<td>1,2:5,6-di-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>MMA</td>
<td>methyl methacrylate</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic anhydride</td>
</tr>
<tr>
<td>HEMA</td>
<td>hydroxylethyl methacrylate</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethyl methacrylate</td>
</tr>
<tr>
<td>TMA-SO₃</td>
<td>trimethyl ammonium sulfite</td>
</tr>
<tr>
<td>6M-IGlc</td>
<td>6-&lt;i&gt;O&lt;/i&gt;-methacryoyl-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>6M-DIGal</td>
<td>6-&lt;i&gt;O&lt;/i&gt;-methacryoyl-1,2:3,4-di-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-galactopyranose</td>
</tr>
<tr>
<td>DIALl</td>
<td>1,2:5,6-di-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-allofuranose</td>
</tr>
<tr>
<td>3Ac-DIGlc</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-acetyl-1,2:5,6-di-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>3M-IXyl</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-methacryoyl-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-xylofuranose</td>
</tr>
<tr>
<td>5M-IXyl</td>
<td>5-&lt;i&gt;O&lt;/i&gt;-methacryoyl-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-xylofuranose</td>
</tr>
<tr>
<td>3Am-DIALl</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-amino-1,2:5,6-di-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-allofuranose</td>
</tr>
<tr>
<td>3M-6S-IGlc</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-methacryoyl-6-&lt;i&gt;O&lt;/i&gt;-sulfonato-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>3M-5S-IGlc</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-methacryoyl-5-&lt;i&gt;O&lt;/i&gt;-sulfonato-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>6M-3,5-DS-IGlc</td>
<td>6-&lt;i&gt;O&lt;/i&gt;-methacryoyl-3,5-&lt;i&gt;O&lt;/i&gt;-disulfonato-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-6M-IGlc</td>
<td>3-methoxyethoxy methyl-6-methacryoyl-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>3M-5,6-DS-IGlc</td>
<td>3-&lt;i&gt;O&lt;/i&gt;-methacryoyl-5,6-&lt;i&gt;O&lt;/i&gt;-disulfonato-1,2-&lt;i&gt;O&lt;/i&gt;-isopropylidene-&lt;i&gt;α&lt;/i&gt;-D-glucofuranose</td>
</tr>
<tr>
<td>Index</td>
<td>Name</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>3M-5,6-DS-IAll</td>
<td>$\alpha$-D-glucofuranose</td>
</tr>
<tr>
<td>6M-3,4-DS-IGal</td>
<td></td>
</tr>
<tr>
<td>3M-5S-IXyl</td>
<td></td>
</tr>
<tr>
<td>5M-3S-IXyl</td>
<td></td>
</tr>
<tr>
<td>6M-3AmS-IAll</td>
<td></td>
</tr>
<tr>
<td>5M-IXyl</td>
<td></td>
</tr>
<tr>
<td>3M-IXyl</td>
<td></td>
</tr>
<tr>
<td>3S-DIGlc</td>
<td></td>
</tr>
<tr>
<td>3S-DIAAll</td>
<td></td>
</tr>
<tr>
<td>3Am-DIGlc</td>
<td></td>
</tr>
<tr>
<td>3AmS-DIGlc</td>
<td></td>
</tr>
<tr>
<td>3Ac-IXyl</td>
<td></td>
</tr>
<tr>
<td>3M-6Trt-IXyl</td>
<td></td>
</tr>
<tr>
<td>3M-6Trt-5MOM-IXyl</td>
<td></td>
</tr>
<tr>
<td>3M-5MOM-IXyl</td>
<td></td>
</tr>
<tr>
<td>3M-6S-5MOM-IXyl</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Chemical Structure</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3M-6TBDMS-IXyl</td>
<td>3-O-methacryoyl-6-O-tributyl dimethyl silyl-1,2-O-isopropylidene-α-D-xylofuranose</td>
</tr>
<tr>
<td>3M-6TBDMS-5S-IXyl</td>
<td>3-O-methacryoyl-6-O-tributyl dimethyl silyl-3-O-sulfonato-1,2-O-isopropylidene-α-D-xylofuranose</td>
</tr>
<tr>
<td>3MEM-DIGlc</td>
<td>3-O-methoxyethoxy methyl-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-IGlc</td>
<td>3-O-methoxyethoxy methyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-6TBDMS-IGlc</td>
<td>3-O-methoxyethoxy methyl-6-O-tributyl dimethyl silyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-6TBDMS-5Bn-IGlc</td>
<td>3-O-methoxyethoxy methyl-6-O-tributyl dimethyl silyl-5-O-benzyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-5Bn-IGlc</td>
<td>3-O-methoxyethoxy methyl-5-O-benzyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-6M-5Bn-IGlc</td>
<td>3-O-methoxyethoxy methyl-6O-methacryoyl-5-O-benzyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>3MEM-6M-IGlc</td>
<td>3-O-methoxyethoxy methyl-6O-methacryoyl-1,2-O-isopropylidene-α-D-glucofuranose</td>
</tr>
<tr>
<td>ms-iso-3mglu</td>
<td>6-O-monosulfonato-isotactic-3-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>ms-syn-3mglu</td>
<td>6-O-monosulfonato-syndiotactic-3-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>ms-iso-6mglu</td>
<td>3-O-monosulfonato-isotactic-6-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>ms-syn-6mglu</td>
<td>3-O-monosulfonato-syndiotactic-6-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>ms-iso-6mgal</td>
<td>3-O-monosulfonato-isotactic-6-O-methacryoyl-galactose</td>
</tr>
<tr>
<td>ms-syn-6mgal</td>
<td>3-O-monosulfonato-syndiotactic-6-O-methacryoyl-galactose</td>
</tr>
<tr>
<td>ds-iso-3mglu</td>
<td>5,6-O-disulfonato-isotactic-3-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>ds-syn-3mglu</td>
<td>5,6-O-disulfonato-syndiotactic-3-O-methacryoyl-glucose</td>
</tr>
<tr>
<td>Symbol</td>
<td>Chemical Structure</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2mm-sulfglu-4mm-sulfgal-2mm</td>
<td>2-O-methyl methacryoyl-sulfonato-glucose-4-O-methyl methacryoyl-sulfonato-galactose-2-O-methyl methacryoyl</td>
</tr>
<tr>
<td>ms-iso-3mxyl</td>
<td>5-O-monosulfonato-isotactic-3-O-methacryoyl-xylose</td>
</tr>
<tr>
<td>ms-syn-3mxyl</td>
<td>5-O-monosulfonato-syndiotactic-3-O-methacryoyl-xylose</td>
</tr>
<tr>
<td>ms-iso-5mxyl</td>
<td>3-O-monosulfonato-isotactic-5-O-methacryoyl-xylose</td>
</tr>
<tr>
<td>ms-syn-5mxyl</td>
<td>3-O-monosulfonato-syndiotactic-5-O-methacryoyl-xylose</td>
</tr>
</tbody>
</table>
Chapter 1

Synthesis and Characterization of
Reactive Methacrylate Monomers Containing
Sulfated Carbohydrates
1.1. Introduction

Naturally occurring sulfated carbohydrate polymers are of wide interest in glycobiology. Therapeutic applications of the glycosaminoglycan heparin and related materials continue to be of great value for clinical applications. However, there are well-documented remaining problems for including bleeding complications, and severe side effects of heparin-induced thrombocytopenia\(^1\). Therefore, development of useful alternatives to heparin is still attractive. An intriguing area for investigation is the synthesis of hybrid polymeric materials related to heparin via synthetic polymers containing specifically sulfated carbohydrates. Surprisingly, very few attempts have been made to synthesize polymers containing sulfated carbohydrates. A survey of the literature showed a number of procedures for preparation of poly sulfated carbohydrate derivatives\(^2\)\(^-\)\(^4\). However, there are no reports of mono or disulfated methyl methacrylate derivatives of carbohydrates. For this reason, our initial goal is to produce structurally defined sulfated carbohydrate derivatives of methyl methacrylate such that, upon polymerization, the position(s) of each sulfate group is known. This should allow a better development of structure-activity relationships between the sulfated carbohydrate polymers and their diverse biological activities. In order to achieve this goal, we have used protective group strategies to provide one or two free hydroxyl groups for subsequent sulfation steps in order to obtain suitable target monomers. We have also used degradative strategies to produce pentose derivatives from readily available hexose sugars.

This chapter describes the synthesis and characterization of a number of novel mono or disulfated carbohydrate containing methacrylate monomers. The position of
sulfate functionality as well as the methacrylate group is varied among these monomers.

Table 1.1 summarizes the structures of target sulfated monomers. Analysis by proton and carbon NMR in conjunction with mass spectrometry were used to confirm the monomer structure and molecular weight. The polymerization of these monomers will be described in chapter 2.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Galactose</th>
<th>Allose</th>
<th>Xylose</th>
<th>Ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="22" alt="Diagram" /></td>
<td><img src="25" alt="Diagram" /></td>
<td><img src="19" alt="Diagram" /></td>
<td><img src="20" alt="Diagram" /></td>
<td><img src="29" alt="Diagram" /></td>
</tr>
<tr>
<td><img src="17" alt="Diagram" /></td>
<td><img src="18" alt="Diagram" /></td>
<td><img src="18" alt="Diagram" /></td>
<td><img src="21" alt="Diagram" /></td>
<td><img src="30" alt="Diagram" /></td>
</tr>
</tbody>
</table>

Table 1.1. Structures of target sulfated monomers
A number of target compounds in Table 1.1 have been synthesized following two different synthetic approaches involving the use of different protecting groups. One strategy was sulfating the potential hydroxyl group(s) directly without use of any protecting groups followed by separation of the mixture of products by chromatography. Another approach was to use blocking groups to protect all potentially active hydroxyl sites except the one to be sulfated. This strategy involved other challenges such as protecting group(s) removal which sometimes resulted in desulfation or demethacrylation. It is very important to know what kinds of reagents and conditions normally cause desulfation. Sulfate esters of sugars are unstable in both acids and bases\(^5\). Since hydrolysis may readily occur when a sugar sulfate is heated under either of these conditions, it is important to monitor conditions very carefully to avoid loss or migration of sulfate functionality. Fortunately, for this research, the sulfate group has been shown to be fairly immune to migration to adjacent free hydroxyl groups under the conditions used.
1.2. Background and Significance

1.2.1. Carbohydrate chemistry

Carbohydrates are known as the most abundant class of bioorganic molecules in nature. They serve organisms as energy sources and as essential structural components. Carbohydrates play an important role in a number of biological processes such as carriers of cellular information, and involvement in infection and inflammation. Carbohydrate polymers are composed of individual units called monosaccharides. Compounds consisting of more than one monosaccharide are classified as disaccharides, oligosaccharides (2-20 units), and polysaccharides (>20 units). The organic chemistry of carbohydrates as multifunctional compounds has fascinated many talented chemists and biochemists from Pasteur and Fischer to Fraser-Stoddart. The highly hydrophilic character as well as their biocompatibility led to an increasing interest in their use in the domain of polymer synthesis for the preparation of biocompatible materials with appropriate pharmacological and biological properties. Carbohydrate-based drugs are known for their advantageous properties such as lack of toxicity and minimal immunogenicity; interest in carbohydrates as drug candidates for therapeutic application remains very high.

1.2.2. Carbohydrates Stereochemistry

When two monosaccharides differ only in stereochemistry at one carbon, they are referred to as epimers. For instance, galactose is the C4 epimer of glucose. Figure 1.1 illustrates two epimers, glucose and galactose.
Due to the reactivity of C1 of the open chain form of a monosaccharide (e.g. glucose), only a fraction of the saccharide remains in the open chain form in solution. Instead, the carbonyl group undergoes ring closure forming a hemiacetal. In cyclic form, C1 is referred to as anomeric center. There are two configurations possible at the anomeric carbons, alpha (α) and beta (β). Figure 1.2 shows anomeric forms of D-glucose.

There are two types of reactions of monosaccharides, those associated with the “anomeric” hydroxyl and the ones with the hydroxyl groups at the other carbons. Carbohydrates with free hydroxyl group at the anomeric carbon (C1) are prone to oxidizing conditions and are known as reducing sugars. Most naturally occurring monosaccharides and disaccharides are reducing sugars. 1,2:5,6-di-O-isopropylidene-D-glucofuranose (DIGlc) is an non-reducing sugar. Figure 1.3 shows the structure of DIGlc.
One of the reactions at the anomeric center is formation of glycosides. This reaction is known as intermolecular condensation at the anomeric carbon. One example of this type of reaction is the linkage of the repeating disaccharide units of heparin by glycosidic bonds. Sugar ester formation is a type of the reaction not involving the anomeric center carbon. One class of biologically important sugar esters is sugar sulfates, such as that present in glycosaminoglycans (GAGs). GAGs are composed of repeating disaccharide units, which consist of an amino sugar and another sugar residue, typically a uronic acid. Most GAGs are sulfated with the exception of hyaluronic acid which contains carboxylate groups. Heparin is a heterogenous copolymer of (1→4) linked glucuronic acid and N-acetyl glucosamine residues with 2-3 sulfates at various positions per repeating unit.

Figure 1.4. Most commonly repeated disaccharide unit of heparin, (linkage of 1→4) in α-D-uronic acid-2-sulfate (1→4) α-D-glucosamine-2,6-sulfate.
1.2.3. Sulfated sugars

Over time, the importance of sugar sulfates for regulation and control of biological functions has become better appreciated. It has been shown that addition of a sulfate group to a carbohydrate can lead to its recognition by a specific receptor\textsuperscript{13}. Sulfates are known to be present in the ATIII binding region of heparin\textsuperscript{13}. Thus, the 3-O-sulfate group\textsuperscript{14-15}, 6-O-sulfate group\textsuperscript{16}, and N-sulfate group\textsuperscript{17} are essential for the biological activity of heparin\textsuperscript{18}. One possible outcome of the research outlined in this thesis is the synthesis of polymers of sulfated sugars that may mimic the structure and biological activity of heparin.

Despite the interesting properties of sugar sulfates, their chemistry is known to be challenging. This is reflected by the work described in this chapter.

1.2.4. Methacrylate-based materials

Approximately two billion pounds of polymeric materials are produced annually from methacrylate monomers which are used in a wide variety of applications\textsuperscript{19}. Methyl methacrylate (MMA), methacrylic acid (MAA), and hydroxylethyl methacrylate (HEMA) have all been proven useful as monomers for the preparation of hydrogels for biomedical applications\textsuperscript{20}. PMMA has been used in hip-joint replacement as cement or grouting material at the bone/joint interface\textsuperscript{21}.

Products containing a PMMA backbone with pendant carbohydrate groups are often referred to as poly (vinyl saccharide)s, since the sugar-based monomers are coupled via a vinyl type polymerization. They are known for their attractive properties for use as biomaterials\textsuperscript{22}. The biocompatibility of PMMA is illustrated by its use as coating agent
for tablets in the pharmaceutical industry. For instance, PMMA is an essential element of the coatings of some drug molecules such as mesylamine (Asacol).
1.3. Experimental Section

1.3.1. Materials

All solvents and reagents were purchased from Sigma-Aldrich and Fluka unless reported otherwise and used without further purifications. 1,2:5,6-Di-O-isopropylidene-D-glucofuranose was purchased from Carbopharm, and 1,2:5,6-Di-O-isopropylidene-D-allofuranose\textsuperscript{23} was synthesized based on a published procedure. The synthesis of 1,2:3,4-Di-O-isopropylidene-\(\alpha\)-D-galactofuranose was novel.

1.3.2. Sulfation methods

Common sulfating agents are chlorosulfonic acid, sulfuric acid, and sulfur trioxide complexed with reagents such as pyridine, trimethyl amine, and triethyl amine. Reactions must often be carried out under anhydrous conditions for the maximum yield. Solvents commonly employed are pyridine, DMF, concentrated sulfuric acid, and liquid sulfur trioxide. Sephadex G-25 followed by Dowex 50 Na\textsuperscript{+} was used to purify final sulfated monomers.

1.3.3. Characterization methods

Melting points were determined with a Fisher-Johns apparatus and are uncorrected. NMR spectra were recorded on a Varian Inova 300 (H\textsubscript{1}: 300 MHz, C\textsubscript{13}: 75 MHz) or 500 MHz (H\textsubscript{1}: 500 MHz, C\textsubscript{13}: 125 MHz) spectrometer at ambient temperature in an appropriate deuterated solvent using TMS as the internal standard. MS spectra were recorded on a Bruker Biotof II for negative electrospray ionization. Column
chromatography was conducted on Silica Gel 60 (60-200 Mesh, Mallinckrodt Chemicals). Thin layer chromatography (TLC) was conducted on precoated Silica Gel 60 F254; compounds were detected by spraying with a 10% solution of o-anisaldehyde or vanillin in EtOH followed by heating. Small impurities in intermediates were sometimes neglected when they did not interfere with the next step.

1.3.4. Monomer synthesis

Due to similarity of a number of steps in the synthetic reactions toward target monomers, general procedures for these steps are reported. Two approaches were used to prepare some of the sulfated monomers such as compounds (22) and (26).

1.3.4.1. General procedure for the methacrylation of carbohydrate derivatives

The glucose and allose methyl methacrylate derivatives were synthesized by a literature procedure with some modifications24. A stirred solution of protected sugar (1.0 g) and methacrylic anhydride (1.0 mL, 1.8 equiv) in pyridine (5.0 mL, 0.76 M) was heated at 65 °C for 4-5 h. Water (2.5 mL) was added and the reaction was stirred for an additional hour at 65 °C. The reaction temperature was lowered to 30 °C and stirred for 18 h. At this point, the reaction mixture was diluted with petroleum ether (20 mL) and washed with 5% NaOH (3x, pH ~ 7.0) and water (10 mL, 3x). The combined organic layers were dried over Na2SO4. 4-Methoxy phenol (0.032 g) was added before the evaporation of the organic solvent as a polymerization inhibitor. The crude product was distilled under vacuum (p = 0.4 mmHg) twice to afford the desired compound in 60% yield as a thick syrup which crystallized upon standing at RT.
1.3.4.2. General procedure for selective deprotection of isopropylidene derivatives

This is a modification of a published procedure\textsuperscript{25}. The protected carbohydrate derivative was dissolved in 50% HOAc and stirred for a period of 18 h at RT. Upon the completion of the reaction, the solvent was removed at reduced pressure and the product was dried under high vacuum.

1.3.4.3. General procedures for sulfation of carbohydrate derivatives

This procedure is based on a published procedure\textsuperscript{26} with some minor modifications. The methacrylated carbohydrate was dissolved in dry DMF (10 mL /g) and treated with TMA-SO\textsubscript{3} (1.5 eq/OH). The resulting mixture was stirred at RT for a period of 72 h. Typically, the mixture becomes homogenous after 36 h. Upon completion of the reaction, the solution was mixed with an excess of 10% NaOAc and concentrated under reduced pressure. The residue was dissolved in deionized water (4x) and concentrated each time. The final residue was desalted by suspending in methanol.
(2x) followed by filtration. The crude product was purified by ion exchange chromatography using Sephadex G-25 followed by Dowex resin Na⁺.

1.3.4.4. Synthesis of 6M-IGlc (10)

This compound was synthesized based on a literature procedure with major modifications. After dissolving 4.0 g of compound (8) in 30 mL of pyridine, 1.0 eq of methacrylic anhydride was added. The resulting solution was heated at 60 °C under a blanket of nitrogen for 4 h. Water (15 mL) was added and the heating was continued for an additional hour. Then, the temperature was lowered to 30 °C and stirred until completion indicated by TLC analysis (17 h). The reaction mixture was diluted with CH₂Cl₂ (30 mL) and washed with 5% NaOH (3x 30 mL), water (3x 30 mL), and brine (1x 30 mL). The organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was recrystallized from 2:1 MeOH: H₂O at RT.

Figure1.7. Synthesis of 6M-IGlc (10)
1.3.4.5. 6M-DIGal (3).

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose (5.0 g) was dissolved in 30 mL of CH$_3$CN and treated with 2 eq of DMAP. The resulting solution was cooled in an ice-bath before the addition of methacrylic anhydride (2 eq) and was then heated at 50°C in acetonitrile (25 mL) for 5 h under slow stream of N$_2$. 4-Methoxyphenol was added as a polymerization inhibitor. Distilled water (12 mL) was added and heating was continued at 50°C for an additional hour. Then the temperature was lowered to 30°C and stirred for 15 h. Upon completion of the reaction, the solution was diluted with EtOAc (30 mL) and washed with 5% NaOH (3x10mL), water (2x10mL), and brine (1x15mL). To remove residual DMAP, evident from NMR of the crude product, 10% HCl (2x10mL) wash followed by water (1x10mL) was conducted. The organic layer was dried over MgSO$_4$ and concentrated under reduced pressure. The clear syrup so obtained was crystallized at RT.

Figure 1.8. Synthesis of 6M-DIGal (3)

1.3.4.6. Synthetic VI (2a)

The synthesis of compound (2a) was accomplished following a modified literature procedure$^{23}$. 1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose (1a) (0.52 g) was dissolved in a mixture of anhydrous DMSO (4.0 mL) and Ac$_2$O (1.0 mL) and stirred at
RT for 24 h under nitrogen blanket. Analysis by TLC (CH$_2$Cl$_2$:CH$_3$OH, 95:5) indicated complete oxidation of the free hydroxyl group. The reaction was then cooled in an ice-bath. Sodium borohydride (0.032 g, 3equiv) was added in portions over a 10-15 min time period while the reaction temperature was kept in the range of 0-5 °C. After complete addition of reducing agent (NaBH$_4$) the resulting mixture was stirred for 1-4 h at 20-25°C or until complete reduction as indicated by TLC. The resulting mixture was diluted with water (10.0 mL) and extracted from CH$_2$Cl$_2$ (15.0 mL, 3X). The combined organic layers were washed with water (10.0 mL, 2X), and brine (10.0 mL, 1X), dried using MgSO$_4$, and concentrated under vacuum to afford a yellowish oil. The oil was dissolved in MTBE (5.0 mL) and extracted with water (10.0 mL, 2X). The combined aqueous layer was extracted with CH$_2$Cl$_2$ (15.0 mL, 2X). The organic layer was dried over MgSO$_4$ and concentrated in vacuo to afford an oily product. Analytically pure desired compound was obtained in 60 % yield after two crystallizations from cyclohexane.

![Figure 1.9. Synthesis of DIAll (2a)](image)

**Figure 1.9. Synthesis of DIAll (2a)**

### 1.3.4.7. Synthesis of 3Ac-DIGlc (4)

The synthesis of compound (4) was based on a modified procedure$^{27}$. 1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose (5.0 g) was dissolved in 100 mL of CH$_2$Cl$_2$ and treated with 3 eq of DMAP and 1.5 eq of acetic anhydride. TEA (3 eq) was added slowly while the reaction flask was kept in an ice bath. After this addition, the reaction was
stirred at RT for a period of 3 h or until completion of the reaction. The resulting solution was quenched with saturated NH₄Cl solution and diluted with H₂O (15 mL) and ether (20 mL). The separated aqueous layer was extracted with ether (2x15 mL). The combined organic layers were dried over CaCl₂ and concentrated under pressure. The crude residue was purified by column chromatography with a gradient of CH₂Cl₂:CH₃OH. (92%);

![Chemical structure](image)

**Figure 1.10. Synthesis of 3Ac-DIGlc (4)**

### 1.3.4.8. Synthesis of 3M-IXyl (11)

This procedure was based on a modified procedure from the literature²⁸. After dissolving compound (5) in 1:1 mixture of MeOH: H₂O, 1.1 eq of NaIO₄ was added and stirred at RT until completion. TLC analysis indicated that the reaction was complete after 90 min when a white precipitate formed. This precipitate was filtered and the pale yellow filtrate was concentrated under reduced pressure. The obtained yellow solution was extracted with CH₂Cl₂ continuously overnight. The combined organic layers were washed with brine and dried over CaCl₂ and concentrated under pressure and dried under high vacuum (5a). The presence of a singlet at 9.8 ppm in proton NMR indicated the presence of aldehyde functionality. The obtained product (5a) (420 mg) was dissolved in 20 mL of CH₂Cl₂ and cooled in an ice bath (0-5°C). Sodium borohydride (3 eq) was
added slowly in small portions. The resulting mixture was stirred at RT until reaction completion. The obtained white mixture was diluted with CH₂Cl₂, washed with H₂O (3x20 mL), brine (2x15 mL) and dried over CaCl₂. The organic phase was concentrated under reduced pressure and further dried under high vacuum. Analysis by ¹H NMR indicated the absence of the aldehyde proton in the spectra.

