Title
Development of Analytical Methods for Trace Impurity Analysis and Structure Determination of Heparin/Heparan Sulfate-Derived Oligosaccharides

Permalink
https://escholarship.org/uc/item/1kb6d9gq

Author
Eldridge, Stacie Liane

Publication Date
2009

Supplemental Material
https://escholarship.org/uc/item/1kb6d9gq#supplemental

Peer reviewed|Thesis/dissertation
Development of Analytical Methods for Trace Impurity Analysis and Structure Determination of Heparin/Heparan Sulfate-Derived Oligosaccharides

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Chemistry

by

Stacie Liane Eldridge

August 2009

Dissertation Committee:
Dr. Cynthia K. Larive, Chairperson
Dr. Dallas Rabenstein
Dr. Wenwan Zhong
The Dissertation of Stacie Liane Eldridge is approved:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

                                          Committee Chairperson

University of California, Riverside
Acknowledgements

I am blessed to have had the opportunity to pursue two careers in my lifetime. Without the love and encouragement from my parents, this would not have been possible. Their unconditional support for me and care for my son in my absence has allowed me to change my place in the world and provide a better life for my family. I will be eternally indebted to them. I would like to thank my son, Mason, for giving me inspiration every step along the way. I love him more than he will ever know. I would also like to thank my brother, Paul, sister-in-law, Denise, and my beautiful nieces, Alexis and Kellirae. Their continual support and interest in my pursuits means the world to me. My best friend, Pattie, has been very instrumental to my journey here. She has always encouraged me to chase my dreams, however big, and is a constant reminder of how powerful unreserved determination can be.

I want to extend sincere thanks to my academic family. First and foremost to my advisor, Dr. Cynthia Larive. Her vision for my future extended far beyond my own. She was patient with me, yet persistent, and taught me that to succeed often times means leaving my comfort zone. I am forever grateful to her for making me a better scientist. To Dr. Albert Korir for taking me under his wing and teaching me (as much as I was willing to learn) the intricacies of cITP and microcoil NMR. I am proud to call him a friend and colleague. To Dr. Bridget Becker, for helping me acclimate to the group in my first year and for her friendship. To the members of the Larive Research Group; Jenn Cruz, for always listening to me without judgment, being there, and supporting me through it all. John Limtiaco, who will make a great NMR spectroscopists someday. Thank you for all
of your general help and advice. Kayla Kaiser, a powerful woman in her own right, thank you for your teaching and presentation suggestions and unrelenting support. You will go far no matter where life takes you. Chris Jones, thank you for your friendship and camaraderie. You could always be counted on, especially for sushi. To my former and present labmates: Christiana Merrywell, Kasie Fang, Greg Barding, Derek Langsley, and Daryl Bulloch, thank you for the laughter and friendships. I would also like to thank my academic grandfather, Prof. Dallas Rabenstein for his unwavering support and interest in both my research and life.

I have an overwhelming sense of gratitude to Dr. Deborah Parsons. She is the person that guided me towards chemistry and sensed an innate ability in me to conquer new frontiers. She saw me through my first criminal justice class, transition to chemistry, my first academic job interview, and acceptance to graduate school. She has always been by my biggest fan, standing on the sidelines, cheering me on, and doing her best to steer me in the right direction. Thank you. I also want to express appreciation to the Chemistry faculty and staff of Cal State University, San Bernardino for helping me get here.

Above all else, I want to thank the love of my life, Lynn Snodgrass, for her unconditional support, understanding, patience, and friendship. For the long nights she spent alone while I wrote this dissertation and the many meals she made for me so I could continue uninterrupted… for listening to me and providing me peace over the last four years. I think Karen Carpenter said it best when she said, “I can take all the madness the world has to give, but I won’t last a day without you”. I love you and cannot thank you enough.
Copyright Acknowledgements

The text and figures in Chapter 1 are a reprint of the material as it appears in *Advances in Chromatography*, **2008**, 46, 351-390. Copyright 2008 by Taylor & Francis Group.

Figure 1.5 in Chapter 1 was reprinted with permission from *Analytical Chemistry*, **1998**, 70, 3280-3285. Copyright **1998** American Chemical Society.

The text and figures in Chapter 2 were reprinted with permission from *Analytical Chemistry*, **2007**, 79, 8446-8453. Copyright **2007** American Chemical Society.

The text and figures in Chapter 3 were reprinted from *Carbohydrate Research*, Vol 343, Eldridge, S. L.; Korir, A. K.; Gutierrez, S. M.; Campos, F.; Limtiaco, J. F. K.; and Larive, C. K., Heterogeneity of depolymerized heparin SEC fractions: to pool or not to pool?, 2963-2970, Copyright **2008**, with permission from Elsevier.

The text and figures in Chapter 4 were reprinted with permission from *Analytical Chemistry*, **2009**, ASAP, DOI 10.1021/ac901218q. Copyright **2009** American Chemical Society.
This dissertation is dedicated to my sons Mason and James Eldridge.

Let this be testament that anything is possible

through hard work and determination.

To my parents for their unwavering love and support

and finally, to Lynn, for providing me love and sanctuary.
ABSTRACT OF THE DISSERTATION

Development of Analytical Methods for Trace Impurity Analysis and Structure Determination of Heparin/Heparan Sulfate-Derived Oligosaccharides

by

Stacie Liane Eldridge

Doctor of Philosophy, Graduate Program in Chemistry
University of California, Riverside, August 2009
Dr. Cynthia K. Larive, Chairperson

The goal of this dissertation is to develop improved analytical methods and approaches that address the difficulties in separating and detecting pharmaceutical impurities and heparin oligosaccharides. The specific methods used were capillary electrophoresis with ultraviolet detection (CE-UV), liquid chromatography-mass spectrometry (LC-MS), with special emphasis given to capillary isotachophoresis-nuclear magnetic resonance (cITP-NMR).

The FDA requires structural identification of all pharmaceutical impurities and degradants present at levels ≥ 0.1% of the parent compound. This poses a significant analytical challenge since these impurities are typically present in trace amounts along with high levels of the parent drug and matrix components. Greater complications arise in the analysis of heparin and heparan sulfate (HS). These highly sulfated linear polysaccharides display a wide range of biological activities through interaction with proteins. The enzymatic modifications that occur during biosynthesis for the purposes of cell adaptation and regulation, contribute to the heterogeneity of heparin and HS, making them a challenge to characterize.
Progress in the areas of impurity and heparin/HS substructure analysis requires the use of NMR for molecular characterization. NMR is a powerful analytical tool despite its intrinsically poor sensitivity when compared to other analytical methods. This limitation becomes magnified when the structure elucidation of concentration or mass-limited compounds is required. This problem is addressed in this work by coupling microcoil NMR to the pre-concentration method cITP. Microcoil NMR is an inexpensive way to increase the intrinsic sensitivity of NMR, and cITP can concentrate analytes up to 2 to 3 orders of magnitude. This makes cITP-NMR ideal for studying analytes that are mass- or volume-limited.

Results are presented illustrating the ability of cITP-NMR to separate and detect charged impurities in the presence of 1000-fold excess of the parent compound. Also, cITP-NMR was used to develop a disaccharide chemical shift database and probe intracapillary pD, providing insight into processes that drive cITP separations. NMR, together with CE-UV and LC-MS, facilitated the characterization of several physico-chemical properties of heparin di- and tetrasaccharides, including functional group pK\textsubscript{a} values, with the goal of advancing the methods for the separation and structural characterization of heparin and HS oligosaccharides.

SUPPLEMENTARY MATERIALS:

Video file for Figure 5.7. Cationic cITP focusing of 100 µM methyl green dye

Video file for Figure 5.8. Anionic cITP focusing of 100 µM bromophenol blue dye

Video file for Figure 5.9. Counter ion migration in cationic cITP

Video file for Figure 5.14. Anionic cITP of bromophenol blue using the TE buffer, ACES
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Copyright Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>viii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xxv</td>
</tr>
<tr>
<td><strong>Chapter One: Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction to NMR Spectroscopy</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1 Fundamentals of NMR</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2 NMR Sensitivity</td>
<td>6</td>
</tr>
<tr>
<td>1.1.3 Microcoil NMR</td>
<td>12</td>
</tr>
<tr>
<td>1.2 On-Line NMR Detection for Separations</td>
<td>17</td>
</tr>
<tr>
<td>1.2.1 Stopped-Flow NMR</td>
<td>23</td>
</tr>
<tr>
<td>1.2.2 Solid-Phase Extraction-NMR (SPE-NMR)</td>
<td>25</td>
</tr>
<tr>
<td>1.2.3 Applications of Hyphenated NMR Techniques</td>
<td>25</td>
</tr>
<tr>
<td>1.2.3.1 Liquid Chromatographic-NMR (LC-NMR)</td>
<td>26</td>
</tr>
<tr>
<td>1.2.3.2 LC-NMR-Mass Spectrometry (LC-NMR-MS)</td>
<td>27</td>
</tr>
<tr>
<td>1.2.4 Electrophoretic Techniques</td>
<td>28</td>
</tr>
<tr>
<td>1.2.4.1 Capillary Electrophoresis-NMR (CE-NMR)</td>
<td>32</td>
</tr>
<tr>
<td>1.2.4.2 Capillary Electrochromatography-NMR (CEC-NMR)</td>
<td>35</td>
</tr>
<tr>
<td>1.2.4.3 Capillary Isotachophoresis-NMR (cITP-NMR)</td>
<td>36</td>
</tr>
<tr>
<td>1.3 Impurity Analysis of Pharmaceutical Substances</td>
<td>38</td>
</tr>
<tr>
<td>1.3.1 Impurities and Drug Stability</td>
<td>39</td>
</tr>
<tr>
<td>1.3.2 Evaluating and Profiling Impurities</td>
<td>40</td>
</tr>
<tr>
<td>1.3.3 Analytical Challenges of Impurity Identification</td>
<td>41</td>
</tr>
<tr>
<td>1.4 Heparin and Heparan Sulfate Analysis</td>
<td>43</td>
</tr>
<tr>
<td>1.4.1 Biological Functions and Protein Binding</td>
<td>44</td>
</tr>
<tr>
<td>1.4.2 Heparin and HS Microstructure and Biosynthesis</td>
<td>46</td>
</tr>
<tr>
<td>1.4.3 Analytical Challenges</td>
<td>51</td>
</tr>
<tr>
<td>1.5 Summary</td>
<td>51</td>
</tr>
</tbody>
</table>
Chapter Two: Development of Cationic cITP-NMR for the Separation and Analysis of Trace Degradation Products in a Pharmaceutical Formulation

2.1 Introduction: Structure Elucidation of Trace Degradants in Acetaminophen Using cITP-NMR

2.1.1 Applying cITP-NMR for the Separation and Detection of Thermal Degradation Products in Acetaminophen Samples

2.2 Experimental Section

2.2.1 Chemicals

2.2.2 Acetaminophen Sample Spiked with 4-Aminophenol

2.2.3 Acetaminophen Degradation Sample in D$_2$O Solution

2.2.4 Acetaminophen Degradation Sample in Aqueous Solution

2.2.5 On-Line cITP-NMR Experiments

2.2.6 Off-Line cITP-NMR Experiments

2.2.7 Capillary Electrophoresis (CE) Experiments

2.2.8 LC-MS Experiments

2.3 Results and Discussion

2.3.1 Analytical Considerations in the Trace Analysis of Degradation Samples: Acetaminophen Model

2.3.2 cITP-NMR Analysis of Spiked Acetaminophen Sample

2.3.3 cITP-NMR Analysis of Forced Degradation Acetaminophen Sample in D$_2$O

2.3.4 Preliminary LC-MS/MS Analysis of the Forced Degradation Acetaminophen Sample in D$_2$O

2.3.5 CE and cITP-NMR Analysis of the Forced Degradation Acetaminophen Sample in H$_2$O

2.3.6 LC-MS/MS Analysis of the Forced Degradation Acetaminophen Sample in H$_2$O

2.4 Summary

2.5 References

Chapter Three: Examining the Heterogeneity of Preparative-Scale SEC Fractions Obtained from Enzymatic Digests of Heparin
## 3.1 Introduction: Depolymerization and SEC Separation of Heparin

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 Experimental Section</td>
<td></td>
</tr>
<tr>
<td>3.2.1 Chemicals</td>
<td>96</td>
</tr>
<tr>
<td>3.2.2 Enzymatic Digest of Heparin</td>
<td>96</td>
</tr>
<tr>
<td>3.2.3 SEC Fractionation</td>
<td>97</td>
</tr>
<tr>
<td>3.2.4 Capillary Electrophoresis</td>
<td>97</td>
</tr>
<tr>
<td>3.2.5 Ultraprformance Liquid Chromatography (UPLC)-UV-MS</td>
<td>99</td>
</tr>
<tr>
<td>3.3 Results and Discussion</td>
<td></td>
</tr>
<tr>
<td>3.3.1 Enzymatic Depolymerization and SEC Separation of Porcine Heparin</td>
<td>100</td>
</tr>
<tr>
<td>3.3.2 CE-UV Analysis of SEC Fractions</td>
<td>103</td>
</tr>
<tr>
<td>3.3.3 Reversed-Phase Ion-Pairing (RPIP)-UPLC-UV-MS</td>
<td>111</td>
</tr>
<tr>
<td>3.4 Summary</td>
<td>116</td>
</tr>
<tr>
<td>3.5 References</td>
<td>117</td>
</tr>
</tbody>
</table>

## Chapter Four: Analysis and Characterization of the Physico-Chemical Properties of Heparin Disaccharides

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction: Structural Properties of Heparin and Heparan Sulfate</td>
<td>120</td>
</tr>
<tr>
<td>4.1.1 Chemical Analysis by Capillary Electrophoresis-UV (CE-UV)</td>
<td>121</td>
</tr>
<tr>
<td>4.1.2 Chemical Analysis by cITP-NMR</td>
<td>124</td>
</tr>
<tr>
<td>4.1.3 Ring Conformations of Heparin Monosaccharides and Mutarotation of GlcN Residues</td>
<td>125</td>
</tr>
<tr>
<td>4.1.4 pKₐ Measurements of Heparin and Heparan Sulfate Substructures</td>
<td>127</td>
</tr>
<tr>
<td>4.2 Experimental Section</td>
<td>129</td>
</tr>
<tr>
<td>4.2.1 Chemicals</td>
<td>129</td>
</tr>
<tr>
<td>4.2.2 Capillary Electrophoresis (CE-UV) Experiments</td>
<td></td>
</tr>
<tr>
<td>4.2.2.1 CE Separation in Formate Buffer</td>
<td>130</td>
</tr>
<tr>
<td>4.2.2.2 CE Separation in Phosphate Buffer</td>
<td>131</td>
</tr>
<tr>
<td>4.2.3 cITP-NMR Experiments</td>
<td>131</td>
</tr>
<tr>
<td>4.2.3.1 Anionic cITP of Heparin Disaccharides IS-IIS, IA-IIIA, and IH</td>
<td>132</td>
</tr>
<tr>
<td>4.2.3.2 Cationic cITP of Heparin Disaccharide IVH</td>
<td>132</td>
</tr>
<tr>
<td>4.2.4 Isolation of Heparin-Derived Tetrasaccharides for Titration</td>
<td>133</td>
</tr>
<tr>
<td>4.2.4.1 Enzymatic Digestion of Heparin</td>
<td>133</td>
</tr>
<tr>
<td>4.2.4.2 SEC Fractionation</td>
<td>133</td>
</tr>
<tr>
<td>4.2.4.3 Separation and Isolation of Heparin-Derived Tetrasaccharides</td>
<td>135</td>
</tr>
</tbody>
</table>
4.2.4.4 Removal of Tributylammonium Ion From Tetrasaccharide Isolates .................................................. 136

4.2.5 Solution Preparation for Indicator Titrations .......................................................... 137
4.2.6 Solution Preparation for Heparin Disaccharide Titrations ............................................. 139
4.2.7 Solution Preparation for Heparin Tetrasaccharide Titrations .......................................... 140
4.2.8 $^1$H NMR Experiments ................................................................. 140
   4.2.8.1 Two-dimensional DQF-COSY (double-quantum filtered COSY) ............................. 143
   4.2.8.2 Two-Dimensional Total Correlation Spectroscopy (TOCSY) ..................................... 145
   4.2.8.3 Water Attenuation by Transverse Relaxation (WATR) .......................................... 146

4.3 Results and Discussion ................................................................. 147
4.3.1 Determination of Heparin Disaccharide $pK_a$ Values .............................................. 147
   4.3.1.1 $^1$H NMR pD Titration of the Iduronic Acid Carboxyl Group .................................. 149
   4.3.1.2 $^1$H NMR pD Titration of the GlcN Ammonium Group .......................................... 154
4.3.2 Determination of Heparin-Derived Tetrasaccharide $pK_a$’s Using NMR .......................... 157
4.3.3 Determination of Disaccharide Mutarotation Equilibrium Constants ............................ 163
4.3.4 Development of cITP-NMR Database for Heparin Disaccharides ......................... 163
   4.3.4.1 cITP-NMR Focusing of Disaccharides IS-IIIS, IA-IIIA, and IH ................................. 165
   4.3.4.2 cITP-NMR Focusing of Disaccharide IVH ........................................................ 168
   4.3.4.3 cITP-NMR of Disaccharides IVA and IIH-IIIIH .................................................... 171
4.3.5 CE-UV Separation of Heparin Disaccharides .............................................................. 174
4.3.6 Mobility Measurements of Heparin Disaccharides ..................................................... 178
4.3.7 Insights into the CE Separation of Heparin Disaccharides Using NMR $pK_a$ and Electrophoretic Mobility Measurements .......................................................... 179

4.4 Summary ................................................................................. 184

4.5 References .............................................................................. 187

Chapter Five: Investigating cITP Separation Processes Using CE, NMR, and Stereo Microscopy .......................................................................................................................... 193

5.1 A Closer Look at Capillary Isotachophoresis ................................................................. 193
   5.1.1 Characteristics of the Steady Isotachophoretic State ................................................. 195
   5.1.2 Requirements of Isotachophoresis ........................................................................... 198
   5.1.3 The Role of Counter Ions in cITP ........................................................................... 199
5.1.3.1 Counter Ions in Acid-Base Equilibria........................................ 199
5.1.3.2 Counter Ions in Complex-Forming Equilibria............................ 200
5.1.4 Frontal Migration of H⁺ and OH⁻............................................. 204
5.1.5 Studying Electromigration Behavior of Ions in cITP....................... 205
  5.1.5.1 Using NMR to Study Migration Behavior of Ions During cITP........ 207
  5.1.5.2 Using Microscopy to Study Migration Behavior of Ions During cITP 208
5.1.6 Development of Anionic cITP for Heparin Disaccharides IVA, IIH, and IIIH......................................................... 210
5.1.7 Development of Anionic cITP for Heparin Tetrasaccharides Using Complex-Forming Equilibria................................................. 212

5.2 Experimental Section........................................................................ 214
  5.2.1 Chemicals.................................................................................... 214
  5.2.2 Microscopic Visualization Experiments........................................ 215
    5.2.2.1 Solution Preparation of Steady-State cITP Solutions................. 215
    5.2.2.2 Solution Preparation of Counter Ion cITP Solutions............... 216
  5.2.3 Capillary Electrophoresis (CE-UV) Experiments.......................... 217
    5.2.3.1 CE-UV Mobility Measurements of Heparin Disaccharide Standards at pH 6.50.............................................. 218
    5.2.3.2 Separation of MES and Heparin Disaccharide IVA in Phosphate Buffer................................................................. 219
    5.2.3.3 Separation of ACES and Heparin Disaccharides IVA and IIH in Phosphate Buffer..................................................... 219
  5.2.4 Off-Line cITP Experiments Using Optical Detection..................... 219
  5.2.5 On-Line cITP-NMR Experiments................................................ 220
    5.2.5.1 cITP-NMR of Heparin Disaccharide IVA Using ACES TE Buffer......................................................... 221
    5.2.5.2 cITP-NMR of Formate and Acetate Using ACES TE Buffer to Probe Intracapillary pD............................................. 221
  5.2.6 NMR pD Titration Experiments..................................................... 222
  5.2.7 cITP-NMR Separations of Heparin Tetrasaccharides Using BTP.... 223

5.3 Results and Discussion....................................................................... 224
  5.3.1 Microscopic Visualization of Steady-State cITP............................ 224
  5.3.2 Microscopic Visualization of Counter Ions in Cationic cITP......... 226
  5.3.3 Anionic cITP-NMR of Heparin Disaccharides IVA, IIH, and IIIH 230
    5.3.3.1 Utilizing CE to Investigate the Electrophoretic Mobility of MES................................................................. 230
    5.3.3.2 Utilizing CE to Investigate the Electrophoretic Mobility of ACES........................................................................ 236
    5.3.3.3 cITP-NMR Focusing of Heparin Disaccharide IVA Employing ACES as a TE Buffer............................................. 236
5.3.3.4 Intracapillary pD Determination ................................. 242
5.3.4 Anionic cITP-NMR of Heparin Tetrasaccharides: Exploring Complex-Forming Equilibria Using BTP ........................................... 246

5.4 Summary ........................................................................................................... 257
5.5 References ......................................................................................................... 258

Chapter Six: Conclusions and Future Directions .................................................. 263

6.1 Conclusions ....................................................................................................... 263
6.2 Future Directions ................................................................................................ 265
  6.2.1 Stopped-Flow cITP-NMR ........................................................................... 265
  6.2.2 cITP-NMR Database of Larger Oligosaccharides ....................................... 266
  6.2.3 pKₐ Determination of Tetrasaccharides Containing N-Unsubstituted Glucosamine Residues ......................................................... 267
  6.2.4 Studies of Heparan Sulfate Structures Important for Enzyme Recognition ...................................................................................... 268
  6.2.5 Investigation of Counter Ion Migration in cITP ......................................... 269

6.3 References ......................................................................................................... 271
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1</strong></td>
<td>9</td>
</tr>
<tr>
<td>RF coil geometries and their orientation with respect to the magnetic field, ( B_0 ). (A) a saddle or Helmoltz RF coil and (B) a solenoidal coil, shown here wrapped directly around a separation capillary. For the saddle coil, ( V_s ) and ( V_{obs} ) are the volume of the sample in the NMR tube and the volume observed by the detection coil, respectively.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.2</strong></td>
<td>16</td>
</tr>
<tr>
<td>The homebuilt microcoil (^1)H NMR probe used in this research with the polypropylene bottle cut away to allow a view of the contents. A 14-turn microcoil (magnification shown on right) is wrapped around a polyimide sleeve and stabilized between two shoulders of a U-shaped plastic circuit board. A capillary is threaded through the sleeve for on-line electrophoretic separations. The observe volume of the microcoil is 25 nL.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.3</strong></td>
<td>19</td>
</tr>
<tr>
<td>(A) Representation of a chromatographic peak, ( V_s ), eluted in a volume that closely matches the flow cell of the NMR probe. (B) A chromatographic peak in a volume that is much larger than the NMR flow cell. (C) A chromatographic peak in a volume much smaller than the flow cell of the NMR probe.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.4</strong></td>
<td>30</td>
</tr>
<tr>
<td>Representation of the diffuse double layer responsible for electroosmotic flow in capillary electrophoresis.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.5</strong></td>
<td>34</td>
</tr>
<tr>
<td><strong>Figure 1.6</strong></td>
<td>37</td>
</tr>
<tr>
<td>The cITP separation of charged analytes: (A) The sample is injected between the leading electrolyte (LE) and the trailing electrolyte (TE). (B and C) Upon application of an electric field, the sample ions separate into discrete zones based on their electrophoretic mobilities. (D) A steady-state is reached in which all zones travel uniformly at the same velocity and the mixing of zones is prevented.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.7</strong></td>
<td>48</td>
</tr>
<tr>
<td>Monosaccharide building blocks of heparin and heparan sulfate.</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 1.8</strong></td>
<td>50</td>
</tr>
</tbody>
</table>
(A) Pentasaccharide sequence responsible for anticoagulant activity. (B) Major repeating disaccharide unit of heparin.

**Figure 2.1** Structures of (A) acetaminophen and its major degradant, (B) 4-aminophenol (PAP).

**Figure 2.2** A schematic of the cITP-NMR set-up.

**Figure 2.3** Static 600 MHz $^1$H NMR spectra of (A) acetaminophen, (B) PAP, and (C) matrix resonances from the acetaminophen tablet.

**Figure 2.4** A portion of on-line cITP-NMR spectra showing resonances of 0.1% PAP spiked in the acetaminophen tablet matrix. Each spectrum was produced by averaging 8 scans over 11.2 seconds. Spectra 2.4 A and B contain only the HOD resonance of the deuterated LE. Spectra 2.4 C-G provide a spectral profile of the focused PAP band as it passes through the microcoil detector. Spectra 2.4 H and I again contain only the HOD resonance of the TE.

**Figure 2.5** A portion of on-line cITP-NMR spectra showing resonances of PAP (spectra 2.5 B-F) and unexpected unknown components (spectra 2.5 G-K) from the degradation sample in D$_2$O. Spectra 2.5 A and L were measured for the LE and TE, respectively.

**Figure 2.6** Total ion chromatogram (TIC) of the degradation sample in D$_2$O after direct injection onto the LC-MS following cITP-NMR analysis.

**Figure 2.7** (A) Total ion chromatogram (TIC) of the degradation sample in D$_2$O after separation and concentration using off-line cITP. Unknown degradation products are indicated by an asterisk. (B) MS spectrum of TIC peak with molecular ion $m/z$ 246.127.

**Figure 2.8** CE-UV Electropherograms of (A) a mixture of 4 mM PAP and 6 mM acetaminophen standards and (B) the aqueous degradation sample.

**Figure 2.9** A portion of on-line cITP-NMR spectra showing resonances of the unknown degradation components (spectra 2.9 A-F) from the degradation sample in H$_2$O.
Figure 2.10. (A) MS/MS spectrum of the molecular ion \( m/z \) 243.125. The fragmentation pattern in (A) is complementary to that of (B), \( m/z \) 259.113. (C) MS/MS spectrum of molecular ion \( m/z \) 273.080, which is also similar to (D) \( m/z \) 257.077. The proposed structures for the unknown thermal degradation products are labeled on the far right next to the corresponding parent ion. The structures postulated for specific fragments are also shown for each mass spectrum.

Figure 3.1. Cleavage of heparin by heparinase I. Heparinase I specifically cleaves glycosidic linkages between GlcNS(±6S) and IdoA(±2S) residues of GAG chains via a \( \beta \)-elimination.

Figure 3.2. The preparative SEC-UV chromatogram showing relative absorbance versus fraction number for the separation of the components produced by ED1.

Figure 3.3. Electropherogram showing the separation of 10 commercially available disaccharide standards from Table 3.1 in 60 mM formic acid buffer at pH 3.43.

Figure 3.4. (A) Electropherogram of fraction 216 from the dp2 peak of the preparative SEC separation of ED1. The peaks migrating at 6.35 and 8.37 minutes were identified as IS and IIIS, respectively, by spiking the fraction 216 sample solution with standards of heparin disaccharide IIIS (B) and IS (C).

Figure 3.5. CE electropherograms of fraction 201 (A) and fraction 219 (B) from the dp 2 peak of the preparative SEC separation of ED1.

Figure 3.6. A plot of the peak area ratios of disaccharides IIIS and IS (□) and relative disaccharide concentration (▲) versus fraction number demonstrating the variability in the abundance of IS and IIIS across the dp 2 peak shown in Figure 3.2.

Figure 3.7. CE electropherograms of the preparative SEC dp 4 fractions 162 (A), 163 (B), 168 (C), and 176 (D) for ED1. The three major tetrasaccharide components are labeled P1, P2, and P3.

Figure 3.8. CE-UV electropherogram of SEC fraction 72 of the dp 4 peak of ED2.
Figure 3.9. RPIP-UPLC-UV chromatogram of fraction 72 measured with UV detection at 232 nm. The major peaks are labeled P1-P5.

Figure 3.10. (A) Extracted mass spectrum of the most intense absorbance peak (P5) from the RPIP-UPLC-UV chromatogram shown in Figure 3.9. The molecular ion, \( m/z \) 575.9639, confirms the identity of this tetrasaccharide as IS-IS. (B) Mass spectrum of P2 and (C) P4 from the same chromatogram.

Figure 4.1. Ring conformations of the monosaccharide residues of heparin and heparan sulfate. (A) The predominant \( ^4C_1 \) chair form of glucuronic acid (GlcA) and glucosamine (GlcNS6S shown). (B) The equilibrium mixture of \( ^4C_1, ^1C_4 \), and \( ^2S_0 \) conformers of IdoA (IdoA(2S) shown). (C) The major, \( ^2H_1 \), and minor, \( ^1H_2 \), form of unsaturated uronic acid residues (\( \Delta UA2S \) shown).

Figure 4.2. Preparative scale SEC-UV chromatogram showing relative absorbance versus fraction for the separation of the heparin oligosaccharides produced by enzymatic digestion with heparinase I. The peak with the greatest absorbance (fractions 357-440) corresponds to the disaccharide components (dp2), the preceding peak (fractions 318-356) corresponds to tetrasaccharides (dp4), followed by hexasaccharides (dp6) and larger oligosaccharides (fractions 186-317).

Figure 4.3. (A) NMR spectra of 1.12 mM heparin-derived disaccharide IS spiked with 2.1 mM tributylammonium acetate. Tributylammonium acetate resonances at 3.167, 1.703, 1.412, and 0.967 ppm were decreased by a factor of 78.4 overall following batch ion-exchange using Na\(^+\) cation resin. (B) Spectra taken after the first ion-exchange step and (C) after the second step.

Figure 4.4. The three major tetrasaccharides derived from enzymatic depolymerization with Heparinase I.

Figure 4.5. NMR pulse sequences for (A) DQF-COSY, (B) TOCSY, and (C) CPMG.

Figure 4.6. (A) \(^1\)H NMR titration plot for formate (p\( K_a \)(D) = 4.04 ± 0.01). The limiting chemical shifts of formic acid and formate are 8.222 and 8.446 ppm, respectively. (B) \(^1\)H NMR
titration plot for imidazole ($pK_a(D) = 7.63 \pm 0.01$). The limiting chemical shifts for imidazolium and imidazole are 8.689 and 7.762 ppm, respectively.

Figure 4.7..........................................................151
Titration of the carboxylate group following the change in chemical shift of the IIIS disaccharide H4’ resonance indicated with a circle. The $pK_a(D)$ value is 3.74 ± 0.01.

Figure 4.8..........................................................155
Titration of the ammonium group following the change in chemical shift of the IVH disaccharide α anomer H2 resonance indicated with a circle. The $pK_a(D)$ value is 8.68 ± 0.01.

Figure 4.9..........................................................156
DQF-COSY spectrum of heparin disaccharide IIH at pH 7.80. Because mutarotation of the GlcN ring is slow on the NMR time scale, the resolved resonances of each anomer can be assigned as shown by the labeled α and β resonances of the H2 proton between 2.5 and 3.5 ppm.

Figure 4.10..........................................................159
UPLC chromatogram showing the separation of the three major tetrasaccharides derived from enzymatic digestion of porcine heparin with heparinase I.

Figure 4.11..........................................................160
Titration of the carboxylate group following the change in chemical shift of the IS-IIIS tetrasaccharide ΔUA2S H4’ resonance as indicated by a circle. The $pK_a(D)$ value is 3.84 ± 0.01.

Figure 4.12..........................................................164
$^1$H NMR spectra showing the GlcN H2 resonances of disaccharides IS, IH, IIIS, and IIIH. The doublet of doublets between 3.2 and 3.4 ppm is the H2 resonance of the α anomer and the triplet between 2.8-3.1 ppm is the H2 resonance of the β anomer. Mutarotation equilibrium constants determined by integration of these resonances are listed in Table 4.2.

Figure 4.13..........................................................166
The cITP-NMR spectra of heparin disaccharide IS. Spectra 4.13 A and B contain the resonances of imidazole (8.558 and 7.432 ppm), HOD (4.807 ppm), and the chemical shift reference, tert-butanol (1.236 ppm). Spectra 4.13 C-F are of IS. Anomeric resonances can be clearly seen at 5.497 and 5.444 ppm, along with the most downfield resonance of the ΔUA2S H4 proton at 5.955 ppm. The resonances of the TE buffer, MES and imidazole are shown in spectra 4.13 G and H.
cITP-NMR spectra of the heparin disaccharide standards IH, IS, and IA obtained by post acquisition coaddition of the individual cITP-NMR spectra.

**Figure 4.15**
CITP-NMR spectra of the heparin disaccharide standards IIIS, IIS, and IS obtained by post acquisition coaddition of the individual cITP-NMR spectra.

**Figure 4.16**
H NMR spectra from the cITP-NMR focusing of heparin disaccharide IVH. NMR spectra 4.16 A-D contain resonances of the α anomer migrating at the front of the focused analyte band as indicated by the H1α and H2α resonances at 5.444 and 3.339 ppm, respectively. Spectra 4.16 E-G contain a mixture of the α and β anomers of IVH, and spectra 4.16 H and I contain only the β anomer as indicated by the H1β and H2β resonances at 4.945 and 3.054 ppm, respectively.

**Figure 4.17**
A portion of the H NMR spectra produced by the cITP-NMR experiment performed to focus heparin disaccharide standard IVA. Spectra 4.17 A-C contains only the LE resonances of imidazole (8.558 and 7.430 ppm), the solvent HOD (4.809 ppm), and tert-butanol (1.236 ppm). Spectra 4.17 D and E contain the additional TE resonances of MES between 2.5 and 4.0 ppm. The absence of spectra in between the LE and TE indicates that the analyte failed to focus.

**Figure 4.18**
Electropherogram of 10 commercially available disaccharide standards from Table 4.1 in 60 mM formic acid buffer, pH 3.43. The inset shows an expansion of the region between 4 and 15 minutes. The peak marked with an asterisk (*) in the electropherogram is benzenesulfonic acid, used as an internal standard.

**Figure 4.19**
Electropherogram of 10 commercially available disaccharide standards from Table 4.1 in 50 mM phosphate buffer, pH 3.50. The inset shows an expansion of the region between 4 and 13 minutes. The peak marked with an asterisk (*) in the electropherogram is benzenesulfonic acid, used as an internal standard.

**Figure 4.20**
Titration conducted in 95% H2O/5% D2O of the carboxylate group following the change in chemical shift of the IS disaccharide H4’ resonance as indicated by a circle. The pKa(H) value is 3.16 ± 0.02.

**Figure 4.21**
Correlation of electrophoretic mobility at pH 3.43 determined by CE and effective net charge-to-mass ratios (q/m) calculated using the pKa(H) values for each disaccharide. The
mobility of disaccharide IVH is not displayed due to its cationic behavior under these conditions.

**Figure 4.22.** Electropherogram of SEC fraction 327 from enzymatically digested heparin using heparinase I. The peaks at 5.43, 5.71, and 5.85 min correspond to tetrasaccharide IS-IS, IS-IIS, and IS-IIIS, respectively. The CE separation was performed under reversed polarity using 50 mM phosphate buffer, pH 3.50.

**Figure 5.1.** Properties of isotachophoretic zones L, X, and T. (A) Distribution of the zones at steady-state, (B) field strength, $E$, (C) conductivity, $\kappa$, and (D) effective mobility $\mu_e$.

**Figure 5.2.** Buffering in isotachophoretic zones. (A) Cationic system – the pH in the sample zone of a weak base, X, is buffered by the weak acid HA and its anion A$^-$, which form the counter ionic system. (B) Anionic system – the pH in the sample zone of a weak acid, HX, is buffered by the weak base, B, and its protonated form BH$^+$, which form the counter ionic system.

**Figure 5.3.** Examples of complex-forming equilibria in isotachophoretic systems. (A) The analyte ion X$^-$ forms a kinetically labile complex with counter ion, M$^+$, of the LE ($X^- + M^+ \leftrightarrow MX$). The terminating ion, T$, is present at the rear boundary for the recombination reaction. (B) A neutral ligand, L$^0$, is present in the LE and upon complexation to form LX$^-$, attenuates the migration of X. (C) Analyte, Y$^+$, is opposite in charge of the LE and TE and is converted to the anion, YT, due to a complex formation reaction with the terminating ion, T$. In all systems M$^+$ is the counter ion of the LE.

**Figure 5.4.** (A) The uncontrolled migration of free OH$^-$ ions in anionic cITP are shown migrating through all boundaries of the isotachophoretic zones. This is demonstrated by the dashed line. (B) The controlled migration of free OH$^-$ ions in anionic cITP. The OH$^-$ ions do not cross the boundary of the terminating ion, T$, and analyte, X$, as shown by the solid line. BH$^+$ and H$^+$ are the free counter ions in the LE available for the recombination reaction at the frontal boundary of the TE.

**Figure 5.5.** Visualization of counter ion migration. A colored counter ion, R$^{2-}$, that is introduced into the LE will form a colored complex, YR$, when it passes through the analyte zone, Y$^+$. 

**Figure 5.6.** Counter ion and dye molecules used in cITP-NMR experiments.
Figure 5.7 ................................................................. 225
Video clips of cationic cITP focusing of 100 µM methyl green dye. The distance between the black lines is 1 mm.

Figure 5.8 ................................................................. 227
Video clips of anionic cITP focusing of 100 µM bromophenol blue dye.

Figure 5.9 ................................................................. 229
Video clips recording the influence of counter ion, mordant blue 9, added to the LE on the cationic cITP focusing of methyl green at three different time points (A-C). Video clips D-F are of the progressive migration of the methyl green zone at time point C.

Figure 5.10 ................................................................. 233
CE electropherogram of 0.5 mM BSFA, 1 mM MES, and 0.5 mM IVA in 20 mM phosphate buffer, pH 6.54 migrating at 6.38, 11.63, and 13.32 min, respectively.

Figure 5.11 ................................................................. 235
CE electropherogram of 0.5 mM BSFA, 1 mM MES, and 0.5 mM IVA in 20 mM phosphate buffer, pH 5.61. Migration times for BSFA and IVA were 6.50 and 13.49 min, respectively. A separate run of MES alone demonstrated that there was an overlap in the migration times for IVA and MES.

Figure 5.12 ................................................................. 237
CE electropherogram of 0.5 mM BSFA, 0.29 mM IVA, and 1 mM ACES in 20 mM phosphate buffer, pH 6.50, migrating at 6.32, 13.28, and 21.00 min, respectively.

Figure 5.13 ................................................................. 238
CE electropherogram of 0.5 mM BSFA, 0.5 mM IIH, and 1 mM ACES in 20 mM phosphate buffer, pH 6.50, migrating at 6.22, 11.83, and 20.38 min, respectively.

Figure 5.14 ................................................................. 240
Video clips of anionic cITP of bromophenol blue using ACES as the TE buffer.

Figure 5.15 ................................................................. 241
Anionic cITP-NMR of IVA and BSFA using ACES as the TE buffer.

Figure 5.16 ................................................................. 243
NMR detected titration curves for (A) ACES and (B) acetate. The $pK_a(D)$ values were determined to be $7.32 \pm 0.00$ and $5.09 \pm 0.00$, respectively.

