Analysis of Human Erythropoietin and Erythropoietin N-Linked Glycans Using Capillary Electrophoresis and Mass Spectrometry

by

Jessica M. Risley

B.Sc., Thompson Rivers University, 2012

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES (Chemistry)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

June 2020

© Jessica M. Risley, 2020
The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Analysis of Human Erythropoietin and Erythropoietin N-Linked Glycans Using Capillary Electrophoresis and Mass Spectrometry

submitted by Jessica Risley in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry

Examiner Committee:
Dr. David Chen, Professor, Chemistry, UBC  
Supervisor
Dr. Dan Bizzotto, Professor, Chemistry, UBC  
Supervisory Committee Member
Dr. Allan Bertram, Professor, Chemistry, UBC  
University Examiner
Dr. Keng Chou, Professor, Chemistry, UBC  
University Examiner
Abstract

Glycoproteins are of interest as therapeutic agents. Characterization of the glycan component of glycoproteins is challenging due microheterogeneity, but necessary as glycans can cause negative immune system reactions. Erythropoietin is a glycoprotein hormone that stimulates the production of red blood cells and is used as a therapy for anemic patients. However, erythropoietin is also used as a doping agent in sports to boost performance by increasing the oxygen-carrying capacity of blood. Techniques for studying the glycans of erythropoietin and for differentiating between endogenous and exogenous erythropoietin are of interest in the scientific community. This thesis presents work on the development of techniques for the study of erythropoietin and its glycans for differentiation between endogenous and exogenous erythropoietin.

Chapter 2 presents a capillary electrophoresis-mass spectrometry method that separates the terminal residue found on glycans, sialic acid. The presence of one type of sialic acid indicates an exogenous origin since humans cannot produce that sialic acid. By manipulating the post-column chemical conditions of the separation, the sensitivity of the method was increased. The most common sialic acid and the sialic acid that cannot be produced by humans were separated, and the feasibility of the method to detect low abundance levels of sialic acids from erythropoietin was shown. A key factor in lowering the LOD was the ability to inject reproducibly from a 1 µL sample volume.

In chapter 3, different sialic acids from the producing conditions of erythropoietin (e.g. in the body or in cell culture) change the isoelectric point. A capillary isoelectric focusing method was developed with ultraviolet and laser induced fluorescence detection to analyze erythropoietin
produced both endogenously and exogenously. The method was able to separate erythropoietins produced from different sources, and the isoelectric points of the various glycoforms of recombinant erythropoietin were determined.

Chapter 4 focuses on simulations of an electro-fluid-dynamic microfluidic device for the feasibility of desalting glycoproteins with the device. Simulations concerning the ability of the device to selectively separate and purify analytes using a single power supply by manipulating the dimensions of the lengths and widths of the collection channels of the device.
Lay Summary

Proteins with attached sugars can be used to treat illnesses. It is important to study the sugar component of the protein because the sugars are often complex and can cause negative immune system responses if the sugars are not compatible with the human body. Erythropoietin is a protein that contains sugars that causes red blood cells to be made. Erythropoietin can be used to help people suffering from anemia, but it can also be used to cheat in sports. Tools called capillary electrophoresis and mass spectrometry have been used to study erythropoietin and the attached sugars to better develop the techniques used to study proteins and any attached sugars.
Preface

This dissertation presents and discusses the experimental and simulated data for the analysis of erythropoietin using capillary electrophoresis and mass spectrometry as implemented in Dr. Chen’s research laboratory at The University of British Columbia. The experiments, data analysis, interpretation, and writing were performed by the author of this thesis. Sections of chapters 1 and 2 are based on material that was published previously, and they are included in this thesis with revision.

Contributions from other researchers:

Chapter 1: Sections in the introduction are based on work that was presented in a book chapter that was co-written by me and Caitlyn De Jong. All other material presented, including all measured data and analyses, were carried out entirely by the author of this thesis. Dr. D.D.Y. Chen directed the work and co-authored the journal articles cited below.

Publications arising from work presented in this dissertation:

A version of section 1.6 of chapter 1 has been published. Risley, J.M., De Jong, C.A.G., and Chen, D.D.Y. (2016) Electrospray Ionization Interface Development for Capillary Electrophoresis–Mass Spectrometry in Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications. Ed: de Jong, G. Wiley-VCH Verlag GmbH & Co. KGaA. pp 7-39. I wrote section 2.3 and put together Table 2.1. Caitlyn wrote section 2.2. We wrote the rest of the book chapter collaboratively (i.e. the other sections have been written by both authors).

A version of section 2.3.2 of chapter 2 has been published. Risley, J.M., and Chen, D.D.Y. (2017) Improved sensitivity by post-column chemical environment modification of CE-ESI-MS using a flow-through microvial interface. Electrophoresis. 38(12):1644-1648. I conducted all of the testing and wrote all of the manuscript.
Ethics approval for use of human serum:

Ethics approval granted by the UBC Clinical Research Ethics Board, certificate number H16-01910.
# Table of Contents

Abstract ........................................................................................................................................ iii  
Lay Summary .................................................................................................................................. v  
Preface .............................................................................................................................................. vi  
Table of Contents ................................................................................................................................. viii  
List of Tables ........................................................................................................................................ xiv  
List of Figures ......................................................................................................................................... xvi  
List of Abbreviations ............................................................................................................................ xx  
Acknowledgements ................................................................................................................................. xxii  
Dedication .............................................................................................................................................. xxiii  

## Chapter 1: Introduction to Glycosylation and the Analysis of the Therapeutic Glycoprotein Erythropoietin

1.1 Glycan Structure and Characteristics ......................................................................................... 1  
1.2 Therapeutic Glycoproteins ........................................................................................................... 3  
1.3 Erythropoietin ............................................................................................................................... 4  
1.4 Established Methods for the Analysis of Erythropoietin .............................................................. 6  
  1.4.1 Differences in EPO Glycans ...................................................................................................... 6  
  1.4.2 The World Anti-Doping Agency (WADA) ............................................................................... 6  
  1.4.3 Other Methods of EPO Analysis ............................................................................................. 7  
1.5 Capillary Electrophoresis: Aqueous Charged Species Separation in an Open Tube ................. 8  
  1.5.1 Instrumentation ......................................................................................................................... 9  
  1.5.2 Principles of Separation .......................................................................................................... 10
1.5.2.1 Electrophoretic Mobility ........................................................................................................ 10
1.5.2.2 Electroosmotic Flow ............................................................................................................. 12
1.5.3 Capillary Inner Wall Coating .................................................................................................. 14
1.6 The Interface Between Capillary Electrophoresis and Mass Spectrometry .................. 15
1.6.1 Electrospray Ionization ........................................................................................................ 15
1.6.1.1 Electrochemical Reactions at the ESI Interface ............................................................... 17
1.6.1.2 Considerations for Interfacing CE and MS ................................................................... 18
1.6.2 Sheath-liquid CE-ESI-MS Interface Design ....................................................................... 19
1.6.3 Sheathless CE-ESI-MS Interface Design ............................................................................ 20
1.6.4 The Flow-Through Microvial ESI Interface ....................................................................... 22
1.7 Mass Spectrometry: Gaseous Charged Species Separation ........................................... 24
1.7.1 Quadrupole Mass Analyzer ................................................................................................... 24
1.7.2 Linear Ion Trap Mass Analyzer ............................................................................................ 25
1.8 Objectives .................................................................................................................................. 26
1.8.1 Method Development for Analyzing Sialic Acids ............................................................... 26
1.8.2 Capillary Isoelectric Focusing for Discrimination between Recombinant and
Endogenous Human Erythropoietin ............................................................................................. 26
1.8.3 Desalting Glycoproteins and Separating N-linked Glycans using the Electro-Fluid-
Dynamic (EFD) Microfluidic Device ............................................................................................. 27

**Chapter 2: Improved Sensitivity for Sialic Acid Detection Using a Flow-Through Microvial
ESI Interface for CE-MS** ............................................................................................................ 28

2.1 Introduction .............................................................................................................................. 28
2.2 Materials and Methods ........................................................................................................... 32
2.2.1 Materials .................................................................................................................. 32
2.2.2 Solution Preparation ............................................................................................... 32
2.2.3 Separation Capillary Preparation ......................................................................... 33
2.2.4 Small Volume Injection Vial Preparation ............................................................ 34
2.2.5 Equipment and Instrumentation .......................................................................... 35
2.3 Results and Discussion ............................................................................................ 36
  2.3.1 Neu5Gc LOD and LOQ Determination ............................................................... 36
  2.3.2 Post-Column Environment Modification ......................................................... 38
  2.3.3 Refinement of BGE and Modifier Solution pH Conditions for Separation and Sensitivity ................................................................. 44
  2.3.4 Small Volume Sample Injections ..................................................................... 46
2.4 Concluding Remarks ............................................................................................... 49

Chapter 3: Separation of Exogenous and Endogenous Human Erythropoietin using Capillary Isoelectric Focusing with UV Absorption and Laser Induced Fluorescence ........50