Figure 1.11. Synthesis of 3M-IXyl (11)

1.3.4.9. Synthetic approaches toward synthesis of 5M-IXyl (14)

Two different synthetic routes were followed in order to prepare compound (14) in which the starting materials in each route were varied. Initially, an attempt was made to synthesize this compound using (1a) in which the experimental yield on the cleavage of 5,6-diol functionality by NaIO₄ was quite low. Therefore, an alternative route was chosen such that the hydroxyl group on C3 was protected by an acetyl group. These two synthetic approaches are described in details in Figure 1.12 and 1.13.

1.3.4.9.1. Synthesis of compound (14) following Route I

Here, compound (1a) was used as starting material. The cleavage of the diol at C5-C6 was accomplished using NaIO₄. The synthetic steps are shown in figure 1.12.
Similar synthetic procedures as described in section 1.3.4.8 were followed to convert compound (3a) to (4a) and compound (4a) to (13). The yields for both steps were substantially low such that not enough material was obtained for further steps toward synthesis of compound (14). A possible mechanistic explanation will be discussed in section 1.6.

1.3.4.9.2. Synthesis of compound (14) following Route II

The following derivatives were synthesized from 3-\textit{O}-acetyl derivative of D-glucose. Upon acetylation of the protected sugar, a similar approach was followed as described in section 1.3.4.8 to obtain 3-\textit{O}-acetyl-1,2-\textit{O}-isopropylidene-xylofuranose. The acetyl functionality was then removed before methacrylation at C5 due to sensitivity of the methyl methacrylate to deacetylation conditions. Typically, 3-\textit{O}-acetyl-1,2-\textit{O}-isopropylidene-xylofuranose was treated with NaOMe at RT for 60 min in methanol. The crude product was then methacrylated at C5 using one equivalent of methacrylic anhydride following a modified procedure published in the literature. The white
crystalline material obtained at RT was analyzed by $^1$H NMR before sulfation.

![Chemical structures](image)

**Figure 1.13. Synthesis of 5M-IXyl (14)**

1.3.4.10. Synthesis of 3Am-DIAII (24)

This compound was synthesized by one pot oxidation and reductive amination of hydroxyl functionality at position C3 following a modified published procedure. A solution of (1a) in DMSO (16 mL) was treated with Ac$_2$O (4 mL) and stirred at RT under N$_2$ for a period of 24 h. The reaction mixture was diluted with 40 mL water and extracted with CH$_2$Cl$_2$ (3x20 mL). The combined organic layers were washed with water (2x20 mL) and brine (1x15 mL) and dried over Na$_2$SO$_4$. After filtering the drying agent, the solvent was removed under reduced pressure. The crude product was dissolved in 1:1 (pyridine:ethanol) (30 mL) and treated with NH$_2$OH.HCl (2.93 g, 5.5 eq) at RT for 18 h. The solvent was then concentrated and the residue was mixed with water (15 mL) and washed with Et$_2$O (4x20 mL). The combined ether layers were dried over Na$_2$SO$_4$ and concentrated under pressure. The obtained crude product was then dissolved in dry THF (100 mL) and treated with LiAlH$_4$ (1.75 g, 7.7 eq) at RT overnight under N$_2$. The N$_2$
pressure should be kept very low to avoid loss of solvent by evaporation. To this mixture, water was added with extreme caution. After all of LAH was destroyed, 50 mL of 30% NaOH was added and the dark black mixture was extracted with ether (2x50 mL). The organic layer was then washed with water (2x20 mL) and dried over Na₂SO₄ and concentrated to dryness. The obtained product was analyzed using ¹H NMR and MS.

Figure 1.14. Synthesis of 3Am-DIAll (24)

1.3.4.11. Alternative routes toward synthesis of 3M-6S-IGlc (22)

1.3.4.11.1. Synthesis of compound (22) using protecting group chemistry, route I

This approach involved using protecting group chemistry to block all hydroxyl functionalities but the one to be sulfated. Varied protecting groups were used in which the ones with best results are reported in this section. An outline of the synthetic steps is shown in scheme 1.1.
Scheme 1.1. Initial Synthetic Steps toward Synthesis of Monomer (22), route I

Compound (1) was synthesized based on procedure outlined in section 1.3.4.1. After selective removal of 5,6-isopropylidene group following the procedure in section 1.3.4.2, the hydroxyl group at C6 was selectively tritylated based on a method from the literature\(^\text{30}\).

Trityl Chloride and DMAP were dissolved in dry CH\(_2\)Cl\(_2\) (20 mL) and stirred under N\(_2\) for one hour. Upon addition of 100 mL diethyl ether, the salt was filtered and dried. Compound (5) was dissolved in 20 mL of CH\(_2\)Cl\(_2\):CH\(_3\)OH (9:1) and treated with 1.2 equivalent of trityl salt at RT for 16 h or until completion as indicated by TLC analysis. The obtained solution was washed with 10% HCl (2x10 mL), water (3x15 mL),
and brine (1x15 mL) and dried over MgSO4. The crude product (5b) was concentrated under reduced pressure and dried under vacuum before analysis by $^1$H NMR.

A solution of compound (5b) in CH$_2$Cl$_2$ was cooled in an ice-bath. Methoxymethyl chloride (MOM-Cl), 1.2 eq, was added dropwise. While the reaction mixture was kept at 0ºC, DIEA (1.2 eq) was added dropwise, monitoring the temperature during the addition. After complete addition of DIEA, the reaction was stirred for one hour when the ice-bath was removed and the solution continued stirring at RT for a period of 8 h or until completion of reaction indicated by TLC analysis. The reaction solution was diluted with CH$_2$Cl$_2$ (20 mL), extracted with water (2x15 mL), brine (1x15 mL), and dried over CaCl$_2$. The crude product (5c) was analyzed by $^1$H NMR before use in the next step.

Removal of trityl group from compound 5c was accomplished based on a published procedure$^{31}$. After dissolving 5c in 20 mL of 9:1 (CH$_2$Cl$_2$:CH$_3$OH), the solution was treated with 10 equivalents of NaHSO$_4$-SiO$_2$ at RT until completion reaction as judged by TLC analysis. The mixture was then filtered and concentrated. The obtained residue was washed with hexane and purified by column chromatography using 80:20 (CH$_2$Cl$_2$:CH$_3$OH) solution as eluent. The pure fraction containing the desired product was a very small percentage (7%) of the total product. The purity of the detrityl compound (5d) was analyzed by $^1$H NMR.

The synthesis of compound (5e) was accomplished following procedure given in section 1.3.4.3. A reasonably good yield (62%) of the sulfated product was obtained.

Finally, an attempt was made to remove the methoxymethyl functionality using thiophenol and trifluoroboron etherate (BF$_3$.Et$_2$O). Analysis of the crude product by $^1$H
NMR indicated a mixture of products. After purification, a second analysis by $^1$H NMR and MS indicated that the majority of the product contained no sulfate or methacrylate groups. A very small yield of desired product (22) was obtained. Apparently, both sulfate and methyl methacrylate groups were removed by this deprotection reaction.

**Figure 1.15. Synthesis of 3M-6S-IGlc (22)**

**1.3.4.11.2. Synthesis of compound (22) without using protecting group chemistry, route II**

In this synthetic route, compound (22) was synthesized without using protecting group chemistry except the isopropylidene functionality on C1-C2. This synthetic scheme was based on different reactivity of primary and secondary hydroxyl groups toward sulfation reaction. An outline of steps is shown in scheme 1.2.

**Scheme 1.2. Synthetic steps for preparation of compound (22), route II**

After selective isopropylidene group removal from C5-C6 on compound (1) as explained in section 1.3.4.2, the primary hydroxyl group on C6 was sulfated following
procedure outlined in section 1.3.4.3. The final product was purified as described above and analyzed by $^1$H NMR and MS for purity.

1.3.4.12. Synthesis of 3M-5S-IGlc (25)

Compound (25) was synthesized based on steps outline in scheme 1.3 using protecting group chemistry.

Scheme 1.3. Synthesis of 3M-5S-IGlc (25)

The selective protection of primary hydroxyl functionality by TBDMS-Cl was accomplished using a published procedure$^{32}$. A solution of 2.0 g (5) in 25 mL DMF was treated with 0.33 eq of DMAP and 2.2 eq of TBDMSCl and purged under N$_2$ for 30 min at RT. After cooling the reaction flask using an ice-bath, TEA (1.2 eq) was added dropwise while the reaction was kept at or below 0ºC. The obtained mixture was stirred at RT for 16 h. The mixture was diluted with EtOAc (40 mL) and extracted with H$_2$O (20 mL). The organic layer was washed with H$_2$O (2x10 mL), saturated NH$_4$Cl (1x15 mL), brine (1x15 mL), and dried over Na$_2$SO$_4$. The solvent was removed under reduced
pressure and the crude product was dried under high vacuum. The analysis by \textsuperscript{1}H NMR indicated the desired pure product (5f).

The outlined of the sulfation of (5f) is explained in section 1.3.4.3. A very small yield (10\%) of compound (5g) was obtained with low purity.

A solution of (5g) in THF was treated with 10 eq of TBAF and stirred at RT for a period of 2 h. The resulting mixture was washed with H\textsubscript{2}O (2x), brine (1x), and dried over CaCl\textsubscript{2}. The solvent was then removed under pressure. The crude product was purified by column chromatography using CH\textsubscript{2}Cl\textsubscript{2}:CH\textsubscript{3}OH (9:1). \textsuperscript{1}H NMR and MS analysis indicated a small amount (<5\%) of desired product is present in the product mixture with no TBDMS present. Attempts to repeat the purification to remove the residue of TBDMS resulted in loss of methyl methacrylate group.

Figure 1.16. Synthesis of 3M-5S-IGlc (25)
1.3.4.13. Alternative synthetic steps toward preparation of 6M-3,5-DS-IGlc (26)

1.3.4.13.1. Synthesis of compound (26) using protecting group chemistry, route I

In this approach, all hydroxyl functionalities but the one to be sulfated were blocked using suitable protecting group. Outline of the synthesis is shown in scheme 1.4.

Scheme 1.4. Initial Synthetic Steps toward Synthesis of Monomer (26)

This protection reaction is based on a published procedure. A solution of (1a) in dry THF was cooled to 0°C in an ice-bath. Sodium hydride (1.3 eq) was added very
slowly while keeping the temperature of the reaction mixture at 0°C over approximately 10-15 minutes. The resulting mixture was treated with methoxy methyl chloride (MEM-Cl) near 0°C. Upon complete addition of MEM-Cl, the solution was stirred at 0°C for an additional hour and then stirred at RT until reaction completion as indicated by TLC (18-20 h). The resulting solution was then diluted with CH₂Cl₂ and washed with H₂O. The aqueous layer was extracted with CH₂Cl₂ (2x). The combined organic extracts were then washed with H₂O (2x) and brine (1x) and finally dried over CaCl₂ and concentrated under reduced pressure. The crude residue was purified by column chromatography using gradient of CH₂Cl₂:CH₃OH collecting the pure fraction at 95:5 ratio of CH₂Cl₂:CH₃OH. The final pure product (1b) with 84.5% yield was analyzed by ¹H NMR.

After selective deprotection of 5,6-isopropylidene group following the procedure described in section 1.3.4.2, the primary hydroxyl was selectively protected using TBDMSCl based on the procedure in section 1.3.4.12.

The benzylation of this compound was accomplished based on a modified procedure in literature³⁴. A 60% oily dispersion of NaH (1.92 eq) was suspended in dry DMF and cooled to 0°C using ice-bath. A solution of (1d) in DMF was then added dropwise over a period of 30 min. The resulting mixture was then stirred at 50°C for 1 h and then cooled down to 0°C. The resulting solution was then treated with benzyl bromide (BnBr) very slowly and stirred at RT for 16 h. After completion of the reaction (TLC analysis), the solvent was removed under reduced pressure and the obtained residue was diluted with CH₂Cl₂, washed with saturated NaHCO₃ (3x), H₂O (2x), and brine (1x). The organic layer was dried over CaCl₂ and concentrated under pressure. Final
purification by column chromatography using CH$_2$Cl$_2$: CH$_3$OH gradient afforded pure product (1e) which was analyzed by $^1$H NMR.

Removal of the TBDMS protecting group was accomplished based on the procedure given in section 1.3.4.12. Methacrylation of C6 hydroxyl was done by the procedure outlined in section 1.3.4.1, which afforded compound (1h) in 67% yield. Debenzylation of this compound was accomplished based on a modified procedure from the literature$^{35}$. A solution of compound (1h) in ethanol was treated with 10% Pd/C and stirred under a balloon containing H$_2$ at RT and atmospheric pressure until completion (TLC analysis) for approximately 15 h. The resulting mixture was then filtered through Celite and the solvent was removed under reduced pressure. $^1$H NMR analysis indicated a very small percentage (0.7%) of the desired product with loss of benzyl functionality and a large amount of product with loss of both benzyl group and methacrylate group, undesired product (1j).

![Figure 1.17. Synthesis of 3MEM-6M-IGlc (1i)](image)

Due to the considerable small yield in synthesis of compound (1i), the synthesis of compound (26) was not accomplished following procedure outline in Scheme 4.

1.3.4.13.2. Synthesis of compound (26) without protecting group chemistry, route II

In this synthetic route, compound (26) was synthesized without protecting groups except isopropyldiene functionality. This scheme was based on methacrylation of a
primary hydroxyl in preference to secondary, followed by disulfation of C3 and C5. An outline of the synthetic steps is shown in scheme 1.5.

Scheme 1.5. Synthesis of 6M-3,5-DS-IGlc (26)

After selective deprotection of 5,6-\(O\)-isopropylidene group following the procedure in section 1.3.4.2, the primary hydroxyl functionality was preferentially methacrylated with one equivalent of methacrylic anhydride. Disulfation at C3 and C5 was accomplished based on the general procedure outlined in section 1.3.4.3 and the obtained product was purified by Sephadex column (G-25) followed by ion exchange column (\(\text{Na}^+\)). The final product was analyzed by \(^1\)H NMR and MS.

1.3.4.14. Synthesis of 3M-5,6-DS-IGlc (17)

After selective deprotection of 5,6-\(O\)-isopropylidene group from compound (1), disulfation of (5) was accomplished following the procedure described in section 1.3.4.3. The desired product (17) was purified as explained above and analyzed by \(^1\)H NMR and MS before polymerization. Outline of this procedure is shown in scheme 1.6.
1.3.4.15. 3M-5,6-DS-DIAll (18)

Selective deprotection of the 5,6-\(O\)-isopropylidene group from compound (2) followed by disulfation of (6) based on procedures described in section 1.3.4.2 and 1.3.4.3, respectively, afforded product (18) in 80% yield. The desired product (18) was purified as explained above (section 1.3.4.13) and analyzed by \(^1\)H NMR and MS before polymerization. An outline of this procedure is shown in scheme 1.7.

1.3.4.16. Synthesis of 6M-3,4-DS-IGal (19)

The synthesis of this monomer was outlined in scheme 1.8. After deblocking of the isopropylidene group from C3 and C4 as outlined in section 1.3.4.2, intermediate (7) was disulfated using trimethyl amine sulfate following the general procedure of sulfation described in section 1.3.4.3. The yield of this reaction was very low such that not enough
material was obtained for polymerization. Further experimentation is necessary to optimize the yield.

Scheme 1.8. Synthesis of 6M-3,4-DS-IGal (19)

1.3.4.17. Synthesis of 3M-5S-IXyl (20)

This monomer was obtained by sulfation of (11) following the procedure given in section 1.3.4.3. A summary of the reaction conditions is shown in Figure 1.18.

Figure 1.18. Synthesis of 3M-5S-IXyl (20)

1.3.4.18. Synthesis of 5M-3S-IXyl (21)

Sulfation of (14) using trimethyl amine sulfate was done as explained in section 1.3.4.3. An outline of the reaction conditions is shown in Figure 1.19.
1.3.4.19. Attempt for preparation of 6M-3AmS-IAll (28)

Sulfation of (24) was accomplished by the general sulfation procedure of section 1.3.4.3 resulting in the intermediate (27). An attempt at deprotection of (27) using 50% aqueous acetic acid was resulted in desulfation of the product. Other selective deblocking of 5,6-isopropylidene functionality is necessary in order to synthesize the target compound (28).

The synthetic route is shown in scheme 1.9.

Scheme 1.9. Synthetic route toward synthesis of 6M-3AmS-IAll (28)

1.3.4.20. Deprotection of 1,2-O-isopropylidene group

The final step of the synthesis of desired sulfated carbohydrate derivatives of methacrylate monomers is deprotection of 1,2-O-isopropylidene functionality. A survey of literature procedures gives a few methods for removal of 1,2-O-isopropylidene group from a number of polymers\(^{36-38}\). The goal of this work is to deprotect 1,2-O-isopropylidene group from sulfated monomers. Therefore, a number of reagents (e.g., HCl and HCOOH acids) and conditions such as temperature (e.g. RT and 60°C) and time (4-24 h) were used in order to
find the best procedure for this deprotection step. $^1$H NMR spectroscopy was used as a tool to monitor a number of conditions for this reaction. The results are shown in section 1.3.5.

1.3.4.21. Temperature control NMR experiment to separate $\alpha$ and $\beta$ anomers

After removal of 1,2-$O$-isopropylidene functionality, it is of interest to determine relative amount of $\alpha$ and $\beta$ anomers in the final compound. $^1$H NMR spectroscopy is a useful tool to differentiate between two anomic protons. Therefore, NMR spectrum from an authentic sample (2,3-disulfated glucosamine) was obtained in order to separate the $\alpha$ and $\beta$ anomic protons in the sample and calculate the relative ratios of two anomers. Five NMR spectra at different temperatures (22ºC to 60ºC) were collected in order to monitor the $H_\alpha$ and $H_\beta$ chemical shifts. The results are shown in section 1.3.5. This experiment indicates that it is possible to determine the ratio of $\alpha$ and $\beta$ anomers. In case of overlap with other protons present in the spectrum, changing the temperature allows the exact location of the signals to be varied.
1.4. Results

The majority of compounds synthesized in section 1.3.4 have been characterized by TLC, m.p., $^1$H NMR, $^{13}$C NMR, and MS. 2D NMR has been used to characterize some compounds where necessary.

1.4.1. 3M-DIGlc (1)

Prepared from 1a; 30.0 g (60%); white crystals; mp 32-33 °C, lit.$^{24}$ 34-35 °C; R$_f$ 0.81 (9:1 CH$_2$Cl$_2$-MeOH); $^1$H NMR (CDCl$_3$): $\delta$ 6.15 (d, 1 H, 1.2 Hz, CH$_2$=CCH$_3$), 5.92 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.65 (d, 1 H, 3.0 Hz, H-1), 5.32 (bs, 1 H, H-3), 4.57 (d, 1 H, 0.6 Hz, H-2), 4.28 (d, 2 H, 1.2 Hz, H-4, H-5), 4.17 (dd, 2 H, Hz, H-6), 1.98 (s, 3H, CH$_3$C=CH$_2$), 1.55 (s, 3H, CCH$_3$), 1.43(s, 3H, CCH$_3$), 1.31 (2s, 6H, C(CH$_3$)$_2$); $^{13}$C NMR (CDCl$_3$): $\delta$ 18.1 (CH$_3$C=CH$_2$), 25.0-26.6 (4C, C(CH$_3$)$_2$), 67.0 (C-6), 72.3 (C-5), 76.5 (C-3), 79.7 (C-4), 83.1 (C-2), 104.9 (C-1), 109.1 (CCH$_3$), 112.1 (CCH$_3$), 126.4 (CH$_2$=CCH$_3$), 135.6 (C=CH$_2$), 165.8 (C=O).

1.4.2. DIAll (2a)

Prepared from 1a; 1.5 g (40%); white crystals; mp 103 °C; R$_f$ 0.81 (9:1 CH$_2$Cl$_2$-MeOH); $^1$H NMR (CDCl$_3$): $\delta$ 5.85 (s, 1H), 4.65 (d, 1H), 4.34 (t, 1H), 4.10 (dd, 2H), 4.05 (dd, 1H), 3.85 (t, 1H), 2.16 (bs, OH), 1.61 (s, 3H), 1.53 (s, 3H), 1.46 (s, 3H), 1.38 (s, 3H).

1.4.3. 3M-DIAll (2)

Prepared from 2a; 2.0 g (60%); white crystals; mp 52-55 °C; R$_f$ 0.70 (9:1 CH$_2$Cl$_2$-MeOH); $^1$H NMR (CDCl$_3$): $\delta$ 6.18 (d, 1 H, 1.2 Hz, CH$_2$=CCH$_3$), 5.87 (d, 1 H, 3.3 Hz,
\( \text{CH}_2=\text{CCH}_3 \), 5.65 (d, 1 H, 1.5 Hz, H-1), 4.90 (d, 2 H, 5.1 Hz, H-2, H-3), 4.33 (t, 1 H, 2.1 Hz, H-6), 4.21 (t, 1 H, 4.3 Hz, H-6), 4.09 (t, 1 H, 1.8 Hz, H-4), 3.94 (dd, 1 H, 5.4 Hz, H-5), 1.98 (s, 3H, CH\(_3\)C=CH\(_2\)), 1.52-1.58 (2s, 6H, C(CH\(_3\))\(_2\)), 1.44(s, 3H, CCH\(_3\)), 1.35 (s, 3H, CCH\(_3\)); \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 18.0 (CH\(_3\)C=CH\(_2\)), 25.0-26.6 (4C, C(CH\(_3\))\(_2\)), 67.1 (C-6), 72.7 (C-5), 75.0 (C-3), 77.4 (C-4), 104.0 (C-2), 109.8 (C-1), 112.9 (2C-CCH\(_3\)), 126.5 (CH\(_2=CCH_3\)), 135.3 (C=CH\(_2\)), 166.2 (C=O)

1.4.4. 6M-DIGal (3)

Prepared from 3a: (60%); white crystals; mp 42-45 °C; R\(_f\) 0.86 (9:1, CH\(_2\)Cl\(_2\):MeOH); \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 6.13 (d, 1 H, 0.6 Hz, CH\(_2=CCH_3\)), 5.56 (t, 1 H, 1.5 Hz, H-1), 5.53 (d, 1 H, 5.1 Hz, CH\(_2=CCH_3\)), 4.62 (dd, 1 H, 2.7 Hz, H-3), 4.36 (d, 1 H, 4.8 Hz, H-5), 4.32 (dd, 1 H, 2.4 Hz, H-6), 4.27 (t, 1 H, 4.5 Hz, H-4), 4.24 (d, 1 H, 2.1 Hz, H-2), 4.05 (t, 1 H, 4.8 Hz, H-6), 1.94 (s, 3 H, CH\(_3\)C=CH\(_2\)), 1.49 (s, 3 H, CCH\(_3\)), 1.45 (s, 3 H, CCH\(_3\)), 1.32 (s, 6 H, C(CH\(_3\))\(_2\)); \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 18.1 (CH\(_3\)C=CH\(_2\)), 24.2-25.8 (4 C, C(CH\(_3\))\(_2\)), 63.4 (C-6), 65.7 (C-2), 70.3-70.9 (3 C, C-3, C-4, C-5), 96.1 (C-1), 105.4 (CCH\(_3\)), 108.6 (CCH\(_3\)), 125.7 (CH\(_2=CCH_3\)), 135.9 (C=CH\(_2\)), 164.4 (C=O).