Figure 5.17 ................................................................. 245
cITP-NMR of acetate and BSFA using ACES as the TE buffer to probe intracapillary pD.

Figure 5.18 ................................................................. 247
cITP-NMR of tetrasaccharide fraction 342 and BSFA using 160 mM NaCl as the LE and 160 mM MES as the TE.

**Figure 5.19** A continuation of the cITP-NMR spectra shown in Figure 5.18: (A) the spectrum of tetrasaccharide 342, (B) the boundary of fraction 342 and BSFA, (C-E) spectra of BSFA, and (F) the boundary of BSFA and MES (TE buffer).

**Figure 5.20** cITP-NMR of tetrasaccharide fraction 342 and BSFA using 160 mM DCl / 20 mM BTP as the LE and 160 mM MES as the TE. Every fifth spectrum is plotted.

**Figure 5.21** cITP-NMR of tetrasaccharide 342 and BSFA using 160 mM DCl / 50 mM BTP as the LE and 160 mM MES as the TE.

**Figure 5.22** cITP-NMR of heparin tetrasaccharide IS-IS and BSFA using 160 mM NaCl / 80 mM imidazole as the LE and 160 mM MES as the TE. The cITP-NMR spectra of BSFA are not shown.
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>94</td>
</tr>
<tr>
<td>Legend for the structures of commercially available heparin disaccharide standards: IA-IVA, IS-IIIS, and IH-IIIH used in this work.</td>
<td></td>
</tr>
<tr>
<td>Table 4.1</td>
<td>123</td>
</tr>
<tr>
<td>Legend for the structures of commercially available heparin disaccharide standards: IA-IVA, IS-IIIS, and IH-IVH.</td>
<td></td>
</tr>
<tr>
<td>Table 4.2</td>
<td>153</td>
</tr>
<tr>
<td>pK_a(D) values, mutarotation equilibrium constants and electrophoretic mobilities determined for the heparin disaccharides.</td>
<td></td>
</tr>
<tr>
<td>Table 4.3</td>
<td>161</td>
</tr>
<tr>
<td>pK_a(D) values determined for the heparin-derived tetrasaccharides.</td>
<td></td>
</tr>
<tr>
<td>Table 4.4</td>
<td>170</td>
</tr>
<tr>
<td>^1H NMR chemical shifts (ppm) of heparin disaccharide standards (pH = 6.5) obtained from cITP-NMR experiments. Under the anionic cITP-NMR conditions stated in section 4.2.3.1, disaccharides IVA, IIH, and IIIH failed to form a focused isotachophoretic zone. Disaccharide IVH focused successfully using cationic cITP-NMR.</td>
<td></td>
</tr>
<tr>
<td>Table 4.5</td>
<td>182</td>
</tr>
<tr>
<td>pK_a(H) values calculated for the heparin disaccharides.</td>
<td></td>
</tr>
<tr>
<td>Table 5.1</td>
<td>232</td>
</tr>
<tr>
<td>Electrophoretic mobilities determined for the heparin disaccharides using 20 mM phosphate buffer at pH 6.50 and 25 °C.</td>
<td></td>
</tr>
<tr>
<td>Table 5.2</td>
<td>254</td>
</tr>
<tr>
<td>Summary of the cITP-NMR experiments performed to improve the focusing of heparin tetrasaccharides (fraction 342) using complex-forming equilibria. BTP was added to the LE as the counter ion.</td>
<td></td>
</tr>
</tbody>
</table>
Chapter One

Introduction

The research presented in this dissertation focuses on the development of capillary isotachophoresis-nuclear magnetic resonance (cITP-NMR), capillary electrophoresis with ultraviolet detection (CE-UV), and liquid chromatography-mass spectrometry (LC-MS) for analysis of pharmaceutical impurities and heparin-derived oligosaccharides. The FDA requires structural identification of all pharmaceutical impurities and degradants present at levels ≥ 0.1% of the parent compound. This poses a significant analytical challenge since these impurities are typically present in trace amounts along with high levels of parent drug and matrix components such as excipients. Even greater complications arise in the analysis of heparin and heparan sulfate (HS). Due to the enormous structural diversity and heterogeneity of these biopolymers, obtaining sufficient quantities of pure oligosaccharides, especially those that constitute rare protein binding motifs, is a tedious and laborious process often involving several separation steps. Since heparin and HS are at the forefront of drug discovery, both as targets and as candidates for new therapeutics, assembling a more complete knowledge of the microstructure of these biopolymers is crucial.

The ability of modern NMR experiments to reveal elements of structure makes it an essential tool for trace impurity analysis and the chemical analysis of heparin oligosaccharides. However, the Achilles’ heel of NMR is its poor limits of detection when compared to other analytical methods. This limitation becomes magnified when the structure elucidation of concentration or mass-limited compounds is required. The
development of solenoidal microcoil NMR probes with nanoliter detection volumes has greatly improved the mass limits of detection of NMR. However, this technique still suffers from poor concentration sensitivity and manual introduction of the sample into the miniaturized detection coils of these probes can be difficult. A strategy developed to overcome these limitations is the on-line coupling of microcoil NMR to the sample pre-concentration technique, capillary isotachophoresis (cITP-NMR). The ease of sample introduction and the ability to concentrate analytes up to 2 to 3 orders of magnitude, makes this method ideal for studying analytes that are mass- or volume-limited. Throughout this work, special emphasis is given to the use and further development of cITP coupled with microcoil NMR detection.

The goal of this dissertation is to propose improved analytical methods and approaches that address the difficulties in separating and detecting pharmaceutical impurities and heparin components and this is accomplished through the following objectives:

**Objective 1:** Explore cationic cITP-NMR and LC-MS/MS for the separation and analysis of trace degradants in a pharmaceutical formulation (Chapter 2).

**Objective 2:** Employ analytical techniques to probe the heterogeneity of depolymerized heparin SEC fractions for use in subsequent structural analysis (Chapter 3).
**Objective 3:** Characterize several of the physico-chemical properties of heparin di- and tetrasaccharides. (Chapter 4).

**Objective 4:** Use CE, NMR, and stereo microscopy to gain insights into the cITP separation processes of heparin di- and tetrasaccharides. (Chapter 5).

The first part of this introductory chapter presents background information about hyphenated chromatographic NMR techniques. This is followed by a discussion of the application of these techniques to the challenges of impurity analysis and structural characterization of heparin and HS oligosaccharides.

### 1.1 Introduction to NMR Spectroscopy

NMR spectroscopy is a powerful tool for chemical analysis and is often used to identify impurities or degradation products in a sample. NMR detection can be used for both qualitative and quantitative measurements, but is primarily used for structure elucidation. NMR is an important method for investigating structure with the ability to unambiguously determine connectivity and spatial arrangements of the atoms of a molecule. In addition, it is nondestructive, facilitating the possibility of subsequent analysis by other analytical techniques such as mass spectrometry (MS), which can provide complementary structural information.

Despite its many attributes, NMR detection suffers from two significant disadvantages. Although direct analysis of mixtures is possible where resonances of the
species of interest can be resolved, for complex mixtures analytes of interest must be separated prior to detection. For this reason, it is often necessary to subject sample components to a separation or extraction step prior to NMR analysis. These separation methods may include high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFE), or capillary electrophoresis (CE). Although fraction collection following a separation step can permit tube based off-line NMR measurements, there is interest in hyphenation of NMR with separation methods. Direct coupling of separations with NMR detection eliminates unnecessary transfer steps and minimizes possible sample loss, contamination, and degradation. The second disadvantage of NMR detection is its inherently poor sensitivity when compared with many other spectroscopic detection methods. This may hinder the analysis of samples that are mass-limited or components present at extremely low concentrations. In order to better understand how different NMR parameters affect sensitivity, it is instructive to review some of the basic principles of NMR spectroscopy.

1.1.1 Fundamentals of NMR

A spectrum is generated by absorption of a quantized unit of energy that causes a transition from a lower energy ground state to a higher energy excited state. The difference in energy between the two states is proportional to the frequency, $\nu$, and Planck’s constant, $h$,

$$\Delta E = h \nu. \quad (1.1)$$
In NMR spectroscopy, these energy differences are observed in the radio-frequency (rf) region of the electromagnetic spectrum and are extremely small, such that the populations in the ground and excited spin states are almost equal at room temperature. Energy differences in optical spectroscopy are much greater due to the higher frequency of the transition. For example, in ultraviolet (UV)-visible absorption spectroscopy, a wavelength of 500 nm corresponds to a frequency of $600 \times 10^{12}$ Hz, whereas NMR frequencies range from 60 to 900 MHz, corresponding to wavelengths on the order of 5 m to 30 cm, depending on the strength of the applied magnetic field. Since frequency is directly proportional to energy, the higher frequencies in optical spectroscopy lead to increased sensitivity when compared to NMR.

In NMR, the sensitivity is proportional to the difference in the populations of the upper and lower energy states, as given by the Boltzmann distribution,

$$\frac{N_l}{N_u} = e^{\frac{\Delta E}{k_B T}} = e^{\frac{\gamma B_0 \hbar}{k_B T}},$$  \hspace{1cm} (1.2)

where $N_l$ and $N_u$ are the populations of the lower and upper energy states, respectively. $\Delta E$ is the energy difference between the lower and upper states, $k_B$ is the Boltzmann constant ($1.38 \times 10^{-23}$ J/K), $\gamma$ is the magnetogyric ratio (a constant for a given type of nucleus), $B_0$ is the applied magnetic field in units of Tesla, $\hbar$ is Planck’s constant divided by $2\pi$ ($1.05 \times 10^{-34}$ J · s), and $T$ is temperature in units of Kelvin. The magnitude of the resonance frequency, $\nu$, and thus $\Delta E$, is proportional to the magnetic field strength

$$\nu = \left(\frac{\gamma}{2\pi}\right) B_0.$$  \hspace{1cm} (1.3)
Therefore, NMR is more sensitive at higher magnetic field strengths. However, even at the highest magnetic field strengths currently available, the energy difference between the upper and lower spin states is small. Aside from this fundamental limitation, there are also experimental parameters and hardware options that contribute to the sensitivity of an NMR measurement, as described in the following section.

### 1.1.2 NMR Sensitivity

In analytical chemistry, sensitivity is traditionally defined as the slope of the calibration curve resulting from a plot of the measured signal versus the amount of analyte being detected. Because sensitivity in NMR experiments depends on many different factors, such as type of probe, data acquisition and processing parameters, tuning, matching, digital resolution, and number of scans, NMR spectroscopists use the signal-to-noise ratio, $S/N$, as a figure of merit for sensitivity comparisons. Sensitivity is an inherent property of each NMR probe and is defined under a set of optimized conditions. Thus, the $S/N$ can be used as a direct indication of the relative sensitivity for comparisons between NMR probes or spectrometers from two different vendors.\(^2\) The $S/N$ is calculated for an NMR experiment as the peak signal divided by the rms noise, given by Equation 1.4, and is directly related to the performance of the rf coil. It was first succinctly defined by Hoult and Richards\(^5\) through the expression

$$S / N = \frac{k_{_B}(B_i / i)V_s N_S \gamma \hbar^2 I(I + 1)\alpha_0^2 / 3k_B T \sqrt{2}}{V_{\text{noise}}}.$$  \hspace{1cm} (1.4)
This equation demonstrates that the S/N for a given NMR resonance is proportional to the square of the nuclear precession frequency ($\omega_0$, rad/s), the magnitude of the transverse magnetic field ($B_i$) induced in the RF coil per unit current ($i$), the number of spins per unit volume ($N_S$), the sample volume ($V_s$), the spin angular momentum quantum number ($I$), and ($k_0$) a scaling constant that accounts for magnetic field inhomogeneities. The $S/N$ is inversely proportional to the thermal noise generated in the RF receiver and also from the sample ($V_{\text{noise}}$) as described by the Nyquist formula\textsuperscript{5, 6},

$$V_{\text{noise}} = \sqrt{4k_B T R_{\text{noise}} \Delta f}, \quad (1.5)$$

where $R_{\text{noise}}$, measured over a specified spectral width ($\Delta f$, Hz), is a representation of conductive losses from the coil itself, and magnetic (eddy current) and dielectric losses in the sample and surroundings. If the constants in Equation 1.4 are combined into a single term ($\eta$), the equation is condensed to

$$S / N = \eta \frac{\omega_0^2 B_i V_s}{V_{\text{noise}}}, \quad (1.6)$$

and these variables can be adjusted to improve the sensitivity of the NMR measurement.

As Beer’s law in absorption spectroscopy has a path length dependence, the observe volume, $V_{\text{obs}}$, of an NMR probe (also termed the active volume) is also an important determinant of the sensitivity of NMR measurements. The observe volume is the volume of analyte within or very near the coil which returns a signal and is often a fraction of the total sample volume, $V_{\text{tot}}$, injected into a flow system or placed in an NMR tube (proportional to $V_s$ in Equation 1.4). In the case of a tube (non-flow) probe, $V_{\text{tot}}$ is the
volume in the NMR tube; in the case of a flow probe, $V_{tot}$ is the flow cell volume plus the volume of sample in its connected plumbing. Assuming the sample is present at a uniform concentration, the relationship between these volume terms can be expressed in the following equation,

$$f_0 = \frac{V_{obs}}{V_{tot}},$$

where $f_0$ is known as the observe factor and defines the fraction of analyte that is actually in the coil and contributing to the NMR signal. Optimally, $f_0 = 1$ for maximum sensitivity ($V_{obs} = V_{tot}$). However, due to magnetic susceptibility differences at the solution/air and solution/container interfaces leading to local distortions of the magnetic field, the sample volume usually extends beyond the boundaries of the coil ($f_0 < 1$) in order to obtain good line shape. The best example of this is the required sample volume of 600-750 µL for a conventional 5 mm NMR probe. The actual $V_{obs}$ region inside the coil is only about 250 µL. A greater sample volume is needed than is actually detected, which presents a significant challenge in the case of mass-limited samples. A related parameter is the fill factor, $f_{fill}$, defined as the fraction of volume inside the observe coil that can be occupied by the sample,

$$f_{fill} = \frac{V_{obs}}{V_{coil}},$$

where $V_{coil}$ is the internal volume of the coil. Again, it would be expected that if $f_{fill} = 1$, sensitivity would be heightened. However as shown in Figure 1.1A, magnetic susceptibility effects generally require that the sample be maintained at some distance
Figure 1.1. RF coil geometries and their orientation with respect to the magnetic field, $B_0$; (A) a saddle or Helmholtz RF coil and (B) a solenoidal coil, shown here wrapped directly around a separation capillary. For the saddle coil, $V_s$ and $V_{obs}$ are the volume of the sample in the NMR tube and the volume observed by the detection coil, respectively.
from the coil to achieve sufficiently narrow and homogeneous line shape. In most probes, there is an air gap between the coil and the sample tube such that \( f_{\text{fill}} < 1 \). The walls of the sample container can further reduce \( f_{\text{fill}} \) as they increase the distance of the sample from the coil.

To assess the sensitivity of NMR probes and maximize the \( S/N \) for mass-limited samples, mass sensitivity (\( S_M \)) can be normalized according to the number of moles of analyte (\( mol \)) within \( V_{\text{obs}} \) during the acquisition time (\( t \)),

\[
S_M = \frac{S/N}{mol \sqrt{t_{\text{acq}}}}. \tag{1.9}
\]

This is a more commonly used figure of merit for microcoil probes due to the ability to associate the \( S/N \) directly to the number of detected nuclei. For experiments in which the sample concentration is fixed, for example by solubility, the concentration sensitivity, \( S_C \), can be calculated by substituting the number of moles, \( mol \), in the above equation with the concentration of the analyte, \( C \),

\[
S_C = \frac{S/N}{C \sqrt{t_{\text{acq}}}}. \tag{1.10}
\]

For a fixed acquisition time, \( S_C \) is the \( S/N \) per unit concentration and is directly proportional to \( V_{\text{obs}} \) through the sample volume term, \( V_S \), in Equation 1.4. Therefore, if a 4 mm diameter sample cell is replaced with a 3 mm diameter cell, keeping the length of the coil the same, both the concentration sensitivity and the observe volume will decrease by a factor of 1.78 because a smaller sample volume will be interrogated by the coil.

To heighten the sensitivity of NMR and lower the limits of detection the
traditional approach of signal averaging can provide significant gains, with spectral $S/N$ increasing as the square root of the number of transients coadded. However, there are practical limits on the gains that can be obtained, typically leveling off at acquisitions of 24-48 hours. In addition, signal averaging is not useful for real-time, online detection of separations.

Another approach for improving $S/N$ is the use of higher strength magnetic fields (e.g. 900 MHz or 21.1 Tesla) since sensitivity increases as $B_0^{7/4}$. Increasing the magnetic field has the advantage that in addition to improving sensitivity, it also increases the dispersion of NMR resonances and produces spectra with less second order coupling.

Improvements to RF coil design is another approach to improving NMR sensitivity. Since temperature and resistance are significant contributors of noise, some advances in coil design center around using superconducting materials and/or constructing coils that have small volume capacities. The former has resulted in the development of cryoprobes in which the coil and the preamplifier are cooled to ~20-25 K, while keeping the sample at room temperature. Through this technology it is possible to increase the $S/N$ by a factor of ~4 for samples with low conductivity by reducing the thermal noise generated by the coil and associated circuitry. However, for high salt or “lossy” samples, such as those from biological sources, practical improvements in the $S/N$ obtained with cryoprobes, and thus sensitivity, is significantly less. In recent years, the desire to characterize mass-limited as well as various volume-limited biological samples using NMR technology has prompted the development of solenoidal microcoils, which
also provide the dual advantage of significantly increasing the \( S/N \) of NMR measurements.

### 1.1.3 Microcoil NMR

Due to the present-day demands of microanalysis, advances in NMR detection have moved towards accommodating samples available in limited quantities, such as natural products, metabolites, or pharmaceutical impurities. This has stimulated the development of extremely small microcoils, capable of analyzing nanoscale amounts of material. There is no firm definition of the term “microcoil”, but it is generally used to refer to coils with \( V_{\text{obs}} \) on the order of a few microliters to as small as 1 nanoliter. This technology provides an inexpensive way to improve the sensitivity of NMR probes.

In contrast to the traditional saddle or Helmholtz coil that is vertically housed in the NMR probe, microcoil probes usually contain a solenoidal coil that is placed horizontal to the magnetic field, \( B_0 \), and in most cases, contains an observe volume of 1 \( \mu \)L or less (Figure 1.1B). There are both practical and theoretical benefits to constructing microscale NMR coils with a solenoidal geometry. From a practical point-of-view, it is easier to construct a small solenoidal coil with good homogeneity properties and it can be wound directly onto a separation capillary or flow cell offering an improved \( f_{\text{fill}} \). Winding the coil around a hollow polyimide sleeve that allows for the exchange of capillaries has also been reported. However, this can decrease \( f_{\text{fill}} \) depending on the dimensions of the capillary and sleeve. From a theoretical standpoint, it is useful to consider how the RF coil sensitivity scales with coil size. As seen in Equation 1.6, the term \( \frac{B_1}{i} \) represents the
sensitivity of the RF coil through the magnitude of the transverse magnetic field, $B_1$, produced by a given current, $i$, flowing through the windings of the coil.\textsuperscript{5,9} For a saddle coil, the coil sensitivity corresponds to,

$$\frac{B_1}{i} = \frac{n\mu_0\sqrt{3}}{\pi} \left[ \frac{2dh}{(d^2+h^2)^{3/2}} + \frac{2h}{d\sqrt{d^2+h^2}} \right], \quad (1.11)$$

where $n$ defines the number of turns in the coil, $\mu_0$ is the permeability of free space, and $h$ and $d$ are the height and diameter of the coil, respectively. For a solenoidal coil, sensitivity can be defined as

$$\frac{B_1}{i} = \frac{\mu_0n}{d_{coil}\sqrt{1+(h/d)^2}}, \quad (1.12)$$

providing $\frac{h}{d}$ remains constant. If the volume of the coil is designed to match the volume of the sample, the result is enhanced sensitivity and an increase in the generated signal. This holds true when coil diameters are between 3 mm and 100 $\mu$m. When the diameter is below 100 $\mu$m, improvement in $S/N$ with reduced coil diameter is minimal.\textsuperscript{2,6} It can be shown theoretically, and has been demonstrated experimentally, that the sensitivity of a solenoidal coil is about three times that of a saddle coil of the same size.\textsuperscript{5} In relation to the $S/N$ in Equation 1.4, it is also important to note that the major contributor of noise for NMR microcoils having a diameter less than 3 mm is from the resistance of the coil itself. The noise from the sample, even lossy biological samples, is negligible.\textsuperscript{12}

In order to obtain greater $S/N$, significant consideration must be given to the design of the microcoil. The geometry of the coil must be optimized with respect to the wire diameter, number of turns, and the interturn spacing.\textsuperscript{13} Another consideration is that
as the coil size becomes smaller, the external components of the coil (copper coating, adhesive, air) and surrounding circuitry contribute to the noise generated in the signal.\(^2\) Examples include transmission lines and impedance matching capacitors that can cause line broadening due to magnetic susceptibility variations in the local field near the sample. Following the work of Olson et al. a strategy used to counteract magnetic susceptibility effects is the use of a matching fluid that surrounds the RF coil.\(^2\) In our lab, we construct microcoils that are housed inside a 10 mL, low-density polyethylene bottle filled with a perfluorinated organic fluid, FC\(^\text{®}\) -43 (3M), consisting of a mixture of C12 branched fluorinated tributylamines with an average molecular weight of 670 g/mol. This fluid has a magnetic susceptibility closely matching that of copper, allowing the acquisition of high resolution spectra with line widths around 1-2 Hz.

Recently, a novel microcoil design has been introduced based on planar stripline technology, in which a thin strip of metal, in close contact with the sample generates RF \(B_1\) radiation by inserting the metal inside an external static field, \(B_0\).\(^{14}\) The RF radiation is confined by grounding metallic planes above and beneath the strip. The sensitivity of this design has been shown to be intrinsically higher than solenoidal coils for both liquid and solid samples.\(^{15}\) Earlier this year, Bart and co-workers integrated a microreactor to an NMR microfluidic chip containing a stripline coil.\(^{16}\) They were able to probe the reaction times of carbamate formation from toluene diisocyanate and pure ethanol, as well as, the acetylation of benzyl alcohol with acetyl chloride in the presence of N,N-diisopropylethylamine. In addition, they were able to test the suitability of the microfluidic stripline probe for metabolomics studies, successfully detecting 0.7 nmol of
alanine in a 600 nL sample of cerebrospinal fluid. This technology looks to be promising for microfluidic NMR applications.

The cITP-NMR spectra acquired in this research were generated using a microcoil probe built in-house (Figure 1.2). The microcoil consists of 50 µm polyurethane coated copper wire that is wrapped around a polyimide sleeve having an outer diameter of 430 µm and an inner diameter of 370 µm. This 14-turn microcoil is constructed with no spacing between the turns and is approximately 1 mm in length with a 25 nL observe volume. The microcoil is stabilized between two shoulders of a U-shaped plastic circuit board that contains grooves for inserting the polyimide sleeve and is secured with epoxy. The circuit board with the mounted coil is housed inside of a polypropylene bottle filled with matching fluid. The leads of the coil protrude through holes drilled in the bottle located parallel to the coil and are soldered to a nonmagnetic variable tuning capacitor. The capacitor is supported by two 20 AWG stranded wires, one of which is impedance matched to the 50-Ω RF transmission line. The microcoil is then impedance matched to 50 Ω at 600 MHz using an Agilent RF network analyzer 8712ET (300 kHz – 1,300 MHz) by measuring the input reflection coefficient ($S_{11}$).

1.2 On-Line NMR Detection for Separations

Several factors regarding sample handling and detection need to be considered when designing a hyphenated NMR experiment. First, many conventional HPLC separations require the use of high flow rates (mL/min) to achieve good chromatographic
Figure 1.2. The homebuilt microcoil $^1$H NMR probe used in this research with the polypropylene bottle cut away to allow a view of the contents. A 14-turn microcoil (magnification shown on right) is wrapped around a polyimide sleeve and stabilized between two shoulders of a U-shaped plastic circuit board. A capillary is threaded through the sleeve for on-line electrophoretic separations. The observe volume of the microcoil is 25 nL.
resolution and as a result, use large volumes of mobile phase. Coupling these techniques to NMR can be economically unfeasible due to the high cost of deuterated solvents. The consequence of using protonated solvents in HPLC-NMR is that the intense signals they produce lower the dynamic range of the analyte signals. This can be remedied through the use of solvent suppression methods, however these methods also reduce peaks of interest in close proximity to the solvent resonances, making resonance assignment difficult, particularly for unknown compounds. Introduction of capillary liquid chromatography columns (CapLC) and capillary electrophoresis (CE) coupled with on-line NMR detection has greatly reduced the amount of solvent required for these separations, permitting the use of deuterated solvents at reasonable cost.

Another factor to consider with hyphenated NMR experiments using chromatographic separations is the dilution of the analyte peak during the separation process. The chromatographic dilution, $D$, of a compound can be estimated by the equation,

$$D = \frac{C_0}{C_{\text{max}}} = \frac{r^2 \varepsilon \pi (1+k) \sqrt{2LH \pi}}{V_{\text{inj}}}, \quad (1.13)$$

where $C_0$ is the initial concentration of the analyte, $C_{\text{max}}$ is the final analyte concentration at the peak maximum, $r$ is the column radius, $\varepsilon$ is the column porosity, $k$ is the retention factor, $L$ is the column length, $H$ is the column plate height, and $V_{\text{inj}}$ the injected sample volume. As indicated by this equation, the diameter of the column significantly dictates the volume and thus, concentration of the eluting analyte peak. By using columns with smaller diameters the separation efficiency is increased. Separation efficiency in
chromatography is measured in terms of the number of theoretical plates, $N$, which is proportional to $L$ and inversely proportional to $H$. Decreasing the column diameter reduces bandbroadening by lowering the plate height and minimizing the adverse effects of mass-transfer processes, in turn, eluting peaks with a higher concentration of analyte. Since the sensitivity of NMR detection increases with a decrease in coil size, microscale LC columns with size-matched coils can provide superior $S_C$ of the analyte. It has been shown that a decrease in LC column i.d. from standard (4.6 mm) to capillary (0.32-0.18) size can increase the $S_C$ and peak intensity more than several 100-fold for a fixed (1 nmol) injection volume.17, 18

This effect of LC column i.d. on $S_C$ can be understood by relating the size of a chromatographic or electrophoretic peak to the observe volume of the NMR detection cell. Maximum sensitivity is achieved when the volume of the eluting peak, $V_s$, closely matches the detection volume of the NMR flow cell. To avoid peak distortion due to chromatographic effects and/or an improper $f_0$, the detection volume should be about one-third of the eluting peak volume.17 This situation is represented diagrammatically in Figure 1.3A, where the most intense portion of the chromatographic peak is centered in observe volume of the NMR flow cell. Figure 1.3B shows the case in which an eluting peak is much larger than the NMR flow cell. The consequence is loss in peak intensity and thus reduced detection sensitivity due to dispersion of the analytes. Similarly, separated analyte peaks that are much smaller than the flow cell of the NMR probe (Figure 1.3C) result in an insufficient $f_0$ ($f_0 > 0.33$), producing broad NMR resonances due to poor magnetic field uniformity. The flow cell volumes of commercial NMR
Figure 1.3. (A) Representation of a chromatographic peak, $V_s$, eluted in a volume that closely matches the flow cell of the NMR probe. (B) A chromatographic peak in a volume that is much larger than the NMR flow cell. (C) A chromatographic peak in a volume much smaller than the flow cell of the NMR probe.
probes range from about 5 $\mu$L (CapNMR™ Protasis/MRM Corp., Savoy, IL) to 120 $\mu$L for a typical LC-NMR flow probe sold by Varian or Bruker. However, the $V_{obs}$ of the conventional flow probe is 60 $\mu$L, so if a chromatographic peak with a width of 0.3 min elutes at a flow rate of 1.0 mL/min, the peak volume would be 300 $\mu$L. This results in the detection of only 1/5$^{th}$ of the peak.

When using NMR as a detector for on-line separations, consideration must also be given to how the sensitivity is affected by the movement of nuclei past the detection coil. Aside from the physical hardware setup, the chromatographic and spectroscopic parameters also play a role in the quality of the resulting data. Optimization of flow rate, solvent composition, and residence and acquisition times can result in better quality data. For online LC-NMR experiments, there is an inverse relationship between NMR sensitivity and chromatographic resolution. For example, if the flow rate is lowered, the residence time of the analyte in the detection cell is increased, allowing for a greater number of scans to be acquired. However, due to the diffusion of peaks in the transfer tubing between the LC column and the NMR probe, chromatographic resolution can be compromised.

The signal intensity and line shape are significantly affected by continuous-flow separation methods and, in the case of CE, additional broadening is produced by the electrophoretic current. The amount of time that a nucleus spends in the NMR flow cell or the residence time, $\tau$, is related to the observe volume and the applied flow rate, $F$,

$$\tau = \frac{V_{obs}}{F}. \quad (1.14)$$
For example, if $V_{\text{obs}}$ has a fixed volume of 1 µL (defined by the NMR coil) and an $F$ of 6 µL/min is used, the residence time for a pulsed spin is 10 seconds. By increasing the flow rate to 12 µL/min, the residence time is decreased to 5 seconds. This decreases the number of NMR acquisitions that can be recorded for a particular spin, thus decreasing the $S/N$ in the resulting NMR spectrum.

The residence time of flowing nuclei is also related to both the effective spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times

\[
\frac{1}{T_{1\text{flow}}} = \frac{1}{T_{1\text{static}}} + \frac{1}{\tau}, \quad (1.15)
\]

\[
\frac{1}{T_{2\text{flow}}} = \frac{1}{T_{2\text{static}}} + \frac{1}{\tau}, \quad (1.16)
\]

where $T_{1\text{flow}}$ and $T_{2\text{flow}}$ are the relaxation times in a flowing system and $T_{1\text{static}}$ and $T_{2\text{static}}$ are relaxation times for spins in a stationary system. In a flowing system, the $T_1$ relaxation time of the sample nuclei is reduced due to pulsed spins being constantly replaced by unpulsed spins in the active volume of the NMR detector. This allows for the use of a faster pulse repetition time, resulting in a greater $S/N$. However, the $T_2$ relaxation time of a sample spin may also be reduced. If the residence time ($\tau$) of the observed spin is too short to give sufficient time for full signal decay before it leaves the detection region, line broadening will occur. Because the NMR line width is inversely related to $T_2$

\[
W_{1\text{static}}^{1/2} = \frac{1}{\pi T_{2\text{static}}}, \quad (1.17)
\]

and in-turn, $\tau$, in a flow system,
faster flow rates result in broader line shapes and ultimately a decrease in $S/N$.\textsuperscript{20} The optimum flow rate for on-line NMR detection is a compromise based on the desired outcomes with respect to $S/N$, resonance line width, and chromatographic resolution.\textsuperscript{21}

In electrophoretic separations, the line width of an NMR resonance is also affected by current-induced magnetic field gradients accompanied by thermal effects. Since solenoidal microcoils are placed perpendicular to the static magnetic field, $B_0$, a second local magnetic field gradient is induced due to electrophoretic current.\textsuperscript{17, 22, 23} This induced magnetic field, $B_i$, can be described by the following equation,

$$B_i = \frac{\mu_0 i r}{2\pi R^2},$$

where $i$ is the electrophoretic current, $r$ is the radial distance from the center of the capillary, and $R$ is the internal diameter of the capillary. This local magnetic field adds to the disruption of the homogeneity in the overall magnetic field and can rarely be recovered by shimming. Since the local magnetic field produced by the electrophoretic current is directly proportional to the magnitude of the current, performing separations at higher voltages increases the magnetic field inhomogeneity resulting in increased line broadening.

\subsection{1.2.1. Stopped-Flow NMR}
One way to increase peak resolution and reduce the flow effects in chromatographic separations and current induced gradients of electrophoretic separation systems is to halt the flow during NMR detection. This strategy is often used for sample components that are mass-limited or present at low concentrations since the separated analyte is held in the NMR coil allowing extensive signal averaging. Stopped-flow LC requires careful calibration of the time it takes for a chromatographic peak to travel between the UV detector and the flow cell of the NMR probe. Automated LC-NMR systems have this ability integrated in the software. Once the peak of interest reaches the UV detector, a signal is activated that turns off the pump after the allotted transit time. In contrast to the on-flow system, once the peak is inside the detection cell, unlimited acquisitions can be conducted. However, a disadvantage of this approach is that during this acquisition, diffusion of separated peaks that are still in the transfer tubing or column can occur. This results in poorer chromatographic resolution and thus, reduced S/N in the subsequent NMR spectra of these later eluting peaks. Advancement in this area includes the development of a capillary LC-NMR (CapNMR) probe that can be coupled to capillary HPLC separations. This microcoil NMR probe utilizes small diameter capillaries in lieu of traditional PEEK (polyetheretherketone) tubing. Compared to conventional LC-NMR, this results in less band broadening in stopped-flow experiments. In addition to extended signal averaging, stopped-flow experiments allow the acquisition of the time-consuming 2D experiments that are usually required for structure elucidation.

An alternative approach to stopped-flow LC-NMR is the incorporation of a loop collector into the LC-NMR set-up. Once analytes are separated and then detected by the
UV detector, the operator can transfer selected peak(s) of interest into individual storage loops of \(~240\) µL each for subsequent NMR analysis. An advantage of loop collection is that several analytes can be directed individually to the NMR for analysis from a single chromatographic run. Also, some loop collectors contain several hundred storage loops allowing for many consecutive chromatographic separations to occur in parallel with NMR detection, essentially decoupling the separation and analysis steps. However, over time dilution of the analyte by diffusion within the loop will occur, which may reduce sensitivity when compared to stopped-flow experiments.

In CE-NMR, one way to employ stopped-flow techniques is to temporarily reduce or turn off the voltage once the analyte has reached the detection coil, so that NMR information can be obtained under static conditions. This retards or terminates the current induced magnetic field gradient, improving NMR sensitivity because of the improved line shape and the longer acquisition time. Stopped-flow CE was demonstrated by Olson et al. in which a separation voltage was applied for 15 s then switched off for 1 minute to acquire NMR data.\(^2^2\) Additionally, field-amplified sample stacking techniques were used by Olsen et al. to preconcentrate the analyte several fold during the separation process.\(^2^2\) Another approach to stopped-flow detection using CE was demonstrated by Wolters et al. through the use of an NMR probe constructed with multiple solenoidal microcoils for continuous-flow capillary electrophoresis.\(^2^3\) The electrophoretic flow from a single separation capillary was split into multiple outlets, each with its own detection coil. The outlets were cycled between electrophoretic flow and NMR detection to allow for continuous CE with stopped-flow detection.
1.2.2. Solid-Phase Extraction-NMR (SPE-NMR)

In lieu of using storage loops to trap chromatographic peaks, SPE is an alternative method for sample introduction in LC-NMR experiments. In this method, the eluent from the chromatographic separation is directed into SPE cartridges where the analyte of interest is trapped. Mobile phase solvents can then be removed by drying the cartridges with a suitable gas, such as nitrogen, before eluting the analyte with a small volume of deuterated organic solvent. Using LC-SPE-NMR for on-line ‘peak parking’ allows the use of protonated solvents during the chromatographic separation, saving valuable deuterated solvents of which only small volumes are needed for transfer from the SPE cartridge to the flow cell of the NMR probe. In addition, the need for solvent suppression is significantly reduced or no longer necessary. It is also possible to further increase the concentration of the analyte by performing multiple separations and trapping the same analyte repeatedly on an SPE cartridge. The major disadvantage of using SPE for peak trapping is loss of the most polar compounds due to poor retention by the SPE packing material and difficulty in eluting very hydrophobic compounds from the cartridge. Even so, many compounds suitable for LC-NMR analysis will have physicochemical properties amenable to analyses using SPE.

1.2.3. Applications of Hyphenated NMR Techniques

1.2.3.1. Liquid Chromatographic-NMR (LC-NMR)
Conventional LC-NMR includes the coupling of liquid chromatographic separations to NMR detection. This implies one of two designs; a direct connection between the HPLC column and the NMR detector via tubing with a diameter smaller than that of the column to reduce band broadening, or the use of storage loops in which the separated analytes are temporarily stored prior to NMR acquisition. Many of the practical considerations of this technique have been discussed in the above sections and several reviews can be found in the literature.\textsuperscript{2, 17, 27-29} Since the first publication on LC-NMR in 1978\textsuperscript{30}, significant improvements in probe design have helped to establish LC-NMR as an accepted method of choice for small volume or mass-limited samples. The most recent generation of commercially available LC-NMR probes includes the capillary NMR probe, which uses micron capillaries to connect the column to the flow cell and contains extremely small (~5 µL) flow cells that are oriented perpendicular to the static magnetic field.

Applications of LC-NMR include, but are not limited to, structure elucidation of natural products, pharmaceutical impurities, plant extracts, and analysis of biological fluids such as blood and urine.\textsuperscript{1} A recent application of LC-NMR studied the compositional analysis of human amniotic fluid (HAF) and identified metabolites that could not be detected by NMR alone due to significant spectral overlap of the NMR resonances.\textsuperscript{31} Approximately 60 metabolic compounds were detected including 5 that were identified in HAF for the first time. This enabled the expansion of the HAF database used in the detection of biomarkers for disorders during pregnancy. Another recent study reported the use of LC-NMR to chemically profile secondary metabolites from a marine
organism known as the Southern Australian Sponge. On-flow HPLC-NMR allowed the initial identification of major constituents in a dichloromethane extract of the sponge and stopped-flow experiments resulted in the partial identification of pentaprenylated $p$-quinol. In this study, the first complete assignment of this metabolite was achieved through subsequent heteronuclear 2D NMR experiments.

1.2.3.2 LC-NMR-Mass Spectrometry (LC-NMR-MS)

Hyphenated LC-NMR systems that incorporate MS detection exploit the greater sensitivity of MS, while gaining complementary structural information from NMR. Applications of LC-NMR-MS include the analysis of natural products, identification of drug metabolites, and the impurity analysis of pharmaceuticals. There are two possible configurations for this hyphenated technique depending upon the desired results. One possibility aligns the LC, NMR, and MS in series permitting NMR detection to occur first, followed by subsequent MS analysis. This configuration has several disadvantages; NMR is a less sensitive method than MS and NMR data collection can take hours or days to complete. Also, the build-up of backpressure at the interface to the MS can exceed that tolerated by most NMR flow probes causing leaks in the system. It is, therefore, more practical to configure MS and NMR detection in parallel. Following chromatographic separation, this design employs a splitter, which sends a small amount of sample (typically 5%) to the MS with the rest directed to the NMR flow cell. The MS data can be used to complement the NMR data for the purposes of structure elucidation.
or in lieu of UV detection to initiate stopped-flow NMR spectral acquisition, for example, in cases where the analyte of interest does not contain a UV chromophore.