3.1 Introduction ................................................................................................................ 50
3.2 Materials and Methods ........................................................................................... 53
  3.2.1 Materials ............................................................................................................. 53
  3.2.2 Solution Preparation .......................................................................................... 54
  3.2.3 HPC-Coated Separation Capillary Preparation ............................................... 55
  3.2.4 Sample Preparation .......................................................................................... 56
  3.2.5 EPO Extraction and Desalting ......................................................................... 57
  3.2.6 Ampholyte Photobleaching for cIEF-LIF ......................................................... 58
  3.2.7 Protein Labelling with Chromeo P503 Labelling Reagent .................................. 58
3.2.8 Instrumentation and Instrumental Analysis .......................................................... 58
  3.2.8.1 CZE-UV for Desalted EPO Detection ......................................................... 58
  3.2.8.2 cIEF-UV .................................................................................................... 59
  3.2.8.3 cIEF-LIF .................................................................................................... 59

3.3 Results and Discussion .......................................................................................... 60
  3.3.1 Optimization of Voltage, Pressure, and Time for cIEF Focusing and Mobilization
    Steps .................................................................................................................... 60
  3.3.2 Comparison of HPC and PVA Coated Capillaries ............................................ 62
  3.3.3 Sample Solution Optimization for Glycoprotein Separation ......................... 63
    3.3.3.1 Glycerol Content of Sample and Solutions ............................................ 64
    3.3.3.2 Urea Concentration of Sample ................................................................. 65
    3.3.3.3 Ampholyte Content of Sample ................................................................. 67
    3.3.3.4 Comparison of Broad and Narrow Range Ampholytes ............................ 68
    3.3.3.5 Comparisons of Ratios of Complex Ampholyte Mixtures ....................... 71
    3.3.3.6 Ampholyte Photobleaching for Reduced Background Signal using LIF
        Detection ......................................................................................................... 74
  3.3.4 Sensitivity and Glycoform Resolution Determination of Developed cIEF-LIF
    Method using Ovalbumin ..................................................................................... 76
  3.3.5 Detection of rhEPO and hEPO by cIEF-LIF .................................................... 77
    3.3.5.1 Detection of Desalted EPO ................................................................. 77
    3.3.5.2 cIEF-LIF Profiles of rhEPO and Serum hEPO ....................................... 78
    3.3.5.3 Determination of Isoelectric Points of Recombinant Human EPO Glycoforms
        by cIEF-LIF .................................................................................................... 81
Chapter 4: Theoretical Glycoprotein Desalting and N-Linked Glycan Purification Using the Electro-Fluid-Dynamic Microfluidic Device

4.1 Introduction

4.2 Materials and Methods
  4.2.1 Instrumentation and Software
  4.2.2 Materials
  4.2.3 Simulation Parameters and Models
  4.2.4 Chip Design and Fabrication
  4.2.5 Chip Testing

4.3 Results and Discussion
  4.3.1 EFD Chip Design and Fabrication
  4.3.2 First Generation Chip Chemical Tests
  4.3.3 Theoretical Modelling of Glycoprotein Desalting by EFD Device
  4.3.4 Collection Channel Dimension Manipulation Simulations
    4.3.4.1 Manipulation of Channel Width Simulations
    4.3.4.2 Manipulation of Channel Length Simulations
    4.3.4.3 Theoretical Modelling of Analyte Selectivity
    4.3.4.4 Theoretical Modelling of the Effects of Multiple Collection Channels on Electric Field Strength and Flow Velocity

4.4 Concluding Remarks

Chapter 5: Conclusion and Future Work

5.1 Concluding Remarks
5.2 Future Research ....................................................................................................................... 109

5.2.1 Developing Internal Standards to Quantify Free Sialic Acids using CE-MS ........ 109

5.2.2 Developing a cIEF-ESI-MS Method for Human Serum EPO Detection and
Quantification ............................................................................................................................. 110

5.2.3 Desalting Extracted EPO on the EFD Device ................................................................. 110

5.2.4 Comparing the Glycobiograms of Recombinant Human EPO Produced in Human
Embryonic Kidney Cells against Endogenous Human EPO ................................................. 110

Bibliography .............................................................................................................................. 112

Appendices ................................................................................................................................ 136

Appendix A .................................................................................................................................. 136
Appendix B .................................................................................................................................. 137
List of Tables

Table 2.1: Conditioning and application of HPC to bare fused silica capillary for neutral coating ................................................................. 34
Table 2.2: Optimized conditions for SRM of Neu5Ac and Neu5Gc ................................................................. 36
Table 2.3: Neu5Gc standards analyzed using CE-MS ................................................................................. 37
Table 2.4: Comparison of migration times, peak areas, peak heights, and peak widths of Neu5Ac, Neu5Gc, and SiaLac under different BGE pH conditions ......................................................... 45
Table 2.5: Comparison of migration times, peak areas, peak heights, and peak widths of Neu5Ac, Neu5Gc, and SiaLac under different modifier pH conditions ................................................. 46
Table 2.6: Results for injections from sample volumes of 1.0 µL with and without mineral oil . 48
Table 3.1: Conditioning and application of HPC to bare fused silica capillary for neutral coating ................................................................................................. 56
Table 3.2: Typical cIEF method used in EPO method development tests........................................... 59
Table 3.3: Comparison of PVA and HPC capillaries for sensitive and well-resolved cIEF-UV of proteins and glycoproteins ........................................................................................................ 62
Table 3.4: Comparison of resolution and glycerol content for cIEF-UV of proteins and glycoproteins ............................................................................................................... 65
Table 3.5: Comparison of urea concentration for sensitive and well-resolved cIEF-UV of proteins and glycoproteins ........................................................................................................ 66
Table 3.6: Comparison of ampholyte content for sensitive and well-resolved cIEF-UV of proteins and glycoproteins ........................................................................................................ 68
Table 3.7: Comparison of ampholyte pH range for sensitive and well-resolved cIEF-UV of proteins and glycoproteins

Table 3.8: Comparison of sensitivity and resolution for analyses of glycoproteins using different ampholyte ratios in cIEF-UV

Table 4.1: COMSOL Multiphysics simulation parameters used for the geometry and models
List of Figures

Figure 1.1: The three types of N-linked glycans and the four common cores of O-linked glycans. Republished with permission of Future Medicine Ltd., from Bacterial glycosidases in pathogenesis and glycoengineering, Sjögren, Jonathan; Collin, Mattias, 9(9), 2014; permission conveyed through Copyright Clearance Center, Inc. ................................. 2

Figure 1.2: Diagram of capillary electrophoresis instrument ................................................................. 10

Figure 1.3: Schematic of the flow-through microvial interface used in this thesis. ............................... 23

Figure 2.1: Calibration curve of Neu5Gc .................................................................................................. 37

Figure 2.2: Schematic of the flow-through microvial interface ............................................................. 40

Figure 2.3: Calibration curves of peak height are shown for Neu5Ac analysis using acidic or basic modifier conditions .................................................................................................................. 41

Figure 2.4: Extracted ion electropherograms and mass spectra are shown for two additional example analytes. ........................................................................................................................................ 43

Figure 3.1: Electropherograms (left) and current profiles (right) of the optimized conditions of cIEF-UV ........................................................................................................................................ 62

Figure 3.2: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 10%, (B) 15%, and (C) 20% glycerol in the sample, anolyte, catholyte, and mobilizer. Traces offset for clarity ........................................................................................................................................ 64

Figure 3.3: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 4 M, (B) 5 M, and (C) 6 M urea in the sample. Traces offset for clarity. ............................ 66

Figure 3.4: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 0.5%, ........................................................................................................................................ 67
Figure 3.5: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) broad range (pH 3-10), (B) a 1:1 mix of broad and narrow range, (C) a 1:2 mix of broad and narrow range, (D) a 2:1 mix of broad and narrow range, and (E) narrow range (pH 2.5-5) ampholytes in the sample. Traces offset for clarity. ................................................................. 69
Figure 3.6: cIEF-UV electropherograms of beta-lactoglobulin A and ovalbumin using (1) a range of different ampholyte ratios with three highlighted analyses of pH range 3-10:2.5-5:4-6.5:5-8 in ratios of (A) 0:0:1:1 (B) 1:0:1:0 and (C) 1:0:1:1. The three highlighted analyses are enlarged in (2). Traces offset for clarity. ........................................................................................................ 72
Figure 3.7: cIEF-UV electropherograms of beta-lactoglobulin A and ovalbumin samples containing (A) 1%, (B) 2%, (C) 3%, and (D) 4% ampholytes. Traces offset for clarity......... 74
Figure 3.8: cIEF-LIF electropherogram of baseline signal from 15 – 25 min (after focusing but before protein detection) for (A) no photobleaching, (B) 1 hour of photobleaching, (C) 2 hours of photobleaching, (D) 3 hours of photobleaching, (E) 4 hours of photobleaching, and (F) 5 hours of photobleaching. Traces offset for clarity. ........................................................................................................ 75
Figure 3.9: CZE-UV electropherogram of seven glycoforms of desalted BRP EPO. BGE used contains putrescine and CE conditions were described in section 3.2.8.1................................. 78
Figure 3.10: cIEF-LIF electropherogram of recombinant EPO with each glycoform numbered. 79
Figure 3.11: cIEF-LIF electropherogram of EPO extracted from pooled human serum.......... 79
Figure 3.12: cIEF-LIF electropherogram of (A) EPO extracted from pooled human serum, (B) beta-lactoglobulin A, (C) physicochemical test recombinant EPO, and (D) a pI 3.6 marker peptide. ............................................................................................................................ 80
Figure 3.13: Calibration curve of isoelectric point using pI markers. ........................................ 81
Figure 3.14: Electropherogram of recombinant EPO with the pI of each glycoform labelled. .... 82
Figure 4.1: Schematic of a multi-branched EFD device with a sample inlet, I, at the top of the main channel, pressure P applied at the bottom of the main channel, and analyte outlets, O, at the ends of the collection channels. A positive potential is applied to the sample inlet and each collection outlet, while another side channel is grounded. Adapted with permission from Anal. Chem. 2014, 86, 22, 11380–11386. Copyright (2014) American Chemical Society

Figure 4.2: EFD microfluidic masks with collection channels of (A) the same widths (100 µm) and lengths (14.14 mm), (B) different widths (100 and 200 µm), and (C) different lengths (4.95 mm, 10.60 mm, and 19.80 mm).