1.4.5. 3Ac-DIGlc (4)

Prepared from 1a: (92%); white solid, R\(_f\) 0.77 (9:1, CH\(_2\)Cl\(_2\)::CH\(_3\)OH); \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 5.82 (d, 1 H, 3.9 Hz, H-1), 5.18 (d, 1 H, 2.4 Hz, H-2), 4.44 (d, 1 H, 3.6 Hz, H-5), 4.15 (d, 2 H, 2.7 Hz, H-3, H-4), 4.01 (dd, 2 H, 5.4 Hz, H-6), 2.04 (s, 3 H, CH\(_3\)C=O), 1.45 (s, 3 H, CCH\(_3\)), 1.34 (s, 3 H, CCH\(_3\)), 1.24-1.25 (2s, 6 H, C(CH\(_3\))\(_2\)).
1.4.6. 3M-IGlc (5)

Prepared from 1: (97%); thick clear syrup; $^1$H NMR (CD$_3$OD): δ 6.17 (bs, 1 H, CH$_2$=CCH$_3$), 5.92 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.68 (d, 1 H, 1.5 Hz, H-1), 5.32 (d, 1 H, 2.4 Hz, H-3), 4.6 (d, 1 H, 3.9 Hz, H-2), 4.22 (dd, 1 H, 6.0 Hz, H-5), 3.84 (dd, 1 H, 3.0 Hz, H-4), 3.74 (d, 1 H, 5.4 Hz, H-6), 3.69 (dd, 1 H, 1.2 Hz, H-6), 1.95 (s, 3 H, CH$_3$C=CH$_2$), 1.52 (s, 3 H, CCH$_3$), 1.31 (s, 3 H, CCH$_3$)

1.4.7. 3M-IAll (6)

Prepared from 2: (93%); clear syrup; $^1$H NMR (CD$_3$OD): δ 6.11 (d, 1 H, 0.9 Hz, CH$_2$=CCH$_3$), 5.80 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.63 (d, 1 H, 1.5 Hz, H-1), 4.21 (d, 2 H, 8.1 Hz, H-3), 3.80 (t, 1 H, 2.1 Hz, H-6), 3.55-3.29 (2d, 2 H, 5.1 Hz, H-2, H-6), 3.49-3.46 (2d, 2 H, 7.2 Hz, H-4, H-5), 1.92 (s, 3H, CH$_3$C=CH$_3$), 1.47 (s, 3H, CCH$_3$), 1.27 (s, 3H, CCH$_3$)

1.4.8. 6M-IGal (7)

Prepared from 3: (85%); clear syrup; $^1$H NMR (CD$_3$OD): δ 6.04 (d, 1 H, 1.5 Hz, CH$_2$=CCH$_3$), 5.56 (d, 1 H, 1.5 Hz, H-1), 5.42 (d, 1 H, 4.8 Hz, CH$_2$=CCH$_3$), 4.60 (d, 1 H, 2.4 Hz, H-3), 4.31 (d, 1 H, 2.1 Hz, H-6), 4.23 (d, 1 H, 1.8 Hz, H-2), 4.21 (d, 1 H, 3.9 Hz, H-6), 4.15 (d, 1 H, 5.1 Hz, H-5), 4.06 (t, 1 H, 4.0 Hz, H-4), 1.98 (s, 3 H, CH$_3$C=CH$_2$), 1.43 (s, 3H, CCH$_3$), 1.27 (s, 3H, CCH$_3$)
1.4.9. IGlc (8)  
Prepared from 1a: (90%); white crystals; \(^1\)H NMR (CD\(_3\)OD): \(\delta\) 5.84 (d, 1 H, 3.6 Hz, H-1), 4.52 (d, 1 H, 3.9 Hz, H-3), 4.14 (d, 1 H, 2.7 Hz, H-6), 3.91 (d, 1 H, 2.7 Hz, H-2), 3.75 (t, 1 H, 3.3 Hz, H-6), 3.61 (d, 1 H, 2.7 Hz, H-5), 3.48 (dd, 1 H, 6.0 Hz, H-4), 1.34 (s, 3 H, CCH\(_3\)), 1.18 (s, 3 H, CCH\(_3\)); \(^1\)C NMR (CD\(_3\)OD): \(\delta\) 27.5-27.9 (2 C, C(CH\(_3\))\(_2\)), 65.9 (C-6), 70.8 (C-5), 76.0 (C-3), 82.1 (C-2), 86.8 (C-4), 107.1 (C-1), 115.1 (C(CH\(_3\))\(_2\)).

1.4.10. 3Ac-IGlc (9)  
Prepared from 4: (94%); white crystal; \(^1\)H NMR (CD\(_3\)OD): \(\delta\) 5.91 (d, 1 H, 3.9 Hz, H-1), 5.25 (d, 1 H, 3.0 Hz, H-2), 4.58 (d, 1 H, 3.6 Hz, H-3), 4.21 (dd, 1 H, 3.0 Hz, H-4), 3.82 (d, 1 H, 3.0 Hz, H-6), 3.78 (d, 1 H, 2.7 Hz, H-5), 3.64 (dd, 1 H, 6.3 Hz, H-6), 2.13 (s, 3 H, CH\(_3\)C=O), 1.52 (s, 3 H, CCH\(_3\)), 1.35 (s, 3 H, CCH\(_3\)).

1.4.11. Synthesis of 6M-IGlc (10)  
Prepared from 1a: (30%); white shiny crystals; mp 130-134 °C; R\(_f\) 0.77 (9:1 CH\(_2\)Cl\(_2\)-MeOH); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 6.17 (d, 1 H, 0.6 Hz, CH\(_2\)=CCH\(_3\)), 5.97 (d, 1 H, 2.1 Hz, CH\(_2\)=CCH\(_3\)), 5.63 (d, 1 H, 0.9 Hz, H-1), 4.54 (d, 1 H, 2.1 Hz, H-3), 4.50 (d, 1 H, 1.5 Hz, H-6), 4.38 (d, 1 H, 1.5 Hz, H-2), 4.33 (d, 1 H, 0.6 Hz, H-6), 4.30 (d, 1 H, 2.1 Hz, H-5), 4.11 (d, 1 H, 1.5 Hz, H-4), 1.96 (2, 3 H, CH\(_3\)C=CH\(_2\)), 1.48 (s, 3 H, CCH\(_3\)), 1.32 (s, 3 H, CCH\(_3\)); \(^1\)C NMR (CDCl\(_3\)): \(\delta\) 13.3 (CH\(_3\)C=CH\(_2\)), 26.0-26.6 (2 C, C(CH\(_3\))\(_2\)), 66.2 (C-6), 69.1 (C-5), 75.4 (C-3), 79.1 (C-2), 85.0 (C-4), 104.8 (C-1), 111.7 (C(CH\(_3\))\(_2\)), 126.6 (CH\(_2\)=CCH\(_3\)), 135.6 (CH\(_3\)C=CH\(_2\)), 167.9 (C=O), HMQC indicated the correct correlations between protons and carbons.
1.4.12. 3M-IXyl (11)

Prepared from 1: (68.5 %), Rf 0.22 (9:1 CH₂Cl₂-MeOH); ¹H NMR (CDCl₃): δ 6.15 (bs, 1 H, CH₂C=CH₃), 5.96 (d, 1 H, 3.6 Hz, CH₂C=CH₃), 5.66 (d, 1 H, 1.2 Hz, H-1), 5.27 (d, 1 H, 2.7 Hz, H-3), 4.62 (d, 1 H, 3.6 Hz, H-2), 4.44 (d, 1 H, 3.0 Hz, H-4), 3.81 (dd, 1 H, 6.0 Hz, H-5), 3.64 (dd, 1 H, 6.0 Hz, H-5), 1.95 (s, 3 H, CH₃C=CH₂), 1.47 (s, 3 H, CCH₃), 1.30 (s, 3 H, CCH₃).

1.4.13. 5M-IXyl (14)

Prepared from 4: (50 %), Rf 0.62 (9:1 CH₂Cl₂-MeOH); ¹H NMR (CDCl₃): δ 6.09 (d, 1 H, 0.6 Hz, CH₂=CCH₃), 5.86 (d, 1 H, 3.6 Hz, CH₂=CCH₃), 5.59 (d, 1 H, 0.6 Hz, H-1), 5.25 (d, 1 H, 0.6 Hz, H-3), 4.50 (d, 1 H, 3.6 Hz, H-2), 4.30 (d, 2 H, 6.3 Hz, H-5), 4.00 (dd, 1 H, 1.5 Hz, H-4), 2.00 (s, 3 H, CH₃C=CH₂), 1.50 (s, 3 H, CCH₃), 1.36 (s, 3 H, CCH₃); ¹³C NMR (CDCl₃): δ 18.0 (CH₃C=CH₂), 26.6 (2 C, C(CH₃)₂), 67.0 (C-5), 72.3 (C-3), 79.9 (C-4), 83.1 (C-2), 104.8 (C-1), 109.1 (C(CH₃)₂), 126.1 (CH₂=CCH₃), 135.6 (CH₃C=CH₂), 165.7 (C=O).

1.4.14. 3S-DIGlc (15)

Prepared from 1a: (95 %), Rf 0.25 (8:2 CH₂Cl₂-MeOH), mp > 200 °C; ¹H NMR (CD₃OD): δ 5.85 (d, 1 H, 3.6 Hz, H-3), 4.85 (d, 1 H, 3.9 Hz, H-1), 4.66 (d, 1 H, 3.0 Hz, H-2), 4.32 (t, 2 H, 4.8 Hz, H-6), 4.01 (dd, 1 H, 6.3 Hz, H-5), 3.94 (dd, 1 H, 5.7 Hz, H-4), 1.42 (s, 3 H, CCH₃), 1.34 (s, 3 H, CCH₃), 1.27 (s, 6 H, C(CH₃)₂); ¹³C NMR (CD₃OD): δ ; negative ESI-MS: m/z 339.2 (M + H).
1.4.15. 3S-DIAll (16)

Prepared from 2a: (83 %), Rf 0.34 (8:2, CH₂Cl₂:MeOH); ¹H NMR (CD₂OD): δ 5.75 (d, 1 H, 2.4 Hz, H-3), 4.80 (d, 1 H, 3.3 Hz, H-1), 4.61 (d, 1 H, 2.1 Hz, H-2), 4.02 (d, 2 H, 2.4 Hz, H-6), 3.90 (d, 1 H, 4.2 Hz, H-5), 3.58 (dd, 1 H, 2.1 Hz, H-4), 1.42-1.44 (2s, 6 H, C(CH₃)₂), 1.24-1.26 (2s, 6 H, C(CH₃)₂); ¹³C NMR (CD₂OD): δ 25.5 (C(CH₃)), 36.6 (C(CH₃)₃), 44.5 (2C, C(CH₃)₂), 60.2 (C-6), 65.9 (C-5), 70.3 (C-4), 73.8 (C-3), 80.3 (C-2), 93.4 (C-1), 113.6 (2C, C(CH₃)₂); negative ESI-MS: m/z 338.9 (M + H).

1.4.16. 3M-5,6-DS-IGlc (17)

Prepared from 5: (93 %), Rf 0.45 (8:2 CH₂Cl₂-MeOH), mp > 250 °C; ¹H NMR (CD₂OD): δ 6.14 (d, 1 H, 0.9 Hz, CH₂=CCH₃), 5.90 (d, 1 H, 2.4 Hz, CH₂=CCH₃), 5.61 (d, 1 H, 2.1 Hz, H-1), 5.28 (d, 1 H, 1.8 Hz, H-5), 4.77 (d, 1 H, 0.9 Hz, H-2), 4.60 (t, 1 H, 2.1 Hz, H-3), 4.57 (d, 1 H, 1.5 Hz, H-4), 4.54 (d, 1 H, 2.1 Hz, H-6), 4.19 (dd, 1 H, 2.4 Hz, H-6), 2.0 (s, 3 H, CH₃C=CH₂), 1.50 (s, 3 H, CCH₃), 1.30 (s, 3 H, CCH₃); ¹³C NMR (CD₂OD): δ 61.4 (C(CH₃)₂), 135.6 (C=CH₂), 143.9 (C=CH₂), 127.5 (C=CH₂), 126.0 (C=CH₂), 123.6 (C=CH₂), 117.4 (C=CH₂), 114.0 (C=CH₂), 113.0 (C=CH₂), 112.0 (C=CH₂), 111.0 (C=CH₂), 110.0 (C=CH₂), 109.0 (C=CH₂), 108.0 (C=CH₂), 107.0 (C=CH₂), 106.0 (C=CH₂), 105.0 (C=CH₂), 104.0 (C=CH₂), 103.0 (C=CH₂), 102.0 (C=CH₂), 101.0 (C=CH₂), 100.0 (C=CH₂), 99.0 (C=CH₂), 98.0 (C=CH₂), 97.0 (C=CH₂), 96.0 (C=CH₂), 95.0 (C=CH₂), 94.0 (C=CH₂), 93.0 (C=CH₂), 92.0 (C=CH₂), 91.0 (C=CH₂), 90.0 (C=CH₂), 89.0 (C=CH₂), 88.0 (C=CH₂), 87.0 (C=CH₂), 86.0 (C=CH₂), 85.0 (C=CH₂), 84.0 (C=CH₂), 83.0 (C=CH₂), 82.0 (C=CH₂), 81.0 (C=CH₂), 80.0 (C=CH₂), 79.0 (C=CH₂), 78.0 (C=CH₂), 77.0 (C=CH₂), 76.0 (C=CH₂), 75.0 (C=CH₂), 74.0 (C=CH₂), 73.0 (C=CH₂), 72.0 (C=CH₂), 71.0 (C=CH₂), 70.0 (C=CH₂), 69.0 (C=CH₂), 68.0 (C=CH₂), 67.0 (C=CH₂), 66.0 (C=CH₂), 65.0 (C=CH₂), 64.0 (C=CH₂), 63.0 (C=CH₂), 62.0 (C=CH₂), 61.0 (C=CH₂), 60.0 (C=CH₂), 59.0 (C=CH₂), 58.0 (C=CH₂), 57.0 (C=CH₂), 56.0 (C=CH₂), 55.0 (C=CH₂), 54.0 (C=CH₂), 53.0 (C=CH₂), 52.0 (C=CH₂), 51.0 (C=CH₂), 50.0 (C=CH₂), 49.0 (C=CH₂), 48.0 (C=CH₂), 47.0 (C=CH₂), 46.0 (C=CH₂), 45.0 (C=CH₂), 44.0 (C=CH₂), 43.0 (C=CH₂), 42.0 (C=CH₂), 41.0 (C=CH₂), 40.0 (C=CH₂), 39.0 (C=CH₂), 38.0 (C=CH₂), 37.0 (C=CH₂), 36.0 (C=CH₂), 35.0 (C=CH₂), 34.0 (C=CH₂), 33.0 (C=CH₂), 32.0 (C=CH₂), 31.0 (C=CH₂), 30.0 (C=CH₂), 29.0 (C=CH₂), 28.0 (C=CH₂), 27.0 (C=CH₂), 26.0 (C=CH₂), 25.0 (C=CH₂), 24.0 (C=CH₂), 23.0 (C=CH₂), 22.0 (C=CH₂), 21.0 (C=CH₂), 20.0 (C=CH₂), 19.0 (C=CH₂), 18.0 (C=CH₂), 17.0 (C=CH₂), 16.0 (C=CH₂), 15.0 (C=CH₂), 14.0 (C=CH₂), 13.0 (C=CH₂), 12.0 (C=CH₂), 11.0 (C=CH₂), 10.0 (C=CH₂), 9.0 (C=CH₂), 8.0 (C=CH₂), 7.0 (C=CH₂), 6.0 (C=CH₂), 5.0 (C=CH₂), 4.0 (C=CH₂), 3.0 (C=CH₂), 2.0 (C=CH₂), 1.0 (C=CH₂), 0.0 (C=CH₂).

1.4.17. 3M-5,6-DS-DIAli (18)

Prepared from 6: (67 %), Rf 0.47 (8:2, CH₂Cl₂:MeOH); ¹H NMR (CD₂OD): δ 6.02 (d, 1 H, 4.5 Hz, CH₂=CCH₃), 5.82 (d, 1 H, 2.1 Hz, CH₂=CCH₃), 5.61 (d, 1 H, 2.7 Hz, H-1), 4.85 (d, 1 H, 1.8 Hz, H-3), 4.82 (d, 1 H, 2.1 Hz, H-2), 4.15 (ddd, 1 H, 3.9 Hz, H-6), 4.03 (d, 1 H, 3.0 Hz, H-6), 3.68 (d, 1 H, 3.0 Hz, H-5), 3.55 (dd, 1 H, 2.4 Hz, H-4), 1.94 (s, 3 H, CH₃C=CH₂), 1.36 (s, 3 H, CCH₃), 1.18 (s, 3 H, CCH₃); ¹³C NMR (CD₂OD):
δ 17.0 (CH₃C=CH₂), 25.3-25.6 (2C, C(CH₃)₂), 68.0 (C-6), 71.6 (C-4), 71.7 (C-3), 77.2 (C-2), 78.7 (C-5), 104.1 (C-1), 113.7 (C(CH₃)₂), 128.0 (CH₂=CHCH₃), 134.7 (CH₂=CHCH₃), 167.9 (C=O); negative ESI-MS: m/z 469.2 (M + Na), 450.2 (M + 2H), 367.2 (M – C(CH₃)₂).

1.4.18. 6M-3,4-DS-IGal (19)

Prepared from 7: (1 %), Rf 0.52 (8:2, CH₂Cl₂:MeOH); ¹H NMR (CD₃OD): δ ; negative ESI-MS: m/z 450.3 (M + 2H), 367.3 (M – SO₃⁻), 339.3 (M – CH₂=CHCH₃ – SO₃⁻).
The amount of product was not sufficient for NMR studies. In the future, this procedure should be optimized.

1.4.19. 3M-5S-IXyl (20)

Prepared from 11: (58 %), Rf 0.60 (8:2 CH₂Cl₂:MeOH); ¹H NMR (CD₃OD): δ 6.16 (d, 1 H, 0.9 Hz, CH₂C=CH₂), 5.94 (d, 1 H, 4.5 Hz, CH₂C=CH₂), 5.64 (d, 1 H, 0.9 Hz, H-1), 4.78 (d, 1 H, 1.5 Hz, H-2), 4.48 (d, 1 H, 2.4 Hz, H-3), 4.39 (dd, 1 H, 3.9 Hz, H-4), 4.33 (dd, 1 H, 3.6 Hz, H-5), 4.28 (dd, 1 H, 6.3 Hz, H-5), 1.97 (s, 3 H, CH₃C=CH₂), 1.43 (s, 3 H, CCH₃), 1.27 (s, 3 H, CCH₃); negative ESI-MS: m/z 336.9 (M + H).

1.4.20. 5M-3S-IXyl (21)

(63 %), Rf 0.35 (8:2 CH₂Cl₂: MeOH); ¹H NMR (CD₃OD): δ 6.12 (bs, 1 H, CH₂=CCH₃), 5.69 (d, 1 H, 1.5 Hz, CH₂=CCH₃), 5.21 (d, 1 H, 3.0 Hz, H-3), 4.56 (d, 1 H, 3.9 Hz, H-1), 4.31 (d, 1 H, 4.8 Hz, H-4), 4.22 (d, 1 H, 3.0 Hz, H-5), 4.09 (dd, 1 H, 8.4 Hz, H-2), 3.97 (dd, 1 H, 4.8 Hz, H-5), 1.93 (s, 3H, CH₃C=CH₂), 1.48 (s, 3 H, CCH₃), 1.38 (s,
$^3$H, CCH$_3$): $^{13}$C NMR (CD$_3$OD): $\delta$ 17.5 (CH$_2$=CCH$_3$), 24.8-25.4 (2C, C(\text{CH}_3)$_2$), 66.3 (C-5), 73.7 (C-4), 81.1 (C-3), 85.1 (C-2), 104.9 (C-1), 111.7 (C(CH$_3$)$_2$), 125.8 (CH$_2$=CCH$_3$), 135.8 (CH$_2$=CCH$_3$), 165.8 (C=O); negative ESI-MS: m/z 338.9 (M + H), 299.0 (M - C$_3$H$_4$).

1.4.21.3M-6S-IGlc (22)

(45 %), Rf 0.43 (8:2 CH$_2$Cl$_2$: MeOH); $^1$H NMR (CD$_3$OD): $\delta$ 6.10 (bs, 1 H, CH$_2$=CCH$_3$), 5.87 (d, 1 H, 3.9 Hz, H-1), 5.63 (d, 1 H, 11.4 Hz, CH$_2$=CCH$_3$), 5.24 (d, 1 H, 2.7 Hz, H-3), 4.52 (d, 1 H, 3.9 Hz, H-6), 4.45 (d, 1 H, 3.3 Hz, H-2), 4.28 (d, 1 H, 8.1 Hz, H-6), 3.98 (d, 1 H, 8.1 Hz, H-5), 3.28 (d, 1 H, 1.5 Hz, H-4), 1.91 (s, 3H, CH$_3$C=CH$_2$), 1.46 (s, 3 H, CCH$_3$), 1.27 (s, 3 H, CCH$_3$): $^{13}$C NMR (CD$_3$OD): $\delta$ 16.9 (CH$_2$=CCH$_3$), 24.9-25.4 (2C, C(CH$_3$)$_2$), 63.6 (C-6), 68.4 (C-3), 75.8 (C-4), 78.1 (C-2), 83.0 (C-5), 105.1 (C-1), 111.7 (C(CH$_3$)$_2$), 125.6 (CH$_2$=CCH$_3$), 135.9 (CH$_2$=CCH$_3$), 166.1 (C=O); negative ESI-MS: m/z 367.1 (M + H), 299.0 (M - C$_3$H$_4$).

1.4.22. 6M-3,5-DS-IGlc (26)

(30 %), Rf 0.25 (8:2, CH$_2$Cl$_2$:MeOH); $^1$H NMR (CD$_3$OD): $\delta$ 5.99 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.84 (d, 1 H, 2.7 Hz, CH$_2$=CCH$_3$), 5.56 (d, 1 H, 3.9 Hz, H-1), 4.54 (d, 1 H, 3.6 Hz, H-3), 4.27 (dd, 1 H, 10.5 Hz, H-2), 4.15 (d, 1 H, 8.7 Hz, H-6), 4.11 (d, 1 H, 3.9 Hz, H-5), 4.01 (d, 1 H, 7.5 Hz, H-6), 3.60 (bs, 1 H, H-4); negative ESI-MS: m/z 468.9 (M + Na), 367.0 (M – SO$_3$). The carbon NMR studies will be accomplished in the future.
1.4.23. 3Am-DIGlc (24)

This compound was synthesized by one pot oxidation and reductive amination of hydroxyl functionality at position C3. (19.6 %); mp 55-58°C; $^1$H NMR (CDCl$_3$): δ 5.95 (d, 1 H, 3.9 Hz, H-1), 4.56 (d, 1 H, 3.9 Hz, H-2), 4.36 (d, 2 H, 1.2 Hz, H-4, H-5), 4.19 (dd, 1 H, 6.3 Hz, H-6), 4.15 (dd, 1 H, 0.6 Hz, H-6), 4.02 (d, 1 H, 0.9 Hz, H-3), 1.55 (s, 3 H, CCH$_3$), 1.44 (s, 3 H, CCH$_3$), 1.36 (s, 3 H, CCH$_3$), 1.32 (s, 3 H, CCH$_3$); positive ESI-MS: m/z 284.0 (M + Na).