In a recent study, a nanoSplitter was employed at the MS interface that delivered only a fraction of the HPLC eluent (<0.1%) to the MS and to rest to an automated microdroplet NMR loading system for microcoil NMR analysis. The system was evaluated using a mixture of four commercial drugs and then applied to the analysis of a bioactive cyanobacterial extract demonstrating its potential in natural product discovery. However, for most laboratories, it is more practical to perform LC-MS or LC-NMR analyses in parallel or to collect fractions following LC-NMR analysis and then subject them to off-line MS analysis. Although LC-MS-NMR is a valuable tool for obtaining MS and NMR information on the same chromatographic peak, this hyphenated instrumentation is not required for most analyses, especially considering the sensitivity differences between NMR and MS and the timing of the analytes, which depends on the slower NMR technique.

1.2.4 Electrophoretic Techniques

Electrophoretic separation methods are based on differences in electrophoretic mobility, \( \mu_e \), which depends on the charge-to-mass ratio of each species and the frictional retarding factors encountered as ions migrate in solution through the electric field, \( E \). Positively charged cations will migrate toward the cathode (-) and negatively charged anions will migrate toward the anode (+). If the masses of the ions are identical, those possessing a higher net charge will migrate faster. Similarly, those with a smaller mass
will migrate faster if charges are equivalent. Neutral molecules are not influenced by the electric field and travel at the same rate as the bulk solution. The speed at which the analytes travel is called the migration velocity \( \nu \) and is a measurement of how far an ion travels per unit time. This value can be calculated from the electrophoretic mobility \( \mu_e \) multiplied by the strength of the electric field \( E \) using the equation,

\[
\nu = \mu_e E.
\]  

(1.20)

Other considerations for successful electrophoretic separations are the distance that the analyte must travel to the detection region and the magnitude of the applied potential.

In addition to movement of ions based on electrophoretic mobilities, CE separations have an additional contribution from electroosmotic flow (eof), giving an overall migration velocity defined by the equation,

\[
\nu = (\mu_e + \mu_{\text{eof}})E,
\]  

(1.21)

where \( \mu_{\text{eof}} \) is the mobility due to electroosmotic flow. Generally, CE is carried out using a continuous buffer system inside of capillary tubes with extremely small diameters (<100 µm I.D.), which increase the cooling efficiency so as to minimize problems associated with thermal effects. The most common type of capillary used is fused silica, which contains silanol (Si-OH) groups on the internal surface that are ionized at pH > 3. As demonstrated in Figure 1.4, due to the presence of these Si-O\(^-\) groups, there is an attraction from the positively charged species in solution. This forms a layer of positive charge along the inner wall referred to as the fixed layer. However, this fixed layer is not sufficiently dense enough to completely alter the negative charge on the inner wall of the capillary, so a second layer of cations, called the mobile layer, forms. Both layers
**Figure 1.4.** Representation of the diffuse double layer responsible for electroosmotic flow in capillary electrophoresis.
together are termed the diffuse double layer of cations. Since the outer layer of cations is not bound tightly to the Si-O$^-$ groups, in the presence of an electric field they migrate toward the cathode, dragging bulk solvent with them and generating eof. In the normal polarity mode of CE, all ions migrate toward the cathode, eluting in the order: cations, neutral molecules, anions. Although the electrophoretic velocity of an anion is toward the anode, the greater velocity generated by the eof carries it toward the cathode and past the detector. For neutral molecules $\mu_e = 0$, therefore they elute as a group migrating at a velocity determined by the eof. Separated molecules are analyzed by placing a detector near the end of the capillary. A plot, called an electropherogram, can be constructed from detector response versus time.

The extremely high separation efficiency of CE is largely due to the absence of band broadening mechanisms typical of chromatographic methods.$^{39}$ If the radius of a capillary is greater than seven times the double layer thickness, then a flat flow profile is expected.$^{40}$ Although frictional drag occurs from the diffuse double layer along the inner walls of the capillary, adverse effects on the flat profile are minimal. This flat flow profile causes all of the solute molecules to experience the same velocity, regardless of their radial position inside the capillary. This means that each separated solute elutes as a narrow band, producing narrow peaks of high efficiency and giving CE a significant advantage over other separation techniques that rely on pressure-driven flow, which produces parabolic flow profiles.
Capillary electrophoresis was first introduced in the early 1980’s when small, fused-silica capillaries (<100 µm I.D.), protected by an external layer of polyimide coating, became available.\textsuperscript{41, 42} Although the first commercial CE instrument was not introduced until 1989, over 36,000 publications have appeared on this topic encompassing a wide range of disciplines and application areas.

1.2.4.1 Capillary Electrophoresis-NMR (CE-NMR)

The hyphenation of CE and NMR combines a powerful separation method with an information rich detection method. CE-NMR has the potential to impact a variety of applications in pharmaceutical, food, forensics, environmental, and natural products analysis because of the high information content and low sample requirements of this method.\textsuperscript{43-45} In addition to standard capillary electrophoresis, two other types of CE separations have become increasingly important in CE-NMR; capillary electrochromatography (CEC) and capillary isotachophoresis (cITP). CEC utilizes a packed LC column, instead of an open capillary, to provide the separation efficiency of CE with the selectivity of LC. The concentrating effect of cITP makes it beneficial for the investigation of analyte samples that are mass-limited and present at low concentrations; compensating for the inherently poor sensitivity of NMR detection. Small volume (5 nL-1 µL) NMR probes have been introduced that provide the successful coupling of these separation methods to NMR detection. The detection coil is typically attached directly to the flow cell or capillary and these probes provide highly resolved spectra that are comparable to those obtained using 5 mm tubes.
A typical instrumentation set-up for CE-NMR is given in Figure 1.5. Because small diameter capillaries are used in CE-NMR, detection is typically accomplished using low volume microcoil NMR probes. A solenoidal microcoil is either wrapped around the capillary itself or wrapped around a sleeve through which a capillary is inserted. As mentioned in section 1.1.2, the closer the detection coil is to the sample, the greater the S/N. For this reason, the former design is preferred in which the capillary acts as both the sample holder and the coil form. CE-NMR can also be performed using small saddle coils, although they tend to be larger than solenoidal coils because of the difficulty of their fabrication.

In CE-NMR experiments, a voltage is applied across the capillary maintaining the flow of charged species. The analyte ions are then separated based on their electrophoretic mobilities and detected as they flow through the coil. Buffers are usually contained in plastic vials and Pt electrodes can safely be used near the magnet. A high power voltage supply source is needed (up to 30 kV) and in some cases, a syringe pump or other equipment can be used. These and other magnetic objects must be kept a sufficient distance from the magnet. Since the introduction of shielded magnets, some separation equipment can be moved as close as 1 meter from the magnet.

There are typically two modes of injection used in CE-NMR: hydrodynamic and electrokinetic. Hydrodynamic injection either uses pressure or vacuum at one end of the capillary, while the other end is submersed in the sample solution. This injection mode is non-selective as the composition of the analyte injected is the same as the sample solution. Electrokinetic injection is induced by the application of potential with the
Figure 1.5. Scheme of the instrumentation for the coupling of capillary separation techniques with NMR. (Reproduced from Pusecker, K. et al. *Anal. Chem.* 1998, 70, 3280-3285. Copyright (1998). Used with the permission of the American Chemical Society.)
injection end of the capillary immersed in the sample. Using this method, the composition of the sample plug depends on the mobility and charge of the ions injected. In either case, the amount of analyte loaded into the capillary must be kept small (1 to 10 nL) in order to maintain the high separation efficiency of CE.

1.2.4.2 Capillary Electrochromatography-NMR (CEC-NMR)

Instrumentation for CEC-NMR is similar to CE-NMR except that a packed LC column is used instead of an open capillary. The separation is electroosmotically driven rather than pressure driven and the higher sample loading capacity compared with CE increases the sensitivity of NMR detection. Because electroosmotic pumping leads to plug rather than hydrodynamic flow, greater separation efficiencies are achieved compared with conventional LC. Two mechanisms for separation can occur in CEC, either in combination or separately. One is the development of partition equilibria between the stationary phase of the column and the mobile phase. The other is differences in charge among the analyte ions. These features increase the plate efficiency, and thus the selectivity, providing an added advantage over CE or HPLC, depending upon the application. A disadvantage of CEC separations is the potential for bubble formation within the sorbent bed, although this can be minimized by pressurizing the buffer vials (~500 psi) or using low conductivity buffers like MES and Tris.

1.2.4.3 Capillary Isotachophoresis-NMR (cITP-NMR)
cITP-NMR is another mode of CE-NMR, that utilizes a discontinuous buffer system and a capillary modified to minimize electroosmotic flow. Charged analytes in the sample separate into discrete zones based on their electrophoretic mobilities, as demonstrated in Figure 1.6. The sample is sandwiched between two different ionic buffers: the leading electrolyte (LE) with the highest mobility and the trailing electrolyte (TE) with the lowest mobility (Figure 1.6A). Upon the initial application of a high voltage (e.g. 15 kV) across the capillary, the mobility, and consequently conductivity or resistance, varies along the separation axis due to the inhomogeneity of the electric field. Between the LE ions and the ions from the sample, local resistance and the electric field increase. This causes the faster moving sample ions to accelerate toward the LE ions to prevent a non-conductive gap at the boundary. In turn, they act as a leading electrolyte for the slower moving sample ions (Figure 1.6B-C). Over time a steady-state condition is reached such that each ion type is separated into its own discrete zone, flanked by ions possessing higher mobility on one side and lower mobility on the other (Figure 1.6D). All zones will travel uniformly at the same velocity and the mixing of these zones is prevented. This progression is based on Ohm’s law and the requirements of electroneutrality as stated by the Kohlrausch regulating function.\textsuperscript{48, 49} After the formation of discrete zones, an adjustment of ion concentration in each zone occurs, known as the concentrating effect.\textsuperscript{50} Through careful selection of the leading and trailing buffers, cITP separations result in very narrow sample zones of high concentration. cITP can concentrate analytes by 2-3 orders of magnitude, which is ideal for on-line microcoil NMR detection.
Figure 1.6. The cITP separation of charged analytes: (A) The sample is injected between the leading electrolyte (LE) and the trailing electrolyte (TE). (B and C) Upon application of an electric field, the sample ions separate into discrete zones based on their electrophoretic mobilities. (D) A steady-state is reached in which all zones travel uniformly at the same velocity and the mixing of zones is prevented.
1.3 Impurity Analysis of Pharmaceutical Substances

Hyphenated instrumentation such as LC-NMR and LC-MS are frequently used in pharmaceutical development for stability testing and the structure elucidation of degradation products and impurities.\(^{51,52}\) In the pharmaceutical industry, impurity analysis is an essential part of drug development. Whether the aim is to develop new drugs from natural products or synthesize them chemically, the final active pharmaceutical ingredient (API) should be as pure as possible. However, trace impurities that develop during formulation or upon degradation of the active ingredient, typically remain with APIs. The presence of these unwanted impurities even in small amounts can influence the safety of the drug. The structure elucidation and quantitative determination of impurities and degradation products is of major importance for assessing the risks and side effects of the pharmaceutical product. In the case of unknown impurities and degradants, the isolation, identification, and quantification requires analytical methods that are both sensitive and selective. This poses a significant analytical challenge since most impurities are present at trace levels.

The authorities responsible for setting impurity guidelines are the International Conference on Harmonization (ICH), the US Food & Drug Administration (FDA), the World Health Organization (WHO), and the European Committee for Directorates. In particular, the FDA and ICH have outlined principles for controlling the limits of individual impurities taking into consideration the toxicity of the impurity and the daily dose of the drug.\(^{53}\) Impurities are typically classified into three groups: 1) organic, which usually arise from starting materials, synthetic intermediates, byproducts, or degradation
products; 2) inorganic, which usually originate from the equipment used or reagents; and 3) residual solvents.\textsuperscript{54} This last group is broken up into three classes based on the risk to human health. Class 1 solvents are known or suspected to be human carcinogens and are hazardous to the environment. The use of these substances during drug production should be strictly controlled below pre-set concentration limits or avoided altogether. Class 1 solvents include benzene (2 ppm limit) and carbon tetrachloride (4 ppm limit). Class 2 solvents are non-genotoxic carcinogens and are controlled according to the PDEs (Permitted Daily Exposure) limits set by the ICH. Examples of Class 2 solvents are methylene chloride (600 ppm limit), methanol (3,000 ppm limit), and acetonitrile (410 ppm limit). Class 3 solvents are known to have the lowest potential for human toxicity and residual amounts ($\leq 50$ mg per day) are acceptable without justification. These include substances such as acetone, ethanol, and acetic acid.

### 1.3.1 Impurities and Drug Stability

During drug development, several physical and chemical properties of the new drug must be evaluated. In particular, establishing the stability of a new drug is an important requirement. This includes a detailed investigation in which the changes to pertinent properties of the drug substance and formulated drug product are quantitatively detected over time. This stability indicating assay is carried out in two stages. First, in the early developmental stages the bulk drug is subjected to various conditions such as, exposure to UV-light, adverse temperatures, pH fluctuations, and humidity. The results from these studies impart knowledge of the drug degradation behavior and are then further evaluated and validated. In the second stage, similar experiments are carried out
with the drug, but in the presence of its excipients or formulation constituents. The data generated from stability testing helps to establish regulations for the drug in question, such as expiration dates, shelf-life, and any special requirements for shipping and handling. The FDA and ICH require characterization of impurities and degradants at concentration levels greater than or equal to 0.1% of the parent compound. Depending on their toxicological profiles, impurities may be regulated at even lower levels in approved drugs. The analysis and structure elucidation of impurities at this level can be a challenging task.

1.3.2 Evaluating and Profiling Impurities

In profiling impurities, the overall goal is to elucidate the chemical structure and possible mechanisms of impurity formation. Depending upon their nature and complexity, the identification of an unknown impurity could involve a simple procedure such as matching the chromatographic retention time, NMR and mass spectral profiles to that of a standard. In some cases, a more involved approach requiring a team of scientists to work through a process for days or even months to elucidate the structure of an unknown compound is needed. Typical analysis of drug impurities begins after they are detected in test samples using techniques such as thin layer chromatography (TLC), HPLC, GC, or CE. The retention times of the impurities are compared with reference impurity standards, if they are available. If they are not comparable with the reference standard impurity samples, then preparative TLC, HPLC, or CE can be used to separate the impurities from the samples, followed by detection using UV, NMR, or MS.
Although an absolute unknown is atypical in the pharmaceutical industry, the structure of most unknown impurities or degradants can be identified and reported using this protocol. In contrast, if an impurity remains unidentified, then hyphenated techniques such as LC-NMR, LC-MS/MS, and GC-MS can be used to elucidate the structure. These techniques are capable of analyzing trace amounts of impurities at the identification threshold levels. In all cases the structure, chromatographic and spectroscopic properties of the drug substance are known. In most cases, comparison of this information and that of an unknown impurity obtained from a systematic investigation using a hyphenated technique, a structure can be proposed. The next step is to synthesize the proposed drug impurity and re-run the selected chromatographic and spectroscopic methods to confirm identification. If the structure of the impurity is a match, then the impurity material is used as a standard reference in the development of analytical methods for future determination of impurities in the drug substance and/or products.

1.3.3 Analytical Challenges of Impurity Identification

The most common hyphenated technique used for identification of an unknown impurity is LC-MS. The sensitivity of LC-MS often allows direct detection of trace impurities at $\leq 0.1\%$ of the parent compound, which eliminates the need for additional separation and isolation or sample enrichment steps. However, careful consideration must be given to the modifiers or buffers being used in the LC method. Many non-volatile buffer components, such as phosphate, borate, and citrate are not amenable to common mass spectrometry ionization sources, such as electrospray ionization (ESI), and may
cause a build-up of salt deposits on the source. The ability of mass spectrometry to provide accurate structural information is contingent upon the type of ionization technique employed and the compatibility of the analyte solutions. Although LC-MS has good sensitivity compared to many other methods, not all impurities are efficiently ionized and therefore some may be missed entirely in the LC-MS analysis. In some cases, LC-MS results only indicate that the impurity of interest is drug related, has structural similarities with the bulk drug, or with other known impurities. Mass spectrometry does not always provide sufficient information for complete structure elucidation.

Analysis of impurities using LC-NMR presents different analytical challenges. First, protic solvents typically used in LC methods must be replaced with their deuterated counterparts to avoid elimination of large spectral regions by solvent suppression routines. The use of deuterated solvents can be expensive due to the large volumes of solvent required in LC separations. More important is the challenge of dealing with the relative poor sensitivity of NMR. Many times the concentration of the impurity of interest is too low to conduct full characterization using LC-NMR and when steps are taken to concentrate the impurity, further degradation can occur. In recent years, improvements to LC-NMR have been made with the incorporation of new coil designs, better RF systems for multiple solvent suppression, and the reduction in flow cell volume to increase on-line sensitivity.27, 56

Although LC-MS and LC-NMR are effective methods for separation and detection of unknown pharmaceutical impurities and degradants, there is clearly a need for an improved hyphenated NMR technique that is sufficiently sensitive to perform rapid
analysis of trace impurities and/or unstable compounds. Chapter 2 explores the use of cITP-NMR for the separation and detection of trace degradants in a pharmaceutical formulation. Analysis of an acetaminophen thermal degradation sample using cITP-NMR revealed resonances of several degradation products in addition to its major degradant, 4-aminophenol. Subsequent LC-MS/MS analysis provided complementary information for the structure elucidation of the unknown degradation products.

1.4 Heparin and Heparan Sulfate Analysis

Hyphenated techniques can also be useful for the separation and detection of complex biological materials, such as heparin and heparan sulfate-derived oligosaccharides. Heparin and heparan sulfate (HS) are members of the glycosaminoglycan (GAG) family. They are unbranched polysaccharides that vary in length and are primarily located in the extracellular space of connective tissues and associated with cell surfaces. They are involved in many normal and pathological processes related to human health. Heparin is most well known for its anticoagulant activity and is second only to insulin as a natural therapeutic agent.\textsuperscript{57, 58} It has become one of the top-selling anticoagulant drugs worldwide with annual sales reaching just over 4 billion dollars, and a projected 15\% annual growth rate in the United States alone. Pharmaceutical heparin is extracted from the tissues of animals used for consumption (i.e., porcine intestine), purified, and administered as an anticoagulant. It is estimated that 30 to 40 tons of heparin are prepared each year worldwide and extracted from 400 to 700 million pigs.\textsuperscript{59} The purification and safety of heparin has become a major concern due to
the recent contamination of several sample lots of heparin by oversulfated chondroitin sulfate (OCS).\textsuperscript{60-62} OCS is structurally related to heparin and also exhibits anticoagulant activity. Due to non-specific routine quality control tests, this contaminant went undetected and resulted in 81 deaths and nearly 1000 cases of allergic reactions in the US and Germany.\textsuperscript{62} This crisis has heightened awareness about the need for improved separation and detection techniques for heparin purity analysis.

There is also growing interest in the protein binding properties of heparin and heparan sulfate for use in cancer therapy and in the treatment of viral infections.\textsuperscript{58, 63-67} However, most of the sub-structures responsible for these activities are not known. This is mainly due to the structural diversity and heterogeneity of heparin and heparan sulfate, complicated by the lack of specific and sufficiently sensitive analytical techniques. As a result, the secondary structures responsible for binding to specific proteins have been fully characterized for only a few heparin and HS sequences.

### 1.4.1 Biological Functions and Protein Binding

Heparin is mainly found in the cytoplasmic granules of arterial wall mast cells. Mast cells are directly involved in the immune response of animals and in the pathogenesis of human allergic reactions. In contrast, HS GAGs are typically found on the surface of mammalian cells and in the extracellular matrix. Therefore, many of their functions involve the regulation of cell signaling and entry. One of heparin’s primary functions is to act as an inhibitor of blood coagulation by binding to the protein antithrombin III through a specific pentasaccharide sequence.\textsuperscript{68, 69} This binding induces a
conformational change in antithrombin enhancing its affinity for the key coagulation protease factor, Xa, which forms an inactive complex upon binding with thrombin, inhibiting the blood coagulation cascade. Heparin and HS also bind a number of growth factors and chemokines, and low molecular weight heparin (LMWH, average molecular weight 3-6 kDa) has been shown to be effective for the treatment and prevention of venous thromboembolism and tumor angiogenesis. Heparin and HS also interact with lipid or membrane binding proteins, such as the annexins and apolipoprotein E (apoE). As an integral component of mammalian cell-surface receptors, HS is a target of many viral pathogens as a route to cell insertion.

Heparin and HS are linear polysaccharides that exist primarily as helical structures and, unlike proteins, are not known to fold into any particular tertiary structure. The helical structure of heparin and HS positions the negatively charged carboxylate and sulfate groups along the outside surface of the polysaccharide backbone. This feature along with the conformational flexibility of the residues, and the orientation of the glycosidic linkages all contribute to the binding specificity of heparin and HS to proteins. The chain length typically required for binding is between 3-9 disaccharide units, although longer sequences have also been shown to participate in binding. Hundreds of proteins have been identified that bind to heparin and HS and their binding domains typically contain large numbers of the basic residues lysine, arginine, and to a lesser extent, histidine. Other amino acid residues, such as asparagine and glutamine, are known to bind to heparin through hydrogen bonding interactions. Although it is well known that hydrogen bonding and hydrophobic forces are responsible for the binding of
heparin to some proteins, the most prominent interactions are ionic. Electrostatic interactions occur when positively charged, basic amino acid residues of proteins bind to the negatively charged sulfo and carboxylate moieties of heparin. Since heparin is a highly charged linear polyanion, the high, repulsive energy of these negatively charged groups promotes binding to counter ions (i.e. Na\(^+\), K\(^+\), Mg\(^{2+}\)) to reduce the net negative charge density of the molecule. The binding of cations to a polyelectrolyte, such as heparin, is entropically unfavorable. Upon binding to the positively charged amino acid residues of a protein, the release of these heparin-bound counter ions results in an entropically favorable process overall. This is known as the polyelectrolyte effect and it contributes to the free energy of interaction between heparin and proteins.

1.4.2 Heparin and HS Microstructure and Biosynthesis

The structural complexity of heparin and HS is related to their general sequence, conformation, molecular weight, charge density, and chain flexibility. These intricacies can be considered at various biological stages, beginning at the proteoglycan level where numerous polysaccharide chains, that vary in length and contain random sequences, can be attached to the core protein. The biosynthesis of heparin takes place predominately in the Golgi apparatus and is a three stage process beginning with chain initiation. Chain initiation begins with the step-wise addition of a xylose (Xyl) residue, 2 galactose (Gal) residues and a glucuronic acid (GlcA) residue that attach to serine residues of serglycin (core protein of heparin) to form the tetrasaccharide linker \(\beta\)-GlcA-(1\(\rightarrow\)3)-\(\beta\)-Gal-(1\(\rightarrow\)3)-\(\beta\)-Gal-(1\(\rightarrow\)4)-\(\beta\)-Xyl-Ser. This stage is the same for HS, however HS has several different
core proteins; the syndecans (transmembrane proteins), the glypicans (cell membrane proteins), and other core proteins located in the extracellular matrix.

The second stage is polymerization where alternating GlcA and N-acetylated glucosamine (GlcNAc) monosaccharide residues attach via a 1→4 linkage to the linker tetrasaccharides to form heparin polysaccharide chains ([GlcA-(1→4)-GlcNAc]ₙ). The third stage of heparin and HS biosynthesis is polymer modification, which is the origin of heterogeneity in heparin and HS. Both are constructed from the same basic monosaccharides shown in Figure 1.7. These uronic acid (either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA)) and glucosamine residues (GlcN) alternate in the polysaccharide chain and form multi-substituted uronic acid-(1→4)-glucosamine disaccharide building blocks. This process begins with the sequential modification of the [GlcA-(1→4)-GlcNAc]ₙ chains by a series of enzyme-catalyzed reactions. N-deacetylase removes the N-acetyl groups from GlcNAc and N-sulfotransferase replaces them with sulfate groups (GlcNS). This modification is initiated randomly along the polymer chains and it is believed that these two enzymes perform in concert in vivo. This step occurs in a more localized fashion in HS resulting in a smaller ratio (~1:1) of GlcNS/GlcNAc than in heparin (>4:1). This N-deacetylation/N-sulfation is followed by the conversion of GlcA to IdoA through the action of C5 epimerase. The requirement for this modification is that the GlcA must be attached to the reducing end of a GlcNS residue.¹⁷,⁸⁵ IdoA residues are then 2-O-sulfated by the enzyme 2-O-sulfotransferase and this reaction is limited by the substrate specificity of the enzyme. 2-O-sulfotransferase cannot bind to IdoA residues that contain a C1-attached GlcNS(6S) (IdoA-(1→4)GlcNS(6S)).⁵⁸,⁸⁶ This suggests that
Figure 1.7. Monosaccharide building blocks of heparin and heparan sulfate
the 2-\(O\) modification must occur first, followed by \(O\)-sulfation at the C6 position of the GlcNS and unmodified GlcNAc residues by 6-\(O\)-sulfotransferase. Finally, certain GlcNS(6S) residues can act as a substrate for 3-\(O\)-sulfotransferase. The resulting 3-\(O\)-sulfation (GlcNS(3,6S)) is a modification that is required in the pentasaccharide sequence responsible for the anticoagulant activity of heparin (Figure 1.8A). \(O\)-sulfation can also occur at other sites, such as the 2-\(O\)-sulfation of GlcA residues and 6-\(O\)-sulfation of isolated GlcNAc residues, but sulfation at these sites occurs to a much smaller extent. Overall, HS contains fewer \(O\)- and \(N\)-sulfo modifications compared to heparin. The number of \(O\)- and \(N\)-sulfates per disaccharide ranges from 0.2-0.7 for HS and 2.0-2.5 for heparin.\(^58, 87\) The negative charge density of heparin is the highest of any known biological macromolecule and is due to the abundance of charged \(O\)- and \(N\)-sulfo and carboxylate groups.\(^86\) As a result, the most common sub-structure of heparin is the tri-sulfated disaccharide, IdoA(2S)-(1\(\rightarrow\)4)-GlcNS(6S) shown in Figure 1.8B. Although the enzymatic modifications are performed in an organized and regulated way, not all of the potential substrate residues are modified. Thus, the resulting mature polysaccharide chains are structurally complex and heterogeneous. It is still not clear what additional factors play a role in the regulation of these enzymes along the biosynthetic pathway.

Following polymer modification, the mature polysaccharide chains of heparin are cleaved at some of the GlcA residues by an \textit{endo-\(\beta\)}-\(\text{D}\)-glucuronidase to yield shorter, unbound chains of heparin. Because cleavage does not occur at all GlcA residues, heparin is polydisperse with regard to chain length and molecular weight. This cleavage also occurs for HS chains, but to a lesser degree. Most HS chains remain attached to their core
Figure 1.8. (A) Pentasaccharide sequence responsible for anticoagulant activity. (B) Major repeating disaccharide unit of heparin.
proteins and function as part of the HS proteoglycan. The additive effect of microheterogeneity and polydispersity in heparin and HS is the major contributor to the difficulty in evaluating the important binding motifs that are responsible for their biological function and significance.

1.4.3 Analytical Challenges

Ideally, a complete structural characterization of heparin requires the identification of each variable substituent and the conformation of each alternating uronic acid and glucosamine monosaccharide. This would be followed by their sequence determination in the polysaccharide chain. However, the polydispersity of these biopolymers makes isolation and purification a very labor intensive task and GAG motifs that are important for protein binding are typically present in low abundance. Since the biosynthesis of heparin and HS is not a template driven process, there are currently no amplification techniques available for producing them in larger amounts. This makes the identification of their microstructure a significant challenge due to the lack of sufficiently sensitive and specific analytical techniques. There is clearly a need for development of improved on-line separation methods coupled to NMR detection, which can facilitate structure characterization of mass-limited heparin- and HS-derived oligosaccharide fragments.

1.5 Summary
Hyphenation of chromatographic separations with NMR detection allows the acquisition of data sets high in information content. This is especially useful for structure elucidation or identification of new or unknown compounds present in complex sample matrices, for example, natural products, biological materials, or degradation products of formulated pharmaceuticals.

Capillary techniques such as CE-NMR and cITP-NMR are especially useful for analyzing mass- and concentration-limited samples. Despite the intrinsically low sensitivity of NMR detection, microcoil-NMR and new coil designs, hold promise for overcoming this limitation. In addition, a new hyphenated, capillary technique has emerged onto the scene, GC-NMR, demonstrating that even two-dimensional NMR experiments are possible in the gas phase.\textsuperscript{88, 89}

Despite the clear advantages of NMR spectroscopy, interpreting NMR spectra of pharmaceutical impurities and heparin and heparan sulfate oligosaccharides can be a challenging task, due to the complexity of degradation product mixtures or the preponderance of isomers, respectively. The low sensitivity of NMR further complicates the characterization of these small quantity samples by limiting the ability to conduct two-dimensional NMR experiments. Thus, additional improvements in hyphenated NMR techniques are needed. As a goal of this dissertation, this need is addressed through the development and application of cITP-NMR methods for improving the sensitivity of NMR and advancing the study of pharmaceutical impurities and heparin components.
1.6 REFERENCES


54. ICH; Q3C, Residual solvents. **2002**.


Chapter Two
Development of Cationic cITP-NMR for the Separation and Analysis of Trace Degradation Products in a Pharmaceutical Formulation

Based on a research paper published in Analytical Chemistry¹

This Chapter describes the effectiveness of cITP-NMR to isolate and detect 4-aminophenol (PAP) and several unknown degradation products in an acetaminophen thermal degradation sample. Subsequent LC-MS/MS analysis provided complementary information for the structure elucidation of the unknown degradation products, which were dimers formed during the degradation process.

2.1 Introduction: Structure Elucidation of Trace Degradants in Acetaminophen Using cITP-NMR

Hyphenated NMR techniques, such as LC-NMR, are widely used in the pharmaceutical industry as powerful tools for structure elucidation of trace impurities and pharmaceutical degradation products. However, a limitation of using NMR for the structure elucidation of these concentration- or mass-limited samples is the relatively poor sensitivity of this method compared with other techniques.¹ As mentioned in Chapter 1, there are a number of approaches to increase the sensitivity of NMR experiments, such as signal averaging, employing higher magnetic field strengths, or

using cryogenically cooled probes.\textsuperscript{2} An alternative strategy for improving NMR sensitivity in trace analysis is through the use of solenoidal microcoils.\textsuperscript{3} Microcoil NMR probes have higher mass sensitivity, but poorer concentration sensitivity than probes based on the traditional Helmholtz coil design.\textsuperscript{4-6} They can be constructed to allow easy on-line coupling of microscale separations such as capillary HPLC, CE, and capillary isotachophoresis (cITP).\textsuperscript{7-11} cITP is covered in greater detail in Chapter 5, but briefly can be described here as a mode of CE capable of preconcentrating analytes by 2 to 3 orders of magnitude. A discontinuous buffer system is employed that is comprised of a leading electrolyte (LE) with high electrophoretic mobility and a trailing electrolyte (TE) with low electrophoretic mobility.\textsuperscript{10-13} The analyte of interest has an intermediate electrophoretic mobility and is sandwiched between the LE and TE. Separation is based on the differences in electrophoretic mobilities of charged analytes under the influence of a high electric field across the capillary.\textsuperscript{14} This approach was used to isolate and detect PAP in an acetaminophen sample spiked at the 0.1\% level, with no interference from the parent compound. Analysis of an acetaminophen thermal degradation sample revealed resonances of several degradation products in addition to PAP, confirming the effectiveness of on-line cITP-NMR for trace analyses of pharmaceutical formulations.

### 2.1.1 Applying cITP-NMR for the Separation and Detection of Thermal Degradation Products in Acetaminophen Samples

Coupling cITP to NMR (cITP-NMR) has been demonstrated to be an effective tool for the detection of microgram quantities of material.\textsuperscript{10, 11, 15-17} In this study, we explore the use of cITP-NMR for the structure elucidation of trace impurities in
pharmaceutical formulations through the separation and detection of thermal degradation products in acetaminophen samples. Acetaminophen (paracetamol) is an analgesic and antipyretic that is a widely used over-the-counter (OTC) drug for pain management in a variety of patients.\textsuperscript{18} Although pure acetaminophen is stable under dry conditions at temperatures up to 45 °C, if exposed to excess heat and humidity it thermally degrades to PAP.\textsuperscript{19} Therefore, the presence of PAP in a pharmaceutical formulation could arise as a synthetic intermediate or as a hydrolytic thermal degradation product. The structures of acetaminophen and PAP are shown in Figure 2.1. Because it has significant nephrotoxicity and teratogenic effects, the United States,\textsuperscript{20} British,\textsuperscript{21} and European\textsuperscript{22} pharmacopeia limits for PAP, are 50 ppm (0.005% w/w) in the drug substance.\textsuperscript{23, 24} For acetaminophen, the sample degradation process is characterized by a slow color change of the sample solution from colorless to light brown resulting from the further degradation of PAP to form additional dimeric products.\textsuperscript{25-28}

2.2 Experimental Section

2.2.1 Chemicals

All chemicals and reagents were used as received without any further purification. For the NMR experiments, deuterium oxide (D\textsubscript{2}O, 99.9% D low paramagnetic) and deuterated acetic acid were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and deuterated sodium acetate and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water was purchased from Burdick & Jackson (Muskegon, MI, USA). Methyl green and sodium
Figure 2.1. Structures of (A) acetaminophen and its major degradant, (B) 4-aminophenol (PAP).
acetate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The Millex GP filter (0.22 µm) was purchased from Millipore Corp., (Billerica, MA, USA). Tylenol™ tablets (acetaminophen) 325 mg and 500 mg were purchased over the counter, control numbers JAA188 and LCA367, respectively.

2.2.2 Acetaminophen Sample Spiked with 4-Aminophenol

In the first step of this study an acetaminophen tablet matrix was spiked with 0.1% of its primary degradant, 4-aminophenol. This was achieved by dissolving a 325 mg Tylenol™ tablet in 8.56 mL of D₂O. To this solution, 43 µL of a 50 mM solution of PAP was added to yield a solution with a 250 µM concentration of 4-aminophenol (0.1% of the acetaminophen molar concentration). The resultant solution was carefully filtered using a 0.22 µm Millipore Express PES membrane filter. The pD of the solution was adjusted to 3.0 using deuterated acetic acid. If the pH electrode is calibrated using aqueous buffers, as in these experiments, the pH meter reading (pH*) can be corrected to compensate for the deuterium isotope effect at the glass electrode using the relationship

\[ pD = pH^* + 0.4 \]

2.2.3 Acetaminophen Degradation Sample in D₂O Solution

An attempt was made to develop a thermally degraded acetaminophen sample and compare the analysis results to those of the spiked sample. This was done by dissolving a 325 mg Tylenol™ tablet in 10 mL of D₂O. The resultant solution was carefully filtered using a 0.22 µm Millipore Express PES membrane filter. The pD of the solution was
adjusted to 3.0 using deuterated acetic acid. The acetaminophen tablet solution was left in the oven at 70 °C for 120 days to undergo thermal degradation. The sample was filtered with a 0.22 µm Millipore Express PES membrane filter prior to analysis.

2.2.4 Acetaminophen Degradation Sample in Aqueous Solution

To account for the chemical exchange of deuterium with the phenolic protons of the degradation products in the LC-MS results, another acetaminophen sample was thermally degraded using H₂O as the solvent. For this experiment, a 500 mg Tylenol™ tablet was dissolved in 15 mL of HPLC-grade water. The pH was adjusted to 3.0 using glacial acetic acid and filtered as previously described. The acetaminophen solution was forced to undergo thermal degradation in the aqueous solution after being placed in the oven at 70 °C for 108 days. Confirmation of degradation was observed by the characteristic brown color accompanied by dark precipitate. The solution was filtered and lyophilized prior to analysis.

2.2.5 On-Line cITP-NMR Experiments

A “Zero EOF” fused silica capillary (180 µm i.d. and 340 µm o.d.) purchased from Polymicro Technologies (Phoenix, Arizona, USA) was used for the cITP separations. The capillary surface was internally modified by MicroSolv Technologies (Long Branch, NJ) using a proprietary method to eliminate electroosmotic flow (eof). The external surface of the capillary was cleaned with acetone and passed through the polyimide sleeve upon which the homebuilt NMR microcoil was wound.
The cITP buffers were prepared in D$_2$O. A 160 mM deuterated sodium acetate solution was used as the LE. The pD of the LE solution was adjusted by addition of 99% deuterated glacial acetic acid to achieve a pD of 4.74. A solution containing 160 mM deuterated acetic acid, pD 2.55, was used as the TE. The use of deuterated sodium acetate and acetic acid was required in these experiments to avoid overlap with the acetaminophen acetyl resonance in the on-line NMR experiments. The spiked and degradation samples were analyzed directly without additional purification. Samples were introduced into the separation capillary using hydrodynamic injection at a height differential of 18 cm. In the cITP run, the capillary was first filled with LE. The injection protocol for these experiments involved injection of TE for 1 minute, sample for 6 minutes, followed by TE for an additional 7 minutes. After completion of the injection protocol described above, the voltage was then applied at 15 kV for the duration of the cITP experiment. An illustration of the cITP-NMR set-up is given in Figure 2.2.

$^1$H NMR spectra for the cITP-NMR analysis of the degradation sample in aqueous solution were recorded using a Bruker Avance spectrometer operating at 600.113 MHz. All other cITP-NMR experiments were conducted using a Varian Unity spectrometer operating at 599.741 MHz. Once a steady current was reached in the cITP-NMR experiments, NMR acquisition was initiated to acquire an array of $^1$H NMR spectra using 45° pulses with an acquisition time of 1.41 s and a spectral width of 7196.8 Hz. Each spectrum was acquired by coaddition of 8 transients with a total acquisition time of 11.28 s. Line broadening equivalent to 2.0 Hz and zero-filling to 128 K points were applied prior to Fourier transformation.
Figure 2.2. A schematic of the cITP-NMR set-up.
2.2.6 Off-Line cITP-NMR Experiments

To reduce matrix interferences prior to LC-MS analysis, off-line cITP experiments were conducted for both the deuterated and aqueous degradation samples. Methyl green was added for visualization of the focused degradant and to facilitate collection of the analyte band. Once the focused band reached the cathodic end of the capillary, the voltage was switched off, the end of the capillary was immediately removed from the buffer vial, placed into a 0.5 mL Eppendorf tube, and brought to a level that was below that of the anodic end. This aided in hydrodynamic expulsion of the focused band, which was collected as a single drop in the Eppendorf tube. This experiment was repeated a total of 10 times for each of the degradation samples to collect a sufficient quantity for LC-MS analysis. The cITP bands collected were combined in an Eppendorf tube, diluted to a final volume of 60 µL using 0.1% formic acid aqueous solution, and 5 µL of this solution was then used for LC-MS analysis.