Figure 4.3: Fabricated EFD chips with collection channels of (A) the same widths and lengths, (B) different widths, and (C) different lengths.

Figure 4.4: A phenol red dye colour change of red to yellow as electrolysis occurs at high applied potential (2000 V) across the chip, and the pH of the BGE changes.

Figure 4.5: COMSOL simulation, with an applied potential of 1000 V and an applied flow rate of 9x10^{-12} m^3/s, showing (A) the collection of a negatively charged analyte (electrophoretic mobility: 9x10^{-13} s · mol/kg) at the sample inlet, (B) the collection of a positively charged analyte (electrophoretic mobility: 5x10^{-14} s · mol/kg) in the first collection channel, (C) no collection of a second positively charged analyte (electrophoretic mobility: 9x10^{-13} s · mol/kg) at first collection channel, and (D) the migration of the second positively charged analyte in the second collection channel and also down towards ground.

Figure 4.6: Plot of the relative electric field in the collection channel (EFCC) over the electric field in the main channel (EFMC) against collection channel width (left y axis) for an applied potential of 1000 V (at both the sample inlet and collection channel outlet), and a plot of the relative flow velocity in the collection channel (vf,CC) over the flow velocity in the main
channel (vf,MC) against collection channel width (right y axis) for an applied flow rate of 9x10^{12} m^3/s. Collection channel width varied from 10 to 500 μm in steps of 10 μm, while the main channel width remained 100 μm.

Figure 4.7: Plot of the relative electric field in the collection channel (EFCC) over the electric field in the main channel (EFMC) against collection channel length (left y axis) for an applied potential of 1000 V (at both the sample inlet and collection channel outlet), and a plot of the relative flow velocity in the collection channel (vf,CC) over the flow velocity in the main channel (vf,MC) against collection channel length (right y axis) for an applied flow rate of 9x10^{12} m^3/s. Collection channel length varied from 3000 to 30,000 μm in steps of 1000 μm, while the main channel length from sample inlet to collection channel junction remained 14000 μm.

Figure 4.8: Coloured contour plots showing (A) the concentration in the collection channel of an analyte (electrophoretic mobility: 5x10^{-14} s · mol/kg) as applied potential ranges from 200 to 1000 V in steps of 100 V, and applied flow rate ranges from 5x10^{-13} to 1x10^{-12} m^3/s in steps of 5x10^{-14} m^3/s, (B) the difference plot of the concentration of the first analyte (electrophoretic mobility: 5x10^{-14} s · mol/kg) and a second analyte (electrophoretic mobility: 5.25x10^{-14} s · mol/kg) over the same applied potential and flow rate ranges, (C) the difference plot of the concentration of the first analyte and a second analyte (electrophoretic mobility: 6x10^{-14} s · mol/kg) over the same applied potential and flow rate ranges, and (D) the difference plot of the concentration of the first analyte and a second analyte (electrophoretic mobility: 1x10^{-13} s · mol/kg) over the same applied potential and flow rate ranges.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>BRP</td>
<td>Biological Reference Preparation</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>cFFE</td>
<td>Continuous free flow electrophoresis</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>cIEF</td>
<td>Capillary isoelectric focusing</td>
</tr>
<tr>
<td>CQAs</td>
<td>Critical quality attributes</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>EDL</td>
<td>Electric double layer</td>
</tr>
<tr>
<td>EFD</td>
<td>Electro-Fluid-Dynamic</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FCCE</td>
<td>Flow counterbalanced capillary electrophoresis</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width half maximum</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>hEPO</td>
<td>Human erythropoietin</td>
</tr>
<tr>
<td>HPC</td>
<td>Hydroxypropylcellulose</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICH</td>
<td>International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser induced fluorescence</td>
</tr>
<tr>
<td>LIT</td>
<td>Linear ion trap</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>N-glycolylneuraminic acid</td>
</tr>
<tr>
<td>OHP</td>
<td>Outer Helmholtz Plane</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>rhEPO</td>
<td>Recombinant human erythropoietin</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>SAR-PAGE</td>
<td>Sodium N-lauroylsarcosinate (sarcosyl) polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SiaLac</td>
<td>Sialyllactose</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>SRM</td>
<td>Single reaction monitoring</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
</tr>
</tbody>
</table>
Acknowledgements

First, I would like to thank Dr. David Chen, my doctoral degree supervisor, for his support and kindness throughout my degree.

My fellow research group members have helped me to grow and develop my skills and ideas throughout my degrees. They have provided understanding and support through both the fun and difficult times that we experience in academia. My particular thanks go to Caitlyn and she knows why.

I would also like to thank Dr. Kingsley Donkor, my undergraduate research supervisor, and Evangeline Lu, my best friend, for encouraging my interest in research and giving me my beginnings in science.

The funding for my research projects came from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Partnership for Clean Competition, and for that, I would like to thank those organizations.

My love and appreciation goes to my husband, Dr. Nicholas McGregor and our cat, James, as well as my dad, Charley, my mom, Karen, and my sister, Deanna. Without them and their support, I would not be where I am today.
To my family
Chapter 1: Introduction to Glycosylation and the Analysis of the Therapeutic Glycoprotein Erythropoietin

1.1 Glycan Structure and Characteristics

Within the context of this thesis, glycans are the complex branched oligosaccharides which are covalently linked to protein molecules. Composed of different monosaccharide units of varying atomic composition and stereochemistry, glycan structures differ from one another in their monosaccharide composition, sequence, and in the many, often branching, ways by which the sequence of monosaccharides are linked together.\(^1\) The range of variations naturally found within glycans gives rise to microheterogeneity within an isolated sample of a glycosylated polypeptide, or “glycoprotein”. Each of the components of this microheterogeneous mixture which differ only in their glycosylation is termed a “glycoform”.

Glycoproteins are polypeptides with one or more glycans covalently attached, usually via “N-linkages” or “O-linkages”.\(^2\)\(^-\)\(^4\) This division between classes of glycans is defined by the amino acid residue to which the glycan is attached and, by extension, the type of bond between the glycan and the amino acid. O-linked glycans are covalently linked to proteins at a serine or a threonine residue through a bond between the reducing end of the glycan and the primary alcohol of the amino acid side chain. N-linked glycans (or N-glycans), which are found on approximately 90% of glycoproteins\(^5\) and are the focus of this thesis, are attached to the primary amide of asparagine amino acid side chains found in the sequence Asn-X-Ser/Thr (where X is any amino acid except proline) via covalent N-glycosidic bonds.\(^2\)\(^,\)\(^3\) It should be noted that not all Asn-X-Ser/Thr amino acid sequences bear N-glycans and it is not trivial to predict which sites will bear glycans.
The three types of N-glycans (see Fig. 1.1) found in animal cells share a common core composed of five monosaccharide units: two N-acetylglucosamine residues and three mannose residues. The three types of N-glycans found in animal cells are called “oligomannose”, “complex”, and “hybrid” (see Fig. 1.1). Oligomannose (or high-mannose) N-glycans contain only complex branching patterns of mannose residues beyond the common core. Complex N-glycans are characterized by branches, or “antennae”, that extend from the common core and contain N-acetylglucosamine and galactose residues. These are often capped with a sialic acid residue. Hybrid N-glycans have some mixed oligomannose branching and complex branching.

Figure 1.1: The three types of N-linked glycans and the four common cores of O-linked glycans. Republished with permission of Future Medicine Ltd., from Bacterial glycosidases in pathogenesis and glycoengineering, Sjögren, Jonathan; Collin, Mattias, 9(9), 2014; permission conveyed through Copyright Clearance Center, Inc.

N-glycans are an essential part of many therapeutic glycoproteins, carrying out a variety
of biological functions. One key role played by N-glycans is promotion of proper protein folding. Improperly folded proteins are unable to function correctly and are likely to aggregate which can result in the destruction of the misfolded protein or, in some cases, the development of more serious detrimental effects. N-linked glycans also act as signals or tags on the surface of a protein dictating the fate of the glycoprotein within the cell. N-glycans are also receptors for the recognition and binding of glycoproteins. The presence of N-glycans on glycoproteins can help to stabilize the glycoprotein and allow it to remain active in vivo for longer periods of time. Given the wide range of impacts that glycosylation has, defects in glycosylation can cause disease, and therapeutic glycoproteins can be powerful tools used to treat disease.