1.4.24. 3AmS-DIGlc (27)

$^1$H NMR (CDCl$_3$): δ 5.86 (d, 1 H, 2.4 Hz, H-1), 4.47 (d, 1 H, 2.1 Hz, H-2), 4.31 (dd, 1 H, 4.2 Hz, H-4), 4.23 (d, 1 H, 1.5 Hz, H-5), 4.07 (dd, 2 H, 3.9 Hz, H-6), 3.96 (dd, 1 H, 3.6 Hz, H-3), 1.49 (s, 3 H, CCH$_3$), 1.39 (s, 3 H, CCH$_3$), 1.33 (s, 3 H, CCH$_3$), 1.30 (s, 3 H, CCH$_3$); $^{13}$C NMR (CD$_3$OD): δ 24.9-25.4 (4C, 2 sets of C(CH$_3$)$_2$), 65.8 (C6), 74.2 (C5), 75.1 (C3), 80.7 (C4), 84.3 (C2), 105.3 (C1), 113.0 (C(CH$_3$)$_2$), 118.3 (C(CH$_3$)$_2$); negative ESI-MS: m/z 339.2 (M + H).

1.4.25. 3M-5S-IGlc (25)

(5%), Rf 0.32 (8:2, CH$_2$Cl$_2$: MeOH); $^1$H NMR (CD$_3$OD): δ 5.97 (d, 1 H, 0.9 Hz CH$_2$=CCH$_3$), 5.86 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.54 (bs, 1 H, H-1), 4.73 (d, 1 H, 3.9 Hz, H-3), 4.28 (2d, 2 H, 3.3 Hz, H-2), 4.14 (d, 1 H, 3.0 Hz, H-5), 4.13 (d, 1 H, 2.7 Hz, H-4), 1.88 (s, 3 H, CH$_3$C=CH$_2$), 1.34 (s, 3 H, CCH$_3$), 1.15 (s, 3 H, CCH$_3$); negative ESI-MS: m/z 367.0 (M +H).
1.4.26. 3M-5Ald-IGlc (5a)

Prepared from (5): 67 %, Rf 0.64, $^1$H NMR (CDCl$_3$): $\delta$ 9.63 (s, 1 H, H=O), 6.10 (d, 1 H, 0.9 Hz, CH$_2$=CCH$_3$), 6.07 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.61 (bs, 1 H, H-1), 5.52 (d, 1 H, 3.6 Hz, H-3), 5.29 (d, 1 H, 3.0 Hz, H-4), 4.61 (d, 1 H, 3.3 Hz, H-2), 1.90 (s, 3 H, CH$_3$C=CH$_2$), 1.48 (s, 3 H, CCH$_3$), 1.30 (s, 3 H, CCH$_3$).

1.4.27. 3Ac-5Ald-IXyl (9a)

Prepared from (9): 67 %, Rf 0.72, $^1$H NMR (CDCl$_3$): $\delta$ 9.62 (s, 1 H, H=O), 5.96 (d, 1 H, 3.6 Hz, H-1), 5.28 (d, 1 H, 1.5 Hz, H-3), 5.24 (d, 1 H, 2.7 Hz, H-2), 4.52 (d, 1 H, 3.9 Hz, H-4), 2.01 (s, 3 H, CH$_3$C=O), 1.48 (s, 3 H, CCH$_3$), 1.28 (s, 3 H, CCH$_3$).

1.4.28. 3Ac-IXyl (12)

Prepared from (9a): 52 %, Rf 0.48, $^1$H NMR (CDCl$_3$): $\delta$ 5.87 (d, 1 H, 3.6 Hz, H-1), 4.45 (d, 1 H, 3.9 Hz, H-3), 4.26 (d, 1 H, 1.5 Hz, H-5), 4.17 (d, 1 H, 2.5 Hz, H-2), 4.09 (bs, 2 H, H-4, H-5), 2.03 (s, 3 H, CH$_3$C=O), 1.42 (s, 3 H, CCH$_3$), 1.27 (s, 3 H, CCH$_3$).

1.4.29. IXyl (13)

Prepared from (12): 85%, Rf 0.23, $^1$H NMR (CDCl$_3$): $\delta$ 5.87 (d, 1 H, 3.6 Hz, H-1), 4.45 (d, 1 H, 3.9 Hz, H-3), 4.26 (d, 1 H, 1.5 Hz, H-5), 4.17 (d, 1 H, 2.5 Hz, H-2), 4.09 (bs, 2 H, H-4), 1.42 (s, 3 H, CCH$_3$), 1.27 (s, 3 H, CCH$_3$).
1.4.30. 3M-6Trt-IXyl (5b)

Prepared from (5): 72%, Rf 0.83, \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.43 (d, 6 H, 2.1 Hz, o-H-C\(_6\)H\(_3\)), 7.23-7.41 (m, 9 H, m- & p-H-C\(_6\)H\(_3\)), 6.16 (d, 1 H, 0.9 Hz, CH\(_2\)=CCH\(_3\)), 5.94 (d, 1 H, 3.5 Hz, CH\(_2\)=CCH\(_3\)), 5.66 (d, 1 H, 3.8 Hz, H-1), 5.31 (d, 1 H, 3.0 Hz, H-2), 4.80 (d, 1 H, 1.2 Hz, H-3), 4.23 (d, 1 H, 0.9 Hz, H-4), 3.72 (d, 1 H, 2.1 Hz, H-5), 3.04 (d, 2 H, 1.8 Hz, H-6), 1.92 (s, 3 H, CH\(_3\)C=CH\(_2\)), 1.50 (s, 3 H, CCH\(_3\)), 1.31 (s, 3 H, CCH\(_3\)).

1.4.31. 3M-6Trt-5MOM-IXyl (5c)

Prepared from (5b): 37 %, Rf 0.56, \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.43 (d, 6 H, 2.1 Hz, o-H-C\(_6\)H\(_3\)), 7.23-7.41 (m, 9 H, m- & p-H-C\(_6\)H\(_3\)), 6.16 (d, 1 H, 0.9 Hz, CH\(_2\)=CCH\(_3\)), 5.94 (d, 1 H, 3.5 Hz, CH\(_2\)=CCH\(_3\)), 5.66 (d, 1 H, 3.8 Hz, H-1), 5.31 (d, 1 H, 3.0 Hz, H-2), 4.68 (d, 2 H, OCH\(_2\)OCH\(_3\)), 4.56 (d, 1 H, 1.2 Hz, H-3), 4.23 (d, 1 H, 0.9 Hz, H-4), 3.48 (d, 1 H, 2.1 Hz, H-5), 3.39 (d, 1 H, 1.8 Hz, H\(_6\)-6), 3.09 (d, 1 H, 1.8 Hz, H\(_6\)-6), 2.86 (s, 3 H, OCH\(_2\)OCH\(_3\)), 1.92 (s, 3 H, CH\(_3\)C=CH\(_2\)), 1.50 (s, 3 H, CCH\(_3\)), 1.31 (s, 3 H, CCH\(_3\)).

1.4.32. 3M-5MOM-IXyl (5d)

Prepared from (5c): 7 %, Rf 0.24, \(^1\)H NMR (CD\(_3\)OD): \(\delta\) 6.16 (d, 1 H, 0.9 Hz, CH\(_2\)=CCH\(_3\)), 5.94 (d, 1 H, 3.5 Hz, CH\(_2\)=CCH\(_3\)), 5.66 (d, 1 H, 3.8 Hz, H-1), 5.31 (d, 1 H, 3.0 Hz, H-2), 4.68 (d, 2 H, OCH\(_2\)OCH\(_3\)), 4.56 (d, 1 H, 1.2 Hz, H-3), 4.23 (d, 1 H, 0.9 Hz, H-4), 3.37 (d, 1 H, 2.1 Hz, H-5), 3.25 (d, 1 H, 1.8 Hz, H\(_6\)-6), 3.09 (d, 1 H, 1.8 Hz, H\(_6\)-6), 2.86 (s, 3 H, OCH\(_2\)OCH\(_3\)), 1.92 (s, 3 H, CH\(_3\)C=CH\(_2\)), 1.50 (s, 3 H, CCH\(_3\)), 1.31 (s, 3 H, CCH\(_3\)).
1.4.33. 3M-6S-6MOM-IXyl (5e)

Prepared from (5d): 72 %, Rf 0.15, $^1$H NMR (CD$_3$OD): $\delta$ 6.16 (d, 1 H, 0.9 Hz, CH$_2$=CCH$_3$), 5.94 (d, 1 H, 3.5 Hz, CH$_2$=CCH$_3$), 5.66 (d, 1 H, 3.8 Hz, H-1), 5.31 (d, 1 H, 3.0 Hz, H-2), 4.68 (d, 2 H, OCH$_2$OCH$_3$), 4.56 (d, 1 H, 1.2 Hz, H-3), 4.23 (d, 1 H, 0.9 Hz, H-4), 3.28 (d, 1 H, 2.1 Hz, H-5), 3.16 (d, 2 H, 1.8 Hz, H-6), 2.86 (s, 3 H, OCH$_2$OCH$_3$), 1.92 (s, 3 H, CH$_3$C=CH$_2$), 1.50 (s, 3 H, CCH$_3$), 1.31 (s, 3 H, CCH$_3$).

1.4.34. 3M-6TBDMS-IXyl (5f)

Prepared from (5): 98 %, Rf 0.51, $^1$H NMR (CDCl$_3$): $\delta$ 6.12 (bs, 1 H, CH$_2$=CCH$_3$), 5.82 (d, 1 H, 6.0 Hz, CH$_2$=CCH$_3$), 5.60 (d, 1 H, 5.7 Hz, H-1), 5.14 (d, 1 H, 2.7 Hz, H-2), 4.5 (d, 1 H, 3.6 Hz, H-4), 4.23 (d, 1 H, 2.7 Hz, H$_a$-6), 4.06 (d, 1 H, 2.7 Hz, H$_b$-6), 3.79 (d, 1 H, 3.0 Hz, H-5), 3.62 (d, 1 H, 6.3 Hz, H-3), 1.90 (s, 3 H, CH$_3$C=CH$_2$), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$), 0.82 (s, 9 H, C (CH$_3$)$_3$), 0.11 (s, 6 H, Si (CH$_3$)$_3$).

1.4.35. 3M-6TBDMS-5S-IXyl (5g)

Prepared from (5f): 37 %, Rf 0.28, $^1$H NMR (CD$_3$OD): $\delta$ 6.12 (bs, 1 H, CH$_2$=CCH$_3$), 5.82 (d, 1 H, 6.0 Hz, CH$_2$=CCH$_3$), 5.60 (d, 1 H, 5.7 Hz, H-1), 5.13 (d, 1 H, 2.7 Hz, H-2), 4.50 (d, 1 H, 3.6 Hz, H-4), 4.23 (d, 1 H, 2.7 Hz, H$_a$-6), 4.06 (d, 1 H, 2.7 Hz, H$_b$-6), 3.35 (d, 1 H, 3.0 Hz, H-5), 3.14 (d, 1 H, 6.3 Hz, H-3), 1.90 (s, 3 H, CH$_3$C=CH$_2$), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$), 0.82 (s, 9 H, C (CH$_3$)$_3$), 0.11 (s, 6 H, Si (CH$_3$)$_3$).
1.4.36. 3MEM-DIGlc (1b)

Prepared from (1a): 47 %, Rf 0.75, $^1$H NMR (CDCl$_3$): $\delta$ 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.53 (d, 1 H, 3.6 Hz, H-2), 4.19 (d, 1 H, 3.0 Hz, H-4), 4.04 (dd, 1 H, 3.0 Hz, H-5), 3.98 (d, 1 H, 1.9 Hz, H$_a$-6), 3.68 (d, 1 H, 4.5 Hz, H$_b$-6), 3.51 (d, 1 H, 3.0 Hz, H-3), 3.40 (dd, 4 H, 2.1 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.33 (s, 3 H, CH$_2$O (CH$_2$)$_2$OCH$_3$), 1.43 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$), 1.24 (s, 6 H, CCH$_3$).

1.4.37. 3MEM-IGlc (1c)

Prepared from (1b): 95 %, Rf 0.46, $^1$H NMR (CD$_3$OD): $\delta$ 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.53 (d, 1 H, 3.6 Hz, H-2), 4.19 (d, 1 H, 3.0 Hz, H-4), 4.04 (dd, 1 H, 3.0 Hz, H-5), 3.98 (d, 1 H, 1.9 Hz, H$_a$-6), 3.68 (d, 1 H, 4.5 Hz, H$_b$-6), 3.51 (d, 1 H, 3.0 Hz, H-3), 3.40 (dd, 4 H, 2.1 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.33 (s, 3 H, CH$_2$O (CH$_2$)$_2$OCH$_3$), 1.43 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$).

1.4.38. 3MEM-6TBDMS-IGlc (1d)

Prepared from (1c): 81 %, Rf 0.31, $^1$H NMR (CDCl$_3$): $\delta$ 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.63 (d, 1 H, 3.6 Hz, H-2), 4.29 (d, 1 H, 3.0 Hz, H-4), 4.09 (d, 2 H, 3.0 Hz, H-6), 3.85 (d, 1 H, 3.0 Hz, H-5), 3.79 (d, 14H, 1.8 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.71 (d, 1 H, 2.1 Hz, H-3), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$), 0.82 (s, 9 H, C (CH$_3$)$_3$), 0.11 (s, 6 H, Si (CH$_3$)$_3$).
1.4.39. 3MEM-6TBDMS-5Bn-IGlc (1e)

Prepared from (1d): 80 %, Rf 0.87, $^1$H NMR (CDCl$_3$): $\delta$ 7.43 (m, 5 H, C$_6$H$_5$), 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.63 (d, 1 H, 3.6 Hz, H-2), 4.52 (s, 2 H, CH$_2$C$_6$H$_5$), 4.29 (d, 1 H, 3.0 Hz, H-4), 4.09 (d, 2 H, 3.0 Hz, H-6), 3.85 (d, 1 H, 3.0 Hz, H-5), 3.79 (d, 14H, 1.8 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.71 (d, 1 H, 2.1 Hz, H-3), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$), 0.82 (s, 9 H, C (CH$_3$)$_3$), 0.11 (s, 6 H, Si (CH$_3$)$_3$).

1.4.40. 3MEM-5Bn-IGlc (1f)

Prepared from (1e): 50 %, Rf 0.71, $^1$H NMR (CDCl$_3$): $\delta$ 7.43 (m, 5 H, C$_6$H$_5$), 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.63 (d, 1 H, 3.6 Hz, H-2), 4.52 (s, 2 H, CH$_2$C$_6$H$_5$), 4.29 (d, 1 H, 3.0 Hz, H-4), 4.09 (d, 2 H, 3.0 Hz, H-6), 3.85 (d, 1 H, 3.0 Hz, H-5), 3.79 (d, 14H, 1.8 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.71 (d, 1 H, 2.1 Hz, H-3), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$).

1.4.41. 3MEM-6M-5Bn-IGlc (1h)

Prepared from (1f): 63 %, Rf 0.79, $^1$H NMR (CDCl$_3$): $\delta$ 7.43 (m, 5 H, C$_6$H$_5$), 6.62 (d, 1 H, 0.9 Hz, CH$_2$=CCH$_3$), 6.18 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.63 (d, 1 H, 3.6 Hz, H-2), 4.52 (s, 2 H, CH$_2$C$_6$H$_5$), 4.29 (d, 1 H, 3.0 Hz, H-4), 4.09 (d, 2 H, 3.0 Hz, H-6), 3.85 (d, 1 H, 3.0 Hz, H-5), 3.79 (d, 14H, 1.8 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.71 (d, 1 H, 2.1 Hz, H-3), 1.95 (s, 3 H, CH$_2$=CCH$_3$), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$).
1.4.42. 3MEM-6M-IGlc (1i)

Prepared from (1h): Prepared from (1f): <1 %, Rf 0.56, $^1$H NMR (CDCl$_3$): $\delta$ 6.62 (d, 1 H, 0.9 Hz, CH$_2$=CCH$_3$), 6.18 (d, 1 H, 3.6 Hz, CH$_2$=CCH$_3$), 5.81 (d, 2 H, 4.2 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 4.76 (d, 1 H, 2.7 Hz, H-1), 4.63 (d, 1 H, 3.6 Hz, H-2), 4.29 (d, 1 H, 3.0 Hz, H-4), 4.09 (d, 2 H, 3.0 Hz, H-6), 3.85 (d, 1 H, 3.0 Hz, H-5), 3.79 (d, 14H, 1.8 Hz, CH$_2$O (CH$_2$)$_2$OCH$_3$), 3.71 (d, 1 H, 2.1 Hz, H-3), 1.95 (s, 3 H, CH$_2$=CCH$_3$), 1.49 (s, 3 H, CCH$_3$), 1.25 (s, 3 H, CCH$_3$).

1.4.43. Deprotection of 1,2-\textit{O}-isopropylidene group

Analysis of $^1$H NMR indicated that reaction of monomer (1) in HCl (5 and 10 %) and HCOOH (50 and 96 %) at RT for any period of time did not remove isopropylidene functionality from C1-C2. However, when the temperature was raised to 60°C, the NMR peaks for the isopropylidene group on C1-C2 at 1.20-1.40 ppm disappeared when HCl (5 or 10 %) was used. Formic acid was also able to remove a reasonable percentage of isopropylidene group but the reaction mixture contained unknown side products in solution with some remaining starting material. Figure 1.20 demonstrates the disappearance of isopropylidene groups. Unfortunately, the same procedure followed in order to remove 1,2-isopropylidene in final sulfated carbohydrate methacrylate derivatives was unsuccessful since sulfate groups are very sensitive to any high acidic or basic conditions at high temperature. Therefore, the sulfated monomers will not be deprotected at this stage. Details of $^1$H NMR is shown in Figure 1.20.
Peaks of methacrylate group at 5.6 & 5.9 ppm are missing.
Peaks of isopropylidene at 1.2-1.4 ppm are missing.

Figure 1.20. Deprotection of 1,2-\textit{O}-isopropylidene group using 5% HCl at 60°C
1.4.44. Temperature control experiment to separate α and β anomers

In this experiment, α and β anomers were characterized using temperature changes to shift NMR peak positions. It was indicated that the peak representing D$_2$O slightly shifts as the temperature of the sample increases from 22ºC to 60ºC such that the peak representing β-hydrogen appeared at 50ºC and 60ºC. The chemical shifts (σ) as well as coupling constant (J) values were in agreement with the literature values for typical α and β hydrogens in carbohydrates as shown in Figure 1.21.

\[
\begin{align*}
\text{H}_\alpha &= 5.3 \text{ ppm} \\
\text{H}_\beta &= 4.7 \text{ ppm} \\
J_{\text{H}_\alpha} &= 3 \text{ Hz} \\
J_{\text{H}_\beta} &= 8 \text{ Hz}
\end{align*}
\]

Figure 1.21. Difference between H$_\alpha$ and H$_\beta$ chemical shift (σ) and coupling constant (J) in 2,3-disulfated glucosamine
Figure 1.22 shows a $^1$H NMR of an authentic sample of 2,3-disulfated glucosamine at RT. The anomeric proton, $H_\beta$, is hidden under D$_2$O peak of the NMR solvent. However, after heating the NMR sample tube in the instrument, the peak representing D$_2$O was shifted toward left such that $H_\beta$ appear when temperature reached 50 °C in NMR tube. Figure 1.23 (a & b) shows the presence of $H_\beta$ at the temperatures of 50 °C (a) and 60 °C (b).
Figure 1.23. Chemical shift changes of D$_2$O in 2,3-disulfated glucosamine with temperature and appearance of H$_\beta$ at 50 °C (a) and 60 °C (b)
1.5. Discussion

Desired target monomers were synthesized following the synthetic routes shown in schemes above. These monomers were characterized for their purity using $^1$H NMR, $^{13}$C NMR, and MS. Synthesis of monomer (22), (25), and (26) were attempted using selective protection and deprotection of primary and secondary hydroxyl groups in order to provide free hydroxyl functionality in a specific position for further sulfation. The synthetic routes for these compounds are shown in section 1.3.

Even though a good strategy often employed for obtaining a specifically sulfated product is to use blocking groups in which all potentially active hydroxyl sites but the one to be sulfated is protected, later removal blocking groups may result in desulfation. It is important to know what kinds of conditions normally cause desulfation. It is known that sulfate esters of sugars are unstable in both acids and bases\textsuperscript{5}. Since hydrolysis of sugar sulfates in high temperatures of acids and bases could occur, it is important to monitor sulfation conditions very carefully. Figures 1.15 and 1.20 explain how removing some protecting groups after sulfation could result in desulfation of desired products (22) and (25).

Therefore, a second strategy was employed to obtain compound (22). This strategy is based on the order of sulfation preference for sugars in varied solutions. It is known that the order of sulfation for carbohydrates is free amino, primary hydroxyl, then secondary hydroxyl, except in strongly acidic solvents, in which case primary hydroxyl sulfation is favored over N-sulfation\textsuperscript{5}. Therefore, compound (22) was prepared following the procedure shown in scheme 2 above.
Monomer (26) was also prepared using two strategies. The first involves the use of protecting groups for all hydroxyl functionalities but the one to be methacrylated. The synthetic steps for this plan are shown in scheme 4 above. This plan was faced with challenges of finding the suitable protecting group such that deblocking step would not cause demethacrylation. Therefore, a second strategy was selected in which the methacrylation preference of primary hydroxyl group over secondary one was used in order to methacrylate the desired position in the carbohydrate of choice.

As it was described in section 1.3.4.9.1, synthesis of compound (14) through route I using starting material (3a) was unsuccessful due to the mixture of products obtained in the initial step of synthetic route (cleavage of vicinal diol by NaIO₄). Figure 1.24 describes the mechanism of diol cleavage using NaIO₄ toward formation of desired intermediate (4a).

![Figure 1.24. Mechanism of cleavage of vicinal diol in (3a) by NaIO₄](image)

However, the analysis of intermediate (4a) by ¹H NMR spectroscopy indicated that there is a mixture of products in the reaction flask. After purification of the crude product by column chromatography, a considerably small yield of the desired product was obtained. Figure 1.25 demonstrates two possible mechanisms for formation of side products in this reaction.
Figure 1.25. Possible mechanisms for formation of side products in cleavage of diol in (3a) using NaIO₄: (a) NaIO₄ attacks the OH group on C₃ and isopropylidene, (b) NaIO₄ attacks OH group on C₃ and aldehyde functionality on C₅.

Therefore, a second synthetic route was followed in order to prepare compound (14) such that hydroxyl group on C₃ was protected with some functionality such as acetyl group. The yield of formation of intermediate (4a) in the second route was increased considerably.