2.2.7 Capillary Electrophoresis (CE) Experiments

CE analysis of the degraded acetaminophen samples was also performed. To begin, stock solutions of 25 mM acetaminophen and PAP were prepared in 20 mM sodium phosphate buffer (pH 3.4). A sample mixture containing 6 mM acetaminophen and 4 mM PAP was prepared from the stock solutions and subjected to CE analysis. Second, the migration times of the components contained in the aqueous degradation sample were compared to those of the stock solution. All CE separations were performed with a Beckman Coulter ProteomeLab™ PA800 automated system equipped with a diode
array detector, fluid-cooled column cartridge and automatic injector. Fused silica capillaries (Beckman) of 50 cm length (length to detector 42.5 cm), 75 μm i.d. and 375 μm o.d. were used. New capillaries were first rinsed with 1 M sodium hydroxide for 1 min, then HPLC-grade water for 5 min, followed by a rinse with 20 mM phosphate buffer (pH 3.4) for 2 min, all at high pressure (20 psi). The capillary was equilibrated with the buffer by applying a separation voltage of 18 kV for 5 min. Between experiments, the capillary was first rinsed with HPLC-grade water for 2 min then with buffer for 2 min, both at a pressure of 50 psi. Samples were pressure injected at 0.5 psi for 7 s and the separation voltage was 18 kV with an applied pressure of 0.5 psi. Absorbance was monitored at 280 nm and data were collected and processed using the 32 Karat software.

2.2.8 LC-MS Experiments

Chromatographic separations were performed using an AQUITY™ Ultra Performance Liquid Chromatography system (Waters Corp., Milford, MA, USA). The separation was carried out in a 2.1 × 100 mm column, packed with 1.7 μm bridged ethylsilica hybrid particles maintained at 40 ºC. The sample was eluted at 0.3 mL/min with a linear gradient of 0-80% B for 10 minutes, where A = 0.1% formic acid in HPLC grade water and B = methanol in 0.1% formic acid.

Mass spectrometry was performed using a Micromass Quadrupole-Time-of-Flight (Q-TOF) instrument (Waters Corp., Milford, MA, USA). The data acquisition software used was MassLynx NT, version 4.0. Mass spectra were obtained in positive ion mode. The nebulization gas was set to 630 L/hr at a temperature of 350 ºC, the cone gas set to
50 L/hr, and the source temperature was kept at 120 °C. The capillary and cone voltages were set to 3000 V and 30 V, respectively. The Q-TOF Micro acquisition rate was set to 0.5 s, with a 0.1 s interscan delay. Argon was employed as the collision gas at a pressure of 17 psi and a collision energy of 10 V. All analyses were acquired using the lockspary, set at a frequency of 10 s, to ensure mass accuracy and reproducibility. Leucine-enkephalin was used as the lock mass ($m/z = 556.2771$) at a concentration of 2 ng/µL and a flow rate of 5 µL/min. Data was collected in continuum mode and averaged over 30 scans.

2.3 Results and Discussion

2.3.1 Analytical Considerations in the Trace Analysis of Degradation Samples: Acetaminophen Model

Due to their structural similarities, the $^1$H NMR spectra of a synthesis impurity or degradation product are typically similar to those of the parent compound, which is present in large excess. The static $^1$H NMR spectra of acetaminophen and PAP, shown in Figure 2.3 A and 2.3 B, indicate that the primary difference in the spectra of these compounds is the acetyl peak of acetaminophen at 2.132 ppm. Upon closer inspection, there are also slight differences in the chemical shifts of the PAP aromatic resonances (6.984 and 7.278 ppm) and those of acetaminophen (6.892 and 7.239 ppm). An additional trace impurity is present in the PAP standard (2.081 ppm). Because PAP is expected at trace levels in acetaminophen degradation samples, it is not possible to detect the PAP resonances in the static $^1$H NMR spectra of degradation samples due to the high degree of overlap with the much more intense acetaminophen aromatic signals. For this reason, it is
Figure 2.3. Static 600 MHz $^1$H NMR spectra of (A) acetaminophen, (B) PAP, and (C) matrix resonances from the acetaminophen tablet.
typically necessary to incorporate a separation prior to NMR detection to resolve the resonances of pharmaceutical impurities and provide insights into the structure of these compounds.

Another consideration in the trace analysis of degradation samples is the presence of excipients, fillers, and other formulation constituents. These matrix components can also complicate the interpretation of NMR data measured for degradation samples. For example, in the static $^1$H NMR of acetaminophen in the tablet matrix, shown in Figure 2.3 C, the resonances between 2.6 and 4.2 ppm can be attributed to inactive ingredients, most likely sodium starch glycolate which is present as a filler.

Because cITP is based on the separation of charged species, with careful choice of the buffer system, PAP and other cationic degradation products can be separated from acetaminophen and tablet matrix components. The PAP amino group has a $pK_a$ of 5.28 and hence is positively charged at the operating pH of the cITP buffers used, whereas acetaminophen remains neutral at this pH and will not be concentrated in the cITP process. Therefore, it should be possible to selectively concentrate and detect PAP in the presence of a large excess of the parent compound, acetaminophen, and other components such as cellulose, magnesium stearate, and sodium starch glycolate present in the formulated tablet.

### 2.3.2 cITP-NMR Analysis of Spiked Acetaminophen Sample

To demonstrate the capability of cITP to selectively concentrate analytes and reduce matrix interferences that generally arise from the inactive ingredients or excipients
in the tablet formulation, 0.1% PAP was spiked into the acetaminophen tablet matrix and
the solution analyzed by cITP-NMR with the results shown in Figure 2.4. In this
experiment a spectrum was acquired every 11.28 s producing a spectral profile of the
focused analyte band as it travels through the microcoil. In spectra 2.4 A and 2.4 B, prior
to detection of the analyte, only the HOD resonance of deuterated LE is observed. The
PAP resonances begin to appear in spectrum 2.4 C and become more intense in spectra
2.4 D-F. In spectrum 2.4 G, the PAP resonances begin to disappear and by spectra 2.4 H-
I, the analyte band has exited the microcoil and only the HOD resonance of the
deuterated TE is detected. Through correct choice of pD and the LE/TE buffers, cITP-
NMR was able to selectively concentrate, separate, and detect PAP in the presence of a
1000 fold excess of the parent compound and tablet matrix components. Another
important aspect to consider is the sensitivity of cITP-NMR. In this experiment only
0.213 µg of PAP was injected into the separation capillary, revealing the power of cITP-
NMR to analyze trace impurities.

### 2.3.3 cITP-NMR Analysis of Forced Degradation Acetaminophen Sample in D$_2$O

After the success achieved in the cITP-NMR analysis of the PAP-spiked
acetaminophen sample, the analysis of a forced degradation sample in D$_2$O was
attempted. The forced degradation conditions used were based on literature studies,
which document that the degradation of acetaminophen is accelerated at high
temperatures and low pH.\(^{27}\) Figure 2.5 shows the cITP-NMR spectra obtained for
thermally degraded acetaminophen in the tablet matrix. The aromatic resonances of the
Figure 2.4. A portion of on-line cITP-NMR spectra showing resonances of 0.1% PAP spiked in the acetaminophen tablet matrix. Each spectrum was produced by averaging 8 scans over 11.2 seconds. Spectra 2.4 A and B contain only the HOD resonance of the deuterated LE. Spectra 2.4 C-G provide a spectral profile of the focused PAP band as it passes through the microcoil detector. Spectra 2.4 H and I again contain only the HOD resonance of the TE.
Figure 2.5. A portion of on-line cITP-NMR spectra showing resonances of PAP (spectra 2.5 B-F) and unexpected unknown components (spectra 2.5 G-K) from the degradation sample in D$_2$O. Spectra 2.5 A and L were measured for the LE and TE, respectively.
focused PAP begin to appear in spectrum 2.5 A and become more intense in spectra 2.5 B-F. In spectra 2.5 G-K a set of resonances are observed from unknown degradation products stacked behind the PAP. The changes in relative resonance intensity in spectra 2.5 G-K suggest that these spectra are produced by more than one component. By spectrum 2.5 L the focused analyte band has left the NMR microcoil and only the HOD resonance of TE is detected. In this experiment cITP-NMR was able to cleanly concentrate, separate and detect trace amounts of PAP in a real degradation sample, and provide spectral evidence suggesting the presence of additional degradation products. Although the unknown compounds in spectra 2.5 G-K cannot be fully characterized from these spectra, some of their structural properties can be inferred. They are positively charged at the operating pD, have a lower electrophoretic mobility than PAP, and therefore, likely a higher molecular weight. These compounds have aromatic resonances more complicated than PAP, suggesting dimerization as a possible mechanism for their formation, and at least one component retains an acetyl moiety, as indicated by the resonance at 2.61 ppm. Further characterization of these unknown components can be achieved through isolation and analysis by mass spectrometry (MS) to obtain complementary structural information.

2.3.4 Preliminary LC-MS/MS Analysis of the Forced Degradation Acetaminophen Sample in D₂O

Following cITP-NMR analysis, the deuterated degradation sample was injected directly onto the LC-MS and subjected to electrospray ionization (ESI) in positive mode. This resulted in a complicated total ion chromatogram (TIC) that was difficult to interpret.
due to the presence of multiple degradation products (including those that may have been neutral or negatively charged under the conditions of the cITP-NMR experiment), matrix peaks and, of course, acetaminophen, present in large excess (Figure 2.6). To exploit the ability of cITP to selectively concentrate and separate the cationic degradation products and reduce matrix interferences, off-line cITP experiments were conducted. The cITP-focused analyte bands, visualized with methyl green, were collected and pooled for 10 replicate separations prior to ESI-MS analysis. The TIC shown in Figure 2.7 contains the molecular ions, [M+H]^+, of the expected degradation product, PAP (m/z 110.095), eluting at 0.93 minutes as well as a small peak from the parent acetaminophen (m/z 152.123) eluting at 3.91 minutes. Although acetaminophen should not have been focused by cITP, a small amount of acetaminophen contaminates the collected sample because sample collection was performed by hydrodynamic expulsion. The most intense peak in the chromatogram was that of the methyl green dye (5.80 min, m/z 200.711) used for visualization of the focused analytes during cITP. Four additional unknown peaks were also observed with molecular ions, [M+H]^+, m/z 262.120 (3.67 min), 246.127 (4.14 min), 275.092 (4.53 min), and 259.094 (5.09 min). Since these observed masses are higher than either PAP or acetaminophen, they could indicate dimerization during the conditions of the forced degradation. Tandem mass spectrometry (MS/MS) analysis revealed a similar fragmentation pattern for molecular ions m/z 262.120 and 246.127, which differ by 16 mass units, attributed to elemental oxygen. Molecular ions m/z 275.092 and 259.094 also exhibited similar MS/MS fragmentation patterns as well as an m/z difference of 16. Because the degradation sample was originally constituted in D_2O, the MS/MS spectra
Figure 2.6. Total ion chromatogram (TIC) of the degradation sample in D$_2$O after direct injection onto the LC-MS following cITP-NMR analysis.
Figure 2.7. (A) Total ion chromatogram (TIC) of the degradation sample in D$_2$O after separation and concentration using off-line cITP. Unknown degradation products are indicated by an asterisk. (B) MS spectrum of TIC peak with molecular ion $m/z$ 246.127.
were complicated by chemical exchange of the phenolic protons with deuterium precluding unambiguous structure determinations based on the fragmentation patterns and exact masses. An example MS spectrum is shown in Figure 2.7 B for peak with molecular ion \( m/z \) 246.127.

### 2.3.5 CE and cITP-NMR Analysis of the Forced Degradation Acetaminophen Sample in \( \text{H}_2\text{O} \)

To facilitate the structure elucidation of the unknown components the above experiments were repeated using a degradation sample constituted in aqueous solution. A Tylenol™ tablet was thermally degraded in \( \text{H}_2\text{O} \) under similar conditions as used for the deuterated sample. CE analysis was performed prior to analyzing the aqueous degradation sample by cITP-NMR and LC-MS/MS. The electropherogram measured for a standard mixture of 4 mM PAP (5.7 min) and 6 mM acetaminophen (13.2 min) is shown in Figure 2.8 A. The electropherogram measured for the aqueous degradation sample (Figure 2.8 B) contains a peak due to PAP as well as a large acetaminophen peak. Additional unknown degradation products were detected in the electropherogram in the form of two peaks at 7.3 and 7.6 minutes. The migration order of PAP and the unknown degradation products observed in the electropherogram shown in Figure 2.8 B is consistent with the cITP-NMR data obtained for the deuterated degradation sample. The unknown products again have a lower electrophoretic mobility eluting after PAP and are not well resolved in the electropherogram, consistent with the inability of cITP-NMR to completely resolve these components. The cITP-NMR spectra measured for the aqueous
**Figure 2.8.** CE-UV Electropherograms of (A) a mixture of 4 mM PAP and 6 mM acetaminophen standards and (B) the aqueous degradation sample.
Figure 2.9. A portion of on-line cITP-NMR spectra showing resonances of the unknown degradation components (spectra 2.9 A-F) from the degradation sample in H₂O.
degradation sample, shown in Figure 2.9, are similar to the cITP-NMR spectra shown in Figure 2.5 for the deuterated degradation sample.

### 2.3.6 LC-MS/MS Analysis of the Forced Degradation Acetaminophen Sample in H₂O

Isolation of the focused analyte in off-line cITP experiments was carried out as described above for the aqueous degradation sample. Pooled isolates from 10 replicate separations were dissolved in 60 µL of 0.1% formic acid and 5 µL of this sample were injected into the LC-MS. The TIC contained the same pattern of peaks detected in the deuterated degradation sample shown in Figure 2.7 A with PAP (1.00 min, m/z 110.050), acetaminophen (4.06 min, m/z 152.065), methyl green (5.79 min, m/z 200.616) and four additional molecular ions m/z; 259.104 (3.74 min), 243.092 (4.20 min), 273.073 (4.69 min), and 257.049 (5.15 min). Because the deuterium exchange was avoided by using water as the solvent, compared with Figure 2.7 A the m/z for each peak was lower by masses ranging from 2-3 Da, and the mass spectra were greatly simplified. The LC-MS/MS results for the four unknown compounds, along with their proposed structures, are presented in Figure 2.10. The unknown products appear to comprise two pairs of compounds with similar structures, 243.125 and 259.113 (Figures 2.10 A and B), and 273.080 and 257.077 (Figures 2.10 C and D), with a mass difference of 16 separating the members of each pair. The MS/MS fragmentation patterns for each pair of compounds are highly complementary. The fragment ions m/z 65.032, 93.029, 134.057, and 150.055 observed in Figures 2.10 A and B are well known acetaminophen fragments suggesting that these products may retain a part of the acetaminophen structure.\(^{19, 26, 27}\) The MS/MS
Figure 2.10. (A) MS/MS spectrum of the molecular ion $m/z$ 243.125. The fragmentation pattern in (A) is complementary to that of (B), $m/z$ 259.113. (C) MS/MS spectrum of molecular ion $m/z$ 273.080, which is also similar to (D) $m/z$ 257.077. The proposed structures for the unknown thermal degradation products are labeled on the far right next to the corresponding parent ion. The structures postulated for specific fragments are also shown for each mass spectrum.
fragmentation patterns for peaks 273.080 and 257.077 are also complementary (Figures 2.10 C and D, respectively). As mentioned previously, it is well accepted that the thermal degradation of acetaminophen yields numerous oxidative and dimeric products depending upon the experimental conditions. The structures shown in Figure 2.10 for the four dimeric degradation products detected by LC/MS are consistent with the LC-MS/MS fragmentation patterns observed as well as our cITP-NMR results.

2.4 Summary

This work demonstrates the successful application of cITP-NMR for the separation and detection of 0.1% PAP as a spiked impurity in the presence of a 1000 fold excess of acetaminophen and tablet matrix components. It is significant to note that only 0.213 µg of PAP was injected and detected in this experiment, significantly less than would be required for conventional NMR measurements. cITP-NMR experiments for a thermal degradation sample revealed the presence of PAP as well as several other unanticipated cationic impurities. Following sample clean-up using off-line cITP, LC-MS/MS analysis allowed the structural identification of four dimeric decomposition products.

This problem was well suited to demonstrate the selective concentration and separation of cationic impurities by cITP-NMR since the parent compound, acetaminophen, and most of the matrix components were charge-neutral. Although this analytical strategy will be easiest to apply in cases where an ionic trace impurity is selectively concentrated and separated from a neutral parent compound, in theory this
method should be effective as long as the components have sufficiently different electrophoretic mobilities. Although off-line cITP experiments were elected as a means to prepare the degradation samples for LC-MS analysis, on-line cITP-MS experiments have been demonstrated and could be used when dictated by sample load.32-34 In laboratories where the NMR and MS instruments are located in close proximity, hyphenated cITP-NMR-MS experiments would also be possible.

The results described in this chapter indicate that cITP-NMR would also be an effective method for the separation and detection of mass-limited biological samples, such as heparin-derived oligosaccharides. Because heparin oligosaccharides are negatively charged and the amount of sample required is dramatically reduced, cITP is amenable for the separation of chemically- or enzymatically-derived heparin fragments. The coupling of NMR detection to this separation provides the potential to gain new insights important for understanding relationships between the many substructures of heparin and their function. Because the structural analysis of intact heparin is complicated, efforts toward this objective require that it be broken down into smaller fragments. In our lab, this is accomplished by enzymatic cleavage using heparin lyases. This procedure is described in Chapter 3 along with the important observations made during the process.
2.5 REFERENCES


Chapter Three

Examining the Heterogeneity of Preparative-Scale SEC Fractions Obtained from Enzymatic Digests of Heparin

Based in part on a research paper published in Carbohydrate Research\textsuperscript{2}


In order to begin the challenging task of elucidating substructures of heparin, it is typically desirable to depolymerize intact heparin into smaller fragments. In this chapter, two enzymatic depolymerization reactions were performed followed by preparative size-separation using size-exclusion chromatography (SEC). Rather than conducting the common practice of pooling like-sized fractions, the heterogeneity of individual fractions was examined with regard to component composition and relative concentration. Since the structural features and biological activity of heparin and HS are discussed in detail in chapters One and Four, a brief introduction to enzymatic depolymerization and size-separation is given as an introduction to this study.

3.1 Introduction: Depolymerization and SEC Separation of Heparin

Presently structural analysis of heparin or HS begins by depolymerizing glycosaminoglycan pools that are extracted from the lungs or intestines of the animal.

\textsuperscript{2}Carbohydr. Res., 2008, 343, 2963-2970
Depolymerization cleaves the heparin chains into smaller fragments and can be performed using enzymatic or chemical methods. Chemical methods include the use of nitrous acid, copper/peroxide, or base to achieve oxidative depolymerization. Enzymatic digestion is performed using heparin lyases. The three most common heparin lyase enzymes used are Heparinase I, II, and III, derived from *Flavobacterium heparinum*. Heparinase I specifically cleaves glycosidic linkages between GlcNS(±6S) and IdoA(2S) residues of GAG chains via a β-elimination reaction shown in Figure 3.1. The substrates required for Heparinase II and III are less specific. Heparinase II cleaves the glycosidic linkage of either 2-O-sulfated or 2-OH-L-iduronic acids. Heparinase III demonstrates a high selectivity for linkages in unsulfated regions of heparin that contain 2-OH-D-glucuronic acids and, to a lesser degree, 2-OH-L-iduronic acids. Digestion with enzymes results in the unsaturation of the C4-C5 bond of the uronic acid at the non-reducing end of the terminating uronic acid residue, thus allowing direct UV detection at 232 nm (Figure 3.1). This provides a practical way to monitor the depolymerization process and detect the relative abundance of like oligosaccharides in subsequent separations. A complete digestion will render an abundance of disaccharides in addition to lesser amounts of tetra-, hexa-, and octasaccharides. A digestion that yields primarily larger fragments can be achieved by shortening the time of the enzymatic reaction. Table 3.1 lists ten of the more common, commercially available disaccharide fragments used in this work.

The complete structural characterization of heparin and HS requires determination of the identity of each disaccharide subunit, including the orientation of the glycosidic
Figure 3.1. Cleavage of heparin by heparinase I. Heparinase I specifically cleaves glycosidic linkages between GlcNS(±6S) and IdoA(±2S) residues of GAG chains via a β-elimination.

Heparinase I

R = SO₃⁻ or H
Y = SO₃⁻, COCH₃, or H
Table 3.1. Legend for the structures of commercially available heparin disaccharide standards: IA-IVA, IS-IIIS, and IH-IIIH used in this work.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>R₁</th>
<th>R₂</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>SO₃⁻</td>
<td></td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>IIS</td>
<td>H</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>IIIIS</td>
<td>SO₃⁻</td>
<td>H</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>IA</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
<td>COCH₃</td>
</tr>
<tr>
<td>IIA</td>
<td>H</td>
<td>SO₃⁻</td>
<td>COCH₃</td>
</tr>
<tr>
<td>IIIA</td>
<td>SO₃⁻</td>
<td>H</td>
<td>COCH₃</td>
</tr>
<tr>
<td>IVA</td>
<td>H</td>
<td>H</td>
<td>COCH₃</td>
</tr>
<tr>
<td>IH</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
<td>H</td>
</tr>
<tr>
<td>IIH</td>
<td>H</td>
<td>SO₃⁻</td>
<td>H</td>
</tr>
<tr>
<td>IIIH</td>
<td>SO₃⁻</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>
linkage and the sequence in which the disaccharide building blocks are assembled in the polysaccharide chain. This sequence information can be obtained by analysis of more complex mixtures of larger oligomers resulting from incomplete enzymatic depolymerization. Heparin-derived oligosaccharide fragments obtained by enzymatic depolymerization are usually separated first according to size by and then by degree of negative charge. Methods include strong anion exchange high performance liquid chromatography (SAX-HPLC)\(^5\), \(^7\), and capillary electrophoresis (CE)\(^8\), \(^9\), although reversed-phase ion-pairing methods are growing in popularity.\(^10\)-\(^12\) The size separation involves size-exclusion chromatography (SEC)\(^13\) and can be conducted at the preparative or analytical scales. Fractionation of microgram quantities of heparin or HS demands the use of high-pressure analytical-scale SEC to avoid dilution effects inherent in low-pressure methods.\(^13\) Preparative-scale SEC is a low-pressure method used to separate bulk quantities of GAGs such as heparin extracted from animal tissue. In preparative-scale SEC, fractions are collected and UV absorbance is measured as a means to follow the separation, allowing the investigator to identify the fractions containing the disaccharides, tetrasaccharides, hexasaccharides, and larger oligosaccharides.

It has been customary in GAG analysis to combine or pool like-sized preparative SEC fractions due to the limited amount of oligosaccharides contained in each fraction.\(^14\)-\(^18\) Ziegler and Zaia have recently demonstrated the chromatographic resolution of the individual components IS, IIS, IVS, and IVA comprising the disaccharide peak obtained by analysis of a heparin digest with high-pressure analytical-scale SEC.\(^13\) As the focus of this chapter, we examine whether partial fractionation also occurs in preparative-scale
SEC of heparin digests and explore the possibility that the practice of pooling fractions prior to analysis may complicate efforts to identify and/or isolate rare structures.

3.2 Experimental Section

3.2.1 Chemicals

Heparin disaccharide standards I-IIIS, IA, I-IVH, heparinase I (EC 4.2.2.7), heparinase II (EC number not yet assigned), heparinase III (EC 4.2.2.8) isolated from Flavobacterium heparinum, the sodium salt of porcine intestinal mucosal heparin, sodium acetate, calcium acetate, and tributylammonium acetate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Heparin disaccharide standards IIA-IVA were purchased from V-Labs, Inc. (Covington, LA). HPLC-grade water was purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, ammonium bicarbonate, acetonitrile, and sodium hydroxide were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

3.2.2 Enzymatic Digestion of Heparin

In the first enzymatic depolymerization (ED1), 130 mg of porcine intestinal mucosal heparin was dissolved in a 4 mL buffer solution containing 20 mM calcium acetate and 50 mM sodium acetate and digested with 50 U of heparinase I (Sigma units, where 1 U is defined as the quantity of enzyme that will form 0.1 µmol of unsaturated uronic acid product per h at pH 7.5 and 25 °C). The depolymerization reaction was performed at 37 °C at an initial pH of 7.5. The progression of the digestion was followed by performing
UV measurements (232 nm) on 6 µL of the digest diluted to a total volume of 600 µL with buffer. All UV measurements were performed using a Varian Cary 50 Bio spectrophotometer (Palo Alto, CA USA). Because the desired level of depolymerization was not reached during the initial reaction, after 30 h a second aliquot of heparinase I enzyme (50 U) was added. The total time for the depolymerization was 50 hours. A second enzymatic depolymerization (ED2) was performed, following the above procedure, on 130 mg of porcine intestinal mucosa heparin using 50 U each of heparin lyases I, II, and III.

3.2.3 SEC Fractionation

Both heparin-derived oligosaccharide solutions were size fractionated separately on a column containing Bio-Rad Bio-Gel P-6 resin (3 cm × 47 cm) and eluted with 0.5 M ammonium bicarbonate at a flow rate of 0.5 mL/min. The total volume contained in each fraction for ED1 and ED2 was 0.5 mL and 1.0 mL, respectively. The progress of both separations was monitored by measurement of UV absorption off-line. Each fraction was lyophilized, reconstituted in 500 µL of HPLC-grade water, and then lyophilized again to reduce the ammonium bicarbonate content. The lyophilized powders were stored at -80 °C until further use.

3.2.4 Capillary Electrophoresis

The CE separation method was optimized using a standard mixture of heparin disaccharides IA-IVA, IS-IIIS, and IH-IVH dissolved in 1.5 mL of 60 mM formic acid
buffer (pH 3.43). Structures for ten of the standards are defined in Table 3.1 and their concentrations ranged from 0.13 to 0.75 mM. CE separations were performed on a Beckman Coulter ProteomeLab™ PA800 instrument equipped with a diode array detector, fluid-cooled column cartridge and automatic injector. The fused silica capillaries supplied by MicroSolv Technology Corporation (Eatontown, NJ) were 50 cm length (length to detector 42.5 cm), 75 µm i.d. and 375 µm o.d. The detection window was formed by removal of ~1 mm of polyimide coating from the outside of the capillary using a MicroSolv Window Maker™. Capillaries were conditioned by rinsing with 0.1 M sodium hydroxide for 1 min, HPLC-grade water for 5 min, followed by a rinse with 60 mM formic acid buffer (pH 3.43) for 5 min, all at high pressure (20 psi). The capillary was equilibrated with the buffer by applying a separation voltage of 18 kV in reverse polarity for 7 min without pressure. Between experiments, the capillary was rinsed with HPLC-grade water for 2 min then with buffer for 3 min, both at a pressure of 50 psi. The separation voltage was 20 kV. Samples were pressure injected at 0.5 psi for 7 s. For the first 18 minutes of the separation a pressure of 0.1 psi was applied, after which the pressure was 0.5 psi for the duration of the run (12 min). Absorbance was monitored at 232 nm and the data was collected and processed using the 32 Karat software (Beckman Coulter, Inc., Fullerton, CA). The lyophilized, heparin-derived fractions were reconstituted in 600 µL of HPLC-grade water and CE separations were performed on each fraction to investigate the oligosaccharide content and purity. During the development and optimization of the standard separation method, the interday reproducibility of migration times remained within 1%. Over the course of performing
separations on the digested fractions, we observed occasional drift in the migration time of the components. This was probably due to the presence of residual enzyme or other matrix components that adsorbed to the capillary wall altering the electroosmotic flow. A neutral marker can be used to account for changes in electroosmotic flow and aid in the identification of analyte peaks.\textsuperscript{20, 21} However, because of the reduction in electroosmotic flow in reversed polarity CE experiments, the use of a neutral marker would require a significant increase in the total separation time. Therefore, spiking with standards was employed to confirm the identity of peaks in the electropherograms in lieu of a neutral marker.

3.2.5 Ultraperformance Liquid Chromatography (UPLC)-UV-MS

Chromatographic separations were performed on an AQUITY UPLC system (Waters Corp., Milford, MA) using a 2.1 × 100 mm Acquity UPLC BEH C\textsubscript{18} column packed with 1.7 µm particles and a guard column containing the same material. Analytes were detected using both UV absorbance at 232 nm and negative-ion mass spectrometry. The column temperature was maintained at 40 °C and the samples were eluted at 0.5 mL/min. A binary solvent system composed of 5% acetonitrile (Buffer A) and 65% acetonitrile (Buffer B) was used. Both buffers contained 2.5 mM tributylamine (Bu\textsubscript{3}N) and 17.5 mM ammonium acetate buffer at pH 7.00. The separation was performed using a gradient of 69% to 31% A over 10 min. This was increased to 100% B over 1 min and held at 100% B for an additional 1 min before returning to the initial composition of 69%
B over 1 min. This composition was retained for 2 min to allow for re-equilibration before the next injection.

Mass spectrometry was performed on-line using a Micromass quadrupole-time-of-flight (Q-TOF) instrument (Waters Corp., Milford, MA). Although tributylammonium ion is used as an ion-pairing reagent for the UPLC separation, prominent tributylammonium adduct ions were not detected in the mass spectra. The data acquisition software used was MassLynx NT, version 4.1. Mass spectra were obtained in the negative-ion mode. The capillary and cone voltages were set to 3000 and 20 V, respectively. The nebulization gas was set to 650 L/h at a temperature of 200 °C, the cone gas set to 10 L/hr, and the source temperature was kept at 120 °C. These parameters were optimized using a mixture of heparin-derived tetrasaccharide fractions to minimize in source fragmentation and reduce ions resulting from loss of sulfate. The scan time was 0.5 s with a 0.1 s interscan delay. The mass-to-charge (m/z) range was set at 215-1000.

3.3 Results and Discussion

3.3.1 Enzymatic Depolymerization and SEC Separation of Porcine Heparin

The heterogeneity of preparative scale SEC fractions isolated from two enzymatic depolymerizations of intestinal porcine heparin, ED1 and ED2, was examined. Heparinase I was used in ED1 to selectively cleave highly sulfated polysaccharide chains containing 1→4 linkages between GlcN and 2-Ο-sulfated iduronic acid residues. In ED2, a mixture of heparinases I, II, and III was used. Enzymatic cleavage introduces an unsaturated C-4–C-5 bond on the non-reducing end of the terminating uronic acid residue
and the reported molar extinction coefficient of a monounsaturated disaccharide at 232 nm is approximately 5500 M$^{-1}$cm$^{-1}$. This chromophore can be used to monitor the progression of the enzymatic reaction through UV absorbance measurements and to assess the oligosaccharide content of each fraction collected during the subsequent SEC size separation. For ED1, the final absorbance corresponded to cleavage of roughly 1/3 of the available disaccharide bonds based on the nominal mass of an average heparin disaccharide.

Preparative scale SEC was performed following ED1 and ED2. The progress of the size separation was monitored by plotting the UV absorbance versus SEC fraction number, as shown for ED1 in Figure 3.2. In this chromatogram, the peak with the greatest absorbance (fractions 194-246) corresponds to the disaccharide components (dp2) produced by complete enzymatic digestion. The preceding peaks correspond to larger oligosaccharides including tetrasaccharides (dp4), and hexasaccharides (dp6). The resolution of this SEC-UV chromatogram is poorer than could be obtained with high-pressure SEC, but this compromise is necessary for the efficient fractionation of larger masses of material. A similar UV-SEC chromatogram was also constructed for ED2, but the peak with the greatest absorbance (fractions 46-79) corresponded to the tetrasaccharide components.

In a typical heparin analysis utilizing SEC fractionation of oligosaccharide components the next step is to pool like-sized fractions. This is done with the goal of increasing the oligosaccharide content of each size fraction prior to further fractionation using an orthogonal separation method such as anion exchange chromatography. The
Figure 3.2. The preparative SEC-UV chromatogram showing relative absorbance versus fraction number for the separation of the components produced by ED1.
underlying assumption inherent in the pooling of SEC fractions is that a peak containing like-sized oligosaccharides, for example, dp2, is fairly homogeneous across all of the fractions collected. However, if partial fractionation occurs within a SEC peak, the pooling of fractions may complicate efforts to identify and/or isolate rare structures since the individual fractions would not be homogeneous in their composition. In this case, pooling would dilute minor components that might be present in only a few of the collected fractions.

3.3.2 CE-UV Analysis of SEC Fractions

Testing whether indiscriminant pooling of SEC fractions can hinder the isolation of minor components requires an analytical method that can resolve the size-selected components based on another property of the oligosaccharides, such as electrophoretic mobility. CE-UV is an ideal tool for profiling size-selected SEC fractions due to its high resolving power and low sample consumption. Because heparin disaccharides are commercially available, we initiated these experiments using the SEC fractions from the dp2 peak of ED1. The electropherogram shown in Figure 3.3 demonstrates the separation of a mixture containing 11 commercially available disaccharide standards. The majority of the disaccharides in the mixture were completely resolved, even two of the three isomeric sets (IIS/IIS/IH and IIA/IIIA). The IIH/IIH isomeric pair was not as well resolved in part because these compounds have only a fractional negative charge under the separation conditions. In addition, the IIH and IIIH peaks may be broadened due to incomplete separation of the glucosamine anomers. For example, heparin disaccharide IH produced a split peak resulting from the partial separation of its glucosamine anomers.
Figure 3.3. Electropherogram showing the separation of 10 commercially available disaccharide standards from Table 3.1 in 60 mM formic acid buffer at pH 3.43.
Although heparin disaccharide standard IVH was present in the mixture, it is not detected due to its cationic behavior under these conditions. This electropherogram of the disaccharide standards was used to identify the components in each of the dp2 SEC fractions of ED1. The identity of individual components was confirmed in several electropherograms by spiking with authentic standards. A representative example of the CE electropherograms obtained in the spiking experiments is shown in Figure 3.4 for fraction 216 from the preparative SEC separation of ED1.

The CE electropherograms of the dp2 fractions for ED1 revealed that although the components are structurally similar disaccharides, there can be differences in the abundance of various components across the series of SEC fractions. This seems reasonable, since the disaccharides shown in Table 3.1 do not all have the same nominal mass. In addition, the more highly sulfated disaccharides may behave as though they have a larger effective size than anticipated from their molecular formula due to hydrodynamic drag from associated cations. The CE electropherogram (Figure 3.5 A) of fraction 201, taken near the beginning of the SEC dp2 peak, contains a single component corresponding to disaccharide IS. Later in the SEC separation, but also included in the dp2 peak, fraction 219 was collected and analyzed (Figure 3.5 B). This fraction contained several other peaks in addition to the IS peak. Spiking confirmed that the peaks migrating at 11.52 and 11.93 min were heparin disaccharides IIIS and IIS, respectively. The earlier peaks in the electropherogram, migrating prior to IS, are unknown. However, it can be inferred from the migration order that they have a greater charge-to-mass ratio than the disaccharides. It is possible that these components are larger oligosaccharides, perhaps
Figure 3.4. (A) Electropherogram of fraction 216 from the dp2 peak of the preparative SEC separation of ED1. The peaks migrating at 6.35 and 8.37 minutes were identified as IS and IIIS, respectively, by spiking the fraction 216 sample solution with standards of heparin disaccharide IIIS (B) and IS (C).
Figure 3.5. CE electropherograms of fraction 201 (A) and fraction 219 (B) from the dp 2 peak of the preparative SEC separation of ED1.
trisaccharides. CE analysis of all the SEC fractions revealed that overall, the most abundant component in each fraction comprising the dp2 peak is heparin disaccharide IS. Disaccharide IIIS is the next most abundant component, however, as observed in Figure 3.5B, it is present at much lower amounts than IS. Disaccharides IIS and IA appear intermittently as minor components across the dp2 peak.

The plot shown in Figure 3.6 was constructed to illustrate both the variation of the IIIS/IS peak area ratio (□) and the relative disaccharide concentration (▲) across the ED1 dp2 peak. This plot shows that the fractions containing the largest concentration of IIIS were collected in the latter half of the dp2 peak. If the goal of this experiment was to preferentially isolate the IIIS disaccharide, fractions 230-245 should be used. With the usual practice of pooling all of the SEC fractions comprising dp2, the concentration of IIIS would be diluted significantly, making its subsequent isolation more difficult.

Differences in the composition and relative concentration of components in the SEC fractions are also observed for larger oligosaccharides. The heterogeneity across the SEC dp4 peak for ED1 (fractions 160-193) was also examined by CE. The CE electropherograms from fractions 162, 163, 168, and 176 are shown in Figure 3.7. Unfortunately because of the lack of authentic tetrasaccharide standards, we could not positively identify the individual peaks in these electropherograms. The overall tetrasaccharide content for fraction 163 (Figure 3.7 B) is more than twice the concentration of the preceding fraction, 162 (Figure 3.7 A), although there seems to be little difference in the relative abundance of the mixture components. Comparison of
Figure 3.6. A plot of the peak area ratios of disaccharides IIIS and IS ($\square$) and relative disaccharide concentration ($\triangle$) versus fraction number demonstrating the variability in the abundance of IS and IIIS across the dp 2 peak shown in Figure 3.2.
Figure 3.7. CE electropherograms of the preparative SEC dp 4 fractions 162 (A), 163 (B), 168 (C), and 176 (D) for ED1. The three major tetrasaccharide components are labeled P1, P2, and P3.
fractions 163 (Figure 3.7 B), 168 (Figure 3.7 C), and 176 (Figure 3.7 D) reveals interesting trends in the relative abundance of the major components P1, P2, and P3. Assuming that the trends observed for the elution of the disaccharides holds for the tetrasaccharides, we suspect that P1 is the most highly sulfated of the 3 major components, and is most likely the IS–IS tetrasaccharide. Various minor components that migrate after the three major components are also present in all four electropherograms, but are most abundant in fractions 163 and 176. Pooling of all the dp4 fractions would clearly dilute and further complicate isolation of these minor components.

The heterogeneity of the preparative SEC fractions becomes even more pronounced when heparin is digested using a heparin lyase cocktail. The CE–UV profile (Figure 3.8) of SEC fraction 72 of the tetrasaccharide peak of ED2 has greater compositional diversity than observed in Figure 3.7 for the electropherograms derived from ED1. The electropherogram for fraction 72 shows two major components migrating between 4 and 5 min as well as several additional components of intermediate and minor abundance. A major limitation in identification of the tetrasaccharide components in these electropherograms is the lack of structural information available from CE–UV. Furthermore, without standards there is no way to evaluate peak purity, important because peak overlap is a potential problem for complex mixtures. Although CE–MS could potentially provide important structural information about the separated components, such experiments are far from routine.

3.3.3 Reversed-Phase Ion-Pairing (RPIP)-UPLC-UV-MS
Figure 3.8. CE-UV electropherogram of SEC fraction 72 of the dp 4 peak of ED2.
To further investigate the composition of ED2 SEC fraction 72, RPIP-UPLC-UV-MS was employed using the volatile ion-pairing reagent, Bu$_3$N. We have previously utilized this approach to separate and detect the disaccharide standards in Table 3.1. Because the reversed-phase ion-pairing separation mechanism relies on the relative strength of interactions between the negatively charged oligosaccharides and the cationic ion-pairing reagent, highly sulfated compounds are more highly retained by the C$_{18}$ column. As a result, the elution order of the disaccharides in the RPIP-UPLC separation is roughly the inverse of the CE migration order shown in Figure 3.3.