1.2 Therapeutic Glycoproteins

Many diseases and ailments can be treated using therapeutic glycoproteins driving interest in the development of tools for the large-scale production of such glycoproteins. Therapeutic glycoproteins are a class of biopharmaceuticals which have been produced using recombinant DNA technology in animal cell lines. While the sequence of a protein is encoded in DNA, glycosylation is not. Glycosylation is dictated by many factors, including the cell type in which the glycoprotein is produced, the availability of different enzymes in those cells, and the culture or growth conditions.

To ensure the quality, including the safety and efficacy, of therapeutic glycoproteins, critical quality attributes (CQAs) are used to assess and regulate therapeutics. A CQA is defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) as a ‘physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired
product quality’. Because of the impact of glycans on the function and safety of therapeutic glycoproteins, the glycan component of a therapeutic glycoprotein is a CQA. By characterizing and monitoring the glycans of a glycoprotein, batch-to-batch variation can be tracked and minimized, and the half-life, activity, stability, and immunogenicity of the therapeutic glycoprotein can be maintained and even improved.

Glycans analysis generally aims to determine the glycosylation site on the protein, the type of glycosylation, and the glycan structure and composition. Depending on the information required, different analytical techniques can be used to characterize the glycan component of glycoproteins. Glycans can be analyzed as part of an intact glycoprotein, as a fragmented glycopeptide, as an intact glycan that has been released (typically enzymatically) from its glycoprotein, or as monosaccharides. Techniques for glycan analysis include nuclear magnetic resonance and various types of liquid chromatography (reverse-phase, hydrophilic, high-performance anion-exchange) or capillary electrophoresis (CE; capillary zone electrophoresis, capillary isoelectric focusing) coupled with many different detection techniques (fluorescence, UV absorption, pulsed amperometric detection, mass spectrometry (MS)).

1.3 Erythropoietin

Composed of 165 amino acid residues, erythropoietin (EPO) is a small protein with a molecular weight of approximately 30 kDa. While 60% of the molecular weight of EPO is attributed to the protein component of the macromolecule, the other 40% of EPO is composed of glycans. One of the glycans of EPO is an O-linked glycan and three others are N-linked glycans. The glycans found on EPO are an essential part of the biologically active molecule. Links have been found between the N-linked glycosylation of EPO and its proper synthesis and biological
activity.\textsuperscript{21} N-linked glycans have also been shown to increase the structural stability of the protein.\textsuperscript{22}

EPO is a glycoprotein hormone that stimulates erythropoiesis, the production of red blood cells.\textsuperscript{23} In 1906, Carnot and Deflandre observed an increase in red blood cells in healthy rabbits following a blood infusion from anemic rabbits, leading the researchers to hypothesize the existence of a factor found in blood that regulates erythropoiesis.\textsuperscript{24} The presence of such a factor was later confirmed by both Krumdieck\textsuperscript{25} and Erslev\textsuperscript{26}. EPO is typically produced in the kidneys of adults, although a small amount can still be produced in the liver which is the site of production prior to birth.\textsuperscript{19} EPO is primarily produced during tissue hypoxia, when the oxygen levels of tissues are reduced,\textsuperscript{27} and continues to circulate through the body in the blood at low levels.\textsuperscript{28} The role of EPO in the stimulation of red blood cell production makes it an important pharmacological tool in the treatment of certain blood disorders.

EPO was first used to treat anemia in 1987\textsuperscript{29}, and is now commonly prescribed for the treatment of anemia caused by chronic kidney disease.\textsuperscript{30} This prescription EPO is generally produced recombinantly in animal cell lines, making it affordable, safe, and readily available. However, due to its ability to stimulate red blood cell production, the ready availability of recombinant EPO presents an opportunity to be misused by athletes as a performance-enhancing drug. By increasing the red blood cell count in the athlete’s blood, the oxygen-carrying capacity of the athlete is increased, allowing for greater aerobic endurance.

A variety of strategies used by athletes to avoid detection by the current testing routine have been documented. Athletes can follow particular EPO treatment schedules, such as microdosing (sometimes called microdoping).\textsuperscript{31,32} Microdosing is used to boost athlete
performance using sustained injections of tiny quantities of the recombinant EPO which are very
difficult to detect. EPO is also difficult to detect due to low levels of endogenous EPO found in
human blood serum or urine, the high level of similarities between endogenous and recombinant
exogenous EPO,\textsuperscript{30,33} and the short half-life of EPO in the bloodstream.\textsuperscript{33}

1.4 Established Methods for the Analysis of Erythropoietin

1.4.1 Differences in EPO Glycans

EPO, as a therapeutic glycoprotein, is produced recombinantly in mammalian cell lines. The
most well-known cell line used to produce therapeutic glycoproteins is Chinese Hamster
Ovary (CHO) cells.\textsuperscript{34,35} The recombinant glycoprotein produced contains the exact same peptide
sequence as the endogenous EPO glycoprotein. However, due to the use of a cell line that isn’t
human, the post-translational modifications, especially glycosylation, differ slightly between
recombinant human EPO (rhEPO) and endogenous human EPO (hEPO). In this thesis the
differences in the N-linked glycans of rhEPO are studied. Some known differences between the
N-linked glycans of rhEPO and hEPO tend to be the number of "antennae" attached to the core
glycan structure, the presence of core fucosylation, the length of N-acetyl-lactosamine repeats,
and, most significantly, the identity of the sialic acid at the terminus of each antenna.\textsuperscript{35,36}

1.4.2 The World Anti-Doping Agency (WADA)

Direct detection techniques have been developed to differentiate between EPO from
different sources based on differences in glycosylation patterns. Currently, the analysis methods
accepted by the World Anti-Doping Agency (WADA) are based on separation using
polyacrylamide gels and focus on differences in molecular weight or isoelectric point (pI) caused
by differences in glycosylation.\textsuperscript{37} While effective, the need for absolute certainty imposes hard
limitations on the WADA methods for rhEPO detection. Recombinant EPO testing methods are currently permitted to be isoelectric focusing (IEF), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), or sodium N-lauroylsarcosinate (sarcosyl) polyacrylamide gel electrophoresis (SAR-PAGE), and must be preceded by immunopurification and followed by immunoblotting for detection.\textsuperscript{37} Both IEF and SDS-PAGE procedures are time-consuming and require large volumes of sample (20 mL of urine or 2 mL of serum/plasma) to extract sufficient EPO for detection.\textsuperscript{38,39} The addition of immunoblotting prior to detection only adds cost and time. Current analyses can take up to 3 days to complete.

1.4.3 Other Methods of EPO Analysis

As one of the first therapeutic glycoproteins approved for use in humans, EPO is well-studied. Methods have been developed not only to detect EPO in complex biological matrices as part of anti-doping measures or diagnostics, but also to characterize EPO to assess its quality as a therapeutic glycoprotein.

Indirect methods have been developed for the detection of EPO injected more than a week prior to analysis.\textsuperscript{35,40} Indirect methods analyze secondary blood markers, such as the haematocrit level (which measures the ratio of the volume of red blood cells to the volume of whole blood) and the soluble transferring receptor (which reflects the level of red blood cells present in blood), but are not often used due to lack of sensitivity and specificity. The direct measurement of EPO in biological matrices is typically accomplished using IEF or SDS-PAGE due to the ability to differentiate between the endogenous and exogenous EPO and obtain sufficient sensitivity for EPO levels. To characterize EPO, many diverse methods have been developed, including lectin-blotting,\textsuperscript{41} liquid chromatography coupled to mass spectrometry (LC-MS),\textsuperscript{42–44} capillary
electrophoresis (CE) with native fluorescence detection,\textsuperscript{45} capillary zwitterionic-type hydrophilic interaction chromatograph coupled to mass spectrometry (MS),\textsuperscript{46} Raman spectroscopy,\textsuperscript{47} and high-performance anion-exchange chromatography with native fluorescence detection\textsuperscript{48} or with pulsed amperometric detection.\textsuperscript{49,50}

Techniques such as liquid chromatography that separate analytes based on hydrophobicity can struggle to separate glycans or glycoforms of EPO as the hydrophobicity differences between them are subtle.\textsuperscript{51} The high separation efficiency of capillary electrophoresis and the sensitivity of mass spectrometry means that CE-MS has been used to characterize and detect EPO to great effect.\textsuperscript{51–62} Mass spectrometry has been used to detect recombinant EPO glycoforms at a level of 0.08 µg/µL,\textsuperscript{63} which is in the range of the 0.01-0.2 mg/mL detection of intact recombinant EPO found using fluorescence detection.\textsuperscript{48} Because CE-MS is amenable to the analysis of large biomolecules, it is rapidly becoming a widely used technique to characterize glycoproteins and glycans.