Finally, an attempt was made toward deprotection of 1,2-isopropylidene functionality from final sulfated carbohydrate monomers following the procedure described in section 1.4.43 in addition to a number of other methodologies such as the use of K₁₀ Clay with microwave heating. It was found that the sulfate functionality is sensitive in acid and base media such that the deprotected monomer lacked a sulfate group. Therefore, the target monomers were polymerized without further deprotection. Future studies will focus on deprotection of 1,2-O-isopropylidene group using neutral reagents such as ZnBr₂ in dichloromethane³⁹, lithium tetrafluoroborate in acetonitrile⁴⁰, or
NaBrO₃/Na₂S₂O₄ under two phase conditions (ethyl acetate/water)⁴¹. Enzymatic reactions to remove the isopropylidene functionality could also be investigated.
1.6. References


58

38. Wallis, R. R. *Ph.D. Thesis*, University of Minnesota, Minneapolis, MN, **1996**


1.7. Appendix I
Chapter 2

Sulfated Carbohydrate Derivatives of Methacrylate Polymers:
Synthesis, Characterization, and Biological studies
2.1. Introduction

Bacterial translocation across the intestinal wall has been demonstrated to be a major cause of infection in hospitalized patients. The majority of the conditions associated with increased intestinal permeability are hemorrhagic shock, burn wounds, and intestinal obstruction\(^1\). *Staphylococcus aureus* is a major human pathogen causing significant morbidity and mortality in both community- and hospital- acquired infections\(^2\). *Staphylococcus aureus* can colonize the intestine in normal and antibiotic-treated individuals. It causes a diverse group of infections ranging from relatively minor wound infections to more serious and life-threatening diseases such as pneumonia, septic arthritis, and osteomyelitis\(^3-8\). Due to the appearance of antibiotic-resistant strains, there is currently great interest in understanding the mechanism(s) of action of this pathogen at the molecular level and possible defense elements that may either provide protection from, or limits on, the infection.

Since *Staphylococcus aureus* can be internalized by enterocytes and is known to bind heparan sulfate (HS), heparan sulfate proteoglycans (HSPGs) could mediate *S. aureus* interactions with intestinal epithelial cells\(^9\). HSPGs are glycoproteins with heparan sulfate (HS) containing glycosaminoglycans (GAGs) chains covalently attached to a core protein. These HS moieties consist of alternating uronic acids and D-glucosamine residues. The disaccharide sequence and level of sulfation of the HS chains confer the unique binding characteristics of a given HSPG\(^10\). One of the goals of this research is to determine whether synthetic sulfated polymers can act as biomimics of natural bioactive polysaccharides (e.g. heparan sulfate). This chapter describes the synthesis and characterization of polymers containing sulfated carbohydrate. It should be noted that the
position of sulfate as well as the point of attachment of methacrylate is varied among these derivatives in order to facilitate structure-activity comparison. We also report biological studies aimed at better understanding of the interaction of sulfated polymers with bacteria such as *Staphylococcus aureus* and possible effects upon intestinal transport. The structures of the target sulfated homopolymers are shown in the table below.

![Structures of synthesized sulfated carbohydrate polymers](image)

**Table 2.1. Structures of synthesized sulfated carbohydrate polymers**
2.2. Background

2.2.1. *Staphylococcus aureus*

*Staphylococcus* is a type of Gram-positive bacteria that is round and forms grape-like clusters. Although more than 20 species of *Staphylococcus* are known, the majority are harmless and live on the skin and mucous membranes of humans and other organisms\(^\text{11}\). Some species, however, can cause disease in both humans and animals. For instance, Staphylococcal toxins are a common cause of food poisoning. *Staphylococcus aureus*, or “golden staph” from the Latin, is one of the pathogenic species of Staphylococci that can cause wound infections\(^\text{12}\). It is known as one of the four most common causes of post-surgical wound infections and is thought to delay wound healing\(^\text{13}\). These infections can be spread by making contact with pus from an infected wound, and from objects such as towels, sheets, and clothing of the infected person. In infants, *Staphylococcus aureus* can cause a Staphylococcal scalded skin syndrome (SSSS). In patients with cystic fibrosis (CF), *S. aureus* colonizes the airways for extended periods manifested as respiratory infections\(^\text{14-15}\).

2.2.2. Caco-2 and HT-29 enterocytes

Caco-2 and HT-29 cells are human colon epithelial cancer cell lines that mimic characteristic of mature intestinal cells, such as enterocytes or mucus cells. They are known as valuable *in vitro* tools for studies related to intestinal cell function and differentiation. This is due to their ability to express relatively high levels of digestive brush-order enzymes\(^\text{16}\) and to display other structural and functional characteristics of
absorptive villus cells\textsuperscript{17}. They are usually cultured on semipermeable plastic supports that fit into wells of multi-well culture plates. Since intestinal bacteria can be internalized by enterocytes, and bacterial translocation has been associated with increased intestinal permeability\textsuperscript{18}, enterocytes such as Caco-2 and HT-29 have been used as model systems for our studies.

Caco-2 cells express the characteristics of normal small intestinal villus cells\textsuperscript{19}. However, HT-29 cell line does not show any distinguishing characteristics of intestinal cells under standard conditions (in media containing glucose and normal serum). Nevertheless, HT-29 cells exhibit a high degree of differentiation when grown in the absence of glucose, for example when galactose is substituted for glucose as a carbon source. This is the reason we grow the HT-29 cells in the absence of glucose and presence of galactose\textsuperscript{20}.

2.2.3. Sulfated carbohydrate polymers

Naturally occurring polysaccharides have many biological properties including serving as anti-infective agents, chemopreventive agents, anti-oxidants, and enhancers of immunologic response\textsuperscript{21}. Biological activities of a polysaccharide depend mainly on molecular structure, the types and polymerization degree, and the anomeric configuration along chains\textsuperscript{22-23}.

Chemical sulfation of polysaccharides should not only enhance the water solubility but also influence the chain conformation, resulting in alteration of biological activities. Chemical modification of polysaccharides by sulfation can enhance anti-inflammatory, antiviral or antitumor activities\textsuperscript{24-30}. Sulfated polysaccharides are water
soluble polymers, including the well known anticoagulant heparin and related compounds. The degree of sulfation (DS) and molecular weight (Mw) often influence the bioactivities of sulfated polysaccharides\textsuperscript{31-32}.

Sulfate modification provides polysaccharides that actively bind antithrombin III\textsuperscript{33}. Heparan sulfate, a highly sulfated polysaccharide, is present on the surface of mammalian cells known to serve as adhesion receptors for bacteria, viruses, and parasites\textsuperscript{34}. Heparin has also been shown to play a regulatory role in angiogenesis\textsuperscript{34}. It stimulates neovascularization (the growth of new blood vessels) both by its affinity for angiogenic endothelial growth factors, and an ability to potentiate angiogenic steroids\textsuperscript{34}. A goal of this research is to determine whether the target sulfated polymers can serve as mimics of naturally occurring sulfated carbohydrate polymers.

2.2.4. Methacrylate-based polysaccharides

Methacrylate-based polymers have stimulated widespread interest in the field of polymer chemistry because of their excellent physical properties as well as demonstrated biocompatibility. These polymers have had a great impact in the medical field as biomaterials (e.g. in contact lenses and for orthopedic applications.)\textsuperscript{35}.

Polymers of methacryloyl-based sugars are water-soluble compounds of the class poly (vinyl saccharide)s, since the sugar based monomers are coupled via a vinyl polymerization. Products containing a PMMA backbone with pendant carbohydrate groups have been known for their attractive properties for use as biomaterials\textsuperscript{36}. For instance, PMMA, itself, is a common coating of some drug formulations such as that used for delivery of mesylamine (Asacol).
A variety of polymerization methods, including solution, surface, emulsion, and bulk can be used to prepare novel methacrylate based poly(vinyl saccharide)s. The properties of such polymers can be tailored based on the type of polymerization technique such that these polymers will be useful for a wide variety of applications. For instance, solution polymerization methods will allow manipulation of the properties of polymers by selecting the amount and type of monomers used.

2.2.5. Pegylated heparin

Glycosaminoglycans (GAGs) form an important component of connective tissues. Some examples of GAGs are heparin, heparan sulfate, keratan sulfate, and dermatan sulfate. Members of GAGs vary in type of hexosamine, hexose units, and in the geometry of glycosidic linkage. Heparin is the highest negative charge density of any known biological molecule and it’s very highly sulfated. However, heparan sulfate is highly similar in structure to heparin and their disaccharide units are organized into distinct sulfated and non-sulfated domains. The most common disaccharide unit in heparin composed of 2-\(\text{O}\)-sulfated-IA, 6-\(\text{O}\)-sulfated, \(N\)-sulfated glucosamine. The most common disaccharide unit within heparan sulfate is glucuronic acid linked to \(N\)-acetylg glucosamine.

A highly sulfated polysaccharide such as heparan sulfate is known to serve as adhesion receptors for bacteria, viruses, and parasites\(^\text{34}\). Studies have shown that heparan sulfate proteoglycan (HSPG), such as syndecan-1, can mediate some bacterial (such as \textit{Listeria monocytogenes} and \textit{Staphylococcus aureus}) internalization by HT-29 cells that mimic human enterocytes\(^\text{37}\).
Polyethylene glycol (PEG) materials are known for their utility in biomedical applications due to their hydrophilicity, nontoxicity and biodegradability characteristics. They have proven to be attractive agents for therapeutic drug delivery. Recent studies have shown that high molecular weight PEGs may act as a surrogate mucin to protect the intestinal tract from invasion by some groups of bacteria such as *E. coli*\(^{38-40}\). High molecular weight PEGs may limit *E. coli* intestinal overgrowth in the antibiotic treated host and essentially interfere with the interactions of *E. coli* with intestinal epithelium\(^{41-42}\). We sought to determine whether heparan-like compounds such as heparin disaccharides and our sulfated polymers conjugated with high molecular weight PEGs may affect the bacterial internalization in a way that might defeat translocation and infection.
2.3. Experimental Section

2.3.1. Materials

All solvents and reagents were purchased from Sigma-Aldrich and Fluka unless reported otherwise and used without further purifications. All monomers used for polymerization reactions were synthesized in our laboratory by procedures outlined in chapter one. VA044 (a water soluble initiator) was provided from Wako Pure Chemical Industries Ltd. Heparin disaccharides and NH₂-PEG 3000 were provided by Dr. Carol Wells. *Staphylococcus aureus* RN6390 (provided by Dr. Patrick Schlievert, University of Minnesota) is a prototypical virulent strain commonly used to study *S. aureus* interactions with cultured eukaryotic cells, such as human endothelial cells and human pulmonary epithelial cells. Caco-2 and HT-29 enterocytes were obtained from the American Type Culture Collection (ATCC).

2.3.2. Characterization Methods

NMR spectra were recorded on a Varian Inova 300 (H1: 300 MHz, C13: 75 MHz) or 500 MHz (H1: 500 MHz, C13: 125 MHz) spectrometer at ambient temperature in an appropriate deuterated solvent using TMS as the internal standard at ambient temperature. Gel Permeation Chromatography (GPC) was conducted on a Water’s instrument (courtesy of Dr. Wang at University of Minnesota) using a Water’s refractive index detector, Water’s Breeze software for molecular weight determinations of polymers. The system was equipped with a 7.8 x 300 mm SEC column, Hydrogel 250, with sodium nitrate (NaNO₃, 0.1 M) used as the mobile phase.
2.3.3. Polymer synthesis

2.3.3.1. Water soluble radical initiator for polymerization of sulfated monomers

Since the sulfated monomers are water soluble, a water soluble initiator was used for polymerization. Because of instability of sulfated monomers at high temperature, VA044 was used due to its decomposition temperature (10 hour half-life) at 44ºC in water. Figure 2.3 shows the structure of VA044.

\[
\text{2,2'=Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA044)}
\]

Figure 2.1. Structure of water soluble initiator (VA044)

2.3.3.2. General procedure for polymerization of sulfated monomers

Polymerization of sulfated carbohydrate derivatives of methyl methacrylate was accomplished following a published procedure with some modifications. In a typical polymerization, deionized water was sparged under N₂ for 30 min. Each monomer was added such that the concentration of the resulting solution was 0.1 M. VA044 (1%, w/w) dissolved in deionized water was also sparged under N₂ for 30 min and then added to the monomer solution. The resulting solution was stirred under N₂ in sealed flask at 60ºC for 6 h. Upon completion of the reaction, the solution was cooled down to RT and lyophilized. The obtained polymers were analyzed by GPC and ¹H NMR. The synthetic step of one of the polymers is shown in figure 2.4 where monomer is 5,6-disulfonato-3-methacryoyl-1,2-isopropylidene-glucofuranose (17).
2.3.3.3. Deprotection of 1,2-isopropylidene group from sulfated polymers

Due to the instability of sulfate functionality in acid, base media, the protecting group (isopropylidene) could not be deprotected at C1 and C2. Attempts to achieve this deprotection step using a number of procedures (shown below) were all unsuccessful.

Figure 2.3. Deprotection of 1,2-isopropylidene group by acid-base reagents

Figure 2.4. Deprotection of 1,2-isopropylidene group by K_{10} clay
2.3.4. Methods of Biological Studies

The interactions of our sulfated polymers with Staphylococcus aureus was studied in collaboration with Dr. Michelle Henry-Stanley. In a typical experiment, Caco-2 and HT-29 enterocytes were cultivated at 37°C in 9.5% CO2, in the absence of antibiotics, using 24-well plastic dishes seeded at 2x10⁴ cells per well. To obtain mature enterocytes, Caco-2 and HT-29 cells were cultivated 15-18 and 21-24 days respectively. Pure cultures of S. aureus were incubated with enterocytes for 1 h. Enterocytes were then washed with Hanks’s balanced salt solution (HBSS). In some cases, enterocytes were pretreated with calcium-free Krebs Ringers solution for 1 h prior to the addition of S. aureus. Low extracellular calcium reversibly opens enterocyte tight junctions and exposes the lateral enterocyte surface without affecting enterocyte viability. For our experiments, enterocytes were pretreated for 30 min with varying concentrations of our sulfated polymers prior to incubation with S. aureus. Bacterial concentrations were determined by densitometry and confirmed by serial dilution followed by viable plate counts on appropriate agar media. Statistical analyses were performed using StatView 5.0.1. Bacterial numbers were log₁₀ transformed prior to statistical analysis. Experiments were repeated on at least three separate days and on a given day, studies were performed in four different tissue culture wells and the average was considered as one assay value. Fractional data were analyzed by ANOVA plus Fischer’s Post-Hoc with continuity correction. Regression analysis was used to analyze dose response. Statistical significance was P<0.01.
2.3.5. General Procedure for pegylation of heparin disaccharides

The pegylation of heparin disaccharides was accomplished based on a modified procedure published in literature\textsuperscript{46}. Each heparin disaccharide was dissolved in distilled water and treated with 1.2 eq of EDC, 0.3 eq of NHS, and 1.2 eq of NH\textsubscript{2}-PEG 3000 at RT for 18 h. Due to relative small amount of heparin disaccharide available (1 mg) and their sensitivity to acid-base work-up conditions, the obtained mixture was not subjected to any work-up procedure. Upon completion of the reaction, the solution was lyophilized and analyzed by \textsuperscript{1}H NMR spectroscopy. Figure 2.5 shows the synthetic step for this synthesis.

![Diagram showing the synthesis of heparin disaccharides and their pegylation](image)

**Figure 2.5. General synthetic step for preparation of heparin disaccharides (IV-H, II-H, I-S, III-S).**

2.3.6. Interactions of heparin disaccharide PEG-NH\textsubscript{2} with bacteria

Two preliminary experiments have been done to study the effect of pegylated heparin on bacterial internalization of *E. coli* by Caco-2 and HT-29 enterocytes by Dr. Michelle Henry-Stanley and her assistants. Mature HT-29 and Caco-2 enterocytes were preincubated for 1 h with different concentrations of pegylated heparin disaccharides.
(shown in figure 2.5). A large quantity ($10^8$) of *E. coli* was added for 1 h and bacterial internalization was assayed.
2.4. Results

2.4.1. Characterization results for sulfated polymers

Proton NMR was used to determine whether the polymerization reaction has been successfully completed. The disappearance of two doublets representing the vinyl protons at 5.9-6.2 ppm indicates the completion of polymerization reaction.

GPC analysis was conducted to determine the molecular weight of the product sulfated polymers. The system was calibrated using polyethylene glycol standards of molecular weights ranging from $1 \times 10^3$ to $12 \times 10^3$ g/mol. All samples were dissolved in degassed, HPLC grade NaNO$_3$ (0.1 M) at concentrations of 10 mg/ml and filtered using 0.2-µm PTFE filters. The samples were analyzed at a flow rate of 0.5 mL/min, an injection volume of 10 µL, and a column temperature of 25°C. Molecular weight data for the sulfated carbohydrate derivatives of methyl methacrylate is shown in table 2.2. ID number for each polymer represents the monomer from which it has been synthesized from. Details in structures of each polymer are shown in table 2.1.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mn (g/mol)</th>
<th>Mw (g/mol)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P17</td>
<td>6764</td>
<td>6777</td>
<td>1.00</td>
</tr>
<tr>
<td>P18</td>
<td>7200</td>
<td>7436</td>
<td>1.13</td>
</tr>
<tr>
<td>P20</td>
<td>3405</td>
<td>3407</td>
<td>1.00</td>
</tr>
<tr>
<td>P21</td>
<td>4589</td>
<td>4715</td>
<td>1.23</td>
</tr>
<tr>
<td>P22</td>
<td>3452</td>
<td>3502</td>
<td>1.11</td>
</tr>
<tr>
<td>P26</td>
<td>6843</td>
<td>7129</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Table 2.2. Molecular weight determinations for sulfated polymers
2.4.2. Effect of sulfated polymers on the internalization of *Staphylococcus aureus* 6390 by HT-29 and Caco-2 enterocytes

Preliminary experiments were designed to determine whether enterocyte internalization of *S. aureus* could be inhibited by our sulfated polymers. The data, shown in table 2.3., indicated that our sulfated polymer, P17, inhibited *S. aureus* internalization by both HT-29 and Caco-2 enterocytes. This effect was more pronounced mainly after the tight junctions were opened by treatment with calcium-free medium. The internalization of *S. aureus* by both Caco-2 and HT-29 was inhibited in a dose independent fashion. It should be noted that concentrations less than 25 µg/mL produced inconsistent results. Figures 2.6 & 2.7 show the results obtained from triplicate studies.

<table>
<thead>
<tr>
<th><em>S. aureus</em> 6390</th>
<th>Negative control</th>
<th>P17 25 µg/ml</th>
<th>P17 50 µg/ml</th>
<th>P17 100 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29 enterocytes</td>
<td>3.7 ± 0.08</td>
<td>2.6 ± 0.08</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Caco-2 enterocytes</td>
<td>4.5 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>3.8 ± 0.08</td>
<td>3.9 ± 0.09</td>
</tr>
</tbody>
</table>

*Table 2.3. Effect of P17 on internalization of *S. aureus* 6390 by HT-29 and Caco-2*
Figure 2.6. Effect of P17 on internalization of *S. aureus* 6390 by HT-29 enterocytes

Figure 2.7. Effect of P17 on internalization of *S. aureus* 6390 by Caco-2 enterocytes
2.4.3. Effect of heparin disaccharides (HP DS) on internalization of \(S. \text{aureus}\) by HT-29 enterocytes pre-incubated in calcium-free medium

The previous results have indicated that heparin disaccharides (sulfated and non-sulfated) may inhibit enterocyte interactions with \(S. \text{aureus}\) in a statistically significant manner\(^47\).

<table>
<thead>
<tr>
<th>(S. \text{aureus}) 502 A</th>
<th>HP Intact</th>
<th>HP DS I-S</th>
<th>HP DS III-S</th>
<th>HP DS II-H</th>
<th>HP DS IV-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>4.3 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>4.2 ± 0.2</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Heparin supplement</td>
<td>2.0 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>200 fold ↓</td>
<td>63 fold ↓</td>
<td>63 fold ↓</td>
<td>79 fold ↓</td>
<td>50 fold ↓</td>
</tr>
<tr>
<td>(S. \text{aureus}) 6390</td>
<td>HP Intact</td>
<td>HP DS I-S</td>
<td>HP DS III-S</td>
<td>HP DS II-H</td>
<td>HP DS IV-H</td>
</tr>
<tr>
<td>Negative control</td>
<td>4.5 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>4.4 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>Heparin supplement</td>
<td>2.1 ± 0.1</td>
<td>2.7 ± 0.0</td>
<td>3.2 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>250 fold ↓</td>
<td>79 fold ↓</td>
<td>16 fold ↓</td>
<td>25 fold ↓</td>
<td>25 fold ↓</td>
</tr>
</tbody>
</table>

Table 2.4. Internalization of \(S. \text{aureus}\) by HT-29 enterocytes pre-incubated in calcium-free medium ± 25 µg/mL selected heparin disaccharides (HP DS)

The results obtained above indicate that a non-sulfated glycosaminoglycan may be used to inhibit \(S. \text{aureus}\)-enterocyte interactions. This finding may have important clinical implications.

2.4.4. Effect of polyethylene glycol (PEG) on internalization of \(E. \text{coli}\) by Caco-2 and HT-29 enterocytes

The previous studies (Table 2.5) performed by Professor Wells’ research laboratory have shown that high molecular weight polyethylene glycol (HMW PEG)
decreases the internalization of *E. coli* by HT-29 and Caco-2 enterocytes in a dose-dependent manner\textsuperscript{48}.

<table>
<thead>
<tr>
<th></th>
<th>Negative control</th>
<th>PEG 1 %</th>
<th>PEG 2.5 %</th>
<th>PEG 5 %</th>
<th>PEG 10 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29 enterocytes</td>
<td>3.9 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Caco-2 enterocytes</td>
<td>4.5 ± 0.3</td>
<td>4.5 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

Table 2.5. Effect of HMW PEG on internalization of *E. coli* by Caco-2 and HT-29 enterocytes

2.4.5. Effect of pegylated heparin on the internalization of *E. coli* by HT-29 and Caco-2 enterocytes

Preliminary experiments have shown that unlike heparan sulfate (HS) and high molecular weight PEG, pegylated heparin did not have any obvious effect on internalization of *E. coli* by HT-29 and Caco-2 enterocytes.
2.5. Discussion

In this chapter, we have shown that novel sulfated carbohydrate polymers can be synthesized in water using water-soluble initiators such as VA044. The isopropylidene functionality on C1-C2 of these polymers was not removed due to instability of sulfate groups in acid-base media or elevated temperature. The molecular weight of these polymers obtained by GPC is shown to be in the range of 3500-7500 g/mol, usually described as low molecular weight polymers. This characteristic is an important one for their interactions with bacteria such as *S. aureus*. In the biological studies of our sulfated polymers, the inhibition of *S. aureus* internalization by enterocytes (Caco-2 and HT-29) was observed in the presence of poly (5,6-disulfonato-3-methacryoyl-1,2-isopropylidene-glucofuranose (P17). This data suggests that this polymer may act as a receptor or co-receptor in mediating *S. aureus* internalization. We have also found that *S. aureus* internalization by both HT-29 and Caco-2 enterocytes was increased when the basolateral enterocyte surface was exposed using calcium-free media. This result proposes that receptors for *S. aureus* are more readily available when the lateral cell surface is exposed. The data presented here suggests that our sulfated polymer could act as a mediator for *S. aureus* internalization by intestinal epithelial cells and possibly play a role in *S. aureus* colonization and pathogenesis. This could have important implications in the pathogenesis of many infections, as well as in the persistence of antibiotic resistant organisms.
2.6. Reference


33. Wallis, R. R. *Ph.D. Thesis*, University of Minnesota, Minneapolis, MN, **1996**.


47. Henry-Stanley, M. J.; Shepherd, M.; Sivertson, M.; and Wells, C. “Heparin disaccharides affect enterocyte interactions with Staphylococcus aureus”, poster presentation, University of Minnesota.