The RPIP-UPLC chromatogram of fraction 72 measured with UV detection at 232 nm is shown in Figure 3.9. The fully sulfated heparin tetrasaccharide IS–IS should be the most highly retained component in this separation. A portion of the mass spectrum extracted from the prominent peak (P5) is shown in Figure 3.10 A. The doubly charged molecular ion, $m/z$ of 575.9639, confirms the identity of this tetrasaccharide as IS–IS. The mass spectral analysis of heparin-derived oligosaccharides is complicated by the preponderance of isomers. As a result, it is not possible for us to unambiguously assign the remaining components from these data alone. The mass spectra for P2 (Figure 3.10 B) and P1 are very similar with a doubly charged molecular ion of $m/z$ 517.0101, indicating that at least one of the disaccharide components contains an acetylated glucosamine. Similarly, P4 (Figure 3.10 C) and P3 have a doubly charged molecular ion $m/z$ 535.9874, which could be produced by a number of different disaccharide combinations involving IS and IIS, IIIS, or IH. Although additional structural information could be derived for these components from MS/MS and NMR experiments, these are beyond the scope of the
Figure 3.9. RPIP-UPLC-UV chromatogram of fraction 72 measured with UV detection at 232 nm. The major peaks are labeled P1-P5.
Figure 3.10. (A) Extracted mass spectrum of the most intense absorbance peak (P5) from the RPIP-UPLC-UV chromatogram shown in Figure 3.9. The molecular ion, \( m/z \) 575.9639, confirms the identity of this tetrasaccharide as IS-IS. (B) Mass spectrum of P2 and (C) P4 from the same chromatogram.
current study to probe the heterogeneity resulting during SEC fractionation of heparin enzymatic digests.²⁶-²⁸

3.4 Summary

The heterogeneous composition of peaks obtained by SEC separation of heparin digests was demonstrated using CE–UV profiling of collected fractions. Although this was most clearly observed for the disaccharide SEC fractions, peak heterogeneity was also observed for the tetrasaccharide SEC peak. Examination of the preparative SEC fractions of larger oligosaccharides is complicated by their greater complexity and the limited availability of standards. Analysis using RPIP-UPLC-UV-MS can provide needed structural insights and should prove very useful in future studies. The results of these experiments suggest that if the goal of a heparin or HS digestion is the isolation of minor components, analysis of the SEC fractions prior to pooling can allow the intelligent combination of fractions containing an unknown species of interest.
3.5 REFERENCES


24. Korir, A. K.; Limtiaco, J. F.; Gutierrez, S. M.; Larive, C. K., Ultraperformance ion-pair liquid chromatography coupled to electrospray time-of-flight mass spectrometry


Chapter Four

Analysis and Characterization of the Physico-Chemical Properties of Heparin Disaccharides

Based in part on a research paper published in Analytical Chemistry


In this chapter, several physico-chemical properties of heparin disaccharide standards were characterized including electrophoretic mobility, mutarotation equilibrium constants, and pK_a values. These studies were conducted to obtain insight into the nature of GAG-protein interactions and proved useful for the optimization and understanding of the charged-based separations commonly used in GAG analysis.

4.2 Introduction: Structural Properties of Heparin and Heparan Sulfate

Heparin and heparan sulfate (HS) are complex glycosaminoglycans (GAGs) with a basic structure comprised of enzymatically-modified repeating disaccharide units of uronic acid-(1→4)-D-glucosamine. These modifications are the result of enzymatic activity during biosynthesis for the purpose of cell adaptation and regulation. The structural variations that are produced, contribute to the sequence microheterogeneity of heparin and HS and are responsible for their ability to bind specifically to a myriad of proteins thereby mediating a wide range of biological processes. The characterization of

the physical properties of heparin and HS functional groups, for example their $pK_a$ values, can provide insights into the nature of GAG-protein interactions.

As discussed in Chapter Three, structural studies of heparin are typically performed on material isolated from tissues of animals used for consumption (i.e. porcine intestine). HS compositional analysis is more challenging due to its greater structural heterogeneity, the variation of HS structure with tissue type, and its relatively low abundance compared with heparin. Due to the size and complexity of intact heparin and HS, structural analysis generally proceeds by depolymerization of the isolated material into smaller fragments by enzymatic or chemical methods. The resulting component mixture is then separated according to size by size-exclusion chromatography (SEC) followed by a charge-based separation. The charge-based separation methods commonly used are strong anion exchange high-performance liquid chromatography (SAX-HPLC) and capillary electrophoresis (CE). Determination of the $pK_a$ values of the carboxylate and $N$-unsubstituted GlcN amino moieties also allows calculation of the total net charge on a heparin or HS oligosaccharide at a given pH, information useful for optimization of the charge-based separations typically used in GAG analysis.

### 4.2.1 Chemical Analysis by Capillary Electrophoresis-UV (CE-UV)

The primary difficulty in analyzing mixtures of GAG-derived oligosaccharides is their structural complexity; namely the presence of substitutional isomers and both $\alpha$ and $\beta$ glycosidic linkages. In addition, native heparin and HS do not contain strong chromophores that can be easily monitored with UV detection. However, enzymatic
depolymerization of intact heparin and HS using lyases introduces a double bond between the C4 and C5 carbons of the uronic acid residue allowing UV detection at 232 nm.

CE-UV has been shown to be an efficient and sensitive method for the separation of heparin and HS oligosaccharides due to its high resolving power and low sample consumption. CE can be used to evaluate the charge-to-mass ratio (q/m) and purity of size-separated SEC fractions, although a difficulty in developing separation methods for subsequent structural studies is the lack of authentic standards for larger oligosaccharides.

For positive peak identification in CE, it is customary to first develop a separation method for standards in which the migration times can be used for comparison with unknown samples. Of the 12 possible glucosamine-containing heparin disaccharides listed in Table 4.1, 11 are readily available commercially, and have been separated by reversed polarity CE methods using either formate or phosphate buffers, at pH 3.40 and 3.50, respectively. Comparison of the electropherograms reported for these buffer systems revealed differences in the migration order of the disaccharides that were N-acetylated (IA-IVA) and those that contained a primary amine (IH-IVH), suggesting that the migration order of these disaccharides may be determined by the CE buffer components. Our laboratory has been successful in carrying out similar reversed polarity CE separations, however in our hands formate and phosphate buffers produce the same migration order for all heparin disaccharides. These conflicting results, in part, motivated the current study to measure the pKₐ values and electrophoretic mobilities (µₑ) of the set of heparin disaccharides shown in Table 4.1.
**Table 4.1.** Legend for the structures of commercially available heparin disaccharide standards; IA-IVA, IS-IIIS, and IH-IVH.

![Image of disaccharide structures]

<table>
<thead>
<tr>
<th>Type of Disaccharide</th>
<th>R₁</th>
<th>R₂</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisulfated disaccharide</td>
<td>IS</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>Disulfated disaccharides</td>
<td>IA</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td></td>
<td>IIA</td>
<td>H</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>IIIA</td>
<td></td>
<td>SO₃⁻</td>
<td>H</td>
</tr>
<tr>
<td>IIH</td>
<td></td>
<td>H</td>
<td>SO₃⁻</td>
</tr>
<tr>
<td>IIIH</td>
<td></td>
<td>SO₃⁻</td>
<td>H</td>
</tr>
<tr>
<td>Monosulfated disaccharides</td>
<td>IVA</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Nonsulfated disaccharides</td>
<td>IVH</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>
4.2.2 Chemical Analysis by cITP-NMR

cITP-NMR has been shown to be an effective method for the separation and characterization of heparin oligosaccharides.\textsuperscript{14-16} Due to the high negative charge density of heparin polysaccharides, the anionic mode of cITP is best suited for this analysis. Equivalent to reversed polarity in CE, this mode of cITP harbors the challenge of overcoming the opposing migration of cations due to residual electroosmotic flow (eof). This requires the development of suitable buffer systems that aid in suppressing eof and stabilizing the individual analyte zones. In our lab, we have addressed this limitation by utilizing fused-silica capillaries internally modified by MicroSolv Technologies (Long Branch, NJ) with polyvinyl alcohol (PVA). Former group member, Dr. Albert Korir, was instrumental in the preliminary development of optimal anionic cITP separation conditions used for the analysis of heparin oligosaccharides.\textsuperscript{16}

The coupling of cITP with NMR allows the direct structural observation of separated heparin components. The development of a cITP-NMR database containing the \textsuperscript{1}H NMR chemical shifts of commercially available disaccharides could be a valuable tool for streamlining the identification of novel or rare oligosaccharides derived from depolymerized heparin. \textsuperscript{1}H NMR survey spectra of heparin digests could be quickly matched to the database to identify known sequences and provide preliminary structural information for unusual disaccharides. Although traditional one-dimensional experiments could provide similar information, cITP-NMR requires significantly less material (1-2 µg). As shown in Chapter 2, cITP offers the additional advantage of focusing the analyte away from matrix components that may be present in the sample. This is important for
the evaluation of depolymerized heparin fragments isolated from the complex mixture obtained by enzymatic digestion. In the studies reported for this chapter, cITP-NMR was used to focus nanomole amounts of each disaccharide standard. Because successful focusing will produce NMR spectra of the pure component, unambiguous chemical shifts can be determined, even for complex samples.

4.2.3 Ring Conformations of Heparin Monosaccharides and Mutarotation of GlcN Residues

The individual monosaccharide residues within the heparin and heparan sulfate polymer take on three possible conformations: $^1C_4$ chair, $^4C_1$ chair, and $^2S_0$ skew boat (Figure 4.1). As revealed by NMR spectroscopy, the GlcA and GlcN residues prefer the rigid $^1C_4$ chair conformation in both free and protein-bound heparin and in heparin-derived oligosaccharides (Figure 4.1 A).$^{17, 18}$ IdoA is more flexible and it assumes all three conformations depending on the substitution pattern and its relative position in the polysaccharide chain (Figure 4.1 B). When IdoA is internally located in the heparin polymer, an equilibrium exists between the $^1C_4$ and $^2S_0$ conformations.$^{19-21}$ If the IdoA is located at the non-reducing end of an oligosaccharide all three conformations are accessible. However, a preference for the $^2S_0$ conformer has been shown upon binding to specific proteins.$^{22-24}$ The relative proportions of the three IdoA conformers varies as a function of sulfation pattern and oligosaccharide sequence.

In the performed experiments, both the heparin disaccharide standards and heparin-derived tetrasaccharides were enzymatically derived using heparin lyases. As mentioned previously, enzymatic depolymerization reactions produce
Figure 4.1. Ring conformations of the monosaccharide residues of heparin and heparan sulfate. (A) The predominant $^4C_1$ chair form of glucuronic acid (GlcA) and glucosamine (GlcNS6S shown). (B) The equilibrium mixture of $^4C_1$, $^1C_4$, and $^2S_0$ conformers of IdoA (IdoA(2S) shown). (C) The major, $^2H_1$, and minor, $^1H_2$, form of unsaturated uronic acid residues ($\Delta$UA2S shown).
unsaturated uronic acid residues (ΔUA) at the non-reducing terminus of the oligosaccharide fragments. These residues can exist in either the $^2H_1$ or $^1H_2$ conformations (Figure 4.1 C) and the equilibrium between these two forms is controlled by their substitution pattern. However, NMR studies suggest that for solution structures of heparin-derived oligosaccharides, the terminal ΔUA residue resides predominantly in the $^1H_2$ conformation with a minor contribution from $^2H_1$.\textsuperscript{19,26}

On the reducing end of an enzymatically cleaved oligosaccharide fragment, the conformation of the glucosamine residue resides in the $^1C_4$ chair conformation and interconverts between the $\alpha$ and $\beta$ anomeric form. It has been suggested that for free glucosamine residues, the dominant conformer depends upon the differences in hydration.\textsuperscript{27,28} In this chapter, the mutarotation of the GlcN residues located on the reducing end of the heparin disaccharide standards was investigated using NMR spectroscopy. The acid-base and anomeric equilibrium of these residues was determined, which could provide further insight into the protein-binding interactions of heparin and HS.

### 4.2.4 $pK_a$ Measurements of Heparin and Heparan Sulfate Substructures

There are three major types of anionic functional groups in the heterogeneous heparin polyanion; $O$-sulfo, $N$-sulfo, and carboxylate. There is also the possibility of cationic functional groups due to the $N$-unsubstituted GlcN primary amine, which occurs more frequently in HS than in heparin. Previous studies have examined the acidic moieties of heparin, however, the methods used were incapable of distinguishing the
individual pK_a’s of these groups within the heparin polymer and did not provide insights as to how functional group acidity is affected by variations of nearby substituents.\textsuperscript{29-33} In a follow-up study by Wang et al., the acidity of heparin’s uronic acid carboxylate groups in enzymatically derived di-, tetra-, and hexasaccharides was examined using \textsuperscript{13}C NMR spectroscopy.\textsuperscript{34} These authors measured individual pK_a values for the carboxylate groups within each heparin oligosaccharide as well as the intact heparin polymer, and explored factors that contributed to the observed differences in acidity. However, this work explored a limited number of O-sulfation configurations and did not examine structures containing a GlcN ammonium group. To our knowledge, the pK_a values of heparin or HS disaccharides containing N-unsubstituted GlcN residues have not been previously reported. Therefore, the goal of this work, in part, is to gain a better understanding of the CE and cITP separation process by determining the pK_a values of the carboxylate and ammonium moieties of heparin disaccharides through \textsuperscript{1}H NMR detected pH titrations. These pK_a values are used to calculate the effective net charge of the disaccharides and are compared with the electrophoretic mobilities (\(\mu_e\)) measured by CE. Furthermore, defined sequences within the heparin chain responsible for specific protein recognition are known to contain \(\geq 2\) disaccharides.\textsuperscript{24, 35, 36} Therefore, this work is extended to include the pK_a determination of the uronic acid carboxylate groups on three major tetrasaccharides produced by enzymatic digestion with heparinase I. The results of the tetrasaccharide pK_a determinations also set the stage for a portion of the future work discussed in Chapter 6.
4.3  Experimental Section

4.3.1  Chemicals

All chemicals and reagents were used as received without any further purification. The purity of the heparin disaccharide standards with respect to possible nonsaccharide contaminants, such as divalent metal ions, was not evaluated. Imidazole, sodium nitrate, phenol, 2-(N-morpholino)ethanesulfonic acid (MES), heparinase I (EC 4.2.2.7) isolated from *Flavobacterium heparinum*, the sodium salt of porcine intestinal mucosal heparin, sodium acetate, calcium acetate, tributylammonium acetate, and heparin disaccharide standards I-IIIS, IA, I-IVH were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Heparin disaccharide standards IIA-IVA were purchased from V-Labs, Inc. (Covington, LA). Disaccharide IVS was not commercially available at the time that these studies were performed. HPLC-grade water was purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, tert-butyl alcohol, sodium hydroxide (NaOH), benzenesulfonic acid (BSFA), deuterated sodium acetate, ammonium bicarbonate, acetonitrile, and sodium phosphate monobasic were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterium oxide (99.9% D low paramagnetic), deuterated acetic acid (99.5% D), sodium deuteroxide (99.5% D), deuterated methanol (99.8% D), and deuterium chloride (99.5% D) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

4.2.2  Capillary Electrophoresis (CE-UV) Experiments

All CE separations were performed on a Beckman Coulter ProteomeLab™ PA800 instrument equipped with a diode array detector, fluid-cooled column cartridge,
and automatic injector. The fused silica capillaries supplied by MicroSolv Technology Corporation (Eatontown, NJ) were 50 cm in length (length to detector 42.5 cm), 75 µm i.d., and 375 µm o.d. The detection window was formed by removal of ~1 mm of polyimide coating from the outside of the capillary using a MicroSolv Window Maker™. Capillaries were conditioned by rinsing with 0.1 M NaOH for 1 min, HPLC-grade water for 5 min, followed by a rinse with buffer for 5 min, all at high pressure (20 psi). The capillary was equilibrated with the buffer by applying a separation voltage of 18 kV using reversed polarity for 7 min. Absorbance was monitored at 232 nm and the data was collected and processed using 32 Karat software (Beckman Coulter, Inc., Fullerton, CA). All bulk solutions and samples were filtered using a 0.2 µm membrane filter prior to analysis.

### 4.2.2.1 CE Separation in Formate Buffer

A CE separation method was optimized according to the work reported by Ruiz-Calero et al. using a standard mixture of heparin disaccharides IA-IVA, IS-IIIS, and IH-IVH (sodium salts) dissolved in 250 µL of 60 mM formic acid buffer (pH = 3.43).\(^{13}\) The structures of the heparin disaccharides are defined in Table 4.1 and their concentrations ranged from 0.02 to 0.12 mM. A reference marker of 0.5 mM BSFA was used in all CE sample solutions to account for fluctuations in eoe. Between experiments, the capillary was rinsed with HPLC-grade water for 2 min then with buffer for 3 min, both at a pressure of 50 psi. Samples were pressure injected at 0.5 psi for 7 s. The separation
voltage was 20 kV and the total run time was 45 minutes. No pressure was applied during the separation.

### 4.2.2.2 CE Separation in Phosphate Buffer

CE separations using phosphate buffer were carried out using a method similar to that reported by Karamanos et al.\textsuperscript{11,12} A solution containing a standard mixture of heparin disaccharides was prepared as described above in 50 mM phosphate buffer (pH = 3.50). Between experiments, the capillary was rinsed with 0.1 M NaOH, followed by HPLC-grade water for 2 min and phosphate buffer for 5 min, all at high pressure (50 psi). Samples were pressure injected at 0.5 psi for 7 s. The separation voltage was 23.3 kV and total run time was 35 min. No pressure was applied during the separation.

### 4.2.3 cITP-NMR Experiments

The instrumental cITP-NMR setup and operation is similar to that described in Chapter Two. The “Zero eof “ fused silica capillary used in the cITP experiments was 180 cm long with an i.d. of 180 µm and an o.d. of 340 µm (Polymicro Technologies, Phoenix, Arizona, USA). Samples were injected hydrodynamically at a height of 16 cm, giving an injection rate of 1.25 µL/minute. The voltage applied across the capillary for all separations was 15 kV.

On-line cITP-NMR spectra of the heparin disaccharide standards were recorded using a Bruker Avance spectrometer operating at 599.983 MHz. Once a steady current was reached in the cITP-NMR experiments, NMR acquisition was initiated to acquire an
array of $^1$H NMR spectra using 45° pulses with an acquisition time of 1.39 s and a spectral width of 6009.6 Hz. Each spectrum was acquired by coaddition of 8 transients with a total acquisition time of 11.12 s. Line broadening equivalent to 2.0 Hz and zero-filling to 128 K points were applied prior to Fourier transformation.

4.3.3.1 Anionic cITP of Heparin Disaccharides IS-IIS, IA-IIIA, and IH

The buffer system used in the anionic cITP focusing of heparin disaccharides IS-IIS, IA-IIIA, and IH was 160 mM DCl/80 mM imidazole as the leading electrolyte (LE) and 160 mM MES as the trailing electrolyte (TE). Both were adjusted to a pD of 6.90 using NaOD. The LE contained tert-butyl alcohol as a chemical shift reference (1.236 ppm relative to trimethylsilylpropionic acid). Solutions containing 300 µM of each disaccharide were prepared in 10 mM TE and D$_2$O. The injection protocol involved first filling the capillary with LE, followed by a 6 minute injection of sample, then a 7 minute injection of TE. The LE and TE ends of the capillary were placed in their respective buffer vials and the separation voltage was applied via electrical connections to platinum electrodes. The currents produced during the course of the cITP experiments ranged from 81 to 52 µA.

4.3.3.2 Cationic cITP of Heparin Disaccharide IVH

The cITP focusing of heparin disaccharide standard IVH was performed in cationic mode. The LE buffer consisted of 160 mM deuterated sodium acetate adjusted to a pD of 4.74 with 99% deuterated glacial acetic acid and containing tert-butyl alcohol as
the chemical shift reference. The TE buffer consisted of 160 mM deuterated acetic acid, 
pD 2.55. Both buffers were prepared in D$_2$O. A 500 µM sample of IVH was prepared in a 
1:1 solution of TE and D$_2$O. The injection protocol involved first filling the capillary with 
LE, followed by a 1 minute injection of TE, 12 minute injection of sample, then 1 minute 
injection of TE. The currents produced during the course of the cationic cITP 
experiments ranged from 18 to 12 µA.

4.2.4 Isolation of Heparin-Derived Tetrasaccharides for Titration

4.3.4.1 Enzymatic Digestion of Heparin

Enzymatic depolymerization proceeded by dissolving 350 mg of porcine 
intestinal mucosal heparin in a 7 mL buffer solution containing 20 mM calcium acetate 
and 50 mM sodium acetate to which 60 U of heparinase I was added (Sigma units, where 
1 U is defined as the quantity of enzyme that will form 0.1 µmol of unsaturated uronic 
acid product per hour at pH 7.5 and 25 °C). The depolymerization reaction was 
performed at 30 °C at an initial pH of 7.2. The progression of the digestion was followed 
by performing UV measurements (232 nm) on 6 µL of the digest diluted with buffer to a 
total volume of 600 µL. All UV measurements were performed using a Varian Cary 50 
Bio spectrophotometer (Palo Alto, CA, USA). The total time for the depolymerization 
was 12 h.

4.3.4.2 SEC Fractionation
Figure 4.2. Preparative scale SEC-UV chromatogram showing relative absorbance versus fraction for the separation of the heparin oligosaccharides produced by enzymatic digestion with heparinase I. The peak with the greatest absorbance (fractions 357-440) corresponds to the disaccharide components (dp2), the preceding peak (fractions 318-356) corresponds to tetrasaccharides (dp4), followed by hexasaccharides (dp6) and larger oligosaccharides (fractions 186-317).
The heparin-derived oligosaccharide solution was size fractionated on a column (3 cm × 47 cm) containing Bio-Rad Bio-Gel P-6 resin. The heparin oligosaccharide components were eluted with 0.5 M ammonium bicarbonate at a flow rate of 0.7 mL/min. The total volume contained in each fraction was 0.5 mL. The progress of the separation was monitored by measurement of UV absorption off-line using a Thermo Scientific NanoDrop ND-1000 (Wilmington, DE, USA). A plot was constructed of the relative UV absorbance versus SEC fraction number, as shown in Figure 4.2. In this chromatogram, the peak with the greatest absorbance (fractions 357-440) corresponds to the disaccharide components (dp2) produced by complete enzymatic digestion. The preceding peak (fractions 318-356) corresponds to tetrasaccharides (dp4), followed by hexasaccharides (dp6) and larger oligosaccharides (fractions 186-317). Each fraction was lyophilized, reconstituted in 500 uL of HPLC-grade water, and then lyophilized again to reduce the NH₄HCO₃ content. The lyophilized powders were stored at -20 °C until further use.

4.3.4.3 Separation and Isolation of Heparin-Derived Tetrasaccharides

Five SEC fractions containing the heparin-derived tetrasaccharides each were reconstituted in 100 µL of Buffer A (see below) and pooled. Then 8 uL of this solution was injected onto the UPLC, for a total of 33 injections.

Three major tetrasaccharides resulting from enzymatic digestion of heparin with heparinase I were separated using an AQUITY UPLC system (Waters Corp., Milford, MA, USA) using a 2.1 × 100 mm Acquity UPLC BEH C₁₈ column packed with 1.7 µm particles and a guard column containing the same material. A flow rate of 0.5 mL/min
was used and the analytes were detected using UV absorbance at 232 nm. The column temperature was maintained at 40 °C. A binary solvent system composed of 5% acetonitrile (Buffer A) and 80% acetonitrile (Buffer B) was used. Both buffers contained 2.5 mM tributylamine and 17.5 mM ammonium acetate buffer at pH 7.00. The separation was performed using a gradient of 83-69% A over 11 min. This was increased to 100% B over 1 min and held at 100% B for an additional 1 min before returning to the initial composition of 83% A over 1 min. This composition was retained for 2 min to allow for re-equilibration before the next injection. For each injection, the chromatographic peaks corresponding to the three major tetrasaccharide components were manually collected into separate polypropylene storage tubes. The three storage tubes containing the isolated peaks were placed in the laboratory hood overnight under a stream of air to allow the evaporation of the acetonitrile.

4.3.4.4 Removal of Tributylammonium Ion From Tetrasaccharide Isolates

Following the removal of acetonitrile, a large amount of tributylammonium ion (TrBA) remains behind with the tetrasaccharide isolates. In subsequent NMR analysis of the isolates, an increase in the receiver gain is desirable because of their low intensity relative to the baseline. Therefore, TrBA must be removed to avoid a lowering of the receiver amplification due to the overwhelming peak intensities of TrBA relative to the tetrasaccharides. More importantly, possible interference from TrBA can hinder accurate chemical shift assignments.
TrBA was removed from the tetrasaccharide isolates using a batch separation with cation exchange resin Bio-Rad Chelex® 100, 100-200 mesh, sodium form (Hercules, CA, USA). Prior to use the resin was washed 3x with deuterated methanol to remove the free resin polymer. The treated resin beads were reconstituted in 1 mM deuterated acetic acid and allowed to settle.

Each of the tetrasaccharide isolates were reconstituted in 200 µL of D₂O and transferred into 1.5 mL eppendorf tubes. Approximately 6 µL of washed resin was pipetted into the tetrasaccharide solutions. The solutions were vortexed and allowed to sit overnight. The solutions were then centrifuged for 2 minutes at 10,000 rpm, and the supernatant was removed and placed into a clean 1.5 mL eppendorf tube. A second 6 µL aliquot of resin beads were pipetted into each eppendorf tube and the procedure was repeated as described above. The remaining supernatant was freeze dried and stored at -20 °C until further use.

Optimization of this method was conducted utilizing a 1.12 mM disaccharide IS isolate from the ED1 enzymatic digest described in Chapter 3 spiked with 2.1 mM tributylammonium acetate. Following the two-step batch ion-exchange method described above, NMR spectroscopy measurements revealed that the TrBA concentration was reduced by a factor of 78.4. The NMR spectra from these experiments are shown in Figure 4.3.

4.2.5 Solution Preparation for Indicator Titrations
Figure 4.3. (A) NMR spectra of 1.12 mM heparin-derived disaccharide IS spiked with 2.1 mM tributylammonium acetate. Tributylammonium acetate resonances at 3.167, 1.703, 1.412, and 0.967 ppm were decreased by a factor of 78.4 overall following batch ion-exchange using Na⁺ cation resin. (B) Spectra taken after the first ion-exchange step and (C) after the second step.
Formic acid ($pK_a = 3.75$) was used as the indicator for the NMR titration of the uronic acid carboxylic acid group and imidazole ($pK_a = 6.99$) for titration of the GlcN ammonium group.\textsuperscript{38} $^1$H NMR spectra and pD measurements were recorded for D$_2$O solutions containing 20 mM of each indicator, 0.1 M NaNO$_3$ to minimize ionic strength effects, and $\text{tert}$-butyl alcohol as a chemical shift reference. Solution pD values were measured potentiometrically using a 3.5 x 55 mm combination glass electrode (ESA Biosciences, Inc., Boston, MA) as the solution pD was adjusted in ~0.5 unit steps using aliquots of NaOD or DCl diluted in D$_2$O. Because the pH electrode was calibrated with standard aqueous buffer solutions, the pD was calculated using the pH meter reading (pH*) and the equation: pD = pH* + 0.4.\textsuperscript{39} The indicator solution temperature was maintained at 25 °C using a water bath during the potentiometric measurements.

### 4.2.6 Solution Preparation for Heparin Disaccharide Titrations

D$_2$O solutions of the heparin disaccharides IA-IVA, IS-IIIS, and IH-IVH, shown in Table 4.1, were each prepared individually with the following composition: 6 mM indicator species, 1.74 mM $\text{tert}$-butyl alcohol, and 0.1 M NaNO$_3$, to maintain a constant ionic strength. The initial concentration of each heparin standard ranged from 1.5 to 5.9 mM. The pD of each solution was adjusted using aliquots of NaOD or DCl diluted in D$_2$O and the $^1$H NMR spectrum recorded. Since disaccharides IH-IVH contain both functional groups of interest, two separate titration solutions were prepared containing each of the indicators.
A separate titration was performed using an aqueous solution containing 1.5 mM disaccharide IS, 6 mM formic acid, 1.74 mM tert-butyl alcohol, and 0.1 M NaNO₃ dissolved in 95% H₂O/5% D₂O. The pKₐ(H) determined from this titration curve was used to calculate the ΔpKₐ term used to correct for solvent isotope effects in the effective net charge calculations.

4.2.7 Solution Preparation for Heparin Tetrasaccharide Titrations

D₂O solutions of the heparin tetrasaccharides IS-IIIS, IS-IIS, and IS-IS shown in Figure 4.4, were each prepared individually with the following composition: 1 mM formic acid indicator species, 0.1 mM tert-butyl alcohol, and 0.1 M NaNO₃, to maintain a constant ionic strength. The concentration of each tetrasaccharide ranged from 0.5 to 1.9 mM. The pD of each solution was adjusted using aliquots of NaOD or DCI diluted in D₂O and the ¹H NMR spectrum recorded.

4.2.8 ¹H NMR Experiments

All ¹H NMR measurements were performed at 298.0 K with a Bruker Avance spectrometer operating at 599.983 MHz. The resonance of tert-butyl alcohol was used as a chemical shift reference (1.236 ppm relative to trimethylsilylpropionic acid) and chemical shift values were measured in Hertz to obtain the maximum number of significant figures. Spectra for NMR titrations were acquired by coaddition of 64 transients using a 90° pulse, acquisition time of 1.8 s, and a 1.0 s relaxation delay. A series of pD-dependent ¹H NMR spectra were acquired for 600 µL aliquots of both
Figure 4.4. The three major tetrasaccharides derived from enzymatic depolymerization with Heparinase I.
indicator solutions and each of the heparin standard solutions in 5 mm NMR tubes
(Wilmad/Lab Glass, Buena, NJ). For the heparin-derived tetrasaccharide titrations, 500
µL aliquots were used. Graphs of chemical shift versus pD were plotted using Origin 7.5
software (OriginLab, Northampton, MA). A function based on Equation 4.2 (see Results
and Discussion section) was fit to the experimental data to determine the pK\( _a \)(D) of the
carboxylic acid and/or GlcN ammonium groups. The reported uncertainties are fitting
errors determined from the nonlinear least-squares analysis and likely underestimate the
true precision.

To determine the anomeric equilibrium constant for each heparin disaccharide,
fully relaxed \(^1\)H NMR spectra were acquired by coaddition of 192 transients using a 90°
pulse, an acquisition time of 1.64 s, and a relaxation delay of 20.0 s. The concentration of
each disaccharide ranged from 1.0 to 1.5 mM and the pD of each solution was 7.9. Zero-
filling to 65536 points was applied prior to Fourier transformation. The spin-lattice \((T_1)\)
relaxation times for protons of disaccharides IA, IIIH, and IS were determined in separate
experiments using an inversion-recovery pulse sequence. Spectra were measured using
16 different longitudinal relaxation periods \((t_1)\) ranging from 0.05 to 15 s and 192
transients were acquired for each \(t_1\) increment. A spectral width of 7184 Hz was used and
65K data points were collected in F2. \(T_1\) relaxation times were determined by plotting the
signal intensity of the proton resonances versus \(t_1\) using the Origin software and fitting
with an exponential function based on the equation:

\[
I_t = I_0 (1 - 2e^{-t_1/T_1}) \quad (4.1)
\]
Where \( I_0 \) is the peak intensity at equilibrium and \( I_t \) is the intensity following each \( t_1 \) delay. For each of the disaccharides studied the H4’ proton of iduronic acid had the longest \( T_1 \) relaxation time, with the highest value measured for disaccharide IIIH (3.48 ± 0.03 s). Therefore, in determining the anomeric equilibrium constants, \(^1\)H NMR measurements were performed using a 20.0 s relaxation delay to ensure that fully relaxed spectra were acquired.

**4.2.8.1 Two-dimensional DQF-COSY (double-quantum filtered COSY)**

DQF-COSY experiments were also performed for disaccharides IA, IS, and IH-IVH to confirm the resonance assignments of the \( \alpha \) and \( \beta \) anomers. The basic DQF-COSY pulse sequence is shown in Figure 4.5 A. It consists of three 90° radiofrequency pulses, the first two of which are separated by a variable time delay, \( t_1 \). The first 90° pulse creates magnetization on the transverse plane. During the evolution time, \( t_1 \) is incremented systematically to allow coherences to evolve under the chemical shift of the spins. The second 90° pulse is applied and transfers coherence from one spin to J-coupled spins. Application of the third 90° pulse allows the spins to be observed and any elements that did not go through double quantum transitions are removed out through phase cycling. Following Fourier transformation, cross peaks are observed in the DQF-COSY spectrum for nuclei that are directly spin-coupled.

For disaccharides IH, IIH, IIIH, IA, and IIA resonance overlap in the one-dimensional \(^1\)H NMR spectra precluded accurate chemical shift measurements at all pD
Figure 4.5. NMR pulse sequences for (A) DQF-COSY, (B) TOCSY, and (C) CPMG.
values for determination of the pK_a(D) values. As necessary, DQF-COSY spectra were acquired to allow extraction of chemical shift values from the COSY cross peaks for use in the pD titration plots. Phase-sensitive DQF-COSY spectra were obtained by acquisition of a 2K × 256 data matrix, with 16 scans per increment, a relaxation delay of 1.5 s, and a spectral width of 4800 Hz in both dimensions. The 2D spectra were apodized by multiplication with a sine function.

4.2.8.2 Two-Dimensional Total Correlation Spectroscopy (TOCSY)

TOCSY experiments can provide information regarding all scalar coupled protons within a given spin system, not just between 3-bond couplings as in COSY. The TOCSY pulse sequence is shown in Figure 4.5 B. During the TOCSY spin-lock, the magnetization is transferred directly or relayed to up to 5 or 6 bonds through the entire spin system. The number of transfers can be adjusted by changing the spin-lock time.

In the tetrasaccharide pD titrations, the H5 proton of the internal uronic acid residue was followed as a reporter proton for the carboxylate pK_a determination. At higher pD values (>4.30) overlap of the H5 and the HOD resonances became a problem. As needed, TOCSY experiments were performed to obtain chemical shift values for the H5 resonance with an incorporated WET (Water suppression Enhanced through T_1 effects) pulse sequence for solvent suppression. The water resonance (~4.80 ppm) was suppressed using a 15 ms SINC shaped pulse. These spectra were measured by acquiring a 2K × 256 data matrix, with 128 scans per increment, and a relaxation delay of 1.6 s.
between scans. A MLEV-17 spin-lock, with a mixing time of 80 ms, and a spectral width of 4808 Hz in the F2 dimension and 6614 Hz in the F1 dimension was used.

### 4.2.8.3 Water Attenuation by Transverse Relaxation (WATR)

Another very effective method for resolving chemical shift information for resonances that reside under the H$_2$O or residual HOD peak is the Water Attenuation by Transverse Relaxation (WATR) method, developed by Rabenstein and co-workers.$^{41-43}$ This NMR technique selectively eliminates the water resonance by transverse relaxation without the loss of sample resonances typical of other types of solvent suppression sequences. By adding a reagent with exchangeable protons (i.e. NH$_4^+$) to the solution, the apparent spin-spin relaxation time ($T_2^*$) of the water protons is reduced by chemical exchange. The one-dimensional $^1$H NMR spectrum is measured by the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence shown in Figure 4.5 C. Between the 90° pulse that creates transverse magnetization and acquisition of the free induction decay, a 180° pulse train of length $2n\tau$ allows for selective elimination of the water resonance by transverse relaxation. The addition of a proton exchange reagent increases the rate of relaxation of the water protons. However, the intensity of sample resonances will also decrease due to transverse relaxation, so the experiment should be optimized to keep the total transverse relaxation time, $2n\tau$, as short as possible. Because the rate of proton exchange between water and the exchange reagent is pH dependent, so is its effect on the water proton relaxation times.$^{43}$ Therefore, the solution pH or pD must be adjusted to a value that produces sufficient attenuation of the H$_2$O or HOD resonance.
The WATR method was used to resolve the H5 proton of the internal uronic acid
residue of the tetrasaccharides. Ammonium chloride was used as the proton exchange
reagent at a concentration of 0.45 M dissolved in 90% H$_2$O/10% D$_2$O. The
tetrasaccharides were individually reconstituted in the exchange reagent solution and 60
µM tert-butyl alcohol. The pH was adjusted to 6.40 using 0.1 M NaOD. The length of the
transverse relaxation period (2nτ) varied between 0.15 and 0.18 ms. This time was
optimized by acquiring several spectra as a function of the length of the transverse
relaxation period to find the minimum time required to completely eliminate the water
resonance. Because this method is pH dependent, only the H5 chemical shift at pH 6.40
could be measured for all three tetrasaccharides.

4.3 Results and Discussion

4.3.1 Determination of Heparin Disaccharide pK$_a$ Values

The pK$_a$ of an acidic or basic functional group can be determined by monitoring
the NMR chemical shifts of nearby protons that are affected by changes in local electron
density as the functional group is titrated.$^{44, 45}$ For a monoprotic acid, the observed
chemical shift ($\delta_{\text{obs}}$) of an affected nucleus can be related to pH through the equation

$$pH = pK_a + \log \left( \frac{\delta_{\text{obs}} - \delta_{\text{HA}}}{\delta_{\text{A}} - \delta_{\text{obs}}} \right)$$  \hspace{1cm} (4.2)

where $\delta_{\text{HA}}$ and $\delta_{\text{A}}$ are the limiting chemical shifts of the fully protonated and
deprotonated species. Using Equation 4.2, the pK$_a$ can be determined by the nonlinear
least-squares fit of $\delta_{\text{obs}}$ measured as a function of pH.$^{46}$ To reduce the interference of the
solvent resonance, D$_2$O was used as the solvent for these NMR experiments. Therefore, solution pD values were recorded and the measured conditional acidity constants, $K_a(D)$, reflect contributions from the deuterium isotope effect.$^{47}$

Accurate potentiometric measurements of pH (or pD in D$_2$O solution) can be made with a small glass electrode using a few milliliters of solution, providing that the solution is well-stirred and maintained at constant temperature. Because the disaccharides used in this work are fairly expensive and the tetrasaccharide isolates are available in limited quantities, it was desirable to perform the pD titrations using only 600 µL of solution in a 5 mm NMR tube (500 µL for tetrasaccharides), conditions that limit the accuracy of potentiometric pH measurements. This limitation was overcome by addition of NMR pH indicators to the solutions being titrated. Potentiometric titrations were performed on NMR indicator solutions of 5 mL to accurately determine the values of $\delta_{D\text{Ind}}$, $\delta_{\text{Ind}}$, and $pK_a(D)_{\text{Ind}}$ from a plot of the $\delta_{\text{obs}}$ versus pD. Addition of the NMR indicator to the sample solution allows these parameters to be used for the electrodeless determination of sample pD over the effective pD range of each indicator.