1.5 Capillary Electrophoresis: Aqueous Charged Species Separation in an Open Tube

Capillary electrophoresis (CE) is a powerful technique that uses an applied voltage, generating a uniform electric field along the length of a narrow capillary, to separate analytes in a small volume, open-flow system. Historically, CE was first investigated in 1967 by Hjertén who conducted free zone electrophoresis experiments in narrow-bore (1 – 3 mm diameter) linear tubes.\textsuperscript{64} Developments were made in tube material composition and diameter before Jorgenson and Lukacs performed electrophoresis in a 75 µm capillary in 1981,\textsuperscript{65} helping to establish CE as a modern analytical technique.
1.5.1 Instrumentation

As shown in Figure 1.2, the CE instrument is composed of a narrow-bore capillary (typically 25 – 100 µm diameter) that connects two reservoirs that are each filled with an electrolyte solution. The capillary itself is usually composed of fused silica and is coated in polyimide to add flexibility and strength to the fragile capillary. Depending on the desire for separation speed versus resolution, the capillary length can vary from 10 to 100 cm. Near the outlet end of the capillary, a small section of the polyimide coating is removed to form an optically transparent window for detection. Inserted into the two reservoirs are chemically-inert electrodes, typically composed of high purity platinum, which allow for electrical contact between a power supply and the capillary filled with an electrolyte solution. The power supply applies a high voltage across the capillary which generates a uniform electric field along the length of the capillary and powers the separation of analytes. While traditional CE is performed in a benchtop box instrument, the process can also be performed on a smaller scale in the form of a microfluidic device. The principles of separation governing CE apply regardless of whether separations are performed in a capillary or on a microfluidic device.
1.5.2 Principles of Separation

1.5.2.1 Electrophoretic Mobility

The separation of analytes in CE is caused by the differential migration of charged analytes in the presence of a uniform electric field. Analytes are separated based on their charge-to-size ratio as they migrate through the capillary at a rate defined by their electrophoretic mobility.

Electrophoretic mobility is approximated using the Debye-Hückel-Henry equation:

\[ \mu_{ep} = \frac{\vec{v}_{ep}}{E} = \frac{q}{6\pi \eta r} \]

where \( \mu_{ep} \) is the electrophoretic mobility of the analyte, \( \vec{v}_{ep} \) is the electrophoretic velocity of the analyte, \( E \) is the magnitude of the electric field, \( q \) is the net charge on the analyte, \( \eta \) is the viscosity of the medium, and \( r \) is the radius of the hydrated analyte.

The Debye-Hückel-Henry equation can be derived from the acceleration of the charged analyte species in solution under an electric field, driven by electrostatic forces, \( \vec{F}_e \). The
electrostatic forces are proportional to the net charge on the analyte, $q$, and the magnitude of the electric field vector, $\vec{E}$.

\begin{equation}
(1-2) \quad \vec{F}_e = q\vec{E}
\end{equation}

The electrostatic forces are balanced by the drag forces, $F_D$, experienced by the charged analyte as it moves in solution. The drag forces are proportional to Stoke’s friction, determined as the product of the effective radius of the hydrated analyte, $r$, the electrophoretic velocity of the analyte, $\vec{v}_{ep}$, and the viscosity of the medium, $\eta$.\textsuperscript{67,68}

\begin{equation}
(1-3) \quad \vec{F}_D = 6\pi\eta r \cdot \vec{v}_{ep}
\end{equation}

\begin{equation}
(1-4) \quad q\vec{E} = 6\pi\eta r \cdot \vec{v}_{ep}
\end{equation}

\begin{equation}
(1-5) \quad \vec{v}_{ep} = \frac{q\vec{E}}{6\pi\eta r}
\end{equation}

\begin{equation}
(1-6) \quad \mu_{ep} = \frac{\vec{v}_{ep}}{\vec{E}} = \frac{q}{6\pi\eta r}
\end{equation}

In uniform solution conditions and electric field, the electrostatic forces and drag forces equilibrate, becoming equal and opposite. As a result, the charged analyte migrates at a constant electrophoretic velocity.

The direction of analyte migration is defined by its charge. Cationic analytes migrate towards the negatively charged cathode (outlet) of the CE and anionic analytes move towards the positively charged anode (inlet) of the CE, and, by convention, away from the detector. As can be taken from equation (1-5) above, neutrally charged analytes do not migrate under an electric field at all. However, using CE, all analytes, regardless of charge, can be detected due to a second force
that acts equally on all components of the solution in the capillary. This second force is called electroosmotic flow (EOF), and is a form electrically-driven mass transport.

1.5.2.2 Electroosmotic Flow

EOF arises in the capillary upon the application of the electric field due to the nature and treatment of the capillary wall. The inner wall of the capillary is composed of silanol groups that, when treated with a basic solution, become deprotonated, causing the inner capillary wall to become negatively charged. Cationic species from the background electrolyte (BGE) in the capillary solution are attracted to the negatively charged capillary wall. Strong electrostatic forces between the oppositely charged wall and BGE cations give rise to a static layer, called the Inner Helmholtz layer or the Stern layer. Still drawn to the incompletely-neutralized negative charge on the capillary wall, more cations from the bulk solution filling the capillary form a diffuse layer on top of the Stern layer. This second layer is called the Outer Helmholtz Plane (OHP). As the distance from the capillary wall increases, the charge density of the cations decreases linearly through the Stern layer and exponentially through the OHP. Together, the Stern layer and the OHP form an electric double layer (EDL). The potential at the interface between the Stern layer and the OHP is called the zeta potential and it is an important variable in the magnitude of the EOF.

When a voltage is applied to the BGE solution filling the capillary, the diffuse layer of cations of the OHP migrate towards the CE cathode/outlet, dragging with them hydrating water molecules. The hydrogen bonding between the hydrating water molecules and the water molecules in the bulk solution create a bulk flow towards the outlet of the capillary. This bulk flow results in the uniquely planar flow profile of CE. This is in contrast to the parabolic flow profile of pressure-
driven separation techniques such as high performance liquid chromatography (HPLC), which results in more dispersive effects and increased band broadening relative to CE.

EOF is typically defined by the Helmholtz-Smoluchowski formula:

\[
(1-7) \quad \vec{\mu}_{eo} = \frac{\vec{v}_{eo}}{E} = \frac{\varepsilon \zeta}{4\pi \eta}
\]

where \( \mu_{eo} \) is the electroosmotic mobility, \( \vec{v}_{eo} \) is the electroosmotic velocity, \( \varepsilon \) is the dielectric constant of the solution filling the capillary, and \( \zeta \) is the zeta potential of the capillary wall.\(^{68,70}\)

The pH of the BGE affects the zeta potential as the charge density of the capillary wall is dependent on the degree of dissociation of the silanol groups. At a pH of greater than 3, the silanol groups of the capillary wall begin to deprotonate, and at a pH greater than 10, all of the silanol groups are fully deprotonated. The ionic strength of the BGE also plays a role in affecting the zeta potential as a high ionic strength results in a greater number of cations in the EDL which causes a more precipitous drop in potential as the distance from the capillary wall increases, reducing the zeta potential and decreasing the magnitude of the EOF.

As EOF is a bulk flow of solution through the capillary towards the cathode (assuming a negative wall charge), it sweeps all components found in the capillary towards the outlet, and more importantly, towards the detector. The apparent mobility of an analyte, \( \mu_{app} \), is the sum of the electrophoretic mobility and the electroosmotic mobility as shown in equation (1-8) below:

\[
(1-8) \quad \mu_{app} = \mu_{ep} + \mu_{eo}
\]
EOF can be manipulated by changing the viscosity, ionic strength, or pH of the BGE solution filling the capillary, and the rate of EOF can be changed by changing the applied voltage. EOF can also be manipulated by modifying the inner surface of the capillary wall.

1.5.3 Capillary Inner Wall Coating

Some charged analytes interact with the charged inner capillary wall, leading to inefficient separations and poor peak shapes.\(^7\) This interaction may be controlled through the manipulation of BGE pH or ionic strength, but these conditions may also destabilize the analyte(s) of interest, especially proteins or glycoproteins.\(^2,73\) A more effective approach is to coat the capillary wall, which creates an opportunity to manipulate the magnitude and direction of the EOF to improve separation.

When selecting an appropriate capillary wall coating, it is important to consider the compatibility of the coating with the method of detection to be used. A capillary wall coating must be stable with respect to both time and BGE or analyte solution characteristics.\(^7\) Ideally, the coating is also introduced easily into the capillary.\(^75\) Should the detector be a mass spectrometer, it is best if the coating does not slowly slough off of the capillary during analysis, as it can be sprayed into the mass spectrometer,\(^73,76\) leading to analyte ion suppression, MS source contamination, and background noise.\(^76,77\) If an optical detector is used, it is important to select a capillary coating that will not absorb at the detection wavelength.\(^75\)

Capillary wall coatings can be dynamic or static. As the name implies, dynamic coatings are not permanently adhered to the capillary wall.\(^7\) Rather, these types of coatings are applied to the capillary via additions to the BGE and are free to adsorb and desorb while the coating-containing BGE solution flows through the capillary. In this way, the dynamic coating competes
with the analyte to interact with the capillary wall. Static coatings, however, permanently modify the capillary wall by bonding to the wall. Because of the more permanent nature of static coatings, they tend to be the coating of choice for CE-MS analysis.

Static coatings can be achieved through either physical adsorption (static-adsorbed) or chemical reaction (static-covalent) and can be positively charged (to generate a reversed EOF) or neutral (to eliminate EOF). In this thesis, two static-covalent coatings were used and both were neutral. Poly(vinyl alcohol), or PVA, and hydroxypropylcellulose (HPC) were used to coat the inner walls of fused silica capillaries. PVA-coated capillaries are made via thermal immobilization of the compound to the capillary wall and stable in aqueous conditions over a broad pH range. HPC-coated capillaries can be readily prepared based on the method developed by Shen and Smith where a solution of HPC is pressure-rinsed through the capillary and immobilized by thermal treatment to induce hydrogen-bonding between the hydroxylpropyl-groups of the HPC and the capillary wall, resulting in a capillary coating that is stable over a broad pH range.