2.7. Appendix II
Chapter 3

Sulfated Carbohydrate Derivatives of Methacrylate Polymers: Computational Studies: Molecular Docking and Molecular Dynamic Calculations
3.1. Introduction

Fibroblast growth factors (FGFs) form a large family of structurally related proteins that regulate a wide variety of biological responses\(^1\). They mediate cellular functions by binding to transmembrane FGF receptors\(^2\) which are activated by dimerization\(^3\). The cellular receptors for FGFs are tyrosine kinase receptors (FGFR) which are activated by ligand-induced dimerization, requiring heparin or heparan sulfate as co-receptors\(^4\). It now seems clear that the activation process, in addition to receptor and growth factor, requires such heparin-like molecules. Two groups have published crystal structures of this tertiary complex, although, surprisingly, the quaternary structure of the complex is not the same. The biologically relevant complex thus remains unclear\(^5\).

Heparin is a linear sulfated polysaccharide chain that is widely distributed in animal tissues in the form of proteoglycans. Heparin and the structurally related heparan sulfate are involved in many important biological functions such as blood coagulation, angiogenesis, cell growth and differentiation\(^6\). Heparin and heparin-like glycosaminoglycans (GAGs) effectively inhibit the proliferation\(^7\) and migration\(^8\) of smooth muscle cells (SMC) in vitro.

In recent years, there has been considerable effort devoted to the development of anti-angiogenetic agents (compounds that inhibit the formation of new blood vessels) by synthesis of inhibitors of growth factors such as FGF2, VEGF and PDGF\(^4\). Development of these inhibitors could have potential applications in cancer therapy\(^4\).

Sulfated polysaccharides have been identified as specific ligands of glycoproteins and glycosaminoglycans. Due to considerable heterogeneity of structure in naturally occurring sulfated carbohydrates, many studies have investigated whether the physical
and biological properties of these compounds are dependant on factors such as the number and location of the sulfate groups, the nature of counter-ions and the conformation of polysaccharide chains\textsuperscript{9}. Methods are necessary to yield insight into the stereochemical features of sulfate groups and their importance in their biological functions. Such studies could evaluate ligand conformational energies and determine the effect of sulfate rotation. Modeling studies at the molecular level may be useful in providing a better description of the interaction of sulfated carbohydrates with biomolecules\textsuperscript{9} such as FGF.

Computer modeling has been widely used to investigate the interactions of ligands and proteins. Thus, we wish to use simulation methods to gain insight into the possible interactions of synthetic sulfated polymers with heparin binding proteins taking as a prototype a member of the FGF family. This system has been chosen because experimental results from X-ray crystallography are available for the complex of a heparin-model that demonstrates the dimerization of the growth factor\textsuperscript{10}. This system may be investigated using the docking program AUTODOCK3 to determine the docking possibilities for our synthetic polymers and compare these results with that found experimentally. Additionally, energy minimization and preliminary molecular dynamics calculations are described.

Previously, the X-ray crystal structure of a biologically active dimmer of human acidic FGF (aFGF) known as 2AXM in a complex with fully sulfated homogeneous heparin disaccharide was studied\textsuperscript{10}. This crystal structure showed a unique form of ligand induced protein dimerization. The sugar binding site on 2AXM is formed by Asn 18 and by side chains of the surface loop that consists of residues 112-128. Most of the
interactions between aFGF and the decasaccharide are ionic contacts between basic residues and the sulfate and carboxylate groups of heparin. Details of decasaccharide-aFGF interactions are shown in table 3.1 and figure 3.1. The main amino acid-interactions were found between Lys 118, Lys 112, Lys 113, Arg 122, and Lysin 128 and sugar sulfate and carboxylate groups.

<table>
<thead>
<tr>
<th>2AXM chains</th>
<th>Interactions of 2AXM With heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chain A</td>
<td>I3 – Gln 127</td>
</tr>
<tr>
<td></td>
<td>S4 – Asn 18</td>
</tr>
<tr>
<td></td>
<td>I3 – Lys 118</td>
</tr>
<tr>
<td></td>
<td>S6 – Arg 122</td>
</tr>
<tr>
<td></td>
<td>S6 – Lys 128</td>
</tr>
<tr>
<td></td>
<td>S6 – Gln 127</td>
</tr>
<tr>
<td></td>
<td>I5 – Lys 113</td>
</tr>
<tr>
<td></td>
<td>I5 – Asn 114</td>
</tr>
<tr>
<td></td>
<td>I5 – Gln 127</td>
</tr>
<tr>
<td>Chain B</td>
<td>I3 – Lys 112</td>
</tr>
<tr>
<td></td>
<td>S6 – Gln 127</td>
</tr>
<tr>
<td></td>
<td>S6 – Lys 113</td>
</tr>
<tr>
<td></td>
<td>S6 – Lys 118</td>
</tr>
<tr>
<td></td>
<td>17 – Lys 128</td>
</tr>
</tbody>
</table>

Table 3.1. Interactions between aFGF protomers and heparin, N-acetyl glucosamine (S), iduronic acid (I)\(^{10}\)

This chapter summarizes our use of computational techniques to investigate the interactions of our final sulfated polymers with heparin binding proteins such as FGF. We used molecular docking methods particularly AUTODOCK3 to predict binding ability of our final polymers with heparin binding proteins. We also used molecular dynamic simulation, mainly GROMACS to provide a detailed description of these systems at the molecular level.
Figure 3.1. Details of hydrogen bonding between aFGF protomers (ribbons) and heparin (ball and stick) in aFGF dimmers A and C.
3.2. Background

3.2.1. Molecular docking

An understanding of three-dimensional molecular structure of ligand/macromolecule complexes is a foundation for structure-based drug design. Structural data are often available for a ligand and a macromolecule individually, but not for potential complexes. Docking is a simulation process by which two or more partners may be brought together to form such complex. Modern docking methods have been reasonably successful at duplicating known ligand/macromolecule complexes as well as for the study of the binding of molecules for which the exact structure of a complex is unknown. The class of methods uses to study protein-ligand molecular recognition is commonly referred to as “molecular docking”. These methods attempt to place a ligand (e.g. a drug molecule) in the binding pocket of a protein using a variety of methods ranging from rather simple and naïve to very complex and sophisticated. The simplest model is analogous to the classical description by Emil Fischer of the “lock-and-key” concept for explaining the complementarity of an enzyme and its substrate. However, since both ligand and protein are usually flexible, the analogy of “hand-in-glove” is more appropriate and flexibility of both ligand and protein is taken into account in more sophisticated docking programs (refer to Figures 3.2 & 3.3).
Since docking plays such an important role in the rational drug design, considerable efforts has been made to use molecular docking in the pharmaceutical industry in an attempt to limit the number and kind of drug candidates synthesized or tested. We are using this methodology to obtain information about potential complexes of our novel polymeric systems with biomolecules.

In this chapter, the binding behavior of a number of synthetic sulfated carbohydrate polymers with one of the heparin binding protein (HBP), fibroblast growth
factor (FGF) known as 2AXM was studied. This modeling will assist us in understanding relevant protein-ligand interactions and may suggest possible modifications of our polymers in order to make them more useful.

3.2.2. Molecular dynamic simulations

Many important properties of a chemical system depend on the motion of atoms. Thus the binding of a ligand by a protein molecule may require movement of the ligand or the protein or both components during the event.

Although protein molecules are typically shown as static structures, this is due to the fact that the most widely used experimental method for structure determination of proteins is X-ray crystallography. This method usually provides a single molecular structure or, at best, a few additional conformations for parts of the molecule. NMR spectroscopy clearly demonstrates that macromolecules taken on many conformations in solution and an NMR structure is often reported as a family of structures with a number of them, typically twenty, being reported.

Molecular dynamics is a computer simulation technique that takes into account the possibility of molecular motion. One way to study accessible configuration of a molecule is to simulate the motions or dynamics of the molecule which is considered to be a mechanical system of balls and springs of the same type as used for molecular mechanics. Such a system can be described by Newton’s equation of motion for all atoms (i = 1, 2, …., N) of the molecular system.

\[
\frac{dr_i(t)}{dt} = v_i(t) \\
\frac{dv_i(t)}{dt} = \frac{F_i(t)}{m_i}
\]
The atomic coordinates, r, and the velocity, v, of atom, i, with mass, m_i, thus become functions of time. The force, F_i, exerted on atom i by the other atoms in the system is given by the negative gradient of the potential energy function V which in turn depends on the coordinates of all N atoms in system^{13}. Upon calculation of the force on each atom, the position of each atom throughout a specified period of time will be known. The force on each atom can also be calculated from the change in energy with respect to position of the atom^{14}.

\[- \frac{dE}{dr_i} = F_i\]

Energies can be calculated using either molecular mechanics or quantum mechanics methods or by a mixture of the two, so called QM-MM methods.
3.3. Calculation Section

3.3.1. Computational methodologies

The molecular docking of our sulfated polymers into heparin binding protein pocket (2AXM) was accomplished using AutoDock3. Further energy minimization of the complexes and molecular dynamics simulations were done using NAMD.

3.3.1.1. AUTODOCK 3

AutoDock is a system of automated docking tools which is designed for predicting how ligand molecules, typically drug-like molecules, bind to a protein receptor. The protein structure may be available from experimental results or it may be the result of homology modeling. The technique is very fast and provides predictions that allow for ligand flexibility. The program is usually run by making an assumption about the general region of ligand binding, but AutoDock3 has also been shown to be very useful in blind docking when the location of the binding site is unknown\textsuperscript{15}. AutoDock3 has been widely used for a variety of applications including X-ray crystallography, structure-based drug design, lead optimization, virtual screening, combinatorial library design, and chemical mechanism studies\textsuperscript{15}.

The program AutoDock3 was originally written in Fortran-77 in 1990 by David S. Goodsell in Arthur J. Olson’s laboratory. It was designed to perform automated docking of ligands (small molecules like a candidate drug) to their macromolecular targets (proteins, or DNA). In 1991, Garrett M. Morris improved the program by adding better
search methods and improved parameters, better empirical free energy scoring functions, thus making it suitable for real-life problems in medicinal chemistry.

AutoDock consists of two main programs: AutoDock itself which performs the actual docking of the ligand using a set of molecular property grids derived from the protein, and AutoGrid which pre-calculates these grids\textsuperscript{16} for a desired region of the protein. Figure 3.2 illustrates the way that autodock and autogrid was used together.

![The representation of AutoDock and AutoGrid](image)

**Figure 3.4. The representation of AutoDock and AutoGrid**

3.3.1.2. GROMACS

Molecular simulation is an extremely useful, but computationally very expensive tool for studies of chemical and biomolecular systems.

Numerical molecular dynamics (MD) is computationally very demanding. A numerical simulation that covers a significant time-span-to reveal protein unfolding for example- can take up to years even on modern parallel computer. Scientists needing to perform parallel MD can today choose from several packages, some of which are freely available, e.g. GROMACS and NAMD\textsuperscript{17}.
GROMACS is an acronym for Groningen Machine for Chemical Simulation. The name arises from a collaborative project of our group in the department of chemistry at the University of Groningen, The Netherlands, with the Department of Computer Science in Groningen, in the early 1990s, to construct a dedicated parallel computer system for molecular simulation. It is a versatile package to perform molecular dynamics, i.e. simulate the Newtonian equation of motion for systems with hundreds to millions of particles\textsuperscript{18}.

Although GROMACS is primarily designed for biochemical molecules like proteins and lipids, but since GROMACS is very fast at calculating the nonbonded interactions, many groups are also using it for research on non-biological systems, e.g. polymers.

The force field used for intramolecular interactions is in principle not part of GROMACS, but the organization of force and energy evaluations, although versatile, does set limitations to the allowed types of force field. Forces and energies are computed on the basis of three different types of interactions:

i) Bonded interactions between two, three, or four particles, based on predefined, fixed lists.

ii) Nonbonded interactions between particle pairs, based on a regularly updated volatile list of pairs.

iii) Special interactions can be defined to impose position, angle, or distance restraints on the motion of the system\textsuperscript{4}.
3.3.2. AutoDock procedure

There are a number of steps involved in docking a specific ligand into protein binding pocket. The first steps include preparation of the macromolecule (protein), and the ligand followed by determination of the grid parameters. Finally the input parameters for the actual docking run are prepared. These provide all the necessary information to be used for the docking process. Depending on the degree of difficulty of the docking calculation, the number of independent runs, the minimization methods, and the energy tolerance for stopping a run may be specified. The protein in our studies is a heparin binding protein (HBP) which is available as 2AXM in protein data bank. The ligand is one of our sulfated polymers shown in table 3.1.

3.3.2.1. Preparation of the macromolecule file

Since protein data bank (PDB) files typically have a variety of problems including missing atoms or side chains, additional water molecules or other ligands, or chain breaks, the original PDB file often needs modification before it can be used in AutoDock. The group responsible for AutoDock has helpfully provided a graphical user interface, AutoDock tools (ADT), to help solve these sorts of problems. The pdb file containing the target macromolecule (2AXM) was first loaded into ADT and, after extracting protein portion of the file it was saved as a new pdb file using ADT. ADT also computes the necessary charges for this file, the so-called Kollman charges that are necessary for the subsequent grid calculation.
3.3.2.2. Preparation of the ligand file

A file in pdb format for each ligand was obtained using the Dundee PRODRG server for which the x, y, z coordinates and suitable initial charges are assigned. When this file is read into ADT, the charges are checked for consistency and may be adjusted if necessary. Because the ligands being studied have integral negative charges, this check is a good one and helps to spot gross errors. However, the charges tolerances for ADT seem to be tighter than PRODRG and often slight deviations from integral values require rescaling. ADT is used in automatic mode to specify the so-called root of a torsion tree, and rotatable bonds in the molecule identified. The desired active torsions are chosen for the ligand and an output file containing the specifications is saved.

3.3.2.3. Preparation of the grid parameters

AutoGrid3 was used to compute grid maps. The grid parameter file gives information about the type of maps to be computed, and the location and extent of those maps. It also specifies pair-wise potential energy parameters. Usually, only one map is calculated for each element in the ligand plus an electrostatics map. The types of maps produced depend on the elements present in the ligand.

The volume of the grid to be used for the calculation can be problematic. Making it too small – equal to the volume of the ligand – may not give the ligand sufficient room to explore the protein surface. Making it too large may not give a fine enough grid as large volumes require a coarse grid. Generally the volume should be large enough to allow free rotation of ligand, even in its most fully extended conformation. In our calculations, the center of the grid box was set on the center of the macromolecule dimer.
(2AXM) and, using the thumbwheel widgets, the number of points in the x, y, and z dimensions was changed such that the grid box will cover the entire macromolecule. Finally the desired grid map parameter file was written out for input into Autogrid.

3.3.2.4. Preparation of the docking parameters

This step will provide all information needed to dock the ligand of choice into the binding site of the protein. The docking parameter file provides AutoDock with information such as the type of map files, the ligand molecule, the number of torsions, docking algorithm to be used, and the number of runs. First, the macromolecule to be docked was selected. This is followed by setting the ligand parameters (e.g. atom types, number of active torsions, number of torsional degrees of freedom) for the ligand of choice. Then, the genetic algorithm parameters were set which allows us to change the number of energy evaluations. Although both simulated annealing and a genetic algorithm are options in the program, work in our laboratory, as well as many others, indicates that the genetic algorithm is far superior. For our calculations, we used 25,000 evaluations. Finally, the docking run parameters were set as default and the docking file was saved.
3.3.2.5. Starting the AutoGrid and AutoDock

Both AutoGrid and AutoDock are run in directories where the macromolecule, ligand, gpf, and dpf files are to be found. These jobs can be launched from the command line on Linux platforms. A typical command to run an autogrid job is:

```
autogrid3 –p protein.gpf –l protein.glg
```

where the input parameter file is protein.gpf and the resulting log file that describes the grid calculation is protein.glg.

Similarly for autodock:

```
autodock3 –p ligand.dpf –l ligand.dlg
```

Where the file ligand.dpf gives the docking parameter files and the output log file is ligand.dlg. This log file gives the coordinates of the result of each docking run as well as an energy that, if all goes well, describes accurately an empirical energy of interaction. An rmsd value is also calculated. This compares the rmsd of each docked structure with that of the starting ligand. This is useful when the starting ligand is that of an experimental X-ray structure, but meaningless when the initial model is not based on experiment.

3.3.2.6. General procedure for docking sulfated polymers into 2AXM

These dimensional coordinates were obtained for each polymer using Chem 3D and saved in pdb format. Corresponding files containing appropriate charges, pdbq, were prepared using AutoDock tools. The desired number of torsions was selected and a docking parameter file prepared. The output docking log was evaluated as explained in following section 4.
3.3.3. Ligands (Sulfated carbohydrate derivatives of methyl methacrylate polymers)

A number of sulfated polymers were docked into the 2AXM binding pocket. The structures of these polymers are shown in tables below.

<table>
<thead>
<tr>
<th>“ms-iso-3mglu”</th>
<th>“ms-syn-3mglu”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“ds-iso-3mglu”</td>
<td>“ds-syn-3mglu”</td>
</tr>
</tbody>
</table>

Table 3.2. Chem 3D structures of mono or disulfated 3-O-methacryloyl-glucose polymers
Table 3.3. Chem-3D structures of mono-or disulfated 6-O-methacyroyl-galactose polymer
Table 3.4. Chem 3D structures of mono or disulfated 6O-methacryloyl-glucose polymers

<table>
<thead>
<tr>
<th>“ms-iso-6mglu”</th>
<th>“ms-syn-6mglu”</th>
</tr>
</thead>
<tbody>
<tr>
<td>“ds-iso-6mglu”</td>
<td>“ds-syn-6mglu”</td>
</tr>
</tbody>
</table>

Figure 3.5. Chem-3D structure of copolymer of sulfated glucose-sulfated galactose-methyl methacrylate (2mm-sulfglu-4mm-sulfgal-2mm)
Table 3.5. Chem 3D structures of monosulfated methacrylated xylose polymers
<table>
<thead>
<tr>
<th>Name</th>
<th>Chem.-3D file</th>
<th>Pdb file</th>
<th>Tiff file</th>
</tr>
</thead>
<tbody>
<tr>
<td>ms-iso-3mglu</td>
<td>ms-iso-3mglu.C3d</td>
<td>ms-iso-3mglu.pdb</td>
<td>ms-iso-3mglu.tiff</td>
</tr>
<tr>
<td>ms-syn-3mglu</td>
<td>ms-syn-3mglu.C3d</td>
<td>ms-syn-3mglu.pdb</td>
<td>ms-syn-3mglu.tiff</td>
</tr>
<tr>
<td>ds-iso-3mglu</td>
<td>ds-iso-3mglu.C3d</td>
<td>ds-iso-3mglu.pdb</td>
<td>ds-iso-3mglu.tiff</td>
</tr>
<tr>
<td>ds-syn-3mglu</td>
<td>ds-syn-3mglu.C3d</td>
<td>ds-syn-3mglu.pdb</td>
<td>ds-syn-3mglu.tiff</td>
</tr>
<tr>
<td>ms-iso-6mglu</td>
<td>ms-iso-6mglu.C3d</td>
<td>ms-iso-6mglu.pdb</td>
<td>ms-iso-6mglu.tiff</td>
</tr>
<tr>
<td>ms-syn-6mglu</td>
<td>ms-syn-6mglu.C3d</td>
<td>ms-syn-6mglu.pdb</td>
<td>ms-syn-6mglu.tiff</td>
</tr>
<tr>
<td>ds-iso-6mglu</td>
<td>ds-iso-6mglu.C3d</td>
<td>ds-iso-6mglu.pdb</td>
<td>ds-iso-6mglu.tiff</td>
</tr>
<tr>
<td>ds-syn-6mglu</td>
<td>ds-syn-6mglu.C3d</td>
<td>ds-iso-6mglu.pdb</td>
<td>ds-syn-6mglu.tiff</td>
</tr>
<tr>
<td>ms-iso-6mgal</td>
<td>ms-iso-6mgal.C3d</td>
<td>ms-iso-6mgal.pdb</td>
<td>ms-iso-6mgal.tiff</td>
</tr>
<tr>
<td>ms-syn-6mgal</td>
<td>ms-syn-6mgal.C3d</td>
<td>ms-iso-6mgal.pdb</td>
<td>ms-iso-6mgal.tiff</td>
</tr>
<tr>
<td>ds-iso-6mgal</td>
<td>ds-iso-6mgal.C3d</td>
<td>ds-iso-6mgal.pdb</td>
<td>ds-iso-6mgal.tiff</td>
</tr>
<tr>
<td>ds-syn-3mxyl</td>
<td>ds-syn-3mxyl.C3d</td>
<td>ds-iso-6mgal.pdb</td>
<td>ds-syn-6mgal.tiff</td>
</tr>
<tr>
<td>ms-iso-3mxyl</td>
<td>ms-iso-3mxyl.C3d</td>
<td>ms-iso-3mxyl.pdb</td>
<td>ms-iso-3mxyl.tiff</td>
</tr>
<tr>
<td>ms-syn-3mxyl</td>
<td>ms-syn-3mxyl.C3d</td>
<td>ms-iso-3mxyl.pdb</td>
<td>ms-iso-3mxyl.tiff</td>
</tr>
<tr>
<td>ms-iso-5mxyl</td>
<td>ms-iso-5mxyl.C3d</td>
<td>ms-iso-5mxyl.pdb</td>
<td>ms-iso-5mxyl.tiff</td>
</tr>
<tr>
<td>ms-syn-5mxyl</td>
<td>ms-syn-5mxyl.C3d</td>
<td>ms-iso-5mxyl.pdb</td>
<td>ms-iso-5mxyl.tiff</td>
</tr>
<tr>
<td>2mm-sulfglu-2mm</td>
<td>2mm-sulfglu-2mm</td>
<td>2mm-sulfglu-2mm</td>
<td>2mm-sulfglu-2mm</td>
</tr>
<tr>
<td>4mm-sulfgal-2mm</td>
<td>4mm-sulfgal-2mm</td>
<td>4mm-sulfgal-2mm</td>
<td>4mm-sulfgal-2mm</td>
</tr>
</tbody>
</table>

Table 3.6. Name of docked polymers with their generated file for AutoDock
3.4. Results

3.4.1. Interpretation of docking results

The first step in analyzing the results of docking experiments is reading a docking log file which shows up as “ligand.dlg”. The key information in a docking log file are the coordinates for the docked structures at the end of each run, the energies of these docked structures, and their similarities to each other. The similarity of docked structures is measured by computing the root-mean-square-deviation (RMSD) between the coordinates of the atoms. The minimized energy and RMSD was used to predict the best conformation of the ligand into protein binding pocket.

3.4.2. General process for interpretation of docking sulfated polymers into 2AXM

Upon obtaining each “ligand.dlg” file, the file was carefully reviewed such that three of the best runs with lowest cluster energy from RMSD table were selected. A new pdb file from the coordinates of the selected runs was created and viewed in UCSF Chimera. Both the ligand file (ligand.out.pdb) and protein file (2AXM.pdb) was viewed in Chimera simultaneously. The smallest binding distances between the sulfate groups of the sulfated polymers and the protein (residue A and B) was selected as the best polymer-protein interactions. The results are shown in tables below. It must be noted that in each sets of energy calculations, the most significant interactions between polymer-protein is in the range of 2.5-4.0 Å. However, a number of interactions outside this range were also reported.
3.4.3. Docking results of sulfated polymers into 2AXM binding pocket

Tables 3.4-3.12 list the best interactions between sulfate groups of each polymer with residues A and B of protein 2AXM.