From a practical standpoint, an ideal NMR pH (or pD) indicator should have a large deprotonation shift, $\Delta\delta = \delta_{\text{Ind}} - \delta_{\text{Hind}}$, for precise determination of the pH of the sample solution. For our purposes, the indicator should only have a single dissociation step and its $pK_a$ should match as close as possible the analyte $pK_a$ of interest. The further away the pH is from the $pK_a$ of the indicator, the greater the error in the derived pH calculation. Also, the indicator should give rise to only a few peaks in the NMR spectrum and they should not overlap with the resonances of the analyte. Finally, NMR pH
indicators should not interact with other components in the solution. Complexation between the indicator and other substances in the solution could produce errors in the chemical shift measurements.

For these experiments, $^1$H NMR pD titrations were first performed for the formic acid and imidazole indicators separately. For formic acid, the $\delta_{D\text{Ind}}$ and $\delta_{\text{Ind}}$ values of the carbon-bound formate proton were determined to be 8.222 and 8.446 ppm, respectively, with a p$K_a$(D) of 4.04 ± 0.01. This is in good agreement with the value of 4.13 reported in D$_2$O solution, given the different ionic strength conditions employed, as well as that of 3.75 reported for aqueous solutions.\textsuperscript{47-50} The $\delta_{D\text{Ind}^+}$ and $\delta_{\text{Ind}}$ values of the imidazole H2 proton were determined to be 8.689 and 7.762 ppm, respectively, with a p$K_a$(D) of 7.63 ± 0.00. This value is in good agreement with the value of 7.67 reported in deuterated solution,\textsuperscript{51} given the different ionic strength conditions employed, and the p$K_a$ reported in aqueous solutions of 7.15.\textsuperscript{52, 53} The resulting titration curves for both indicators are provided in Figure 4.6.

**4.3.1.1 $^1$H NMR pD Titration of the Iduronic Acid Carboxyl Group**

Titrations to determine the iduronic ring carboxylic acid p$K_a$(D) for disaccharides IA-IVA, IS-IIIS, and IH-IVH were performed using the formic acid NMR indicator. An example titration curve for disaccharide IIIS is shown in Figure 4.7. Because heparin disaccharide IVS was not commercially available at the time these titrations were performed, it was not included in these experiments. pD measurements for the disaccharide carboxylic acid titrations ranged between 1.40 and 7.41. The pD values at
Figure 4.6. (A) $^1$H NMR titration plot for formate ($pK_a (D) = 4.04 \pm 0.01$). The limiting chemical shifts of formic acid and formate are 8.22 and 8.446 ppm, respectively. (B) $^1$H NMR titration plot for imidazole ($pK_a (D) = 7.63 \pm 0.01$). The limiting chemical shifts for imidazolium and imidazole are 8.689 and 7.762 ppm, respectively.
Figure 4.7. Titration of the carboxylate group following the change in chemical shift of the IIIS disaccharide H4’ resonance indicated with a circle. The $pK_a(D)$ value is $3.74 \pm 0.01$. 
the extremes of the titration fall outside the effective range of the formic acid indicator, pD 3.44 (80% formic acid) - pD 4.64 (80% formate). The chemical shift change was small (~9.8%) in the range between ± 80% and ± 90% of the titration curve and it was found that the error in the calculated pD grows rapidly at values > 80% of each indicator species. Outside this effective indicator range, potentiometric pD values were used to construct the disaccharide titration curves. The pK\(_a\)(D) values for each carboxylate group, listed in Table 4.2, were determined by nonlinear least-squares fitting of the plot of δ\(_{\text{obs}}\) for the H4’ proton of the iduronic ring vs. pD. The pK\(_a\)(D) value of 3.76 ± 0.02 determined for disaccharide IS is within reasonable agreement to the 3.95 pK\(_a\)(D) value previously reported by Wang and co-workers.\(^{34}\) The difference in the pK\(_a\)(D) values from these two studies is likely attributed to the different ionic strength conditions employed. Our pK\(_a\)(H) value of 3.16 ± 0.02 for IS is also in fair agreement with 3.15 reported by Mikhailov et al. in deuterated solution.\(^{26}\) However, in their study no corrections were made for isotope effects.

The most significant trend observed in Table 4.2 is the effect of the substituent on the GlcN ammonium group on the acidity of the carboxylic acid. N-sulfated disaccharides have higher pK\(_a\)(D) values than those that are N-acetylated, while the H-series of disaccharides containing a free amine have much lower pK\(_a\)(D) values likely due to charge stabilization of the deprotonated carboxylate group. Within each disaccharide series (S, A, and H) the carboxylic acid pK\(_a\)(D) tracks the degree of sulfation and therefore the degree of overall negative charge. The position of O-sulfo groups also affects the carboxylic acid pK\(_a\)(D), but this appears to be largely a secondary effect.
Table 4.2. $pK_a(D)$ values, mutarotation equilibrium constants and electrophoretic mobilities determined for the heparin disaccharides.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>COO$^-$ $pK_a(D)$</th>
<th>NH$_3^+$ $pK_a(D)$ $\alpha$ anomer</th>
<th>NH$_3^+$ $pK_a(D)$ $\beta$ anomer</th>
<th>$K_{eq}$</th>
<th>$\mu_e$ at pH 3.43 (10$^{-5}$ cm$^2$/s·V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>3.76 ± 0.03$^a$</td>
<td>-</td>
<td>-</td>
<td>0.17</td>
<td>-47.1 ± 0.00$^b$</td>
</tr>
<tr>
<td>IIS</td>
<td>3.67 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.24</td>
<td>-37.5 ± 0.23</td>
</tr>
<tr>
<td>IIIS</td>
<td>3.74 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>0.21</td>
<td>-38.5 ± 0.03</td>
</tr>
<tr>
<td>IA</td>
<td>3.56 ± 0.02</td>
<td>-</td>
<td>-</td>
<td>0.38</td>
<td>-37.2 ± 0.17</td>
</tr>
<tr>
<td>IIA</td>
<td>3.63 ± 0.03</td>
<td>-</td>
<td>-</td>
<td>NR$^c$</td>
<td>-25.1 ± 0.02</td>
</tr>
<tr>
<td>IIIA</td>
<td>3.62 ± 0.02</td>
<td>-</td>
<td>-</td>
<td>0.28</td>
<td>-26.0 ± 0.17</td>
</tr>
<tr>
<td>IVA</td>
<td>3.37 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>NR$^c$</td>
<td>-12.1 ± 0.16</td>
</tr>
<tr>
<td>IH</td>
<td>3.35 ± 0.04</td>
<td>8.78 ± 0.02</td>
<td>8.31 ± 0.01</td>
<td>0.53</td>
<td>-27.7 ± 0.09</td>
</tr>
<tr>
<td>IHH</td>
<td>3.35 ± 0.02</td>
<td>8.71 ± 0.02</td>
<td>8.22 ± 0.02</td>
<td>0.51</td>
<td>-13.4 ± 0.19</td>
</tr>
<tr>
<td>IIIH</td>
<td>3.27 ± 0.03</td>
<td>8.72 ± 0.00</td>
<td>8.27 ± 0.01</td>
<td>0.52</td>
<td>-14.0 ± 0.14</td>
</tr>
<tr>
<td>IVH</td>
<td>3.18 ± 0.04</td>
<td>8.68 ± 0.01</td>
<td>8.17 ± 0.02</td>
<td>0.56</td>
<td>2.02 ± 0.08</td>
</tr>
</tbody>
</table>

a. The reported uncertainties in the $pK_a(D)$ values are fitting errors determined from the nonlinear least-squares analysis.
b. The uncertainties in the mobility measurements are standard deviations calculated from at least three electropherograms.
c. NR = Not reported
4.3.1.2 $^1$H NMR pD Titration of the GlcN Ammonium Group

The GlcN primary amine of disaccharides IH-IVH was titrated over the pD range of 5.68 to 11.38 using imidazole as the indicator. An example titration curve for disaccharide IVH is shown in Figure 4.8. Because complex formation between a highly sulfated heparin tetrasaccharide and imidazole has been previously reported, NMR pD titrations were also performed for these four disaccharides using 3-chlorophenol as the indicator ($pK_a = 9.10$). The disaccharide $pK_a(D)$ values obtained using 3-chlorophenol were in excellent agreement with those measured using imidazole, suggesting that the calculated $pK_a(D)$ values were not affected by binding of imidazole with the disaccharides in these titrations. Imidazole was selected over 3-chlorophenol as the indicator for the determination of the ammonium $pK_a(D)$’s of disaccharides IH-IVH due to its lower $pK_a(D)$. The pD values at the extremes of the titration fall outside the effective range of the imidazole indicator, pD 7.02 (80% imidazolium ion) - pD 8.22 (80% imidazole). Outside the effective indicator range, potentiometric pD values were used to construct the disaccharide titration curves.

DQF-COSY NMR spectra measured at each pD increment were used to verify assignments and facilitated chemical shift measurements for resonances that were not well resolved in the one-dimensional $^1$H NMR spectra. Because mutarotation of the glucosamine ring is slow on the NMR timescale, resonances of the $\alpha$ and $\beta$ anomers could be assigned using the COSY spectra and followed in the pD titration. A
Figure 4.8. Titration of the ammonium group following the change in chemical shift of the IVH disaccharide α anomer H2 resonance indicated with a circle. The pKₐ(D) value is 8.68 ± 0.01.
Figure 4.9. DQF-COSY spectrum of heparin disaccharide IIH at pH 7.80. Because mutarotation of the GlcN ring is slow on the NMR time scale, the resolved resonances of each anomer can be assigned as shown by the labeled $\alpha$ and $\beta$ resonances of the H2 proton between 2.5 and 3.5 ppm.
representative COSY spectrum of heparin disaccharide standard IIH taken at pH 7.80 is shown in Figure 4.9. The chemical shift of the H2 proton, which is adjacent to the GlcN ammonium group, was plotted versus pD and the resulting pK_a(D) values are reported in Table 4.2. The α and β anomers of standard disaccharides IH-IVH each had significantly different pK_a(D) values, with the β anomer being more acidic by about 0.45 in each case. The higher basicity of the α anomer of GlcN was also reported for free glucosamine. In work done by Neuberger and Fletcher, pK_a(H) values were calculated for the α and β anomers of glucosamine using optical rotation measurements and reported as 7.71 and 7.27, respectively. These authors postulated that the closer approach between the ammonium and the 1-OH groups in the β conformation, as suggested by molecular modeling, could increase the relative acidity of the GlcN β anomer. Alternatively, Neuberger and Fletcher postulated that solvent effects could also account for the differences in the pK_a values of the GlcN anomers. They suggested that if the β anomer exists in a normal 1C_4 chair conformation, the 2-NH_2 group is located below the plane of the ring and equatorial to the 1-OH group increasing the regional hydrophobicity due to the presence of 3 axial hydrogens. If the hydroxyl group is below the plane of the molecule with the amine group, this portion of the molecule is less hydrophobic and more susceptible to solvation, making the α anomer the stronger base. This conclusion was later supported by Blaskó et al., who reported a more acidic pK_a(D) for the β-ammonium group of glucosamine (7.87) compared to the α anomer (8.12) determined by NMR titration.
4.3.2 Determination of Heparin-Derived Tetrasaccharide pKₐ’s Using NMR

The tetrasaccharides used in this work were derived from enzymatic digests of porcine heparin, separated as a class by SEC, and isolated using UPLC. The UPLC chromatogram revealed three major components; P1, P2, and P3, as shown in Figure 4.10. Structure elucidation and ¹H resonance assignments for these compounds were performed by John Limtiaco, using a microcoil NMR probe and two-dimensional NMR spectroscopy. The least intense peak, P1 at 3.93 min, corresponds to tetrasaccharide IS-IIIS, P2 at 4.23 min is IS-IIS, and the most abundant peak, P3 at 4.70 min, is IS-IS. To ensure accurate chemical shift assignment of the tetrasaccharide resonances without interference from the TrBA used as an ion-pairing reagent in the separation, the TrBA was removed from the tetrasaccharide samples prior to NMR titration using batch ion-exchange.

To determine the terminal ΔUA2S and internal IdoA carboxylic acid pKₐ(D)’s for tetrasaccharides IS-IS, IS-IIS, and IS-IIIS, titrations were performed using the formic acid indicator. An example titration curve corresponding to the H₄’ reporter proton of the ΔUA2S of the IS-IIIS tetrasaccharide is shown in Figure 4.11. pD measurements for all of the carboxylic acid titrations ranged between 1.79 and 7.36. Again, only the values that fell within the effective range of the formic acid indicator were used. Outside of this range, potentiometric pD values were recorded. The pKₐ(D) values for each carboxylate group, listed in Table 4.3, were determined by nonlinear least-squares fitting of the plot of δₐbs for the H₄’ proton of the ΔUA2S residue and the H₅ proton of the internal IdoA
Figure 4.10. UPLC chromatogram showing the separation of the three major tetrasaccharides derived from enzymatic digestion of porcine heparin with heparinase I.
Figure 4.11. Titration of the carboxylate group following the change in chemical shift of the IS-IIIS tetrasaccharide ΔUA2S H4’ resonance as indicated by a circle. The pK<sub>a</sub>(D) value is 3.84 ± 0.01.
Table 4.3. pKₐ(D) values determined for the heparin-derived tetrasaccharides.

<table>
<thead>
<tr>
<th>Tetrasaccharide</th>
<th>R₁</th>
<th>R₂</th>
<th>ΔUA2S COO⁻ pKₐ(D)</th>
<th>IdoA±2S COO⁻ pKₐ(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-IS</td>
<td>SO₃⁻</td>
<td>SO₃⁻</td>
<td>4.03 ± 0.02</td>
<td>4.48 ± 0.02</td>
</tr>
<tr>
<td>IS-IIIS</td>
<td>SO₃⁻</td>
<td>H</td>
<td>3.84 ± 0.01</td>
<td>4.37 ± 0.03</td>
</tr>
<tr>
<td>IS-IIS</td>
<td>H</td>
<td>SO₃⁻</td>
<td>3.75 ± 0.02</td>
<td>4.17 ± 0.01</td>
</tr>
</tbody>
</table>
residue versus pD. The p\(K_a\)(D) values of 4.03 ± 0.02 and 4.48 ± 0.02 determined for the terminal ∆UA2S and internal IdoA2S residues, respectively, of tetrasaccharide IS-IS are within reasonable agreement to the 3.62 and 4.04 p\(K_a\)(D) values previously reported by Wang and co-workers.\textsuperscript{34} These values are also in good agreement with the p\(K_a\)(D)’s reported by Nguyen of 4.17 ± 0.01 for the ∆UA2S residue and 4.66 ± 0.01 for the IdoA2S residue.\textsuperscript{55} Again, differences in p\(K_a\)(D) values among the three studies is likely due to the different ionic strength conditions employed. The p\(K_a\)(D) values for the ∆UA2S residue of IS-IIS and IS-IIIS are 3.75 ± 0.02 and 3.84 ± 0.01, respectively. The p\(K_a\)(D) value for the IdoA2S residue of IS-IIIS is 4.37 ± 0.03 and for the IdoA residue for IS-IIS is 4.17 ± 0.01. To our knowledge, these values have not been previously reported.

It is expected that as the net negative charge on the molecule increases, the acidity of the carboxylate group will decrease due to charge destabilization. In this study, this was a trend observed for the three tetrasaccharides. Overall, the carboxylate p\(K_a\)(D) values were slightly higher for the tetrasaccharides compared to the disaccharides. Also, the carboxylate p\(K_a\)(D)’s for both the ∆UA2S and IdoA residues, tracked the degree of sulfation and overall negative charge on the molecule, as seen with the disaccharides. However, the position of the O-sulfo groups appears to have a larger effect on the carboxylate p\(K_a\)(D) values of the tetrasaccharides. Although the O-sulfo substituents remain the same for the IS disaccharide residue on all three tetrasaccharides, the p\(K_a\)(D) values of the ∆UA2S carboxylate group decrease based on the sulfate positions of the neighboring disaccharide. This is evidence that the acidity of the carboxylic acid groups
can vary according to the substitution pattern on monosaccharides several residues away. These subtle differences could play an important role in heparin-protein binding events.

### 4.3.3 Determination of Disaccharide Mutarotation Equilibrium Constants

Fully relaxed $^1$H NMR spectra were measured and used to calculate the equilibrium constants ($K_{eq}$) given in Table 4.2 for the mutarotation (expressed as $\beta/\alpha$) for each disaccharide. When possible, the integrated area of the H2 resonance adjacent to the N-substituent of each anomer was used for this calculation. For the A series of disaccharides, it was not possible to use the H2 proton resonances due to resonance overlap, so the H1 resonances were also used. For disaccharides IIA and IVA, resonance overlap of the H1 resonances with other peaks precluded the accurate determination of the mutarotation equilibrium constants. The $\beta$ anomer was most abundant in the H-disaccharides containing a free amine, with intermediate populations observed for the N-acetylated disaccharides, and the disaccharides with N-sulfated GlcN residues having the smallest $\beta$ content. The expansion of the $^1$H NMR spectrum containing the GlcN H2 resonances of the $\alpha$ and $\beta$ anomers of the disaccharide pairs IS and IH, and IIIS and IIIH, shown in Figure 4.12, clearly indicate the prevalence of the $\alpha$ anomer in the N-sulfated disaccharides. For similar molecules, it has been suggested that the $\alpha$ conformation is favored due to the anomeric effect; the axial tendency exhibited by many electronegative groups at the C1 of a tetrahydropyran.$^{27, 56, 57}$ However, the comparison of average $K_{eq}$ values for the series of disaccharides studied herein suggests that the presence of a bulky sulfate or acetyl group at the N-position, may create a steric barrier to mutarotation.
Figure 4.12. $^1$H NMR spectra showing the GlcN H2 resonances of disaccharides IS, IH, IIS, and IIIH. The doublet of doublets between 3.2 and 3.4 ppm is the H2 resonance of the $\alpha$ anomer and the triplet between 2.8-3.1 ppm is the H2 resonance of the $\beta$ anomer. Mutarotation equilibrium constants determined by integration of these resonances are listed in Table 4.2.
4.3.4 Development of cITP-NMR Database for Heparin Disaccharides

4.3.4.1 cITP-NMR Focusing of Disaccharides IS-IIIS, IA-IIIA, and IH

An example of the spectra obtained using cITP-NMR for the heparin disaccharides is given in Figure 4.13 for disaccharide IS. In this plot, each spectrum is the result of 8 transients, coadded. The LE used in these experiments was 160 mM DCl/80 mM imidazole and the imidazole resonances (8.558 and 7.432 ppm), are shown in Figure 4.13 A and B, along with HOD (4.807 ppm), and the chemical shift reference, tert-butanol (1.236 ppm). The imidazole resonances are detected throughout the isotachophoretic zones because imidazole is positively charged and does not focus in anionic cITP. As the LE ion leaves the active volume of the microcoil, heparin disaccharide IS enters and is detected in spectra 4.13 C-F. The anomeric resonances can be clearly seen at 5.497 and 5.444 ppm, along with the most downfield resonance of the ΔUA2S H4 proton at 5.955 ppm. As the disaccharide exits the microcoil, it is replaced by the TE buffer, MES, shown in spectra 4.13 G and H at 3.786, 3.163, 2.928, and 2.702 ppm. Spectrum 4.13 G is measured right at the boundary interface between the analyte and TE as evidenced by the splitting of the imidazole resonances. We have previously observed the splitting of resonances measured at zone boundaries. A detailed discussion of this observation and its significance will be discussed further in Chapter 5.

Similar cITP-NMR spectra of heparin disaccharide standards IIIS, IIIS, IA-IIIA, and IH were also measured. Figure 4.14 shows the spectra obtained by post-acquisition coaddition of the analyte spectra resulting from the cITP-NMR experiments performed for disaccharides IH, IS, and IA. The only difference between these structures is the N-
Figure 4.13. The cITP-NMR spectra of heparin disaccharide IS. Spectra 4.13 A and B contain the resonances of imidazole (8.558 and 7.432 ppm), HOD (4.807 ppm), and the chemical shift reference, tert-butanol (1.236 ppm). Spectra 4.13 C-F are of IS. Anomeric resonances can be clearly seen at 5.497 and 5.444 ppm, along with the most downfield resonance of the ΔUA2S H4 proton at 5.955 ppm. The resonances of the TE buffer, MES and imidazole are shown in spectra 4.13 G and H.
Figure 4.14. cITP-NMR spectra of the heparin disaccharide standards IH, IS, and IA obtained by post acquisition coaddition of the individual cITP-NMR spectra.
substituent on the GlcN residue. However, the $^1$H NMR chemical shift fingerprints are distinctly different. The same is true when a comparison is made within a series of disaccharides. Figure 4.15 shows the spectra obtained by post-acquisition coaddition of the analyte spectra from the cITP-NMR experiments of disaccharides IS, IIS, and IIIS. Again, the spectral fingerprints are distinct, even for the isomeric IIS and IIIS. The cITP-NMR chemical shifts were recorded (pH = 6.50) for 8 of the 11 commercially available disaccharide standards and listed in Table 4.4. This database could aid in the rapid recognition of known disaccharides, allowing emphasis to be directed at isolation and structure elucidation of novel disaccharides.

4.3.4.2 cITP-NMR Focusing of Disaccharide IVH

Due to the absence of sulfate groups, the only moieties of heparin disaccharide IVH that have the potential to be charged, are the carboxylate (COO$^-$) and ammonium groups (NH$_3^+$). The pD of the anionic cITP system used in this work is 6.9, a pD at which the net charge of IVH is neutral, therefore focusing is not possible under these conditions. However, cationic cITP separations are typically performed at lower pH values and using the $^1$H NMR determined p$K_a$(D) values for IVH, a suitable cationic buffer system could be selected to facilitate the focusing of this disaccharide.

The cationic buffer system used for the separation of acetaminophen and 4-aminophenol in Chapter 2 was employed here as a means to focus disaccharide IVH. The LE was deuterated sodium acetate (pD 4.74) and the TE was deuterated acetic acid (2.55). The disaccharide was dissolved in a solution of 1:1 D$_2$O:TE at pD 2.73. Under
Figure 4.15. cITP-NMR spectra of the heparin disaccharide standards IIIS, IIS, and IS obtained by post acquisition coaddition of the individual cITP-NMR spectra.
Table 4.4. $^1$H NMR chemical shifts (ppm) of heparin disaccharide standards (pH = 6.5) obtained from cITP-NMR experiments. Under the anionic cITP-NMR conditions stated in section 4.2.3.1, disaccharides IVA, IIA, and IIIH failed to form a focused isotachophoretic zone. Disaccharide IVH focused successfully using cationic cITP-NMR.
these conditions IVH was calculated to have a net charge of +0.74 using the $pK_a(D)$ values from Table 4.2. The results of the on-line cITP focusing of IVH is shown in Figure 4.16. NMR spectra 4.16 A-D contain resonances of the $\alpha$ anomer migrating at the front of the focused analyte band as indicated by the H1$\alpha$ and H2$\alpha$ resonances at 5.444 and 3.339 ppm, respectively. Spectra 4.16 E-G contain a mixture of the $\alpha$ and $\beta$ anomers of IVH and spectra 4.16 H and I contain only the $\beta$ anomer as indicated by the H1$\beta$ and H2$\beta$ resonances at 4.945 and 3.054 ppm, respectively. These spectra demonstrate that cationic cITP was not only able to facilitate successful focusing of heparin disaccharide IVH, but also provided partial separation of its anomic constituents.

### 4.3.4.3 cITP-NMR of Disaccharides IVA and IIH-IIIH

Heparin disaccharides IVA, IIH, and IIIH did not focus under the anionic cITP conditions used in section 4.3.4.1. Due to the absence of sulfate groups, disaccharide IVA contains an overall net charge of -1 at pH 6.9 owing to the carboxyl group. An example of the cITP-NMR spectra resulting from the attempt to focus this disaccharide is shown in Figure 4.17. Spectra 4.17 A-C contains only the LE resonances of imidazole (8.558 and 7.430 ppm), the solvent HOD (4.809 ppm), and tert-butanol (1.236 ppm), which are detected throughout. Spectra 4.17 D and E contains the additional TE resonances of MES between 2.5 and 4.0 ppm. The absence of spectra between the LE and TE indicates the analyte failed to focus. This was also the scenario for heparin disaccharides IIH and IIIH. At pH 6.9 the overall net charge on both is -1 due to the presence of a sulfate group. At the time this work was conducted, it was not clear why the disaccharides containing a net
Figure 4.16. $^1\text{H}$ NMR spectra from the cITP-NMR focusing of heparin disaccharide IVH. NMR spectra 4.16 A-D contain resonances of the α anomer migrating at the front of the focused analyte band as indicated by the H1α and H2α resonances at 5.444 and 3.339 ppm, respectively. Spectra 4.16 E-G contain a mixture of the α and β anomers of IVH, and spectra 4.16 H and I contain only the β anomer as indicated by the H1β and H2β resonances at 4.945 and 3.054 ppm, respectively.
Figure 4.17. A portion of the $^1$H NMR spectra produced by the cITP-NMR experiment performed to focus heparin disaccharide standard IVA. Spectra 4.17 A-C contains only the LE resonances of imidazole (8.558 and 7.430 ppm), the solvent HOD (4.809 ppm), and tert-butanol (1.236 ppm). Spectra 4.17 D and E contain the additional TE resonances of MES between 2.5 and 4.0 ppm. The absence of spectra in between the LE and TE indicates that the analyte failed to focus.
charge of -1 would not focus under anionic cITP conditions as our lab and others have achieved successful cITP focusing of monovalent anions.\textsuperscript{51, 58, 59} We postulated that perhaps a higher concentration of these disaccharides was needed or that complexation with counter ions in the buffer system might occur, rendering them neutral and unable to focus. However, in later experiments, we discovered that the TE used in these experiments has a higher electrophoretic mobility than these three analytes, violating the basic requirement for an isotachophoretic system and preventing the focusing of the IVA, IIH, and IIIH. The details of these experiments and the results of further attempts to focus these disaccharides are presented in Chapter 5.

### 4.3.5 CE-UV Separation of Heparin Disaccharides

Separation of the 11 disaccharide standards was first performed using 60 mM formic acid buffer at pH 3.43 under conditions of reversed polarity. The resulting electropherogram is shown in Figure 4.18. The peak migrating at 8.14 min corresponds to BSFA, which was used as an internal standard to account for changes in eof and thus fluctuations in migration time. Although CE studies often rely on neutral markers for this purpose, this is not practical here due to the low eof resulting from the reversed polarity conditions used. As expected, the disaccharide having the shortest migration time was the trisulfated IS (5.47 min) followed by IIS, IIS, IA, IH, IIIA, and IIIA. The last peak in the electropherogram corresponds to heparin disaccharide IVA (41.74 min). Migration of the isomeric disaccharides IIIH and IIH between 30 and 33 min resulted in overlapped, split peaks due to partial resolution of the GlcN α and β anomers. Individual CE experiments
Figure 4.18. Electropherogram of 10 commercially available disaccharide standards from Table 4.1 in 60 mM formic acid buffer, pH 3.43. The inset shows an expansion of the region between 4 and 15 minutes. The peak marked with an asterisk (*) in the electropherogram is benzenesulfonic acid, used as an internal standard.
with single disaccharides revealed that IIIH has a slightly faster migration time than IIH. In addition, the concentration of IIH (0.150 mM) was twice that of IIIH (0.075 mM) and this is reflected in the peak intensities. Although disaccharide IVH was present in this sample, it is not detected due to its cationic behavior at the pH used for the CE separation. Heparin disaccharide IVS was not included in these separations, as it was not commercially available at the time these experiments were performed. The migration order determined in our experiments is in good agreement with that reported by Ruiz-Calero et al., who reported a migration order of IS<IIIS<IIIA<IIH<IIA<IVA.\textsuperscript{13} These authors did not include the H-series of disaccharides in their investigation.

The CE separation of the 11 disaccharide standards shown in Table 4.1 was also performed using a 50 mM phosphate buffer at pH 3.50. The migration order in this separation was the same as that obtained in Figure 4.18 using formic acid buffer at a slightly lower pH. In two studies conducted by Karamanos et al., the CE separation of all 12 commercially available heparin disaccharide standards was carried out under the same conditions as was used to obtain the electropherogram in Figure 4.19, although these authors achieved much different results.\textsuperscript{11, 12} In contrast to the separation of Ruiz-Calero et al. and our own results in both formate and phosphate buffers, Karamanos et al. achieved significantly shorter migration times resulting in a baseline-resolved separation of all disaccharides in under 18 minutes. These authors also report a different migration order for the disaccharides that were N-acetylated and those containing an unsubstituted GlcN amine, IS<IIIS<IIH<IA<IVS<IIIH<IIIH<IIIA<IIA<IVH<IVA. When comparing any two of these disaccharides across a series (i.e. IH and IA or IVH and IVA)
Figure 4.19. Electropherogram of 10 commercially available disaccharide standards from Table 4.1 in 50 mM phosphate buffer, pH 3.50. The inset shows an expansion of the region between 4 and 13 minutes. The peak marked with an asterisk (*) in the electropherogram is benzenesulfonic acid, used as an internal standard.
the components containing a primary amine migrate first in the separations reported by Karamanos et al.\textsuperscript{11, 12} Also interesting is the observation of the IVH disaccharide by these authors in the allotted separation time. Factors contributing to the differences in experimental outcomes might be attributed to differences in capillary lengths or voltages used. In our experiments the capillary length was 14.5 cm shorter than in the experiments performed by Karamanos et al., however, to achieve the same voltage drop across the capillary the applied separation voltage was lowered from 30 kV to 23.3 kV. The shorter migration times in their separation suggests that they have lower $\text{eof}$.\textsuperscript{11, 12} The overall order of migration we obtain is also consistent with similar work reported by Pervin et al. using a phosphate buffer at pH 3.48.\textsuperscript{8} However, a comparison of the N-acetylated and GlcN unsubstituted disaccharides cannot be made since the IH-IVH standards were not present in the separations carried out by Pervin et al. Although differences in migration times are not unusual in CE separations as electrophoretic mobility values are dependent upon many variables, such as condition of the capillary, buffer components, pH, instrumental parameters, and temperature,\textsuperscript{60, 61} a change in the migration order is more difficult to understand.

4.3.6 Mobility Measurements of Heparin Disaccharides

The migration order in our CE separation was consistent with mobility measurements conducted in separate experiments under similar conditions using 0.5 mM phenol as a neutral marker (Table 4.2). Since these mobility measurements were performed under reversed polarity conditions, it was necessary to apply a constant
pressure of 0.3 psi throughout each run to counteract the effects of eof. Each disaccharide solution containing phenol was analyzed by CE in triplicate and the migration times for each component recorded ($t_s$ and $t_{nm}$, respectively). The average electrophoretic mobility ($\mu_e$) for each disaccharide was calculated using the equation

$$\mu_e = \frac{L_D L_T}{V} \left[ \frac{1}{t_s} - \frac{1}{t_{nm}} \right]$$

(4.3)

where $L_D$ is the length of the capillary from the injection end to the detector, and $L_T$ is the total length of the capillary over which the separation voltage $V$ is applied.\(^{60}\) Although these measurements were obtained using an external pressure applied throughout the separation, the calculated electrophoretic mobilities and migration order of the disaccharides should not be affected as the disaccharide and neutral marker experience the same pressure effect during the separation.\(^{62,63}\)

### 4.3.7 Insights into the CE Separation of Heparin Disaccharides Using NMR p$K_a$ and Electrophoretic Mobility Measurements

To gain insights into this CE separation and the discrepancy between the migration orders observed in our lab and those reported by Karamanos et al., the p$K_a$(D) values determined from the NMR titrations were used to calculate the effective net charge, $q$, of each disaccharide. To account for differences in the solvents used in the CE ($H_2O$) and NMR ($D_2O$) experiments, the p$K_a$(H) was calculated for each p$K_a$(D) value using Equation 4.4.

$$pK_a(H) = pK_a(D) - 0.6$$

(4.4)
Figure 4.20. Titration conducted in 95% H$_2$O/5% D$_2$O of the carboxylate group following the change in chemical shift of the IS disaccharide H4’ resonance as indicated by a circle. The pK$_a$(H) value is 3.16 ± 0.02.
This calculation is based on the $pK_a$ difference determined from the IS NMR titration conducted in 95% H$_2$O/5% D$_2$O solution (Figure 4.20) and 99.9% D$_2$O. The $pK_a$(D) of the IS carboxylate group was found to be 0.6 units higher than the $pK_a$(H) in these experiments, in good agreement with results obtained for other carboxylic acid containing compounds, including glucuronic acid.$^{34, 39, 47}$ The carboxylic acid $pK_a$(H) values shown in Table 4.5 were used to calculate the overall net charge of each disaccharide at pH 3.43. For the IH-IVH disaccharides, it was assumed that the ammonium group was protonated under our CE separation conditions. Because CE migration is determined by both ionic charge and hydrated ionic radii, the effective net charge for each disaccharide was divided by its molecular weight (as a surrogate for the ionic radius) to calculate a $q/m$ value, which is plotted in Figure 4.21 versus the electrophoretic mobility measured at pH 3.43. This graph reveals a strong positive correlation ($R^2 = 0.9894$) between the calculated $q/m$ value and the electrophoretic mobility of the disaccharides. Disaccharide IVH was not included in Figure 4.21 since it is cationic and does not migrate in the same direction under the reversed polarity conditions used in these experiments. The linear correspondence between the NMR $pK_a$(H) derived $q/m$ values and the CE mobilities validates the migration order observed in our CE separations in either formate or phosphate buffers. The observation that $N$-acetylated disaccharides migrate faster than the corresponding H-series disaccharides containing a GlcN unsubstituted amine is reasonable given the structures of these disaccharides and the expected separation mechanism under reversed polarity CE conditions, however validation using $^1$H NMR
Table 4.5. $pK_a(H)$ values calculated for the heparin disaccharides.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>COO$^-$$pK_a(H)$</th>
<th>$\text{NH}_3^+$$pK_a(H)$ $\alpha$ anomer</th>
<th>$\text{NH}_3^+$$pK_a(H)$ $\beta$ anomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>$3.36 \pm 0.03^a$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IIS</td>
<td>$3.27 \pm 0.01$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IIS</td>
<td>$3.34 \pm 0.01$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IA</td>
<td>$3.16 \pm 0.02$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IIA</td>
<td>$3.23 \pm 0.03$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IIIA</td>
<td>$3.22 \pm 0.02$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IVA</td>
<td>$2.97 \pm 0.01$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IH</td>
<td>$2.95 \pm 0.04$</td>
<td>$8.38 \pm 0.02$</td>
<td>$8.31 \pm 0.01$</td>
</tr>
<tr>
<td>IIH</td>
<td>$2.95 \pm 0.02$</td>
<td>$8.31 \pm 0.02$</td>
<td>$8.22 \pm 0.02$</td>
</tr>
<tr>
<td>IIIH</td>
<td>$2.87 \pm 0.03$</td>
<td>$8.32 \pm 0.00$</td>
<td>$8.27 \pm 0.01$</td>
</tr>
<tr>
<td>IVH</td>
<td>$2.78 \pm 0.04$</td>
<td>$8.28 \pm 0.01$</td>
<td>$8.17 \pm 0.02$</td>
</tr>
</tbody>
</table>

a. The reported uncertainties in the $pK_a(H)$ values are fitting errors determined from the nonlinear least-squares analysis and likely underestimate the true error.
Figure 4.21. Correlation of electrophoretic mobility at pH 3.43 determined by CE and effective net charge-to-mass ratios ($q/m$) calculated using the $pK_a(H)$ values for each disaccharide. The mobility of disaccharide IVH is not displayed due to its cationic behavior under these conditions.
measurements of the functional group $pK_a$ values provides important evidence supporting this molecular view of the separation.

The migration order in the CE separation of heparin tetrasaccharides was also explored using the NMR determined $pK_a$'s in Table 4.3. From the SEC separation in section 4.2.4.2, 36 $\mu$M of fraction 327 (see Figure 4.2) was injected onto the CE and separated using 50 mM phosphate buffer at pH 3.50. The resulting electropherogram is shown in Figure 4.22. The peak at 5.43 min corresponds to tetrasaccharide IS-IS. The peaks at 5.71 and 5.85 min correspond to tetrasaccharides IS-IIS and IS-IIIS, respectively. By determining the $pK_a(H)$ for each tetrasaccharide using Equation 4.4, the $q/m$ of IS-IS, IS-IIS, and IS-IIIS was calculated to be 0.00596, 0.00576, and 0.00562, respectively. Again, the effective net charge on each was divided by the molecular weight, as a surrogate for ionic radius, to calculate a $q/m$ value. Based on the calculations, under reversed polarity the migration order should be IS-IS > IS-IIS > IS-IIIS. This theoretical prediction using the NMR $pK_a$ data was confirmed experimentally in the CE separation of the tetrasaccharides shown in Figure 4.22.

4.4 Summary

Through the use of NMR pH indicators, $^1$H NMR pH titration experiments were performed for 11 commercially available heparin disaccharide standards. It was found that the acidity of the carboxylic acid moiety of each disaccharide is directly affected by the substituent on the GlcN nitrogen and $pK_a$ values could be assigned for both the $\alpha$ and $\beta$ anomers of the GlcN residue of disaccharides IH-IVH. By determining the $pK_a$'s of the
Figure 4.22. Electropherogram of SEC fraction 327 from enzymatically digested heparin using heparinase I. The peaks at 5.43, 5.71, and 5.85 min correspond to tetrasaccharide IS-IS, IS-IIS and IS-IIIS, respectively. The CE separation was performed under reversed polarity using 50 mM phosphate buffer, pH 3.50.
carboxylate and primary ammonium moieties we were able to calculate the effective net charge associated with each disaccharide and gain additional insight into the migration order determined from CE experiments. CE separations in both formate and phosphate buffers resulted in the migration order: IS<IIIS<IIS<IA<IH<IIIA<IIIA<IIH<IIH<IVA<IVH and was consistent with the electrophoretic mobility measurements conducted under similar conditions. In this work, determination of pKₐ values for the major disaccharide components of heparin provided useful information for understanding their CE separation. They also allowed the accurate calculation of the effective net charge on IVH, to facilitate optimization of its focusing in cITP-NMR. Furthermore, the disaccharide pKₐ values provided insight as to how functional group acidity is affected by variations of nearby substituents. This work was extended to study the functional group pKₐ values of larger heparin tetrasaccharides. Similar trends were observed and interestingly for the tetrasaccharides, the O-sulfo groups of the neighboring monosaccharides seem to have a greater effect on the acidity of the carboxylate pKₐ(D)’s on both the ∆UA2S and IdoA±2S residues.
4.5 REFERENCES


Chapter Five

Investigating cITP Separation Processes Using CE, NMR, and Stereo Microscopy

In Chapter 4, cITP-NMR focusing of 11 heparin disaccharide standards was investigated and optimal cITP focusing conditions were determined for some, but not all, compounds. Under the anionic cITP conditions used, the focusing of disaccharides IVA, IIH, and IIIH was not successful, despite their overall net charge of -1. Further experiments were conducted in attempts to focus these disaccharides, as well as tetrasaccharides. This chapter discusses these experiments, the observations made, and the additional research conducted to optimize focusing using methods such as acid-base and complex forming equilibria. NMR spectroscopy and stereo microscopy were employed to gain a better understanding of these mechanisms and CE was used to probe suitable TE buffers. To begin, the basis for analytical cITP separations is discussed in greater depth.