The use of a neutral capillary coating eliminates EOF which can compromise the stability of ESI spray due to a lack of BGE flow. However, CE-MS can be performed without EOF and can be used to extend separations, increasing the resolution between structurally-similar analytes with subtly different electrophoretic mobilities.

1.6 The Interface Between Capillary Electrophoresis and Mass Spectrometry

1.6.1 Electrospray Ionization

Although first observed in the 1960s by Malcolm Dole, electrospray ionization (ESI) became established as an ionization technique in the 1980s after Fenn et al. demonstrated the
use of ESI. Due to the straightforward operational nature of ESI and the adaptability of this technique to ionize a wide range of molecule types, ESI is a commonly used ionization technique when coupling capillary electrophoresis and mass spectrometry.\textsuperscript{87,88} CE and MS were first coupled online in 1987,\textsuperscript{89} taking advantage of the high separation efficiency and the orthogonality of the two techniques.\textsuperscript{76} As a soft ionization technique, ESI results in relatively intact (unfragmented), multiply-charged ions that are very useful in the analysis of biomolecules such as proteins.\textsuperscript{88,90} ESI uses the application of a high voltage over a short distance to generate a high electric field which drives the formation of charged analytes during the transition from the liquid phase to the gas phase.

The process of electrospray ionization can be thought of as three main steps: the production of surface-charged droplets, droplet desolvation during transmission towards the mass spectrometer, and the transition of ions from the solution-phase into the gas-phase.\textsuperscript{91,92} The ESI process begins with the formation of a stable spray. A high voltage (typically 2-5 kV) is applied to a conductive metal tube through which the solution runs, generating an electric field gradient which results in the formation of a Taylor cone.\textsuperscript{93}

The tip of the Taylor cone is the point at which a flow of analyte-containing solution becomes a fine spray composed of micrometer-sized droplets that have an electrically charged surface of the same polarity as the voltage applied to the conductive tube. As the droplets are propelled from the ESI interface towards the mass spectrometer, the solvent of the droplets evaporates and the volume of the droplet decreases, causing the density of the electric charge on the surface of the droplet to increase.\textsuperscript{88} The electric charge density on the droplet surface increases until a critical point, called the Rayleigh stability limit,\textsuperscript{94} is reached and electrostatic repulsion
overcomes the surface tension of each droplet, causing the droplets to break into smaller droplets. The fission of droplets into smaller droplets is accomplished through a process called jet fission, and the process continues until the droplets are nanometer-sized.\textsuperscript{93}

The mechanism behind the final step of the ESI process is somewhat controversial, but has been described by two main models.\textsuperscript{92,93} Dole \textit{et al.}\textsuperscript{84} developed the charge residue model whereby jet fissions occur until the tiny droplets contain only one analyte molecule. When desolvation is complete, the analyte retains the surface charge of the droplet and exists as a free gas-phase ion.\textsuperscript{91} The ion evaporation model, developed by Iribarne and Thompson,\textsuperscript{95} assumes that the when the droplets are small enough, electrostatic repulsion causes analyte ions to be ejected from the small and highly-charged droplets into the gas phase.\textsuperscript{88,90}

\textbf{1.6.1.1 Electrochemical Reactions at the ESI Interface}

The application of a voltage across a system of electrolyte-containing solutions via electrodes causes the inlet and outlet vials of CE to exist as electrochemical half-cells. The major electrochemical reactions occurring are the electrolytic oxidation and reduction of water, as seen in the two half-reactions shown below, resulting in the formation of gas bubbles and causing significant changes in pH.\textsuperscript{91,96}

\begin{align*}
O_2(g) + 4H^+ + 4e^- & \leftrightarrow 2H_2O \\
2H_2O + 2e^- & \leftrightarrow H_2(g) + 2OH^- 
\end{align*}

When CE and MS are coupled, the system contains three electrodes.\textsuperscript{91,96,97} The three electrodes are the CE inlet, the CE outlet which is also the ESI sprayer, and the MS inlet. The two electrical circuits of CE and MS therefore share an electrode (the CE outlet/ESI sprayer), and the
voltage applied to the shared electrode determines whether the electrochemical reactions at that electrode will both be oxidative or reductive, or one oxidative and one reductive.\textsuperscript{96} Regardless, the electrochemical reactions will generate gas bubbles and affect the solution pH. Gas bubbles tend to cause spray instability and degrade the consistency of the baseline during detection, while changes in pH will affect the charge and conformation of analytes.\textsuperscript{91} To overcome the problems associated with electrochemical reactions on CE-ESI-MS analysis, pH buffers or redox buffers are often used as the BGE within a capillary.\textsuperscript{96,97} pH buffers with good buffering capacity can help to reduce changes in pH due to the formation of protons and hydroxide ions within the separation capillary or ESI sprayer, while redox buffers can act as sacrificial molecules that are preferentially oxidized or reduced, reducing the extent of the electrolysis of water.

1.6.1.2 Considerations for Interfacing CE and MS

To successfully couple CE and MS, mismatches in the electrical contact and flow rate requirements of CE and the interface must be overcome. Both CE and ESI require electrical contact between two electrodes to allow current flow, but the need to connect the outlet end of the CE separation capillary to the ESI interface requires that the outlet capillary end be removed from the outlet vial of the CE.\textsuperscript{91,98} The ESI interface then acts as the electrode for both the CE and ESI processes, and the electrochemical reactions described above that occur can affect the ionization of analytes and the stability of the flow between CE and MS. Both CE and MS also require an uninterrupted and steady flow of an electrolyte-containing fluid. The volumetric flow rate (as well as the composition of the fluid) are different (CE flow rates are in the nL/min range whereas MS flow rates tend to be in the µL/min range)\textsuperscript{99} and must be reconciled for a successful and stable connection. The controlled presence of a steady EOF or the elimination of EOF from CE can address some of the mismatch between flow rate requirements, but typically this issue is addressed
through the addition of a sheath-liquid, which will be discussed in the next section. The frequent use of highly concentrated non-volatile components of the BGE can lead to ion suppression in ESI, but the careful selection of MS-friendly BGEs can help to address this problem. The presence of organic solvent in the solution flowing between the CE and the MS also facilitates MS detection by allowing the spray to evaporate more rapidly than a purely aqueous solution. This improves the transition of analytes from liquid to gas phase.

A variety of approaches have been used to overcome the challenges associated with coupling CE and MS, and these have been reviewed thoroughly. There are two main categories of CE-MS interfaces, sheath-liquid and sheathless. The main differences between these two types of interface is the use of a sheath liquid, and the means by which electrical contact is established. Both sheath-liquid and sheathless interfaces can be used to generate either electrospray or nanospray (electrospray at flow rates of nanolitres (nL) per minute). A modified sheath-liquid interface has been used for the work presented in this thesis and will be described in more detail in section 1.5.4. The interface used in this thesis has been optimized to minimize dilution effects common to sheath-liquid interfaces and is more versatile and physically robust than typical sheathless interfaces.

1.6.2 Sheath-liquid CE-ESI-MS Interface Design

As the name indicates, sheath-liquid interfaces use a sheath, or modifier as I will be describing it, liquid that contains an electrolyte to maintain electrical contact and to supplement the volumetric flow rate of the CE effluent to produce steady electrospray. The added benefit of adding a sheath liquid is the ability to modify the CE effluent to make it more compatible with
electrospray. However, the supplemental flow dilutes the CE effluent, reducing the concentration of the analyte in the electrospray source.\textsuperscript{104–106,109}

The most frequently used sheath-liquid interface configuration is the coaxial sheath-flow interface developed by Smith \textit{et al.}\textsuperscript{110} The coaxial design is a triple-tube system where the CE separation capillary is within the larger diameter sheath liquid tube. The sheath liquid is a conductive liquid and forms an electrical contact between the voltage applied to the interface electrode and the CE outlet. The sheath liquid and the CE effluent meet at the end of the CE separation capillary. The third tube of the coaxial system surrounds both the CE separation capillary and the sheath liquid tube, and through this third tube flows the sheath (or nebulizing) gas which facilitates the evaporation of solvent from the electrospray.\textsuperscript{111}

Another example of a sheath-liquid interface is the electrokinetically pumped sheath-liquid nanospray interface developed by the Dovichi group.\textsuperscript{109,112–114} This interface design is composed simply of an insulating cross-junction that connects a sheath liquid reservoir to a separation capillary inside of a borosilicate glass sprayer. EOF is formed inside of the glass sprayer when a voltage is applied to the sheath liquid reservoir which results in stable flow and reduced analyte dilution. Designated as a nanospray interface, Dovichi’s device is both sensitive and reproducible, but the emitter is fragile and the maximum current that the interface can handle is low (less than 10 µA).