3.4.3.1. ms-iso-3mglu

In this polymer, sulfate functionality is on C6 and methyl methacrylate group is on C3. Five different number of active torsions (\(r = 0, 2, 4, 6, 8\)) were chosen to investigate the best interactions of sulfate group of this polymer with heparin binding protein (2AXM). However, the results of each set of torsions were similar. Therefore, only the results of docking the polymer with zero number of torsions were evaluated.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>31.05</th>
<th>31.05</th>
<th>31.06</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S2-Arg 119,4.65</td>
<td>S2-Arg 119,4.64</td>
<td>S2-Arg 119,4.61</td>
</tr>
<tr>
<td></td>
<td>S2-Arg 122,3.89</td>
<td>S2-Lys 112,2.82</td>
<td>S2-Lys 112,2.82</td>
</tr>
<tr>
<td></td>
<td>S2-Lys 112,4.21</td>
<td>S1-Lys 112,4.21</td>
<td>S1-Lys 112,4.20</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S3-Lys 113, 3.17</td>
<td>S3-Lys 113,3.17</td>
<td>S3-Lys 113,2.64</td>
</tr>
<tr>
<td></td>
<td>S4-Lys 118, 2.47</td>
<td>S4-Lys 118,2.47</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7. Docking results of ms-iso-3mglu into 2AXM binding pocket
3.4.3.2. ms-syn-3mglu

The only difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy</th>
<th>72.60</th>
<th>72.62</th>
<th>72.75</th>
</tr>
</thead>
<tbody>
<tr>
<td>(kcal/mol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with residue A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A˚)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3-Ser 116, 3.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5-Arg 119, 2.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S7-Arg 119, 4.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interactions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with residue B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A˚)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2-Arg 122, 4.40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8. Docking results of ms-syn-3mglu into 2AXM binding pocket
3.4.3.3. ds-iso-3mglu

This polymer is disulfated polymer where the sulfate groups are on C5 and C6.

and methyl methacrylate functionality is on C3.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>2 x $10^{12}$</th>
<th>2 x $10^{12}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (A^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S7-Arg 119, 3.56</td>
<td></td>
<td>S1-Arg 119, 3.67</td>
</tr>
<tr>
<td>S7-Arg <strong>122</strong>, 4.16</td>
<td></td>
<td>S2-Arg <strong>122</strong>, 3.37</td>
</tr>
<tr>
<td>S1-Tyr 15, 4.19</td>
<td></td>
<td>S1-Cys 117, 1.97</td>
</tr>
<tr>
<td>S8-Gly 20, 1.72</td>
<td></td>
<td>S2-Pro 121, 3.35</td>
</tr>
<tr>
<td>Interactions with residue B (A^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4-Ser 116, 3.48</td>
<td></td>
<td>S5-Asp 70, 3.88</td>
</tr>
<tr>
<td>S5-Lys <strong>118</strong>, 2.67</td>
<td></td>
<td>S5-Arg 119, 3.09</td>
</tr>
<tr>
<td>S5-Asn <strong>18</strong>, 3.60</td>
<td></td>
<td>S3-Lys <strong>113</strong>, 1.94</td>
</tr>
<tr>
<td>S5-Leu 111, 2.81</td>
<td></td>
<td>S4-Gln <strong>127</strong>, 3.48</td>
</tr>
<tr>
<td>S4-Asn <strong>114</strong>, 2.14</td>
<td></td>
<td>S4-Arg <strong>122</strong>, 1.90</td>
</tr>
<tr>
<td>S4-Cys 117, 4.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.9. Docking results of ds-iso-3mglu into 2AXM binding pocket

Figure 3.7. Docking results of ds-iso-3mglu into 2AXM binding pocket
3.4.3.4. ds-syn-3mglu

This polymer is also disulfated polymer with similar locations for sulfate and methyl methacrylate group and different tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>27084.55</th>
<th>27319.30</th>
<th>29351.40</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interactions with residue A (Å)</strong></td>
<td>S4-Gln 127, 3.67</td>
<td>S4-Asn 114, 3.49</td>
<td>S7-Gln 127, 3.22</td>
</tr>
<tr>
<td></td>
<td>S8-Gln 127, 3.23</td>
<td>S7-Gln 127, 3.18</td>
<td>S7-Gln 127, 3.22</td>
</tr>
<tr>
<td></td>
<td>S6-Arg 35, 2.55</td>
<td>S2-Lys 113, 3.61</td>
<td>S2-Lys 113, 3.69</td>
</tr>
<tr>
<td><strong>Interactions with residue B (Å)</strong></td>
<td>S4-Pro 121, 3.58</td>
<td>S4-Pro 121, 3.53</td>
<td>S8-Lys 118, 2.47</td>
</tr>
<tr>
<td></td>
<td>S8-Lys 118, 2.48</td>
<td>S6-Lys 113, 4.33</td>
<td>S6-Lys 113, 4.33</td>
</tr>
</tbody>
</table>

Table 3.10. Docking results of ds-syn-3mglu into 2AXM binding pocket

Figure 3.8. Docking results of ds-syn-3mglu into 2AXM binding pocket
3.4.3.5. ms-iso-6mglu

The position of sulfate functionality and methyl methacrylate group on this polymer is different than the polymer reported above. In this polymer, C3 contains sulfate and methyl methacrylate is on C6.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>6.91</th>
<th>6.91</th>
<th>6.91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S2-Lys 113, 3.88</td>
<td>S6-Arg 122, 4.12</td>
<td>S2- Lys 113, 3.97</td>
</tr>
<tr>
<td></td>
<td>S3-Lys 113, 3.36</td>
<td>S7-Arg 122, 4.79</td>
<td>S3-Lys 113, 3.40</td>
</tr>
<tr>
<td></td>
<td>S6-Arg 122, 3.02</td>
<td></td>
<td>S6-Arg 122, 3.02</td>
</tr>
<tr>
<td></td>
<td>S7-Arg 122, 4.79</td>
<td></td>
<td>S7-Arg 122, 4.79</td>
</tr>
</tbody>
</table>

| Interactions with residue B (Å) | S3-Lys 112, 3.86 | S3-Lys 112, 2.45 | S5-Lys 113, 3.63 |
|                                | S4-Lys 113, 3.33 | S4-Lys 113, 3.34 | S4-Lys 113, 3.36 |
|                                | S5-Lys 113, 3.60 | S5-Lys 113, 6.60 | S3-Lys 112, 2.44 |

Table 3.11. Docking results of ms-iso-6mglu into 2AXM binding pocket

3.4.3.6. ms-syn-6mglu

The only difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>193.88</th>
<th>194.19</th>
<th>197.81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S3-Asn 114, 4.09</td>
<td>S3-Asn 118, 2.23</td>
<td>S3-Asn 18, 2.24</td>
</tr>
<tr>
<td></td>
<td>S3-Asn 18, 2.23</td>
<td>S3-Lys 118, 4.73</td>
<td>S3-Lys 113, 2.89</td>
</tr>
</tbody>
</table>

| Interactions with residue B (Å) | S2-Lys 113, 2.40 | S1-Arg 122, 2.45 | S5-Lys 113, 1.97 |
|                                | S8-Arg 122, 4.50 | S2-Lys 113, 1.83 | S1-Arg 122, 4.45 |

Table 3.12. Docking results of ms-syn-6mglu into 2AXM binding pocket
3.4.3.7. ds-iso-6mglu

The position of methyl methacrylate group on this polymer is similar to the polymer reported above. In this polymer, C3 and C5 contain sulfate and methyl methacrylate is on C6.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>2 x 10^{12}</th>
<th>2 x 10^{12}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S3-Arg 35, 3.70</td>
<td>S4-Lys 113, 4.17</td>
</tr>
<tr>
<td></td>
<td>S6-Arg 122, 3.17</td>
<td>S6-Arg 119, 3.63</td>
</tr>
<tr>
<td></td>
<td>S4-Asn 18, 3.16</td>
<td>S6-Arg 122, 3.36</td>
</tr>
<tr>
<td></td>
<td>S4-Lys 128, 3.07</td>
<td>S6-Lys 112, 2.93</td>
</tr>
<tr>
<td></td>
<td>S4-Ser 17, 3.82</td>
<td></td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S7-Lys 128, 3.33</td>
<td>S3-Arg 119, 3.12</td>
</tr>
<tr>
<td></td>
<td>S1-Asp 70, 2.82</td>
<td>S5-Asn 18, 2.00</td>
</tr>
<tr>
<td></td>
<td>S3-Asn 114, 2.15</td>
<td>S5-Lys 118, 3.86</td>
</tr>
</tbody>
</table>

Table 3.13. Docking results of ds-iso-6mglu into 2AXM binding pocket

3.4.3.8. ds-syn-6mglu

The only difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>2 x 10^{12}</th>
<th>2 x 10^{12}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S6-Gly 19, 2.36</td>
<td>S7-Arg 119, 3.80</td>
</tr>
<tr>
<td></td>
<td>S6-Ser 17, 2.99</td>
<td>S8-Arg 119, 3.64</td>
</tr>
<tr>
<td></td>
<td>S4-Lys 118, 3.61</td>
<td>S2-Lys 113, 3.79</td>
</tr>
<tr>
<td></td>
<td>S5-Ser 17, 3.14</td>
<td>S1-Ser 17, 2.03</td>
</tr>
<tr>
<td></td>
<td>S5-Lys 128, 3.07</td>
<td>S1-Tyr 94, 3.09</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S2-Asn 18, 2.21</td>
<td>S4-Cys 117, 2.35</td>
</tr>
<tr>
<td></td>
<td>S1-Gln 127, 4.40</td>
<td>S4-Arg 122, 2.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S4-Lys 112, 4.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S6-Gly 126, 3.29</td>
</tr>
</tbody>
</table>

Table 3.14. Docking results of ds-syn-6mglu into 2AXM binding pocket
3.4.3.9. ms-iso-6mgal

This is a monosulfated galactose polymer. The sulfate functionality is on C3 and methyl methacrylate group is on C6.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>90.55</th>
<th>90.64</th>
<th>90.78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S6-Lys 113, 7.11</td>
<td>S3-Lys 128, 4.03 S3-Lys 113, 4.02 S6-Lys 118, 2.58 S7-Arg 122, 3.82</td>
<td>S6-Arg 122, 7.33</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S2-Arg 119, 3.16 S4-Lys 112, 3.38 S4-Arg 122, 3.16 S5-Arg 122, 2.49</td>
<td>None</td>
<td>S2-Arg 119, 3.71 S3-Arg 122, 3.96 S4-Lys 112, 3.32 S4-Arg 122, 3.08 S5-Arg 122, 2.47</td>
</tr>
</tbody>
</table>

Table 3.15. Docking results of ms-iso-6mgal into 2AXM binding pocket

![Figure 3.9. Docking results of ms-iso-6mgal into 2AXM binding pocket](image)
3.4.3.10. ms-syn-6mgal

The difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>59.63</th>
<th>59.77</th>
<th>59.78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å°)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1-Gly 126, 4.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3-Asn 18, 2.34</td>
<td>S3-Lys 118, 3.99</td>
<td>S7-Arg 119, 2.97</td>
<td>S3-Asn 18, 2.31</td>
</tr>
<tr>
<td>S7-Arg 119, 2.97</td>
<td>S7-Arg 119, 2.97</td>
<td>S7-Arg 122, 2.98</td>
<td>S3-Lys 118, 3.99</td>
</tr>
<tr>
<td>S3-Lys 118, 4.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interactions with residue B (Å°)</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 3.16. Docking results of ms-syn-6mgal into 2AXM binding pocket

Figure 3.10. Docking results of ms-syn-6mgal into 2AXM binding pocket
3.4.3.11. ds-iso-6mgal

This is a disulfated galactose polymer. The sulfate functionality is on C3 and C4 and methyl methacrylate group is on C6.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>33.82</th>
<th>51.51</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S1-Lys 128, 2.93</td>
<td>S2-Lys 113, 4.02</td>
</tr>
<tr>
<td></td>
<td>S1-Lys 113, 3.82</td>
<td>S3-Arg 122, 2.41</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S2-Arg 122, 3.51</td>
<td>S4-Lys 113, 3.23</td>
</tr>
</tbody>
</table>

Table 3.17. Docking results of ds-iso-6mgal into 2AXM binding pocket

3.4.3.12. ds-syn-6mgal

The difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>2 x 10^{12}</th>
<th>2 x 10^{12}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S6-Arg 122, 3.06</td>
<td>S6-Lys 118, 3.87</td>
</tr>
<tr>
<td></td>
<td>S4-Ala 129, 2.59</td>
<td>S6-Gln 127, 3.17</td>
</tr>
<tr>
<td></td>
<td>S4-Leu 111, 3.27</td>
<td>S6-Thr 123, 2.96</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S3-Arg 119, 2.22</td>
<td>S4-Asn 18, 3.92</td>
</tr>
<tr>
<td></td>
<td>S5-Lys 113, 4.02</td>
<td>S4-Leu 111, 3.86</td>
</tr>
<tr>
<td></td>
<td>S5-Asn 18, 3.59</td>
<td>S5-Lys 113, 3.75</td>
</tr>
</tbody>
</table>

Table 3.18. Docking results of ds-syn-6mgal into 2AXM binding pocket
### 3.4.3.13. 2mm-sulfglu-4mm-sulfgal-2mm

This is a block copolymer of methyl methacrylate, sulfated glucose, and sulfated galactose. The number of each monomer is shown in the name.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>19.43</th>
<th>21.22</th>
<th>21.25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>Sglu-Lys113,2.76</td>
<td>Sgal-Lys112,2.94</td>
<td>None</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>Sglu-Lys112,2.54</td>
<td>Sgal-Lys113,4.45</td>
<td>Sgal-Gln127,4.16 Sgal-Lys118,3.25</td>
</tr>
</tbody>
</table>

Table 3.19. Docking results of 2mm-sulfglu-4mm-sulfgal-2mm into 2AXM binding pocket

![Docking results of 2mm-sulfglu-4mm-sulfgal-2mm into 2AXM binding pocket](image)

Figure 3.11. Docking results of 2mm-sulfglu-4mm-sulfgal-2mm into 2AXM binding pocket
3.4.3.14. ms-iso-3mxyl

This is a pentose derivative of methyl methacrylate. The position of methyl methacrylate is on C3 and the sulfate functionality is on C5.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>49.32</th>
<th>49.96</th>
<th>51.36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S1-Lys 113, 3.12</td>
<td>S1-Lys 113, 2.58</td>
<td>S1-Lys 113, 4.86</td>
</tr>
<tr>
<td></td>
<td>S1-Lys 128, 4.96</td>
<td>S2-Lys 128, 4.52</td>
<td>S1-Lys 113, 4.86</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S2-Lys 112, 3.29</td>
<td>S2-Asn 114, 3.86</td>
<td>S1-Lys 112, 3.74</td>
</tr>
<tr>
<td></td>
<td>S2-Asn 114, 4.09</td>
<td>S3-Lys 114, 4.07</td>
<td>S3-Lys 113, 3.76</td>
</tr>
<tr>
<td></td>
<td>S4-Lys 113, 2.49</td>
<td>S4-Asn 18, 3.89</td>
<td>S4-Lys 118, 3.58</td>
</tr>
<tr>
<td></td>
<td>S5-Asn 18, 3.87</td>
<td>S5-Lys 118, 3.29</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.20. Docking results of ms-iso-3mxyl into 2AXM binding pocket

3.4.3.15. ms-syn-3mxyl

The only difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>63.75</th>
<th>63.78</th>
<th>63.78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>S3-Lys 113, 4.02</td>
<td>S5-Asn 114, 3.35</td>
<td>S5-Asn 114, 3.35</td>
</tr>
<tr>
<td></td>
<td>S5-Asn 114, 3.33</td>
<td>S3-Lys 113, 4.02</td>
<td>S3-Lys 113, 4.02</td>
</tr>
<tr>
<td></td>
<td>S7-Lys 118, 2.72</td>
<td>S7-Lys 118, 2.73</td>
<td>S7-Arg 122, 3.00</td>
</tr>
<tr>
<td></td>
<td>S7-Arg 122, 2.97</td>
<td>S7-Arg 122, 3.00</td>
<td>S7-Gln 127, 3.56</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S4-Arg 122, 2.23</td>
<td>S4-Arg 122, 2.21</td>
<td>S4-Arg 122, 2.21</td>
</tr>
</tbody>
</table>

Table 3.21. Docking results of ms-syn-3mxyl into 2AXM binding pocket
3.4.3.16. ms-iso-5mxyl

This is a pentose derivative of methyl methacrylate. The position of methyl methacrylate is on C5 and the sulfate functionality is on C3.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>47.55</th>
<th>55.49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with residue A (Å)</td>
<td>None</td>
<td>S6-Lys 128, 3.62 S7-Lys 128, 4.00</td>
</tr>
<tr>
<td>Interactions with residue B (Å)</td>
<td>S4-Arg 122, 2.74 S5-Arg 122, 3.23</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 3.22. Docking results of ms-iso-5mxyl into 2AXM binding pocket
3.4.3.17. ms-syn-5mxyl

The only difference between this polymer and the one above is their tacticity.

<table>
<thead>
<tr>
<th>Docked Energy (kcal/mol)</th>
<th>64.09</th>
<th>64.69</th>
<th>68.62</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with residue A (Å)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S8-Arg 122, 4.05</td>
<td>S8-Arg 119, 3.35</td>
<td>S8-Arg 119, 3.26</td>
<td>S8-Arg 122, 5.74</td>
</tr>
<tr>
<td>S8-Arg 119, 3.35</td>
<td>S7-Arg 122, 3.10</td>
<td>S7-Arg 122, 3.02</td>
<td></td>
</tr>
<tr>
<td>S7-Arg 122, 3.10</td>
<td>S5-Lys 128, 2.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S5-Lys 128, 2.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with residue B (Å)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6-Gln 127, 2.57</td>
<td>S6-Gln 127, 2.52</td>
<td>S5-Arg 119, 3.09</td>
<td></td>
</tr>
<tr>
<td>S6-Lys 118, 3.12</td>
<td>S6-Arg 122, 3.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S4-Asn 114, 4.34</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.23. Docking results of ms-syn-5mxyl into 2AXM binding pocket
3.5. Discussion

3.5.1. Docking the sulfated polymers into 2AXM binding pocket

A number of docking experiments have been performed to investigate the binding behavior of the sulfated carbohydrate polymers with the heparin binding protein (2AXM). The results (Tables 3.5-3.21) show the closest interactions of sulfate groups with amino acid residues from chain A or B of the protein 2AXM. Since the number of active torsions did not have any effect on the docking results, only the results with zero number of active torsions are reported.

It was indicated that glucose polymers have better interactions with 2AXM as opposed to galactose polymers which in most cases, no interactions were found between the sulfate groups and residue B of the protein. The results shown in the tables above indicate that the majority of sulfate groups of the carbohydrate polymers interact with the basic amino acid residues of the protein (2AXM) such as Arginine (Arg), Glutamine (Gln), Lysine (Lys), and Asparagine (Asn). This is analogous to the interactions between aFGF and heparin decasaccharide shown with amino acids bolded in table 3.1. Most of the interactions between aFGF and decasaccharide are ionic bonds between basic residues and the sulfate or carboxylate groups of heparin\textsuperscript{10}. This could be due to the interactions of their amino functionality from their side with sulfate groups. FGF molecules must have different binding behavior with heparin-like sugars to promote biological activity. The long side chains of the basic residues in the binding site provide conformational flexibility to accommodate differences in sugar sulfation and alternative sugar directions\textsuperscript{10}. It was shown that there are preferred binding sites of FGF for sulfate
groups of oligosaccharides which are formed from side chains of residues Asn 18, Lys 118, Gln 127, and Asn 114. This preference was also indicated in the interactions between sulfated carbohydrate polymers and FGF (2AXM).

Previous studies have shown that the molecular modeling could be a useful tool to study the polymer-chain tacticity either as a characterization technique or as a probe technique\textsuperscript{19}. Molecular modeling may offer the potential to reveal microscopic interactions accountable for macroscopic properties. In a study performed by Professor Soldera and colleagues, the experimental $T_g$ values of syndiotactic and isotactic PMMA was shown to be in agreement with the simulated values. It was indicated that syndiotactic form possesses lower intramolecular energies than the isotactic PMMA, as opposed to intermolecular energies\textsuperscript{19}. It was also shown that the tacticity of the polymer affects the binding behavior of the polymer and protein such that in most cases, the syndiotactic polymers have more interactions with the protein. This means that the two substituents (methyl groups and methacrylated sugars) should be on alternate positions along the chain to have better interactions with the polymer. The substituents on the same side of the polymer backbone (isotactic) result in more hindrance in the molecule and less interactions with sulfate groups of the polymers. Syndiotacticity of the polymer provides a molecule with a hand to fit nicely in the glove of the protein.

Disulfated glucose polymers have also shown the most number of interactions compared to galactose and pentose polymers. The least interactions of the sulfated polymer with 2AXM were found in the copolymer of methyl methacrylate, glucose, and galactose which contain small number of sulfate groups.
It could be concluded that sulfate groups are the key elements for interactions of these polymers with heparin binding protein 2AXM. The stereochemistry of the polymer could also be a potential factor for better interactions with HBP such as 2AXM.

3.5.2. Dynamic simulation of sulfated polymers into 2AXM binding pocket

A number of attempts were made toward molecular dynamic simulation of sulfated polymers into the binding pocket of heparin binding protein 2AXM using gromacs. However, even with the assistance of tutorial expert, the simulation of a polymer of interest into 2AXM binding pocket was unsuccessful. Future goal is to utilize other molecular dynamic tools such as NAMD to overcome this challenge.
3.6. References


15. [http://w3.to/autodock](http://w3.to/autodock)

16. [http://autodock.scripps.edu](http://autodock.scripps.edu)


Chapter 4

Surface Polymerization of Methacrylate

Carbohydrate Derivatives
4.1. Introduction

Immobilization of biomolecules such as carbohydrates, proteins, and nucleic acids onto various surfaces is a well-known method of producing bioactive surfaces for use in many applications such as immunoassays, chromatography, cell proliferation, and drug delivery\(^1\).

Carbohydrate immobilization has been reported for derivatives of galactose\(^2\), melibiose\(^2\), mannose\(^3\), lactose\(^4\), starch\(^5\), lactosaminide\(^6\), and heparin\(^7\)-\(^11\), typically achieved by adsorption or covalent binding. Chevolot and colleagues used photochemical immobilization techniques to functionalize diamond surfaces with carbohydrates\(^12\). Xue-Long Sun and colleagues demonstrated the applicability of varied organic reactions such as Diels-Alder and click chemistry for the immobilization of carbohydrates and proteins onto a solid surface\(^13\). Hindsgaul and coworkers have developed a simple and economical procedure for the attachment of reducing sugars to aminated solid supports to serve as an inexpensive therapeutic for bacterial toxin-mediated diarrheal diseases\(^14\).

Adsorption of these biomolecules is typically performed on the surface of microtiter plates made of polystyrene. Nonspecific adsorption of these molecules onto plastic surfaces proceeds due to intermolecular attractive forces. Typically plates receive a proprietary treatment permitting other types of interactions such as hydrogen bonding. Often these procedures suffer from non-specific binding and desorption of biomolecules during incubation and washing steps.

This chapter describes a number of techniques used to polymerize 3-\(O\)-methacryoyl-1,2:5,6-di-\(O\)-isopropylidene-\(\alpha\)-D-glucofuranose (3M-DiGlu) (1) and 6-\(O\)-
methacryloyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (6M-DiGal) (2) on the surface of polystyrene 24-well plates and glass cover slips. Reducing sugar assays were used to determine the amount of carbohydrate containing polymers deposited on these surfaces.

Figure 4.1. Structure of carbohydrate derivatives of methyl methacrylate

Surface polymerization of our reactive carbohydrates on some chemically activated surfaces such as polystyrene 24-well plates and glass cover slips will make these surfaces more hydrophilic due to the presence of free hydroxyl functionalities in pendant sugar residues. The resulting surface modification enhances possible hydrophilic interactions including attachment of hydrogels or partially charged moieties in attempt to match passive protein surfaces.