5.1 A Closer Look at Capillary Isotachophoresis

Isotachophoresis (ITP) is a rapid, high resolution, electrophoretic method for the separation of charged compounds.\textsuperscript{1, 2} The fundamental principles of ITP describe the migration of a moving boundary in a discontinuous electrolyte system, containing a common counter ion, under the influence of an electric field. In the absence of a background electrolyte, the participating ions will arrange such that the ion of the leading electrolyte (LE) with the highest mobility in the system will migrate in front, the sample
ion(s) having an intermediate mobility will migrate behind the leading ion, and the ion of the trailing electrolyte (TE) with the lowest mobility will migrate last. Once the ions are assembled in their respective zones, a steady-state is reached and all ions migrate at the same velocity (the Greek iso = equal, tacho = velocity). The length of each zone is proportional to the amount of its sample constituent and carries a distinct, constant voltage, resulting in a stepwise voltage gradient that increases from the leading electrolyte to the terminating electrolyte. The boundaries between each sample component also demonstrate sudden changes in physical properties such as conductivity, absorbance, temperature, and pH.

Modern day ITP was first described in 1923 by Kendall and Crittenden as the ‘ion migration method’. By using this method, they were able to successfully separate rare earth metals and some simple acids. This was the first report of an electrophoretic method not considered as zone electrophoresis. Later, Kendall became the first to propose following the separation in some practical way, such as utilizing a colored ion that had a mobility intermediate to that of the LE and TE. In this way, the end of the experiment could easily be determined without the need of a detector. Unfortunately, at that time, a colored ion with the required properties had not yet been discovered. Other important pioneers of contemporary ITP include MacInnes and Longsworth who presented a system of differential equations to describe the moving boundary for strong electrolytes. By using the ‘moving boundary method’ they could determine the transference numbers of small ions, an important physico-chemical parameter. Transfer numbers, in combination with the conductivity of the electrolyte solution can be used to calculate ionic mobilities.
Following further advancements of the ITP method, Martin and Everaerts introduced an apparatus consisting of a thin glass capillary in which the separation was performed. Electroosmosis was suppressed through the use of a hydroxyethylcellulose additive. This advancement lead to the birth of modern instrumental design for capillary isotachophoresis (cITP).

### 5.1.1 Characteristics of the Steady Isotachophoretic State

The fundamental principle behind cITP development is the Kohlrausch regulating function. This regulating function describes the position of the ions along the path of migration under the influence of an applied electric field, but is restricted to strong electrolytes. A modification of this function, extended for strong and monovalent weak electrolytes is given by

\[
\omega(x) = \sum_i \frac{\overline{c}_i(x) \cdot z_i}{\mu_i} = \text{const}(x). \tag{5.1}
\]

where the summation is carried out for all the substances, \(i\), present at a given moment along the migration path, \(x\), multiplied by the charge, \(z\), of the substance, and divided by its mobility, \(\mu\). For a given \(x\), the value of \(\omega(x)\) is constant and predetermined by the leading electrolyte arrangement prior to applying current. It does not change during electromigration. The total concentration of ionic species, \(\overline{c}_i\), in the migrating zones of the sample will then adjust to this constant value. Although this adjustment of concentration function is conserved for classical ITP systems, there are systems where the Kohlrausch regulating function is not valid and thus, new functions have been defined.
that control the adjustment mechanism.\textsuperscript{9-11} However, discussion of these non-Kohlrausch functions is beyond the scope of this chapter.

Once steady-state ITP is reached, the concentrations in each of the focused sample zones adjust to the fixed concentration of the leading electrolyte and the boundaries between any two isotachophoretic zones is very sharp, as shown in Figure 5.1 A. Each zone only contains one separated substance and the zones migrate in series with the same velocity. This can be expressed for an example system containing a leading electrolyte ion, \( L \), trailing electrolyte ion, \( T \), and sample substance, \( S \), by the equation,

\[
\overline{\nu}_{iso} = E_L \mu_L = E_S \mu_S = E_T \mu_T ,
\]

where \( E \) is the electric field strength of the corresponding zone, \( \mu \) is the effective electrophoretic mobility of each component, and \( \overline{\nu}_{iso} \) is the linear, macroscopic migration velocity of all zones in the system. If this was not the case, space that was absent of ions would arise between the zones interrupting the electric circuit. This is how separated analytes that have different mobilities travel at the same velocity. In addition, the electric field strengths, \( E \), in different zones are automatically adjusted such that the lower the mobility is of a given substance in a zone, the higher \( E \) is in that zone (Figure 5.1 B). In turn, the trend in conductivity, \( \kappa \), is opposite to that of \( E \), making it proportional to the effective mobility, \( \mu \), of a substance (Figure 5.1 C-D). Thus, the conductivity or electric field strength of a zone has a specific value that is characteristic to the separated
Figure 5.1. Properties of isotachophoretic zones L, X, and T. (A) Distribution of the zones at steady-state, (B) field strength, $E$, (C) conductivity, $\kappa$, and (D) effective mobility $\mu_e$. 
component in that zone. These values can be used to identify an unknown or determine the effective mobility of an analyzed substance.

5.1.2 Requirements of Isotachophoresis

Establishment of steady-state ITP requires that the mobilities of the charged sample substances be intermediate to that of the leading and trailing electrolyte,

$$|\mu_L| > |\mu_S| > |\mu_T|$$  \hspace{1cm} (5.3)

After an electric field has been applied and boundaries begin to form between the sample and electrolytes, self-sharpening of the isotachophoretic boundaries takes place. This occurs due to the abrupt change in concentration of the substances and ions diffusing from their own zones to neighboring zones. For example, the ions in the leading zone, $L$, will slow down and begin to enter into the sample zone, $S$. They are acted upon by a higher electric field, thus increasing their velocity and reentering back into their own zone. Similarly, the ions in zone $S$ will accelerate and enter into zone $L$. Because their velocity is decreased, owing to the lower electric field, the $S$ ions move back into their own zone. Finally, the length of a given zone is proportional to the component concentration and the initial concentration of the leading electrolyte ion.

In order for separation to occur in cITP, or any electrophoretic technique, each sample component is required to have different migration rates or mobilities. Several approaches can be taken in order to increase the difference in mobility between analyzed substances. One obvious approach is to change the pH of the leading electrolyte, exploiting the differences in $pK_a$ values and the corresponding proton transfer reactions.
Another approach is to introduce specific counter ions into the leading electrolyte, which can form complexes with the sample components (see section 5.1.3.2). Mobilities of analytes are also dependent upon temperature, composition of the solvent, and the ionic strength. Although the effects of these physical parameters is less pronounced than those resulting from chemical modifications.

Substance components that do not fulfill the requirements of Equation 5.3, migrate within the leading electrolyte \( (|\mu_L| < |\mu_s|) \) or behind in the trailing electrolyte \( (|\mu_s| < |\mu_T|) \). In these situations, the entire moving sample zone is considered unstable and never reaches a steady-state.

### 5.1.3 The Role of Counter Ions in cITP

#### 5.1.3.1 Counter Ions in Acid-Base Equilibria

In practical cITP separations, the conditions are usually optimized to separate and focus either cations or anions, hence the terms cationic or anionic cITP. This is achieved through careful choice of a buffer system. For either system, a suitable leading ion must be sufficiently ionized and possess a high effective mobility. Similarly, a fully dissociated TE ion must have an effective mobility that is lower than that of the analyte ions. In the case of weak acids or bases, their degree of ionization depends on the pH of the solution. In isotachophoresis, the pH of a certain zone depends on the pH of the LE. Since there is no background electrolyte, the system counter ions serve to buffer the isotachophoretic zones. Counter ions are common to all zones and migrate in the opposite direction to the zones of the analyzed substances. The concentrations of the components that make up the
counter ionic system, and therefore the pH of the zones, varies from one zone to another in a way similar to the magnitudes of the adjusted concentrations of the analytes of interest. The buffering scheme of both cationic and anionic cITP is illustrated in Figure 5.2. Figure 5.2 A illustrates the migration of a cationic weak base, X, in its zone, which participates in acid-base equilibrium and is buffered by the weak acid HA and its anion A-, which form the counter ionic system of the LE. Therefore, the pH of the zone is proportional to the concentration ratio of the counter ionic system, $c_{HA}/c_A$, and to the $pK_{HA}$ value. Similarly in anionic cITP, the pH in the sample zone of a weak acid, HX, is closely related to the concentration ratio of the counter ionic system, $c_B/c_{BH^+}$, of the leading electrolyte as shown in Figure 5.2 B. Since the majority of cITP separations conducted in this chapter were of negatively charged heparin di- and tetrasaccharides, further discussion will be partially limited to anionic cITP systems.

In anionic isotachophoresis, the pH of the migrating zones increases from the leading zone to the terminating zone, $pH_L < pH_X < pH_T$. The more alkaline the LE is (higher ratio of $c_B/c_{BH^+}$, higher $pK_{BH^+}$), the more alkaline the subsequent zones of the analyzed ions and TE. Therefore, ITP separations can be optimized utilizing the acid-base equilibria of the ionic components in the system. If the dissociation constant and ionic mobility of the analyte, LE, and TE are known, a more exact estimation of zone pH can be determined, resulting in a more complete separation of discretely focused zones.

5.1.3.2 Counter Ions in Complex-Forming Equilibria
Figure 5.2. Buffering in isotachophoretic zones. (A) Cationic system – the pH in the sample zone of a weak base, X, is buffered by the weak acid HA and its anion A\(^-\), which form the counter ionic system. (B) Anionic system – the pH in the sample zone of a weak acid, HX, is buffered by the weak base, B, and its protonated form BH\(^+\), which form the counter ionic system.
Ionic species that cannot be successfully separated by manipulating the acid-base equilibria, may be separated through complex-forming or ion-pairing equilibria. This type of separation is characterized by the presence of rapid complex-forming equilibria in the sample zone between a counter ion and the analytes of interest. A simple model for anionic systems is shown in Figure 5.3 A where the analyte ion \( X^- \) is free to form a kinetically labile complex with the counter ion \( M^+ \) of the LE. The anion \( X^- \) undergoes the fast reaction \( X^- + M^+ \rightleftharpoons MX \) on the front zone boundary and the complex \( MX \) simultaneously undergoes this reaction in the opposite direction at the rear zone boundary in order to re-establish equilibrium. The presence of the free terminating ion, \( T^- \), is required at the rear boundary of the zone for the recombination reaction. As a result of the complex-forming equilibrium, the effective mobility of the analyte ion (\( X^- \)) is attenuated by the partial neutralization of its charge. This can be an effective technique if the analyte of interest has a slightly higher mobility than the LE.

Another type of complex-forming equilibria includes the introduction of a neutral ligand, \( L^0 \), into the leading electrolyte (Figure 5.3 B).\(^{21}\) The neutral ligand does not migrate under the influence of the electric field and the overall charge of the analyte does not change upon complexation. However, the migration of the analyte ion is decreased due to an increase in the molecular size of the complex ion.

Complex-formation equilibria can also include utilizing the terminating electrolyte as the counter ion (Figure 5.3 C). In these systems, the analyte, \( Y^+ \), has a charge that is opposite to that of the LE and TE electrolytes. In the example shown, the analyte is converted to an anion due to a complex formation reaction with the terminating
Figure 5.3. Examples of complex-forming equilibria in isotachophoretic systems. (A) The analyte ion $X^-$ forms a kinetically labile complex with counter ion, $M^+$, of the LE ($X^- + M^+ \leftrightarrow MX$). The terminating ion, $T^-$, is present at the rear boundary for the recombination reaction. (B) A neutral ligand, $L^0$, is present in the LE and upon complexation to form $LX^-$, attenuates the migration of $X^-$. (C) Analyte, $Y^+$, is opposite in charge of the LE and TE and is converted to the anion, $YT^-$, due to a complex formation reaction with the terminating ion, $T^-$. In all systems $M^+$ is the counter ion of the LE.
ion, \( T \). If the interaction between the analyte species and the terminating ion is stronger, the effective mobility of the analyte becomes larger due to the increase in opposite charge. The use of this system is effective for assisting with the migration of neutral species or those that have a very small electrophoretic mobility.\textsuperscript{22, 23}

For all complex-forming techniques, optimization requires the careful selection of a suitable counter ion and consideration of the stability of the complexes formed. Alteration of the effective mobilities of the analytes requires that the complex formation reactions are sufficiently fast and the leading and terminating electrolytes suitable to the system.

5.1.4 Frontal Migration of \( \text{H}^+ \) and \( \text{OH}^- \)

For cITP migration, it is necessary to have a state in which each zone contains only one separated component and a counter ion system. The contribution of other ions having the same charge as the substances being separated to the electrical conductivity in a given zone must be insignificant. With this requirement, it is important to remember that in aqueous cITP systems water is always present, therefore the free migration of \( \text{H}^+ \) and \( \text{OH}^- \) ions is a constant concern. Since \( \text{H}^+ \) and \( \text{OH}^- \) ions have the highest mobilities of all the ions, they can migrate much faster than the other ions and their moving boundaries.\textsuperscript{24-26} If the speed of these free ions is faster than the migration velocity through the system, the consequence is uncontrolled migration of \( \text{H}^+ \) and \( \text{OH}^- \) across isotachophoretic boundaries. This is particularly true for unbuffered systems or when free \( \text{H}^+ \) and \( \text{OH}^- \) ions are generated by electrode reactions at the terminating electrode. In
anionic cITP, Figure 5.4 A illustrates the uncontrolled migration of the OH\(^-\) ion from the terminating zone, T\(^-\), through the sample zone, X\(^-\), into the leading zone, L\(^-\). Since OH\(^-\) migrates in the same direction as the anions of interest, it acts as a background electrolyte and the principle of ITP is violated. The uncontrolled migration of H\(^+\) ion is analogous for cationic cITP systems.

To eliminate the disturbing effects of free migration of H\(^+\) and OH\(^-\) ions, a buffering counter ion system can be used (i.e. in anionic systems, the cation of a weak base, BH\(^+\), accompanied by the undissociated free base, B). The free migrating H\(^+\) or OH\(^-\) ions will recombine with the free counter ion of the buffer system and re-establish the dissociation equilibrium. Figure 5.4 B illustrates the controlled migration of OH\(^-\) ions in anionic cITP. The OH\(^-\) ion migrates and interacts with the weak base counter ion, BH\(^+\), and this forms the terminating zone of the system. This is providing that the concentration of the OH\(^-\) ion in the zones is negligible.

### 5.1.5 Studying Electromigration Behavior of Ions in cITP

The migration behavior and separation dynamics of ions in various electrolytic systems has been studied extensively using computer simulations and mathematical solutions of basic physico-chemical laws.\(^{27-31}\) These computations and simulations provide important insights into the possible ion migration and separation mechanisms of cITP. However, most mathematical models use certain simplifications and assumptions that may not be true representations of complex cITP systems. To facilitate a more
Figure 5.4. (A) The uncontrolled migration of free OH\textsuperscript{−} ions in anionic cITP are shown migrating through all boundaries of the isotachophoretic zones. This is demonstrated by the dashed line. (B) The controlled migration of free OH\textsuperscript{−} ions in anionic cITP. The OH\textsuperscript{−} ions do not cross the boundary of the terminating ion, T\textsuperscript{−}, and analyte, X\textsuperscript{−}, as shown by the solid line. BH\textsuperscript{+} and H\textsuperscript{+} are the free counter ions in the LE available for the recombination reaction at the frontal boundary of the TE.
complete understanding of the separation dynamics and electromigration behavior of ions, a suitable detection method is needed.

Numerous detection methods can be employed to study the movement of focused analytes and buffer constituents in ITP. The most common is conductivity detection due to the resistance gradient across the capillary. The output signal is proportional to the specific resistance experienced by the focused analytes. Each zone exhibits a characteristic value that changes in a stepwise fashion across each zone boundary and is proportional to the mobility of the analytes (see Figure 5.1 C and D). Similarly, mobilities can be calculated by measuring the field strength (see Figure 5.1 B) or the temperature across the capillary. For components that have appropriate absorbance characteristics, more selective detection, such as UV or fluorescence, may be used.

Although these detection methods are useful for many analytical applications, they only provide information about specific substances (i.e. chromophores) or a particular property of the migrating zones. Comprehensive analysis of all components in the system may require more than one detection method. More importantly, these methods are limited in their ability to characterize the structure of the constituents in each zone. In order to simultaneously monitor the migration behavior of ions and unambiguously detect analytes in the different zones, a detection method, such as NMR, is ideal.

5.1.5.1 Using NMR to Study Migration Behavior of Ions During cITP

NMR is well known for its ability to provide comprehensive structural information, but it is also a valuable tool for revealing dynamic information about certain
processes such as binding and diffusion.\textsuperscript{32-34} In recent years, the coupling of cITP to NMR detection (cITP-NMR) has facilitated investigation into the electromigration behavior of ions during ITP.\textsuperscript{35-37} As in all electrophoretic separations, temperature gradients generated by Joule heating, can influence the buffer pH (or pD in deuterium oxide solutions), migration times, and separation efficiency. On-line cITP-NMR is a powerful technique for probing the intracapillary conditions, as well as, the degree of isotachophoretic focusing. These insights coupled with knowledge of acid dissociation constants and ionic mobilities can aid in the theoretical prediction of the migration order and separability of ions in ITP systems.

### 5.1.5.2 Using Microscopy to Study Migration Behavior of Ions During cITP

To facilitate the understanding of ion migration behavior and separability, real-time and static imaging of isotachophoretic zones has been performed on microfluidic platforms using either direct or indirect fluorescence detection.\textsuperscript{38-40} The latter employs fluorescent mobility markers for the pre-concentration and separation of non-fluorescent analytes.\textsuperscript{39} Conversely, direct detection applies nonfocusing fluorescent tracers to the LE or TE.\textsuperscript{38} As the tracers enter each zone, their concentration adapts to the local electric field in the zone allowing visualization of ITP processes via fluorescence microscopes or charge-coupled device (CCD) cameras. A drawback to the indirect detection technique for visualization is that a large number of fluorescent markers with known mobilities is required for analysis of complex samples. In addition, fluorescence-based methods are particularly suited and applied to on-chip applications due to the optical access provided
by on-chip channels. It is also noteworthy to mention that up to the current time, real-time visualization of counter ions using fluorescence has not been investigated.

For visualization of the isotachophoretic zones, our laboratory has adopted an approach that uses appropriately charged dye molecules. This off-line method allows evaluation of the correct migration and stability of the isotachophoretic system based on the migration direction and length of the focused analyte dye. Thus, rapid optimization of the injection protocol or buffer system can be performed. However, observations of the zones are made with the naked eye and small changes in zone length or composition often go undetected. Also, if transparent analytes are added to the system, the focusing of the dye may not necessarily equate to focusing of the analytes. In such cases, only the buffer system and dye can be evaluated, the separation and focusing of visually undetectable analytes remains inconclusive.

An expansion of this approach explored in this research employs the use of a stereo microscope and camcorder to record the real-time focusing and migration of charged dye molecules. This allows a microscopic view of the isotachophoretic zones and a more accurate image of the moving boundaries. We would also like to gain additional insight into the migration behavior and pathway of counter ions during the cITP process. By introducing a colored counter ion into the LE that can be observed at low concentrations, it may be possible to investigate the ion’s migration pathway as it passes through the analyte zones into the terminating zone. Ideally, a color change of the counter ion would occur. This could be facilitated through one of two ways: 1) upon experiencing a change in pH when the ion passes through the analyte zone or 2) upon forming a
complex with the analyte, which may also be a colored product. The latter possibility is illustrated in Figure 5.5 under cationic cITP conditions. Studies in this chapter have used stereo microscopy to capture video of steady-state cITP, as well as, unprecedented video capture of possible counter ion migration.

### 5.1.6 Development of Anionic cITP for Heparin Disaccharides IVA, IIH, and IIIH

In anionic cITP, it is possible to focus monovalent anions providing the condition of Equation 5.3 is met. Experiments performed in Chapter 4 revealed that monovalent heparin disaccharide standards IVA, IIH, and IIIH failed to focus using the current anionic cITP buffer system. It was proposed that the TE of 2-(N-morpholino)ethanesulfonic acid (MES) used in these experiments could have a higher mobility than the disaccharides. If so, the TE would migrate faster than the disaccharides and steady-state focusing of these analytes would not occur. In this chapter, this theory was tested utilizing capillary electrophoresis (CE) to probe the mobility of the MES relative to the disaccharides at the same pH conducted in cITP-NMR. These findings illustrate the effectiveness of CE for probing suitable cITP TE buffers.

Since the pH of the cITP zones can influence the effective net charge of the system components, on-line cITP-NMR can be used to assess the intracapillary pH (or pD in deuterated solutions). The changes in chemical shift of an indicator molecule within the range of the $pK_a \pm 1$ can provide the intracapillary pH during a cITP separation. Following the CE experiments, the pD of a new buffer system is evaluated
Figure 5.5. Visualization of counter ion migration. A colored counter ion, $R^{2-}$, that is introduced into the LE will form a colored complex, $YR^-$, when it passes through the analyte zone, $Y^+$. 
and conclusions are made regarding the determined pD values across the analyte zones and its affect on the quality of cITP focusing for the heparin disaccharides.

5.1.7 Development of Anionic cITP for Heparin Tetrasaccharides Using Complex-Forming Equilibria

Previous cITP-NMR experiments to focus heparin tetrasaccharides resulted in successful focusing, however the signal-to-noise ratio (S/N) was poorer than those obtained for heparin disaccharides.\textsuperscript{41, 42} Korir et al reported the cITP-NMR focusing of heparin-derived tetrasaccharide IS-IS (structure given in Chapter 4, Figure 4.4) and proposed that the presence of trace impurities may account for the low S/N. In this research, the cITP-NMR focusing of tetrasaccharides was revisited. Depolymerization using heparinase I yields three major tetrasaccharides, IS-IS (overall net charge = -8), IS-IIS (-7), and IS-IIIS (-7) (see section 4.2.4 of Chapter 4). Although these tetrasaccharides should focus very well under anionic cITP conditions due to their high net negative charge, it is possible that they have a slightly larger mobility than the LE. This would violate the requirements for steady-state ITP, resulting in weak focusing or the absence of focusing, and therefore, a decrease in the signal-to-noise (S/N) of the cITP-NMR spectra.

To lower the amount of overall negative charge of the tetrasaccharides, we employed complex-forming equilibria to attempt to selectively retard their migration and aid in focusing. A schematic of this technique is given in Figure 5.3 A. The divalent counter-ion, 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP, Figure 5.6) has been shown previously to facilitate the successful cITP focusing of low molecular weight heparin-like pharmaceuticals known as synthetic sulfated bis-lactobionic acid amides.\textsuperscript{16}
Figure 5.6. Counter ion and dye molecules used in cITP-NMR experiments.
The authors used this technique in cITP as a pre-concentration method for capillary zone electrophoresis. Therefore, BTP was chosen as the counter ion for use in the cITP-NMR experiments conducted to improve the focusing of heparin tetrasaccharides.

5.2 Experimental Section

5.2.1 Chemicals

All chemicals and reagents were used as received without any further purification. Imidazole, phenol, 2-(N-morpholino)ethanesulfonic acid (MES), N-(carbamoylmethyl)-2-aminoethane sulfonic acid (ACES), methyl green, sodium acetate, ammonium acetate, heparin disaccharide standards IIH and IIIH were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Heparin disaccharide standard IVA was purchased from V-Labs, Inc. (Covington, LA). 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) was purchased from MP Biomedicals (Solon, OH, USA). HPLC-grade water was purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, sodium phosphate dibasic, tert-butyl alcohol, sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), glacial acetic acid, bromophenol blue, mordant blue 9, and benzenesulfonic acid (BSFA) were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterium oxide (99.9% D low paramagnetic), deuterated acetic acid (99.5% D), sodium deuterioxide (99.5% D), ammonium deuterioxide (99% D5), and deuterium chloride (99.5% D) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).
5.2.2 Microscopic Visualization Experiments

Visual observation of cITP focusing was conducted using a stereozoom microscope (Nikon SMZ-2B) having a continuous zoom ranging between 0.8× and 5.0×, including 10× eyepieces. The images were obtained using 50× magnification of the capillary. A multi-position fiber optic illuminator 115 VAC (Cole Parmer Instrument Co., Chicago, IL) was used as the light source. A mini DVD camcorder (Samsung Electronics Co. Ltd., USA) 680,000-pixel video with 10x optical/900x digital zoom was abutted to the microscope eyepiece for recording the cITP separations. The distance between the black lines in all video clips is 1 mm, the videos were recorded in real-time, and no alterations were made to the video or video clips.

The “Zero eof” capillary used was 200 cm long with an inner diameter of 180 µm (Polymicro Technologies, Phoenix, Arizona, USA). Approximately 2 mm windows were formed at 69 cm, 74 cm, and 83 cm by removal of the polyimide coating from the outside of the capillary using a MicroSolv Window Maker™. The window at 83 cm is approximately the same as the distance from the end of the capillary to the detection coil in the cITP-NMR experiments. The window at 69 cm was placed at the point at which the analyte begins to focus at the start of a cITP experiment and the window at 74 cm was formed between the other two windows to film the progression of complexation during counter ion experiments using multiple dyes.

5.2.2.1 Solution Preparation of Steady-State cITP Solutions

Cationic buffers were prepared in H₂O. A 160 mM sodium acetate solution was used as the LE. The pH of the LE solution was adjusted to pH 4.35 using glacial acetic
acid. A solution containing 160 mM acetic acid, pH 2.35, was used as the TE. The cationic dye, methyl green (structure given in Figure 5.6), was prepared at a concentration of 100 µM in 1:1 solution of TE and H₂O. Samples were introduced into the separation capillary using hydrodynamic injection at a height differential of 16 cm, giving an injection rate of 1.25 µL/minute. In the cITP run, the capillary was first filled with LE. The injection protocol for these experiments was TE for 1 minute, sample for 6 minutes, followed by TE for an additional 7 minutes. The separation voltage was applied at 15 kV and currents ranged from 44.2 to 32.5 µA during the course of the experiment.

The anionic cITP buffers were also prepared in H₂O. A 160 mM sodium chloride solution was used as the LE. The measured pH of the solution was 6.20 and no further adjustment was made. A TE solution of 160 mM MES was prepared and adjusted to pH 6.5 using 1 M NaOH. The analyte used in the anionic cITP experiments was 100 µM bromophenol blue (structure given in Figure 5.6) dissolved in 10 mM TE. The injection protocol was described previously in Chapter 4. However, for these experiments a shorter injection time of 8 minutes was used for the sample, followed by TE for an additional 8 minutes. The separation voltage was applied at 15 kV and currents ranged from 74.2 to 56.9 µA during the course of the experiment.

5.2.2.2 Solution Preparation of Counter Ion cITP Solutions

Cationic cITP buffers were prepared as described in section 5.2.2.1, except 800 µM of mordant blue 9 (structure given in Figure 5.6) was added to the sodium acetate LE (pH = 4.34) as a counter ion. A sample of 100 µM methyl green was prepared in a 1:1
solution of TE and H₂O and introduced into the separation capillary using hydrodynamic injection at a height differential of 16 cm. In the cITP run, the capillary was first filled with LE. The injection protocol for these experiments was TE for 1 minute, sample for 12 minutes, followed by TE for an additional 1 minute. The separation voltage was applied at 15 kV and currents ranged from 79.5 to 56.4 µA during the course of the experiment.

5.2.3 Capillary Electrophoresis (CE-UV) Experiments

All CE separations were performed on a Beckman Coulter ProteomeLab™ PA800 instrument equipped with a diode array detector, fluid-cooled column cartridge, and automatic injector. Because the experiments in sections 5.2.3.2 and 5.2.3.3 were performed to probe suitable TE buffers for cITP, “Zero-eof” capillaries were used instead of fused silica (MicroSolv Technology Corporation, Eatontown, NJ). Total capillary length was 58.2 cm (length to detector 49.0 cm), with a 75 µm i.d. and 375 µm o.d. An approximately 1 mm detection window was formed by careful removal of the polyimide coating from the outside of the capillary so as not to damage the internal polymer coating. A reference marker of 0.5 mM BSFA was used in the CE sample solutions to account for fluctuations in eof. Capillaries were conditioned by rinsing with buffer for 20 min, then a rinse with water for 5 min, followed by another rinse with buffer for 5 min, all at high pressure (50 psi). The capillary was equilibrated with the buffer by applying a separation voltage of 18 kV using normal polarity for 5 min. Absorbance was monitored between 190 nm and 232 nm and the data was collected and processed using 32 Karat software.
(Beckman Coulter, Inc., Fullerton, CA). All bulk solutions and samples were filtered using a 0.2 µm membrane filter prior to analysis.

5.2.3.1 CE-UV Mobility Measurements of Heparin Disaccharide Standards at pH 6.50

Separate solutions were prepared each containing 0.5 mM of the heparin disaccharide standard and 2 mM phenol (neutral marker) in 20 mM phosphate buffer (titrated to pH 6.51 with 1 M NaOH). Mobility measurements were performed under normal polarity conditions using a fused silica capillary (total length, 60.2 cm and length to detector, 50.0 cm), with a 75 µm i.d. and 375 µm o.d. It was necessary to apply a constant pressure of 0.3 psi throughout each run to facilitate a reasonable migration time for the later migrating disaccharides. Between each experiment, the capillary was rinsed for 1 min with 0.1 M NaOH, 2 min with HPLC-grade water, followed by 3 min with buffer, all at high pressure (50 psi). Samples were pressure injected at 0.5 psi for 7 s. The separation voltage was 20 kV and the total run time was 20 minutes. Each disaccharide solution containing phenol was analyzed by CE in triplicate and the migration times for each component were recorded ($t_s$ and $t_{nm}$, respectively). The average electrophoretic mobility ($\mu_e$) for each disaccharide was calculated using the equation

$$\mu_e = \frac{L_s L_D}{V} \left[ \frac{1}{t_s} - \frac{1}{t_{nm}} \right]$$

(5.4)

where $L_D$ is the length of the capillary from the injection end to the detector, and $L_T$ is the total length of the capillary over which the voltage $V$ is applied.\textsuperscript{43}
5.2.3.2 Separation of MES and Heparin Disaccharide IVA in Phosphate Buffer

A solution containing 0.5 mM of heparin disaccharide IVA, 1 mM MES, and 0.5 mM BSFA was prepared in 20 mM phosphate buffer (pH = 6.54). Between experiments, the capillary was rinsed 2x with HPLC-grade water for 1 min and phosphate buffer for 3 min, all at high pressure (50 psi). Samples were pressure injected at 0.5 psi for 7 s. The separation voltage was 23.3 kV and the total run time was 20 min. No pressure was applied during the separation. Separate CE runs were also performed using 20 mM phosphate buffer at pH 5.61.

5.2.3.3 Separation of ACES and Heparin Disaccharides IVA and IIH in Phosphate Buffer

A solution containing 0.29 mM of heparin disaccharide IVA, 1 mM ACES, and 0.5 mM BSFA was prepared in 20 mM phosphate buffer (pH = 6.50). A similar solution was also made containing 0.5 mM heparin disaccharide IIH. Between experiments, the capillary was rinsed 2x with HPLC-grade water for 1 min and phosphate buffer for 3 min, all at high pressure (50 psi). Samples were pressure injected at 0.5 psi for 7 s. The separation voltage was 23.3 kV and total run time was 20 min. No pressure was applied during the separation.

5.2.4 Off-Line cITP Experiments Using Optical Detection

To test the operation of the ACES buffer as a TE for use in the cITP-NMR measurements, cITP experiments were conducted in aqueous solutions with optical
detection. The same type of “Zero eof“ capillary described in section 5.2.2 was used in these cITP experiments. The LE consisted of 160 mM HCl and the TE of 160 mM ACES, both at a pH of 6.50. The pH was adjusted using 1 M NaOH. A sample containing 100 µM Bromophenol blue was used for visualization and prepared in 10 mM of the ACES TE. The capillary was first filled with LE, then an 8 minute injection of the sample, followed by an 8 minute injection of the TE. The sample was injected hydrodynamically at a height of 16 cm. The voltage applied across the capillary was 15 kV. The currents produced during this experiment ranged from 29.1 to 25.0 µA. Video was captured looking through the window formed 83 cm from the injection end of the capillary.

5.2.5 On-Line cITP-NMR Experiments

The instrumental cITP-NMR setup and operation is similar to that described in Chapter Two. These experiments used the same type of “Zero eof” capillary as described in section 5.2.2. The capillary used in the cITP-NMR experiments was 200 cm long with an inner diameter of 180 µm (Polymicro Technologies, Phoenix, Arizona, USA). The capillary was first filled with LE, then sample was injected for 13 minutes, followed by a 3 minute injection of the TE. The longer injections used in these experiments were intended to maximize the amount of sample and improve detection. Injections were performed hydrodynamically at a height of 16 cm. The voltage applied across the capillary for all separations was 15 kV.

All on-line cITP-NMR spectra were recorded using a Bruker Avance spectrometer operating at 599.983 MHz. Once a steady current was reached in the cITP-
NMR experiments, NMR acquisition was initiated to acquire an array of $^1$H NMR spectra using 45° pulses with an acquisition time of 1.39 s and a spectral width of 6009.6 Hz. Each spectrum was acquired by coaddition of 8 transients with a total acquisition time of 11.12 s. Line broadening equivalent to 2.0 Hz and zero-filling to 128 K points were applied prior to Fourier transformation.

5.2.5.1 cITP-NMR of Heparin Disaccharide IVA Using ACES TE Buffer

The buffer system used in the anionic cITP focusing of heparin disaccharide IVA was 160 mM NaCl as the leading electrolyte (LE) and 160 mM ACES as the trailing electrolyte (TE). The TE was adjusted to a pD of 6.50 using NaOD and no adjustment was made to the LE. The LE contained tert-butyl alcohol as a chemical shift reference (1.236 ppm relative to trimethylsilylpropionic acid). A solution containing 500 µM of disaccharide IVA and 500 µM of BSFA was prepared 10 mM TE and D$_2$O. BSFA was used as a scout analyte and to monitor the ability of the buffer system to effectively focus analytes. The currents produced during the course of these cITP experiments ranged from 37 to 32 µA.

5.2.5.2 cITP-NMR of Formate and Acetate Using ACES TE Buffer to Probe Intracapillary pH

The first buffer system used in these experiments was identical to that described in section 5.2.5.1. A solution containing 250 µM ammonium acetate, 800 µM formic acid, and 500 µM BSFA was prepared in 10mM TE and D$_2$O.
In the second experiment, the solutions were again identical to those described in section 5.2.5.1 with the exception of the LE. In this experiment, the LE was buffered, starting with 160 mM DCl/80 mM imidazole and titrating to pH 6.52 with 1 M NaOH. The currents produced during the course of the cITP experiments ranged from 41 to 34 µA.

5.2.6 NMR pD Titration Experiments

A detailed description of $^1$H NMR pD titrations is given in Chapter 4. Briefly, the chemical shifts of formate, acetate, and ACES in D$_2$O are related to pD by Equation 5.5,

$$pD = pK_a(D) + \log \left( \frac{\delta_{obs} - \delta_{DA}}{\delta_A^- - \delta_{obs}} \right)$$  \hspace{1cm} (5.5)

where $\delta_{obs}$ is the observed chemical shift, and $\delta_{DA}$, and $\delta_A^-$ are the chemical shifts of the acid and base forms of the molecule.$^{44-46}$ As a reminder, the p$K_a$(D) is the conditional acid dissociation constant measured in D$_2$O solutions. Therefore, NMR pD titrations were performed to determine the conditional p$K_a$(D) values of acetate and ACES in D$_2$O. The p$K_a$(D) value for formate was previously determined in Chapter 4 and is 4.04 ± 0.01.

$^1$H NMR spectra and pD measurements were recorded for a D$_2$O solution containing 20 mM ACES, 5 mM sodium acetate, 0.1 M NaNO$_3$ to maintain a constant ionic strength, and tert-butyl alcohol as a chemical shift reference. Solution pD values were measured potentiometrically using a 3.5 x 55 mm combination glass electrode (ESA Biosciences, Inc., Boston, MA) as the solution pD was adjusted in ~0.5 unit steps using aliquots of NaOD or DCl diluted in D$_2$O. Because the pH electrode was calibrated with
standard aqueous buffer solutions, the pD was calculated using the pH meter reading (pH*) and the equation: pD = pH* + 0.4. The indicator solution temperature was maintained at 25 °C using a water bath during the potentiometric measurements.

5.2.7 cITP-NMR Separations of Heparin Tetrasaccharides Using BTP

To explore the use of complex-forming equilibria to facilitate improved focusing of the heparin tetrasaccharides, cITP-NMR experiments were conducted in deuterated solutions. Concentrations of LE and TE were varied in our attempts to optimize the separation. The LE was deuterium chloride (160 mM or 250 mM) and BTP was the counter ion (20 mM or 50 mM) at pD 6.90. The TE consisted of MES (160 mM or 20 mM) adjusted to pD 6.90. To compare the focusing to the previous anionic cITP-NMR buffer system, an LE of 160 mM sodium chloride was used at pH 6.50. Tetrasaccharide fraction 342 from the SEC separation performed in Chapter 4 was used for these experiments without further separation or purification. The concentration of this fraction was determined using UV absorbance measurements and the molar extinction coefficient of 5500 M⁻¹ cm⁻¹. An aliquot of fraction 342 was used to make a cITP sample consisting of 500 µM tetrasaccharide and 500 µM BSFA, reconstituted in 10 mM TE and D₂O. The injection protocol was as follows for each experiment; the capillary was first filled with LE, then a 13 minute injection of the sample, followed by a 3 minute injection of the TE. This resulted in 16.25 µL of sample in the capillary equating to 9.8 µg of the tetrasaccharide fraction based on the molecular weight of IS-IS (1146.88 g/mol). The
voltage applied across the capillary was 15 kV and the currents produced during the experiments varied in the range of 49.5 to 32.1 µA.

5.3 Results and Discussion

5.3.1 Microscopic Visualization of Steady-State cITP

By coupling stereo microscopy to a camcorder, video was captured of both steady-state cationic and anionic cITP at the window 83 cm from the point of injection. All videos recorded for this chapter are found in the Supplementary Data. Figure 5.7 shows clips of the video taken during off-line cationic cITP. The focused band of methyl green analyte enters into view in Figure 5.7 A. From this view, the front boundary at the LE/analyte interface appears to have a flat profile as the methyl green travels toward the cathode. The boundary at the analyte/TE interface also appears to have a flat profile and this is clearly seen in Figure 5.7 B-C. The hydrodynamic injection of 6 minutes at a height differential of 16 cm corresponds to a 29.5 cm plug of analyte (7.5 µL) before the voltage is applied across the capillary. Over the course of this experiment, the methyl green analyte focused to a length of approximately 0.1 cm, as shown in Figure 5.7 B. Therefore the focusing factor for this experiment was estimated to be 295.