\textbf{1.6.3 Sheathless CE-ESI-MS Interface Design}

Sheathless interfaces achieve higher ionization efficiencies and do not tend to have dilution problems but instead must heavily modify the end of the fragile CE separation capillary to establish electrical contact directly with the CE BGE. Various strategies for establishing direct electrical
contact have been designed. Coating the capillary tip with metal or conductive graphite can be straightforward but the metal can deteriorate under the application of high voltages. Attaching a spray tip to the end of the separation capillary allows the separation capillary to be switched more easily but can introduce relatively large dead volumes. Inserting a wire into or near the separation capillary outlet requires no modification of the capillary itself but gas bubbles and pH fluctuations resulting from electrolysis on the wire can disrupt spray stability.

One interesting example of a sheathless interface is the design of a nanospray sheathless interface with a porous tip, introduced by Moini and commercialized Sciex as an integrated CE-ESI-MS interface. The interface from Sciex is called the CESI8000, while the initial design from Beckman Coulter was called the high-sensitivity porous sprayer. By applying a solution of hydrofluoric acid to the outlet end of the separation capillary, the tip is etched to become porous, allowing ions and electrons (but not liquid) to pass through it to establish electrical contact. The ESI needle surrounds part of the porous capillary end while the tip is extruded towards the MS inlet. The ESI needle contains BGE and is the point at which the voltage is applied. The interface is sensitive, and the design ensures that any electrochemical reactions occur apart from the origin of spray formation. However, it is necessary to replace the BGE inside the ESI needle every 3-4 hours to prevent oxidation in the needle.

A similar interface was designed by Wang et al., called the sheathless porous tip nanoESI emitter. Like the CESI8000, this interface has a hydrofluoric acid-etched porous tip, but this tip is separate from the CE separation capillary. The porous capillary tip is inserted into the end of the separation capillary, and this junction is enclosed in a metal tube filled with BGE. The ionization voltage is applied to the metal tube. In this design, the emitter tip may be replaced
separately from the CE capillary and the narrow diameter of the porous tip allows the interface to operate in the nanospray region. However, the porous tip must be coated to disrupt EOF formation and the connection between the porous tip and the separation capillary can leak or be easily blocked.

1.6.4 The Flow-Through Microvial ESI Interface

Developed by the Chen group in 2010, the flow-through microvial sprayer is a type of sheath-liquid interface called liquid junction, and the particulars of this design allow it to overcome the challenges of the mismatches between CE and ESI. The sprayer is made of stainless steel, and consists of a T-junction and a needle that has a beveled tip (see Fig. 1.3). The beveling of the needle tip is an important aspect of the spray needle geometry as it determines the distribution of the electric field. By focusing the electric field at the tip of the beveled needle, a Taylor cone forms that is stable at a lower flow rate (0.1-0.4 µL/min). The asymmetrical nature of the sprayer tip causes the Taylor cone to form at a point slightly separate from the needle orifice, decoupling the CE separation and ESI processes, which increases also spray stability at low flow rates by reducing problems that disrupt fluid flow like electrolysis while maintaining electrical contact and steady electrolyte flow. Operating a stable electrospray at a lower flow rate reduces analyte dilution as a lower volumetric flow rate of modifier solution is required.

The separation capillary from the CE is fed into the T-junction of the sprayer and into the needle.\textsuperscript{87,98} Between the flat end of the capillary and the inside of the tapering steel needle tip, a small space, known as the microvial, acts as the CE outlet vial for electrical contact and mixing of the CE effluent and the modifier solution. The modifier solution is pumped into the T-junction via a syringe pump. The modifier solution is typically composed of a small amount of the volatile BGE used for the CE separation, and a large percentage of organic solvent which allows the solution to more efficiently evaporate during the ESI process.
The flow-through microvial ESI sprayer is simple in design and optimized for functionality. The sprayer is compatible with any commercially available CE and MS and is able to effectively couple the two instruments while maintaining a stable ESI spray at low flow rates.

1.7 Mass Spectrometry: Gaseous Charged Species Separation

Once successfully ionized and delivered from the separation capillary into the gas phase, analyte ions can be separated and detected using a mass spectrometer. Mass spectrometry (MS) is a high-performance analytical separation and detection technique that measures the mass-to-charge ratio ($m/z$) of analytes. With high sensitivity and speed, a mass spectrometer can determine the mass, elemental composition, and/or structure of a compound. Despite the variety of mass spectrometer designs, each instrument performs the same basic steps for analysis: (i) analyte ionization in a source, (ii) analyte separation by a mass analyzer based on $m/z$ (and additional analyte fragmentation (MS$^n$) if desired), (iii) analyte detection, and (iv) data processing to convert signals into mass spectra.

1.7.1 Quadrupole Mass Analyzer

Quadrupoles are sets of four parallel rods that are either circular or hyperbolic in shape. The rods are paired such that rods opposite one another have the same polarity of potential applied to them. The potential applied is composed of both radio frequency (RF) and direct current (DC) components. Analyte ions travel through the centre of the space defined by the four rods. By setting the ratio between the RF and DC aspects of the applied potential, analyte ions with specific $m/z$ have stable trajectories between the rods and reach the detector.

Mass spectrometers that incorporate quadrupole mass analyzers can include two other quadrupoles that operate in tandem and are called triple quadrupole mass spectrometers. In this
mass spectrometer instrument configuration, precursor ions are selected in the first quadrupole, fragmented into product ions in the second quadrupole, and separated in the third quadrupole. Analytes fragment in specific ways and the detection of the precursor ion in combination with a specific product ion, called single reaction monitoring (SRM), can be used to determine analytes sensitively.\textsuperscript{129}

Quadrupole mass analyzers offer moderate resolution, and when operated in full scan mode, have a poor duty cycle, but these mass spectrometers are the most sensitive systems for the detection of a specific analyte, due to the high specificity of the triple quadrupole design.

1.7.2 Linear Ion Trap Mass Analyzer

Linear Ion Trap (LIT) mass analyzers operate similarly to quadrupoles, in that an oscillating RF field is used, but the field is designed to trap specific ions in two dimensions rather than to transmit specific ions.\textsuperscript{130} Linear ion traps are composed on quadrupoles with end-cap lenses that reflect ions backwards and forwards within the quadrupole to trap the ions.

Inside the LIT, ions are slowed by collisions with an inert gas such as helium. Ions travel from end to end of the quadrupole while also oscillating in the x- and y-axis directions due to the applied RF-only potential on the quadrupole rods. A DC potential can be applied to the ends of the quadrupole rods to act in conjunction with the electric field applied to the end caps to help better trap ions by reducing ion loss. Trapped ions are focused in different locations inside of the quadrupole based on the symmetry with which potentials are applied. After trapping, ions can be ejected from the LIT either axially, out of the end of the quadrupole, or radially, out from between or through rods, in a mass-selective manner.
Unlike other ion trap designs, the LIT a larger effective trapping volume, giving it a much higher capacity for trapping ions due to the larger volume and trapping along a line rather than a point in space. The high trapping capacity of the LIT translates to well-resolved masses by reducing space charge effects compared to other ion trap mass analyzers, such as the 3D ion trap. LITs are also more efficient at ion trapping, allowing a greater number of ions to be collected and focused and increasing the sensitivity of LITs over other types of ion traps.

1.8 Objectives

1.8.1 Method Development for Analyzing Sialic Acids

Detecting sialic acids from recombinant and endogenous human erythropoietin and comparing levels of different sialic acids could be used to detect blood doping. The sialic acids found on recombinant EPO differ from those found on endogenous EPO in that endogenous EPO should not contain any N-glycolylneuraminic acid (Neu5Gc). Sialic acids can be isolated from glycans and separated using CE-MS. The sensitivity offered by a triple quadrupole mass spectrometer to detect specific analytes makes it a good choice for the detection of two individual sialic acid residues. Developing the use of the flow-through microvial ESI interface for improved ionization efficiency and sensitivity for detecting low abundance sialic acids using a triple quadrupole mass spectrometer is discussed in Chapter 2.

1.8.2 Capillary Isoelectric Focusing for Discrimination between Recombinant and Endogenous Human Erythropoietin

Current methods of blood doping with erythropoietin are time-consuming and lack sensitivity. Using capillary isoelectric focusing (cIEF), the analysis of erythropoietin from human serum can be automated and performed in a timely manner. Using laser induced fluorescence (LIF)
detection, the resolution and sensitivity of glycoproteins like erythropoietin can be optimized. In Chapter 3, cIEF-UV and cIEF-LIF are used to develop a method to analyze model glycoproteins with optimized resolution and sensitivity. This method is applied to the analysis of recombinant erythropoietin from CHO cells and endogenous erythropoietin extracted from pooled human serum.