The attachment of carbohydrates to surfaces may also be useful for immobilization of enzymes such as trypsin, chymotrypsin, glucose oxidase, and lipase(s) for the attraction of certain types of bacteria\(^{15-17}\).
4.2. Background and Significance

4.2.1. Surface modification of polymers

Surfaces of polymers may be modified by methods such as oxidation by flame or corona discharge, use of surfactants, oxidizing agents, and surface grafting. Growing multi-block layers of polymers directly from a surface (surface grafting) as opposed to the classical bulk synthesis offers many advantages including controlled linear chain growth, and the formation of permanent covalent bonds to the surface. Among different techniques for surface modification of polymers, surface grafting usually provides a good control of the nature of the new surface formed which is especially important for biomedical applications. However, since the properties specific for the surface only depend on the top layers, the grafted layer may be very thin and distinct.

Surface modification of polymeric materials such as polystyrene has received a great deal of attention during the past two decades. The goal is to confer a specific surface property such as adhesion or nonthrombogenicity, without affecting the bulk properties of the polymer. It is often desirable to make the surface more hydrophilic for biomedical use, such as surface heparin derivatization for the inhibition of blood coagulation.
4.2.2. Surface grafting

There are two different techniques to perform surface grafting of the polymers.

4.2.2.1. Technique of “grafting to”

Covalent attachment of the polymer chains to surfaces is called the “grafting to” approach which is accomplished via chemical reaction between the functional groups present on the surface and those along polymer chains (usually end-functionality). The advantage of such technique is that the molecular weight and the chain length of the grafted polymer are well-characterized. This technique is limited by the crowding of the chains at the surface hindering the diffusion of the chain ends to surface for further attachment\textsuperscript{20-21}. Therefore, it is difficult to obtain thick layers of polymer and the coating suffers from a low density of grafted chains.

4.2.2.2. Technique of “grafting from”

Covalent attachment of functionalized initiators to the solid substrate is called “grafting from” technique where the linear chains of polymer are grown from the surface using free-radical polymerization\textsuperscript{22-23}. This approach will provide a large number of polymer chains and achieve a high surface density of the chains. The advantages of the “grafting-from” technique include: (1) very thick and dense coatings can be routinely created; and (2) a wide variety of monomers can be polymerized since the monomer or polymer need not have any specific functional groups. The disadvantage of this technique is that the molecular weight and the chain length distributions of polymer chains formed cannot be easily controlled or measured.
4.2.3. Surface polymerization on polystyrene

Polystyrene is a long carbon chain with pendant benzene rings on every other carbon. The structure of polystyrene makes it a very hydrophobic compound and its hydrophobicity is retained when polystyrene is molded into solid objects such as 96 well plates. However, the surface can be easily modified by radiation or other methods that alter surface properties. The polystyrene can also be modified through chemical reactions to allow the covalent attachment of a variety of compounds containing reactive groups that can be used for subsequent covalent immobilization of biomolecules.

Modification of the surface of polystyrene through chemical reactions that allow covalent attachment of reactive groups (e.g. vinyl saccharides) can be used for subsequent covalent immobilization of biomolecules. Subsequently, the surface of
polystyrene can be made hydrophilic by attachment of methacrylated-based sugars in a
two step process which was developed by Bamford and Al-Lamee\textsuperscript{24}. This method
includes the hydroxylation of the polymer surface, using sodium persulfate, followed by
grafting initiated by thermal decomposition of hydroxyl groups at the surface in the
presence of ceric ammonium nitrate.

In the first step, sodium persulfate is used as hydroxylating agent of the polymer
P-H according to the following mechanism:

\[
\begin{align*}
S_2O_8^{2-} & \rightarrow 2SO_4^{-} \quad (1) \\
SO_4^{-} + H_2O & \rightarrow HSO_4^{-} + HO \cdot \quad (2) \\
HO \cdot + P-H & \rightarrow H_2O + P \quad (3) \\
HO \cdot + P & \rightarrow P-OH \quad (4) \\
2HO \cdot & \rightarrow H_2O_2 \quad (5)
\end{align*}
\]

After isolation of the hydroxylated polymer P-OH, decomposition of the hydroxyl groups
at the surface in the presence of ceric ammonium nitrate generates alkoxy radicals P-O which
can initiate the grafting based on the following reaction:

\[
P-OH + Ce^{IV} \rightarrow P-O \cdot + H^+ + Ce^{III}
\]

Hydroxyl radicals can also initiate some homopolymerization close to the surface.

**4.2.4. Polymerization on surface of glass**

The surface grafting of polymers onto a glass plate could be accomplished by the
polymerization of vinyl monomers initiated by initiating groups introduced onto the
surface. It has been reported that azo\textsuperscript{25}, peroxyster\textsuperscript{26}, potassium carboxylate\textsuperscript{27}, and
benzylium perchlorate\textsuperscript{28} groups introduced onto an ultrafine silica surface have abilities
to initiate anionic, cationic, and radical graft polymerization respectively. The wettability of the glass plate surface can be controlled by the grafting of the polymers onto the surface such that the glass plate will show a hydrophilic characteristic by grafting of hydrophilic polymers and vice versa. In this type of polymerization, the grafting of the polymers onto the glass plate surface is initiated by introducing the azo groups onto glass surface. Radical graft polymerization is initiated using azo-treated glass plates, so called “GP-Azo”. Figure 4.2 illustrates the steps in thermal graft polymerization onto glass plate initiated by azo groups.

Figure 4.3. Steps in thermal graft polymerization onto glass plate by azo group initiator
4.2.5. Reducing sugars

Carbohydrates containing a free or potentially free aldehydo or keto group can be oxidized. These sugars are called reducing sugars. Most naturally occurring mono- and disaccharides are reducing sugars. Several colorimetric methodologies have been developed to determine the amount of sugars in samples quantitatively using reducing sugar assays. This total carbohydrate microanalysis could be used to obtain the average degree of polymerization or molecular weight of polysaccharides. This micro-scale reducing sugar assay could also be applied in quantitative determination of carbohydrates immobilized on solid surfaces such as polystyrene or glass plate.

1,2:5,6-di-\(O\)-isopropylidene-\(\alpha\)-D-glucofuranose (DIGlc) is a non-reducing monosaccharide since C1-C2 are protected with isopropylidene group. Thus, 3O-methacryoyl-1,2:5,6-di-\(O\)-isopropylidene-\(\alpha\)-D-glucofuranose (3M-DIGlc) is also considered a non-reducing sugar monomer. Removal of the isopropylidene group from C1-C2 in this monomer or the corresponding polymer gives a reducing sugar.

![Diagrams of DIGlc, 3M-DIGlc, and 3M-Glc](image.png)

**Figure 4.4. Comparison between reducing sugar compounds with non-reducing ones**

Reducing sugar assay is one of the most convenient methods to quantitatively determine the amount of polymer (methacrylated-based sugars) produced on the surface.
of these plates (polystyrene or glass). Removing the blocking group on these polymers afford the required structure for oxidation of C1 by different oxidizing conditions.

This is due to the fact that the resulting polymers obtained on the surface of polystyrene or glass contains only one aldehyde functional group at the reducing end of the polymer. These polymers have the aldehyde functionality at each monomeric repeating unit along the chain. The unique reducing ability of the carbohydrate moiety of the obtained water soluble polymers is used to determine the amount of polymers attached to the surface.

![Chemical structure](image)

Figure 4.5. Oxidation on C1 of polymer (P-3M-DIGlc)
4.3. Experimental Section

4.3.1. Materials

All organic solvents were purchased from Sigma-Aldrich and used without further purifications. Tolylene-2,4-diisocyanate (TDI) and 4,4’-Azobis (4-cyanopentanoic acid) (ACPA) were obtained from Wako Chemical Co and used as received. Disodium 2,2’-bicinchoninate, copper sulfate pentahydrate, L-serine were purchased from Sigma. The synthesis of the monomers and polymers used were based on published procedures29. Tissue-cultured treated and untreated polystyrene plates were purchased from Falcon. The glass plates (glass cover-slips) were obtained from Fisher Scientific.

4.3.2. Methods of analysis

A Synergy HT-1 micro sample plate reader (Bio-Tek), with interference filters was used to measure absorbance of samples in a 24-well polystyrene plate. The use of a plate reader to measure absorbance has several advantages involving ease of sample handling, uniform treatment of many samples, direct transfer of data to a computer, and the speed and convenience of obtaining measurements of many samples in a relatively short time. The latter can improve the precision of the analyses by easily allowing triplicate determinations of each sample. Contents of the plate are conveniently agitated with a vortex mixer.
4.3.2.1. Reducing Sugar determination

A number of methodologies were studied to determine the amount of sugar reduced in glucose, galactose, or their polymers (poly-3M-glucose and poly-6M-galactose) including copper-bicinchoninate and Park-Johnson’s methods. The best assay for this study was found to be the copper-bicinchoninate method. The appropriate concentration of glucose, galactose, or their polymers is crucial for this assay to give accurate results. The concentration range of sugar standards (glucose or galactose) and respective polymer standards being used was 12-87 µM and 25-100 µM respectively.

4.3.2.1.1. Reducing sugar by copper-bicinchoninate

Reducing sugars determination using this reagent was based on a published procedure with some modifications. Two stock solutions were prepared: Solution A consisted of 97:1 mg of disodium 2,2’-bicinchoninate (Sigma Chemical Co.) dissolved in 45 mL of water containing 3.2 g of sodium carbonate monohydrate and 1.2 g of sodium bicarbonate. The final volume was adjusted to 50 mL. Solution B consisted of 62 mg of copper sulfate pentahydrate and 63 mg of L-serine dissolved in 45 mL of water, and the final volume was diluted to 50 mL. The working reagent was prepared daily by mixing equal volumes of Solutions A and B.

The procedure consisted of adding 100 µL of each sample to 100 µL of working reagent in the wells of a 24-well plate. The plate was covered with Saran wrap and heated in the oven at 70°C for 30 min. The plate was then cooled for 15 min and the absorbance measured at 560 nm. Triplicate analyses were performed for each sample.
4.3.2.1.2. Reducing sugar by Park-Johnson’s method

This colorimetric method was based on Park-Johnson’s method with some modifications to enhance the solubility and stability of the chromophore by increasing the concentration of aqueous sulfuric acid and sodium dodecyl sulfate (SDS). Four stock solutions were prepared: Solution A consisted of 0.5 g potassium ferricyanide in 1000 mL distilled water (D.W.). Solution B contained 5.3 g sodium carbonate and 0.65 g potassium cyanide in 1 L D.W. Solution C consisted of 1.5 g ferric ammonium sulfate and 1.0 g SDS in 1 L of 0.05 N sulfuric acid and solution D contained 1.5 g ferric ammonium sulfate and 2.0 g SDS in 1000 mL of 0.2N sulfuric acid. Fresh stock solutions were prepared every 4-6 weeks to assure the stability of the material. Each sample in 150 µL water was mixed with 50 µL of solution A and 50 µL of solution B. The mixture was heated in a boiling water bath for 10 min, cooled to RT, and 250 µL of solution C or solution D was added. The solution was mixed well with a pipette, transferred to a 96-well microplate and the absorbance at 690 nm measured using a microplate reader. Triplicate analyses were performed for each sample.
4.3.3. General procedure for obtaining standard curves

Reducing sugar standards consisted of either 1.4-22.6 µg/mL of glucose or galactose or 15.5-248.2 µg/mL of their corresponding deblocked polymer. Each sample was assayed with both copper bicinchoniniate and Park-Johnson’s methodology and the calibration curve of absorbance versus concentration was obtained. Each experiment was repeated three times with different 24-well plates at different days and triplicate analyses were performed for each sample.

4.3.4. Surface polymerization on polystyrene surface by Bamford’s method

This polymerization was based on a modified procedure published by Bamford and colleagues. This procedure is a two-stage process, of which the first is hydroxylation and the second polymer grafting. Hydroxylation is achieved by reaction in an aqueous solution of an alkali-metal peroxydisulfate at 80ºC with vigorous nitrogen purging. This step is followed by grafting using conventional ceric ion technique at the same temperature for 16 h. It was found that the amount of polymer grafted on the surface is too small after 4 h for reducing sugar assay to detect. Therefore, the results shown in section 4.4 are the experiments completed after 16 h.

Due to the high temperature of the reaction solutions, each experiment was also performed without purging with nitrogen to study the effect of oxygen content in polymerization.
4.3.4.1. General procedure for grafting 3M-DIGlc or 6M-DIGal on surface of polystyrene 24-well plates

The polystyrene plate was soaked in 10% solution of Na₂S₂O₈ and heated at 60°C in the oven with purging N₂ for 20 min. After cooling the plate to RT, the plate was rinsed with D.I. water several times. To each well, 1 mL of varied concentrations of monomer (5, 10, 20% w/v in butanol) and Ce(NH₄)₃NO₃ (1 mL) was added and the plate was heated at 60°C in the oven with a blanket of nitrogen for 16 h. After cooling the plate to RT, it was washed copiously with water and left to dry in air. The polymer obtained on the surface was then treated with 10% HCl at 60°C overnight to deprotect 1,2-isopropylidene functionality. After 16-18 h, deblocked polymer was neutralized with NaOH and assayed with copper-bicinchoninate for reducing sugar. Positive blanks of glucose (28 µM, 0.01 mg/mL) and deblocked polymer (20 µM, 0.1 mg/mL) were also tested against the grafted polymers. Each grafting experiment was repeated three times using different polystyrene plates at different days and triplicate reducing sugar assays were also performed on each sample. It must also be noted that both tissue-culture treated polystyrene plates and untreated polystyrene plates were used to study the effect of material on the plate on results of grafting.
4.3.4.2. General procedure for grafting 3M-DIGlc or 6M-DIGal on surface of glass

The glass plates were washed with chloroform and treated with 1.0 M HCl solution at 50°C for 8 h. The resulting glass plates were repeatedly washed with water and dried in vacuum at 110°C before use. After cooling the glass plates, they were treated with excess TDI (5 mL) in 60 mL DMSO at 60°C for 4 h. Then, the plates were cooled to RT and treated with ACPA (2.0 g) and the reaction continued at RT for 8 h. The plates were washed with methanol 3-4 times and dried in vacuum at RT. One of the plates was put in a 3-neck RB-flask with a small magnetic stir-bar. Into the flask, 100 mg of monomer and 5 mL of benzene was added and purged under a stream of N₂ for 10 min and sealed. The polymerization was carried under stirring at 80°C for 8 h. The glass plate was then washed with THF 3-4 times and dried in vacuum. The deprotection of isopropylidene groups on the polymer obtained on glass plate was achieved with using 10% HCl at 50°C for 16 h followed by neutralization with 50% NaOH solution. The amount of polymer obtained on the glass was determined using Cu-bicinchoninate reducing sugar assay.

Figure 4.7. Structures of two important components of surface grafting onto glass

Tolylene-2,4-diisocyanate (TDI)  4,4' Azobis (4-cyanopentanoic acid) (ACPA)
4.4. Results

4.4.1. Standard Curve for glucose and deblocked methacrylated glucose polymer using copper bicinchoninate assay

Five varied concentrations of glucose and deblocked methacrylated polymer of glucose (poly-3M-glucopyranose) was assayed using copper bicinchoninate colorimetric method. Figure 4.7 illustrates the standard curve obtained from this assay. We have found that covering the plate with Saran wrap was effective in preventing losses by evaporation during the heating step. A linear regression has been obtained using the above concentration range (12-87 µM of glucose and 25-100 µM of deblocked methacrylated glucose polymer). In order to simplify calculation of the amount of polymer grafted on the surface of polystyrene or glass plates, the calibration curve is shown as weight of the polymer in mg versus absorbance.

![Graph showing standard curve for glucose and glucose polymer using copper bicinchoninate assay](image)

Figure 4.8. Standard curve for glucose and glucose polymer using copper bicinchoninate assay
4.4.2. Standard Curve for glucose and deblocked methacrylated glucose polymer using Park-Johnson’s assay

A number of varied concentrations of glucose and deblocked methacrylated polymer of glucose (poly-3M-glucopyranose) was assayed using Park-Johnson’s colorimetric method. Figure 4.8 illustrates the standard curve obtained from this assay. A linear regression has been obtained using the above concentration range (12-87 µM of glucose and 25-100 µM of deblocked methacrylated glucose polymer). The calibration curve is shown as weight of the polymer in mg versus absorbance to simplify the determination of amount of monomer polymerized onto surface of polystyrene or glass plates. Due to unsatisfactory value of $R^2$ and irreproducibility of this calibration curve, this colorimetric method was not used to determine the amount of polymer grafted onto surface of polystyrene or glass plates.

![Abs vs Polymer/Glucose](image)

Figure 4.9. Standard curve for glucose and glucose polymer using Park-Johnson’s method
4.4.3. Calibration curve for galactose and deblocked methacrylated galactose polymer using copper bicinchoninate

Five different concentrations of galactose and deblocked methacrylated polymer of galactose (poly-6M-galactopyranose) was assayed using copper bicinchoninate colorimetric method. Figure 4.9 illustrates the calibration curve obtained for these compounds. A linear regression was also observed using same concentration range as mentioned above (12-87 µM of galactose and 25-100 µM of deblocked methacrylated galactose polymer). The obtained standard curve was used for the quantitative determination of galactose polymers being attached to the surface of polystyrene or glass plate.

Figure 4.10. Standard curve for galactose and galactose polymer using copper-bicinchoninate assay
4.4.4. Calibration curve for galactose and deblocked methacrylated galactose polymer using Park-Johnson’s assay

Varied concentrations of galactose and deblocked methacrylated polymer of galactose (poly-6M-galactopyranose) was assayed using Park-Johnson’s colorimetric method. Figure 4.10 illustrates the calibration curve obtained from this study. A linear regression was also observed using same concentration range as mentioned before (12-87 µM of galactose and 25-100 µM of deblocked methacrylated galactose polymer).

Even though the obtained value of $R^2$ was satisfactory enough, the obtained standard curve was not used for the quantitative determination of galactose polymer attached to the surface of polystyrene or glass plate. This was mainly due to irreproducibility of the data. Future analysis will be necessary to determine a better concentration range of these compounds to obtain more reproducible results.

![Abs vs Polymer / Galactose](image)

**Figure 4.11. Standard curve for galactose and galactose polymer using Park-Johnson’s method**
4.4.5. Surface grafting of glucose monomer onto polystyrene

Upon completion of polymerization of 3M-DIGlc on surface of polystyrene and removal of diisopropylidene functionality using HCl, deblocked polymer was neutralized using sodium hydroxide. This sample (100 µL) was mixed with 100 µL of copper-bicinchoninate reagent (1:1, Reagent A: Reagent B) and assayed for reducing sugar. Positive blanks of glucose (28 µM, 0.01 mg/mL) and deblocked polymer (20 µM, 0.1 mg/mL) was also tested against the grafted polymer. Results shown below are average of triplicate studies. Based on the equation obtained from the calibration curve of standard polymer (y = 5.3293x), the amount of the polymer grafted onto surface of polystyrene was calculated as shown.

<table>
<thead>
<tr>
<th>Monomer loaded (w/v)</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.052</td>
<td>0.085</td>
<td>0.217</td>
</tr>
<tr>
<td>Weight of polymer (µg)</td>
<td>9.8</td>
<td>15.9</td>
<td>40.7</td>
</tr>
</tbody>
</table>

Table 4.1. Polymer glucose grafted on surface of polystyrene
4.4.6. Surface grafting of galactose monomer onto polystyrene

Upon completion of polymerization of 6M-DIGalc on surface of polystyrene and removal of diisopropylidene functionality using HCl, deblocked polymer was neutralized by 50% NaOH solution. This sample (100 µL) was mixed with 100 µL of copper-bicinchoninate reagent (1:1, Reagent A: Reagent B) and assayed for reducing sugar. Positive blanks of galactose (28 µM, 0.01 mg/mL) and deblocked polymer (20 µM, 0.1 mg/mL) was also tested against the grafted polymer. The results of galactose polymer are shown in the table below. Based on the equation obtained from the calibration curve of standard polymer (\(y = 0.5324x\)), the amount of the polymer grafted on the surface was calculated as shown in table 4.2.

The results indicate that the amount of monomer galactose being polymerized on the surface of polystyrene is substantially higher than the amount of glucose polymer which could explain the higher affinity of galactose polymers for surface of polystyrene.

<table>
<thead>
<tr>
<th>Monomer loaded (w/v)</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.032</td>
<td>0.097</td>
<td>0.158</td>
</tr>
<tr>
<td>Weight of polymer (µg)</td>
<td>60</td>
<td>182</td>
<td>297</td>
</tr>
</tbody>
</table>

Table 4.2. Polymer galactose grafted on surface of polystyrene
4.4.7. Surface grafting of glucose and galactose monomer onto glass plate

Upon completion of polymerization of 3M-DIGlc on surface of glass coverslip and removal of diisopropylidene functionality using HCl, deblocked polymer was neutralized using a 50% of sodium hydroxide solution. Since, the monomer could be attached to either side of the glass coverslip, deprotection of isopropylidene groups, as well as neutralization and reducing sugar assays were all completed in a test tube. Table 4.3 shows the results obtained from copper bicinchoninate reducing sugar assay indicating the amount of glucose and galactose polymer attached to surface of glass plate.

<table>
<thead>
<tr>
<th>Type of monomer</th>
<th>3M-DIGlu</th>
<th>6M-DIGal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>0.156</td>
<td>0.060</td>
</tr>
<tr>
<td>Amount of polymer on glass (μg)</td>
<td>29.3</td>
<td>112.7</td>
</tr>
</tbody>
</table>

Table 4.3. Polymer glucose or galactose on surface of glass
4.5. Discussion

This chapter describes a number of techniques used to polymerize 3MPGlu on the surface of polystyrene 24-well plates and glass cover slips. It also explains two different methodologies for quantitative determination of carbohydrates in a solution using reducing sugar assays. The reducing sugar assays were used to determine the amount of polymers attached to the surface of polystyrene or glass plate.

We have shown that surface of polystyrene can be modified through chemical reactions that allow covalent attachment of reactive groups (such as our methacrylated-based sugars, 3M-DIGlu and 6M-DIGal). We have also demonstrated that methacrylated-based sugars can be polymerized onto a glass surface by use of initiators such as azo groups introduced onto the surface prior to polymerization. This modification will switch their hydrophobic environment to a hydrophilic one due to the presence of free hydroxyl functionalities in pendant sugar residues. Due to unsatisfactory results obtained from Park-Johnson’s reducing sugar assay, the amount of polymer attached to surface of polystyrene or glass plate was determined using copper bicinchoninate assay.

The resulting surface modification enhances the hydrophilic interactions including attachment of hydrogels or partially charged moieties in order to interact with protein surfaces. The resulting polymers will be investigated for their ability to immobilize enzymes such as trypsin, chymotrypsin, glucose oxidase, and lipase(s). The amount of enzyme immobilized, their kinetics and activity will be evaluated by well-established and facile assay techniques. Attachment of sulfated methacrylated-based monomers (Carb. Res., In Press) on polystyrene and glass surfaces is in progress.
This study describes efficient method for permanent surface modification of organic and inorganic polymeric materials utilizing concept of primary reactive polymer layer. The method provides nearly universal approach to effective covalent attachment of low molecular weight of polymeric substances such as biomolecules. This methodology may be used to modify surface of materials and devices to improve properties such as biocompatibility, wettability, adhesion, lubrication, resistance, repellency, membrane selectivity, molecular recognition, colloidal stability and dispersivity.
4.6. References


Bibliography

Chapter One


Chapter Two


47. Henry-Stanley, M. J.; Shepherd, M.; Sivertson, M.; and Wells, C. “Heparin disaccharides affect enterocyte interactions with *Staphylococcus aureus*”, poster presentation, University of Minnesota.


Chapter Three


15. http://w3.to/autodock


Chapter Four