The movement of the focused methyl green band through the capillary was not completely even and continuous, but looked as if it undulated forward through capillary. This irregular motion is probably due to the self-sharpening effect of cITP described in section 5.1.2, in which the ions diffuse from their own zone into the neighboring zone and are forced back due to changes in electric field strength. This was also seen in the
**Figure 5.7.** Video clips of cationic cITP focusing of 100 µM methyl green dye. The distance between the black lines is 1 mm.
video filmed of the anionic cITP experiment using the bromophenol blue analyte however, the movement was less pronounced.

Clips from the video of the anionic cITP experiment are shown in Figure 5.8. In Figure 5.8 A, the focused zone of bromophenol blue moves into view and the profile of the LE/analyte boundary was flat. The 8 minute injection corresponds to a 39.4 cm plug length and a higher volume of sample (10 µL) in the capillary. Despite the larger amount injected, the length of the focused zone is relatively smaller (< 1 mm) compared to the cationic cITP experiment, as shown in Figure 5.8 B. This yielded an estimated focusing factor > 400. However, in Figure 5.8 B-C, a more diffuse analyte/TE boundary was recorded. There are many possible reasons for this observation, including the difference in mobilities of the bromophenol blue, the choice of a TE (MES) with too small of an electrophoretic mobility, or disruption of the focused boundary due to the migration of free counter ions.

The video images of microscopic cationic and anionic cITP provide some insights into the effective focusing ability of different buffer systems and the migration behavior of selected cationic and anionic analytes. To obtain a more complete picture of the cITP process, visualization of counter ion migration is desirable. This could provide useful information about the boundary conditions during an experiment and aid in the optimization of the focusing and separation.

5.3.2 Microscopic Visualization of Counter Ions in Cationic cITP
Figure 5.8. Video clips of anionic cITP focusing of 100 µM bromophenol blue dye.
To examine the migration of counter ions in cITP, a divalent anionic dye, mordant blue 9, was introduced into the cationic LE as the counter ion. At pH 4.34, the dye is fully deprotonated, soluble in aqueous solution, and deep red in color. The analyte used in the experiment was methyl green and video was taken before steady-state had been reached at 69 cm from the point of injection. A clip of from the video is shown in Figure 5.9 A. At this point, the boundary of the LE/analyte appears slightly hyperbolic and there is a flat profile at the analyte/TE interface immediately followed by a diffuse tailing of the zone. This tailing likely reflects the fact that a steady-state has not been fully reached at this point in the cITP experiment.

Figure 5.9 B is a clip from the video recorded at 74 cm from the point of injection and approximately 15 minutes following the clip shown in Figure 5.9 A. At this point in the experiment, the tailing at the analyte/TE boundary has receded and the length of the focused zone has decreased slightly. The LE/analyte boundary has acquired a more flat profile and the general characteristics of the zone are closer to those of a steady-state. However, closer observation of the video revealed the formation of small particles forming behind the zone as it passed through the capillary. This becomes more apparent in the next video clip shown in Figure 5.9 C. This was taken approximately 45 minutes after the start of the experiment and the zone had become less than 1/3 of its original length. Figure 5.9 D-F show the progressive movement of the analyte band as it passes through the capillary window. A particle that has formed and was contained in the analyte band, breaks away (Figure 5.9 E) and falls into solution (Figure 5.9 F). From these observations, it appears that the mordant blue 9 anion and the methyl green cation
Figure 5.9. Video clips recording the influence of counter ion, mordant blue 9, added to the LE on the cationic cITP focusing of methyl green at three different time points (A-C). Video clips D-F are of the progressive migration of the methyl green zone at time point C.
are forming a complex that is insoluble in aqueous solutions. This would account for the shortening of the analyte zone over the course of the experiment. It can also be inferred that the particles are a neutral species because they do not migrate under the applied voltage.

Although the counter ions in this experiment could not be visualized directly, the effects that they had on the focused analyte zone were observed with the aid of stereo microscopy. In experiments performed prior to using the stereo microscope the disappearance of the methyl green dye was observed over time. However, the formation of particles behind the analyte zone and the overall shape of the zone throughout the course of the experiment could not be seen with the naked eye. By visualizing the cITP migration of ions using stereo microscopy, a greater understanding of the separation processes can be obtained. This lays the foundation for future investigations to study steady-state cITP and counter ion migration.

5.3.3 Anionic cITP-NMR of Heparin Disaccharides IVA, IIH, and IIIH

5.3.3.1 Utilizing CE to Investigate the Electrophoretic Mobility of MES

Suspecting that the effective electrophoretic mobility, \( \mu_e \), of the TE buffer, MES, might be higher than the heparin disaccharide standards IVA, IIH, and IIIH, in the cITP focusing experiments, the value of the MES mobility was determined using Equation 5.6,

\[
\mu_e = \frac{\mu_a}{10^{(pK_a-pH)} + 1}.
\]  

(5.6)
Because the electrophoretic mobility of the fully deprotonated species, $\mu_d$, of MES (-28×10^{-5} cm^2/sV) and the acid dissociation constant, $pK_a$ (6.15), are well known, the $\mu_e$ of MES under anionic cITP-NMR conditions (pH = 6.50) was calculated to be -19.4×10^{-5} cm^2/sV. Comparing this value to the CE determined $\mu_e$ values for disaccharides IVA, IIH, and IIIH, the MES $\mu_e$ was higher by approximately -0.3 cm^2/sV. The experimentally determined $\mu_e$ values for all heparin disaccharide standards at pH 6.50 are listed in Table 5.1. Under the described conditions, 69% of MES is fully deprotonated. By exploiting the acid-base equilibrium of MES, this percentage can be decreased by lowering the pH, in turn, decreasing the mobility of the TE buffer. Since both the heparin disaccharides and MES buffer contain chromophores, CE-UV was utilized to probe their relative mobilities as a function of pH.

To validate the above calculations and confirm that MES maintains a higher mobility than the disaccharides, CE runs were conducted in triplicate under the same conditions as those used in the cITP-NMR focusing experiments. First, MES and disaccharide standard IVA were ran separately in 20 mM phosphate buffer (pH 6.54) and the average migration times were recorded as 11.50 and 13.36 min, respectively. Heparin disaccharide standard IVA was chosen for these experiments because it has the lowest mobility of the three monovalent disaccharides (-16.0×10^{-5} cm^2/sV) and the mobility of the cITP TE buffer must be lower than this value. A mixture was then injected containing BSFA, IVA, and MES and the resulting electropherogram is shown in Figure 5.10. The migration time of MES (11.63 min) was almost 2 min sooner than IVA (13.32 min).
Table 5.1. Electrophoretic mobilities determined for the heparin disaccharides using 20 mM phosphate buffer at pH 6.50 and 25 °C.

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>$\mu_e$ at pH 6.50 (10^{-5} \text{ cm}^2/\text{s-V})</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>-50.3 ± 0.08\textsuperscript{a}</td>
</tr>
<tr>
<td>IIS</td>
<td>-40.5 ± 0.18</td>
</tr>
<tr>
<td>IIIIS</td>
<td>-44.0 ± 0.18</td>
</tr>
<tr>
<td>IA</td>
<td>-39.7 ± 0.07</td>
</tr>
<tr>
<td>IIA</td>
<td>-29.2 ± 0.18</td>
</tr>
<tr>
<td>IIIA</td>
<td>-29.4 ± 0.05</td>
</tr>
<tr>
<td>IVA</td>
<td>-16.0 ± 0.02</td>
</tr>
<tr>
<td>IH</td>
<td>-30.5 ± 0.01</td>
</tr>
<tr>
<td>IIH</td>
<td>-17.1 ± 0.03</td>
</tr>
<tr>
<td>IIIH</td>
<td>-17.3 ± 0.08</td>
</tr>
<tr>
<td>IVH</td>
<td>2.09 ± 0.12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The uncertainties in the mobility measurements are standard deviations calculated from at least 3 electropherograms.
Figure 5.10. CE electropherogram of 0.5 mM BSFA, 1 mM MES, and 0.5 mM IVA in 20 mM phosphate buffer, pH 6.54 migrating at 6.38, 11.63, and 13.32 min, respectively.
After confirming that the mobility of the MES was higher than IVA under the current cITP-NMR conditions, the mobility of the MES was recalculated at pH 5.61 using Equation 5.6. At this pH, the mobility of MES would be $6.3 \times 10^{-5}$ cm$^2$/sV, which would be significantly lower than the mobility of IVA. However, only 22% of MES would be in the fully deprotonated state. The resulting electropherogram is shown in Figure 5.11. In the CE separation, the migration time of the IVA disaccharide was 13.49 min and it appeared that the MES did not migrate past the detector in the allotted separation time. However, experiments performed for MES alone at pH 5.61 revealed that the MES migrated as a very broad peak between 13 and 17 minutes, confirming an overlap between IVA and MES in Figure 5.11.

ITP separation requires that the degree of ionization for the buffers and separated substances be higher than 10%, fulfilling the condition $\text{pH}_A \geq \text{pK}_{HA} - 1$. By lowering the pH of the MES, the degree of negative charge would decrease, making MES more neutral. This is apparent in the CE experiments performed at pH 5.61 due to the MES peak spanning over a 4 min migration time. Although MES is 22% ionized at pH 5.61, the migration times, and thus mobilities, of MES and IVA are approximately the same. It was clear that MES was not a good buffer choice for the cITP focusing of disaccharides IVA, IIH, and IIIH. To meet the requirements for cITP and provide successful focusing of these monovalent disaccharides, the TE buffer would have to have a lower mobility in the fully deprotonated state ($<-28 \times 10^{-5}$ cm$^2$/sV) and a higher $\text{pK}_a (> 6.15)$, to provide a larger degree of ionization at pH 6.50.
**Figure 5.11.** CE electropherogram of 0.5 mM BSFA, 1 mM MES, and 0.5 mM IVA in 20 mM phosphate buffer, pH 5.61. Migration times for BSFA and IVA were 6.50 and 13.49 min, respectively. A separate run of MES alone demonstrated that there was an overlap in the migration times for IVA and MES.
5.3.3.2 Utilizing CE to Investigate the Electrophoretic Mobility of ACES

ACES buffer was chosen as a potential anionic TE buffer based on its $pK_a$ (6.84) and mobility ($-24.1 \times 10^{-5} \text{ cm}^2/\text{sV}$) values. Separate CE experiments were performed at pH 6.50 to check the mobility of ACES under cITP conditions. The average migration time was 22.46 min, which is sufficiently lower than disaccharide IVA under the same conditions. A solution containing a mixture of the two components was separated at pH 6.50. The resulting electropherogram in Figure 5.12 shows IVA migrating at 13.28 min and ACES migrating at 21.00 min.

To test the compatibility of the ACES buffer with the II- and IIH disaccharides, a CE separation was conducted on a mixture of IIH and ACES at pH 6.50. Due to its lower mobility ($\mu_e = -17.1 \times 10^{-5} \text{ cm}^2/\text{sV}$), IIH was chosen for the separation. The electropherogram is shown in Figure 5.13. The migration time for IIH was 11.83 min, which is shorter than the time for IVA, as expected. The migration time for ACES was 20.38 min. CE experiments comparing the relative mobility of ACES to both disaccharides IVA and IIH demonstrate that its mobility appears to be sufficiently low at pH 6.50 and suggests that ACES should be a suitable TE buffer for the cITP-NMR focusing experiments.

5.3.3.3 cITP-NMR Focusing of Heparin Disaccharide IVA Employing ACES as a TE Buffer

The ACES buffer was first tested as a suitable anionic cITP TE buffer in an off-line cITP experiment, with optical detection, using bromophenol blue as the analyte.
Figure 5.12. CE electropherogram of 0.5 mM BSFA, 0.29 mM IVA, and 1 mM ACES in 20 mM phosphate buffer, pH 6.50, migrating at 6.32, 13.28, and 21.00 min, respectively.
**Figure 5.13.** CE electropherogram of 0.5 mM BSFA, 0.5 mM IIH, and 1 mM ACES in 20 mM phosphate buffer, pH 6.50, migrating at 6.22, 11.83, and 20.38 min, respectively.
ACES provided good focusing of the analyte as shown in Figure 5.14. This is a series of clips taken from the video of the run, which show the focused band to be approximately 0.5 mm in length with fairly discrete boundaries. However, some tailing is present at the analyte/TE boundary as seen previously in the steady-state anionic cITP video clips (Figure 5.8).

Following this off-line experiment, cITP-NMR experiments were performed using ACES buffer (pH 6.50) as the TE. After confirming that this buffer system was successful at focusing BSFA alone (data not shown), a sample was prepared containing 500 µM BSFA and 500 µM heparin standard IVA. Disaccharide IVA was chosen because it is $N$-acetylated, providing an intense acetyl resonance at ~2.2 ppm. Figure 5.15 shows a portion of the on-line cITP-NMR spectra for this experiment. It is expected that at pH 6.50 the BSFA should focus before the IVA due to its higher mobility, confirmed by the CE data. Figure 5.15 A-B contain the spectra showing the resonances of BSFA between 8.0 and 7.0 ppm and t-butanol at 1.239 ppm, used as a chemical shift reference. Figure 5.15 C-D shows the spectra of the BSFA/TE rear boundary. The ACES resonances are located between 3.5 and 2.9 ppm. A spectrum of the ACES TE alone is shown in Figure 5.15 E. The ACES buffer was effective for focusing the BSFA, but not the IVA disaccharide.

Three trials of this cITP-NMR experiment were performed and each time disaccharide IVA failed to focus. During these trials, the pD of the LE and TE buffer vials were measured after each run. Consistently, the LE buffer measured between pD 3.11 and 3.33 and the TE buffer measured between pD 6.77 and 6.85. The low pD of the
Figure 5.14. Video clips of anionic cITP of bromophenol blue using ACES as the TE buffer.
Figure 5.15. Anionic cITP-NMR of IVA and BSFA using ACES as the TE buffer.
LE buffer at the end of each cITP-NMR experiment could indicate that there is an intracapillary pH gradient occurring due to a build up of $\text{H}^+$ or $\text{OH}^-$ ions. If the pD of the frontal boundary between the LE and disaccharide IVA is low, IVA would fail to focus due to its $pK_a(D)$ of 3.37. At a low pH ($\leq pK_a$), the overall net charge of IVA would only be partially negative or neutral.

5.3.3.4 Intracapillary pD Determination

cITP-NMR experiments were performed under the same conditions described in section 5.3.3.3 to probe the intracapillary pD across the focused zones by using the anions, formate and acetate, as NMR pD indicator molecules. A titration curve was also constructed for ACES to provide the pD conditions of the TE and its frontal boundary.

For these experiments, a $^1\text{H}$ NMR pD titration was first performed for acetate and ACES in the same solution. The $\delta_{DA}$ and $\delta_{A^-}$ values of the acetate proton were determined to be 1.906 and 2.087 ppm, respectively, with a $pK_a(D)$ of 5.09 ± 0.00. This is in good agreement with the value of 5.18 reported in $\text{D}_2\text{O}$, as well as that of 4.75 reported in aqueous solution.\cite{1, 41} The $\delta_{DA}$ and $\delta_{A^-}$ values of the ACES CH$_2$ singlet were determined to be 3.999 and 3.346 ppm, respectively, with a $pK_a(D)$ of 7.32 ± 0.00. This value is in good agreement with the values of 6.84 and 6.90 reported in aqueous solutions.\cite{1, 49} The resulting titration curves for both species are provided in Figure 5.16. The titration of formate was conducted previously and these experiments are described in Chapter 4, section 4.3.1.
Figure 5.16. NMR detected titration curves for (A) ACES and (B) acetate. The pKa(D) values were determined to be 7.32 ± 0.00 and 5.09 ± 0.00, respectively.
Following the \textsuperscript{1}H NMR titration of ACES and acetate, a cITP-NMR experiment was performed on a sample containing formate and acetate. Figure 5.17 A is the spectrum of the LE/acetate boundary and the chemical shift of acetate is 1.905 ppm and outside the effective range of the acetate indicator. However, a pD greater than 7.0 can be inferred from the titration curve. Approximately 3.5 minutes later, Figure 5.17 B shows the spectrum of the acetate/BSFA boundary as the acetate is leaving the detection volume of the microcoil. The chemical shift of acetate here is 1.906 ppm and again, outside of the useful range of the indicator. Figure 5.17 C is the BSFA/TE boundary, in which the ACES CH\textsubscript{2} singlet chemical shift is 3.346 ppm. This value is slightly past the inflection point on the titration curve, but a pD $\geq$ 8.5 can be inferred. Finally, Figure 5.17 D is the spectrum of the ACES TE measured 80 s later. The chemical shift of 3.347 ppm indicates that the pD has dropped slightly, but is consistently $\geq$ 8.5. Due to the rise in intracapillary pD across the analyte zones, it is probable that there is a build-up of free OH\textsuperscript{-} ions at the frontal boundary of the TE. It is also interesting to note that the formate failed to focus in these experiments. It is possible that the concentration of formate may not have been sufficiently high for microcoil NMR detection or that the mobility of formate was not intermediate to that of the buffer components. The pD of the LE buffer vial after the cITP-NMR experiment was measured and recorded at 3.25.

It was postulated that the rise in intracapillary pD could be controlled by using a buffered LE. The above experiment was repeated using the same acetate/formate/BSFA solution, a buffered LE consisting of 160 mM DCI and 80 mM imidazole, and 160 mM ACES as the TE. The resulting array of spectra produced similar results (data not shown).
Figure 5.17. cITP-NMR of acetate and BSFA using ACES as the TE buffer to probe intracapillary pD.
The intracapillary pD at the LE/acetate boundary was $\geq 7.0$ as indicated by the acetate chemical shift of 1.905 ppm and climbed to $\geq 8.5$ as indicated by the 3.346 ppm chemical shift of the ACES CH$_2$ singlet. Despite this rise in pD, the LE buffer vial was again measured post-run and found to be 6.42. The pD of the LE in the buffer vial was maintained during the course of the experiment and had only lowered slightly by the end of the run.

It was concluded that the rise in pD across the analyte zones during the cITP-NMR is likely due to a high concentration of free OH$^-$ ions migrating at the front TE boundary. Free OH$^-$ migration may also be the cause of the slight tailing seen in the anionic cITP videos. A pD greater than 7.40 changes the mobility of the ACES buffer to a value that is higher than the IVA disaccharide, violating the cITP requirements to focus this analyte. A completely new buffer system must be explored to allow effective focusing of IVA, IIH, and IIIH. Based on these experiments, the TE must meet the minimum requirements of possessing a low $\mu_a$ ($< -24.1\times10^{-5}$ cm$^2$/sV) and a $pK_a$ in the range of 6.8-7.0 with a greater buffering capacity.

### 5.3.4 Anionic cITP-NMR of Heparin Tetrasaccharides: Exploring Complex-Forming Equilibria Using BTP

Complex-forming equilibria using BTP was investigated as a means to improve the focusing of heparin tetrasaccharides. The first of this series of experiments was conducted using the anionic buffer system of 160 mM NaCl as the leading electrolyte and 160 mM MES as the trailing electrolyte. Figure 5.18 shows a portion of the cITP-NMR
Figure 5.18. cITP-NMR of tetrasaccharide fraction 342 and BSFA using 160 mM NaCl as the LE and 160 mM MES as the TE.
spectra resulting from this experiment. Figure 5.18 A is a spectrum of the LE just before fraction 342 enters the observe volume of the microcoil. The spectra in Figure 5.18 B-H show fraction 342 focusing behind the LE. There were a total of 22 spectra measured for fraction 342. Post acquisition coaddition of these spectra can be performed to enhance the S/N. However, fraction 342 is a mixture of at least three major tetrasaccharides and due to the poor S/N, the boundaries between the tetrasaccharides were not distinct. Coaddition of these spectra would produce a single spectrum containing a mixture of tetrasaccharides. As expected, the BSFA was separated from fraction 342 and focused after the tetrasaccharides due to its net charge of -1. The boundary of fraction 342 and BSFA is shown in Figure 5.19 B, followed by three spectra of the BSFA in Figure 5.19 C-E (4 spectra total). Figure 5.19 F shows the boundary of BSFA and the MES TE buffer. Due to the number of spectra acquired for the tetrasaccharide fraction, it is probable that the focusing was diffuse. However, NMR spectra of the boundaries on the front and rear edge of the tetrasaccharide band did not indicate the presence of mixed zones (i.e. several spectra of LE and tetrasaccharides or tetrasaccharides and BSFA migrating together).

The next set of experiments employed BTP as a counter ion in the LE to bring about selective retardation of the electromigration of the tetrasaccharides. This counter ion had been used previously in cITP to effectively focus and separate a mixture of heparin-like pharmaceuticals that varied in their degree of sulfation. These authors reported using HCl as the LE with 20 mM BTP as the counter ion and 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO) as the TE. We were aware that chemical shift
Figure 5.19. A continuation of the cITP-NMR spectra shown in Figure 5.18: (A) the spectrum of tetrasaccharide 342, (B) the boundary of fraction 342 and BSFA, (C-E) spectra of BSFA, and (F) the boundary of BSFA and MES (TE buffer).
overlap would occur between the resonances of BTP and those of the tetrasaccharides in the NMR spectra. However, this ion was utilized as a means to test the effectiveness of complex-forming equilibria for improving the focusing of heparin tetrasaccharides in cITP.

For these experiments, the LE consisted of 160 mM DCl and 20 mM of BTP and the TE remained the same as in the previous experiments (160 mM MES). Off-line experiments similar to those conducted in section 5.3.3.3 were used to test the new LE buffer and successful focusing of the bromophenol blue dye was demonstrated and detected visually. The on-line cITP-NMR experiment utilizing this buffer system resulted in the following zones; BSFA (4 spectra total), trailed by a mixed zone of BSFA and fraction 342 (13 spectra total), then a zone containing only tetrasaccharide fraction 342 (5 spectra total). The cITP-NMR spectral array from this experiment is shown in Figure 5.20. However, for better visualization, only every fifth spectrum is plotted. The results from this experiment clearly indicated that the BTP interacted with the tetrasaccharides effectively attenuating their electromigration due to the reverse order of migration between the tetrasaccharides and BSFA. Unfortunately, a mixed zone containing BSFA and the tetrasaccharides was present indicating indiscrète focusing of the zones. There also appeared to be ion-pairing between the BTP and BSFA, as indicated by the decrease in intensity of the BSFA resonances once the mixed zone entered the detection volume of the microcoil.

We hypothesized that by increasing the amount of counter-ion in the LE, it may be possible to slow the migration of the tetrasaccharide such that it separates and focuses
Figure 5.20. cITP-NMR of tetrasaccharide fraction 342 and BSFA using 160 mM DCl / 20 mM BTP as the LE and 160 mM MES as the TE. Every fifth spectrum is plotted.
in its own zone behind the BSFA zone. The concentration of BTP was increased to 50 mM and this experiment resulted in 7 spectra of BSFA, the last two of which are shown in Figure 5.21 A-B, followed by only 3 spectra of fraction 342 (Figure 5.21 C-E). The spectra measured for the tetrasaccharides have poor S/N. It is possible that ion-pairing with a higher concentration of BTP reduced the effective net charge on the tetrasaccharides to the extent that they became essentially charge neutral. This would result in their effective mobility being lower than the TE or in an absence of migration.

Two other experiments were also attempted. One utilized a lower concentration of TE (20 mM) and the other, a lower concentration of TE (20 mM) together with a higher concentration of LE (250 mM DCl). Both of these experiments resulted in several NMR spectra of mixed zones containing tetrasaccharides and BSFA due to indiscrete focusing of the zones. A summary of all the experiments discussed in this section are summarized in Table 5.2.

The experiments conducted using the BTP as an ion-pairing reagent proved to be effective for retarding the migration of the tetrasaccharides. However, it did not improve their degree of focusing. The experiments performed in section 5.3.3.4 to probe the intracapillary pD revealed a pH gradient across the analyte zones in anionic cITP. Because the same buffers are typically used to focus tetrasaccharides, it is possible that changes in zone pD may also be hindering their focusing. Although this seems less likely due to the greater overall net negative charge (higher mobility) and their ability to focus to some extent in the cITP experiments.
Figure 5.21. cITP-NMR of tetrasaccharide 342 and BSFA using 160 mM DCI / 50 mM BTP as the LE and 160 mM MES as the TE.
**Table 5.2.** Summary of the cITP-NMR experiments performed to improve the focusing of heparin tetrasaccharides (fraction 342) using complex-forming equilibria. BTP was added to the LE as the counter ion.

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Buffer System</th>
<th>BSFA and Tetrasaccharide Fraction 342</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LE: 160 NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TE: 160 mM MES</td>
<td>22 spectra tetras</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 spectra tetras/BSFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 spectra BSFA only</td>
</tr>
<tr>
<td>2</td>
<td>LE: 160 DCl / 20 mM BTP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TE: 160 mM MES</td>
<td>4 spectra of BSFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 spectra of BSFA/tetras</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 spectra of tetras only</td>
</tr>
<tr>
<td>3</td>
<td>LE: 160 DCl / 50 mM BTP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TE: 160 mM MES</td>
<td>7 spectra of BSFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 spectra of tetras only</td>
</tr>
<tr>
<td>4</td>
<td>LE: 160 DCl / 20 mM BTP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TE: 20 mM MES</td>
<td>13 spectra of BSFA &amp; tetras</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 spectra of tetras only</td>
</tr>
<tr>
<td>5</td>
<td>LE: 250 DCl / 20 mM BTP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TE: 20 mM MES</td>
<td>21 spectra of BSFA &amp; tetras</td>
</tr>
</tbody>
</table>
The free counter migration of cations, such as H\(^+\), can also lower the effective net charge of the tetrasaccharides, thus lowering their mobility and decreasing their ability to focus effectively in cITP. This possibility was explored by using a buffered LE containing NaCl and imidazole as a counter ion. An aliquot of the IS-IS tetrasaccharide isolated in Chapter 4 and BSFA was used as the sample. It appeared that this buffer system resulted in improved focusing of the tetrasaccharide, yielding 4 spectra of IS-IS as shown in Figure 5.22, followed later by 6 spectra of BSFA. Four spectra were also measured with no peaks, suggesting the presence of an unknown NMR transparent analyte that focused after the IS-IS tetrasaccharide and before the BSFA. It is not uncommon for heparin digests resulting from enzymatic depolymerization to contain buffer salts or impurities that also focus during anionic cITP-NMR experiments.\(^{41,50}\) The intracapillary pD was investigated using the chemical shifts of the imidazole resonances using the calibrated titration curve of imidazole from Chapter 4. The intracapillary pD changed slightly across the analyte zones, starting with a pD of 7.20 at the LE/IS-IS boundary and ending with a pD of 8.00 at the BSFA/TE boundary. The free migration of H\(^+\) ions was not evident based on these measurements.

Although the use of a buffered LE proved promising for improving the focusing of heparin tetrasaccharides, further experiments were not performed due to the loss in sensitivity of the probe, resulting from degradation of the home-built microcoil. This degradation is indicated by the presence of very broad resonances throughout the cITP-NMR spectra in Figure 5.22.
Figure 5.22. cITP-NMR of heparin tetrasaccharide IS-IS and BSFA using 160 mM NaCl / 80 mM imidazole as the LE and 160 mM MES as the TE. The cITP-NMR spectra of BSFA are not shown.
5.4 Summary

In this chapter, stereo microscopy and NMR spectroscopy were used to monitor the effective focusing of dye molecules and heparin oligosaccharides during cITP separations. Microscopic video of cITP provided an enhanced perspective of the analyte and buffer components under steady-state conditions that cannot be achieved with the naked eye. Also, in this work, video capture of possible complex formation in cITP, laid the groundwork for future investigations probing counter ion migration. Other experiments using stereo microscopy detection, including the investigation of stopped-flow cITP using manual hydrodynamic backpressure, were also explored. Future work in this area is discussed in Chapter 6. This chapter further extends the utility of cITP-NMR to provide diagnostic information about cITP processes. Unlike other detection methods used to study the electromigration of ions, NMR spectroscopy has the advantage of unambiguously identifying the organic constituents that occupy each electrophoretic zone. NMR can be used to track the intracapillary pH of buffer and analyte zones and evaluate the effectiveness of focusing during cITP. CE-UV was also a valuable tool for surveying the mobilities of buffer components relative to the analytes of interest. By using CE-UV in concert with off-line cITP with optical detection, one can obtain direct assessment of the behavior of transparent analytes and complementary information about the focusing ability of the buffers. Mobilities, sample zone pH, and counter ion migration are all important factors in the prediction and optimization of isotachophoretic focusing of heparin di- and tetrasaccharides. CE-UV, NMR and stereo microscopy methods have been shown to be effective for evaluating these parameters.
5.5 REFERENCES


49. Roy, R. N.; Bice, J.; Greer, J.; Carlsten, J. A.; Smithson, J.; Good, W. S.; Moore, C. P.; Roy, L. N.; Kuhler, K. M., Buffers for the physiological pH range: Acidic

Chapter Six

Conclusions and Future Directions

6.1 Conclusions

This dissertation explores the development of improved analytical methods and approaches that address the difficulties in separating and detecting pharmaceutical impurities and nanomole quantities of charged oligosaccharides. The various methods investigated include CE-UV, LC-MS, with special emphasis was given to cITP-NMR.

CE-UV played an important role in purity analysis. In Chapter 2, CE-UV was employed to verify the presence and migration order of trace impurities and degradants produced by forced degradation of acetaminophen. Assessment of their migration order and thus, inferred electrophoretic mobilities, allowed verification of the cITP-NMR data. In Chapter 3, CE-UV proved ideal for profiling size-selected SEC fractions produced from the enzymatic digestion of porcine heparin. This technique revealed the differences in composition and relative concentration of heparin components in the SEC fractions. CE-UV analysis confirmed the heterogeneous nature of heparin, even among same-sized fragments.

CE-UV is also important for characterizing electrophoretic mobilities and understanding separation processes. In Chapters 4 and 5, the electrophoretic mobilities were measured for fully deprotonated and partially charged heparin disaccharides. These values provided insights into the CE-UV migration order and cITP separations of these disaccharides. In Chapter 5, CE-UV was useful for probing suitable TE buffers for use in
cITP separations based on their mobility relative to the analyte, and demonstrated that CE can help save time during development and optimization of new cITP buffer systems.

LC-MS was also vital in this work for providing complementary structural information, particularly when the structure of the analyte was unknown or when standards were not available. In Chapter 2, LC-MS facilitated the structural identification of four dimeric decomposition products of acetaminophen in a pharmaceutical formulation and demonstrated the effectiveness of off-line cITP experiments for LC-MS sample preparation. In Chapter 3, the use of the ion-pairing reagent, tributylamine, in LC-MS provided an improved method for separating hydrophilic di- and tetrasaccharides. Subsequent MS analysis of the separated heparin components allowed further investigation into the heterogeneity of preparative SEC fractions. Reversed-phase ion-pairing LC was utilized again in Chapter 4, for the quick separation and isolation of heparin tetrasaccharides for use in subsequent $^1$H NMR analysis.

cITP-NMR technology was highlighted throughout this dissertation and shown to be an effective method for enhancing the sensitivity of NMR, which facilitated characterization of concentration- and mass-limited amounts of material. In Chapter 2, cITP-NMR was a valuable analytical technique for separating and concentrating cationic impurities in the presence of 1000-fold excess of the parent compound. Reinforced was the ability of cITP-NMR to separate charge neutral matrix components away from the analytes of interest. This feature of cITP-NMR also important in the focusing and detection of heparin di- and tetrasaccharides in Chapters 4 and 5. In Chapter 4, a disaccharide cITP-NMR chemical shift database was developed using microgram
amounts of material. The purpose of this work was twofold; to streamline profiling of heparin enzymatic digests in the search for novel disaccharides, and to evaluate the focusing ability of the disaccharides with the selected anionic cITP buffers. In Chapter 5, on-line cITP-NMR was used to probe sample zone pH and provide insight into other processes that drive cITP separations, such as mobility and counter ion migration.

Although the results presented in this dissertation demonstrate the capacity of hyphenated techniques for impurity and heparin analysis, there remains a need for the improvement of cITP-NMR methods and instrumentation. Further optimization of the cITP separation protocol for heparin oligosaccharides is needed, including a survey of different buffer systems. Also, further optimization of NMR microcoil design will improve the sensitivity of this method and the resulting quality of the NMR spectra obtained. Other possible future directions for the research presented in this dissertation are discussed in the following section.

6.2 Future Directions

6.2.1 Stopped-Flow cITP-NMR

It should be possible to acquire two-dimensional NMR spectra, such as COSY, if a cITP-focused analyte can reside in the microcoil for a period of at least 5 minutes, providing the conditions of the separation produce reasonable S/N.\textsuperscript{1} It has been suggested that the use of probes with coils in series could facilitate positioning of focused analyte bands in the observe volume of the microcoil.\textsuperscript{2} The first coil in the series acts as a scout to indicate the approaching bands and the second coil is used for peak trapping. However,
problems with this strategy include electronic crosstalk between the adjacent coils resulting in the observation of peaks from the sample in the neighboring coil. Our lab has explored the use of manual hydrodynamic backpressure in off-line experiments for the positioning of a focused analyte dye in the capillary window. The effectiveness of this technique was observed using the stereo microscopy detection approach reported in Chapter 5. Maintaining precise pressure for several minutes was a challenge and the amount of pressure required to position the analyte changes slightly for every experiment. The use of a syringe pump to apply backpressure in very small increments would be an ideal method for trapping the cITP band in the detection volume of a microcoil. Another way to achieve stopped-flow cITP-NMR could use rapid pulses of the high voltage current applied to the capillary once the analyte band enters the microcoil. This was shown to be an effective method for stopped-flow CE-NMR experiments. As demonstrated in Chapter 5, a scout analyte, such as benzenesulfonic acid, could act as an indicator in cITP-NMR for approaching analytes of interest, providing the electrophoretic mobility of the scout analyte is higher.

### 6.2.2 cITP-NMR Database of Larger Oligosaccharides

Because the sequences of heparin responsible for protein recognition are known to contain \( \geq 2 \) disaccharides, the development of a cITP-NMR database of larger oligosaccharides would be desirable. Aside from disaccharides, standards are not commercially available for most heparin structures. However, heparin-derived oligosaccharides that have been isolated and characterized could be used for this purpose.
For example, the tetrasaccharides isolated in Chapter 4, could serve as standards for constructing a cITP-NMR chemical shift database of the major tetrasaccharides produced by enzymatic depolymerization with heparinase I. Following SEC fractionation, acquisition of $^1$H NMR spectra would allow for rapid recognition of known oligosaccharides. Therefore, efforts can be directed toward the separation and isolation of novel sequences or specific oligosaccharides shown to play a part in recognition studies. Due to the current inability to perform two-dimensional cITP-NMR, full characterization of the heparin oligosaccharides would have to be performed off-line. In addition, further development and optimization of the cITP separations for larger oligosaccharides is needed.

### 6.2.3 $pK_a$ Determination of Tetrasaccharides Containing $N$-Unsubstituted Glucosamine Residues

Comparison of carboxylate $pK_a$ values for the three major tetrasaccharides produced by enzymatic digestion with heparinase I revealed the likelihood that $O$-sulfo groups several residues away, influence the acidity of the carboxylate groups. In order to investigate this trend as it applies to the $N$-unsubstituted glucosamine residues of heparin, the $N$-desulfation reaction described by Huang and Kerns\(^6\), could be performed on the tetrasaccharide isolates from Chapter 4. Following separation and isolation of the various products resulting from the reaction, NMR pD titrations could then be performed for both the carboxylate and ammonium moieties of the tetrasaccharides. The carboxylate $pK_a$’s of these structures can be compared to those determined in Chapter 4. It is expected that the carboxylate acidity will increase due to charge stabilization from the ammonium groups.
New trends will likely be discovered regarding the ammonium pK\(_a\)’s when compared to the values determined for the N-unsubstituted disaccharides. It would be particularly interesting to investigate the effect of the position on the pK\(_a\) of the glucosamine ammonium groups. Simultaneous investigation of the mutarotation of the terminating glucosamine residue could also be conducted.

### 6.2.4 Studies of Heparan Sulfate Structures Important for Enzyme Recognition

As an alternative to the N-desulfation of previously isolated oligosaccharides, depolymerization and separation of heparan sulfate sequences could be performed. Because many heparan sulfate (HS) structures contain two ionizable groups (COO\(^-\) and NH\(_3^+\)), determination of these pK\(_a\) values would be important for understanding the structure-function relationships of HS sequences.

Furthermore, it has been shown that the presence of N-unsubstituted glucosamine residues are required for specific recognition by enzymes such as 3-O-sulfotransferase-3A (3-OST-3A).\(^7, 8\) Currently, the structures required for recognition have not been fully characterized. It appears that the recognition site of the 3-OST-3A enzyme is located on a disaccharide containing a GlcNH\(_2\) bound only by a IIIS disaccharide (IdoUA2S (1→4) GlcNS) on the reducing end. However, it is possible that the enzyme may recognize the site when bound by other neighboring disaccharides. This may be investigated further using various HS isolates.

HS is not abundantly found in porcine intestinal mucosa, which was used throughout this work.\(^9\) Therefore, HS would have to be extracted from other sources,
such as bovine kidney or brain. Because HS is structurally much more diverse than heparin, the HS digests should be more complex to analyze. However, with HS there is a greater possibility of isolating oligosaccharides containing unique structures with interesting biological activity.

6.2.5 Investigation of Counter Ion Migration in cITP

Methods introduced in this research revealed the power of stereo microscopy for real-time visualization of counter ions in cITP separations. This method of optical detection can be used to further investigate counter ion migration using multiple dye molecules. Ideal counter ions introduced into the LE at low concentrations, would change color when they passed through the analyte zone, either by experiencing a change in pH or forming a complex with the analyte. An example of the latter in cationic cITP would be the use of mordant blue 9 (charge = -2) as the counter ion of the LE and acridine yellow (charge = +2) as the analyte. Once the mordant blue enters the analyte zone, a green colored complex would form upon binding with the acridine yellow. However, as seen in Chapter 5, the disappearance of the analyte zone is a likely result. An experiment that exploits changes in pH might employ a pH indicator as the counter ion that exhibits a color change over the pH range of the analyte zone. The intracapillary pH of the system could be probed using cITP-NMR prior to addition of the counter ion. Cationic cITP would probably be more suited for these studies due to problems associated with residual electroosmotic flow in anionic cITP. A major obstacle of counter migration studies will
be finding suitable analytes that achieve the desired visual effect and that have reasonably high solubility limits.
6.3 REFERENCES