1.8.3 Desalting Glycoproteins and Separating N-linked Glycans using the Electro-Fluid-Dynamic (EFD) Microfluidic Device

The use of microfluidic devices allows separations to be performed rapidly and on a very small scale. Using a microfluidic design called the Electro-Fluid-Dynamic (EFD) device, infinite resolution can be theoretically achieved using a single power source through the manipulation of channel dimensions. Using an optimized design of the device, simulations of desalting glycoproteins for sample preparation and the separation of N-linked glycans by selective measures have been performed and are discussed in Chapter 4.
Chapter 2: Improved Sensitivity for Sialic Acid Detection Using a Flow-Through Microvial ESI Interface for CE-MS

2.1 Introduction

Erythropoietin (EPO) is a potential doping agent in competitive sports. Due to its ability to stimulate the production of red blood cells and thereby increase the oxygen-carrying capacity of an athlete’s blood, the introduction of exogenous EPO provides a significant competitive advantage to endurance athletes. To maintain a fair playing field and prevent the abuse of performance-enhancing drugs, improved methods for the detection of EPO in blood must be continuously investigated.33,35,132

Exogenous EPO can come from many sources. The essential elements of functional EPO are a correct amino acid sequence with appropriate N- and C-terminal processing, a correct fold, and appropriate post-translational modifications, especially glycosylation.133 To meet these requirements, the exogenous EPO used for doping is generally produced recombinantly in mammalian cell lines, giving a product known as recombinant human EPO (rhEPO). Chinese Hamster Ovary (CHO) cells are often used for this purpose,8 rather than cultured human cells,33,35 because CHO cells have been well-established as producers of human-like glycoproteins, they can be grown at large-scales in a variety of culture media, and they are resistant to viruses able to infect humans.34,133,134 EPO produced in CHO cells is subtly different from genuine human EPO (hEPO).34,35,131

Unlike most mammals, humans do not naturally produce the sialic acid N-glycoly neuraminic acid (Neu5Gc) due to a deletion in one of the genes responsible for Neu5Gc synthesis.135 Thus, the most common sialic acid found on endogenous human glycoproteins, such
as hEPO, is N-acetylneuraminic acid (Neu5Ac). I hypothesize that the detection of Neu5Gc (above a baseline threshold) cleaved from the N-linked glycans of EPO extracted from human blood serum could indicate the presence of exogenous EPO in the body as a result of doping.\textsuperscript{131} For an example of the differences in molecular structure of the sialic acids Neu5Ac and Neu5Gc, see Appendix B, Figure B.2.

In addition to a desire to monitor the blood of athletes to prevent cheating, there is a need to carefully characterize and regulate glycoproteins used as therapeutic agents.\textsuperscript{8,11,136,137} Neu5Gc is known to be immunogenic in humans,\textsuperscript{135} and because rhEPO is often used to treat diseases such as anaemia caused by chronic kidney disease,\textsuperscript{30} elevated levels of Neu5Gc could present a serious risk of adverse reactions. Methods for the characterization of therapeutic glycoproteins are essential to ensure the safety, purity, and efficacy of the biopharmaceutical.\textsuperscript{136} Among these methods, glycan analysis is an important aspect for several reasons. Variations in protein glycosylation can affect key proteins features such as activity and stability. The glycan component is difficult to predict and generally displays high structural diversity, giving rise to a microheterogeneous collection of glycoforms which may have different physiological effects and tolerances.\textsuperscript{8,138} According to the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) regulations for Biotechnological/Biological Products Q6B, which provides guidance on the requirements for recombinant and non-recombinant protein pharmaceutical product development and registration, the sialic acid content of the carbohydrate structure must be determined as part of routine analysis of biopharmaceuticals,\textsuperscript{139} and the relative levels of Neu5Ac and Neu5Gc are considered a critical quality attribute (CQA) for biotherapeutics.\textsuperscript{137}
One commonly used technique to characterize the sialic acid portion of glycoprotein-derived glycans is high-performance anion-exchange chromatography with pulsed amperometric detection,\textsuperscript{140,141} although high-performance liquid chromatography has also been demonstrated with fluorescence\textsuperscript{142} or mass spectrometric detection.\textsuperscript{143} Sialic acids can also be analyzed by simple fluorimetry.\textsuperscript{144} While these commonly used methods are effective, they can lack sensitivity, require meaningful sample volumes and consume large volumes of solvents, and often require derivatization prior to detection. For example, an HPAEC-PAD method reported LODs of Neu5Ac and Neu5Gc of 1 and 0.5 pmol, respectively,\textsuperscript{140} whereas the method developed in this chapter determined an LOD of 90 attomol for Neu5Ac.

Human erythropoietin is found at naturally-occurring low levels in the bloodstream, between 30 and 170 ng/L,\textsuperscript{17,145} and contains three N-linked glycans,\textsuperscript{19} each of which has up to 4 terminal sialic acids, depending on the degree of branching of the glycan.\textsuperscript{52} In one litre of human serum, one could expect a concentration of up to approximately 7 ng/L of sialic acid (almost exclusively Neu5Ac) attributable to hEPO. Therefore, a sensitive and selective method is required to differentiate between and quantify the sialic acids Neu5Ac and Neu5Gc from EPO, especially for anti-doping purposes.

As an increasingly powerful separation technique, capillary electrophoresis coupled online to mass spectrometry via electrospray ionization (CE-ESI-MS) can be used to address more challenging analytical problems such as a need for high sensitivity. CE is capable of reliably injecting plugs of sample from miniscule volumes ($\leq 10 \ \mu$L)\textsuperscript{146,147} allowing samples to be more highly concentrated. The interface between CE and MS establishes an electrical continuity between the instruments while delivering the analyte seamlessly,\textsuperscript{98} and the design of the interface
can allow for the modification of the post-column chemical environment to enhance ionization efficiency.

As has been described in greater detail in Chapter 1 of this thesis, our group designed an ESI interface for CE-MS that uses a modifier solution to smoothly transfer CE eluent to the mass spectrometer with minimal dead volume attributable to the interface. An added modifier liquid dilutes the CE eluent, counterintuitively enhancing sensitivity through the alterations of the chemical composition of the post-separation mixture to increase ionization efficiency and improve transfer of analytes between the aqueous phase of CE separation and the gaseous phase required for MS detection. For example, an increase in organic solvent content prior to ESI improves the evaporation of charged droplets in ESI but its inclusion during separation can negatively impact analyte separation by altering both the viscosity of the BGE and the physicochemical properties of the analyte.

One of the challenges of introducing a modifier is the potential formation of moving ionic boundaries. During CE-MS, sample and co-ions exit the ESI interface and counterions in the modifier enter the separation capillary and migrate towards the CE inlet. The migration of counterions into the CE separation capillary when there is minimal or no electroosmotic flow (EOF), and the counterions of the background electrolyte (BGE) and modifier are not the same, causes moving ionic boundaries to be formed. Moving ionic boundaries cause distorted peak shapes and reduced resolution. To reduce the formation of moving ionic boundaries, ensure EOF is present, add a small pressure difference between the CE inlet and outlet, or ensure that the electrolyte composition of the CE BGE and the modifier are the same. However, provided that the counterions of the BGE and modifier are the same, other solution properties such as pH and
ionic strength of the BGE and modifier solution can vary without forming moving ionic boundaries.

The objectives of this chapter were to determine LOD values for the sialic acids found on hEPO and rhEPO produced in animal cells lines. Due to the low abundance of erythropoietin found in human serum and the miniscule fraction of sialic acid residues that are Neu5Gc, the development of sensitive detection methods were required, and post-column conditions were modified to further reduce sialic acid LODs. An additional means of detecting low concentrations of sialic acid was to use small sample volumes for injection. A goal of 1 µL of sample for repeated and reliable injections was set.

2.2 Materials and Methods

2.2.1 Materials

N-acetylneuraminic acid (Neu5Ac) and 3’-sialyllactose sodium salt (SiaLac) were obtained from Carbosynth (Berkshire, UK). The sialic acid N-glycolylneuraminic acid (Neu5Gc) was obtained from Toronto Research Chemicals (North York, ON, Canada). Ammonium acetate, acetic acid, formic acid, ammonium hydroxide, methanol (HPLC grade), and 2-propanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Hydroxypropyl cellulose (average molecular weight: 100 000) was purchased from Scientific Polymer Products, Inc. (Ontario, NY, USA).

All solutions were prepared using (18.2 MΩ/cm water from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2.2 Solution Preparation

Stock solutions of the sialic acids, Neu5Ac and Neu5Gc, and sialyllactose were each prepared at a concentration of 10 mM in Milli-Q water. Calibration standards for CE-MS analysis
were prepared by diluting stock solution into background electrolyte (BGE) solution. A sample solution of 50 µM Neu5Ac, Neu5Gc, and SiaLac was used to test the selectivity of the BGE pH and the modifier solution pH.

The BGE for Neu5Gc calibration standards was 0.2% formic acid in 50:50 (v/v) methanol:water. The modifier was 10 mM acetic acid at pH 3.1 (pH adjusted using 10 mM ammonium acetate) and 75% of 2:1 (v/v) 2-propanol:methanol.

The BGE for Neu5Ac calibration standards was 50 mM acetic acid at a pH of 3.17 (pH adjusted using 100 mM ammonium acetate) and 10% methanol. The acidic modifier was 10 mM acetic acid at pH 3.17 (pH adjusted using 100 mM ammonium acetate) and 75% 2:1 (v/v) 2-propanol:methanol. The basic modifier was 10 mM ammonium acetate at pH 9.07 (pH adjusted using 100 mM ammonium hydroxide) and 75% 2:1 (v/v) 2-propanol:methanol. The pH was optimized in the acidic range for the BGE (2.5, 2.8, and 3.1) and in the basic range for the modifier (7, 8, and 9). A bare-fused silica (BFS) capillary was used for the pH optimizations of the BGE and modifier as some of the pH values chosen to test were out of the stability range of the HPC capillary coating.

All solutions were filtered through 0.22 µm PTFE filters prior to separation.

2.2.3 Separation Capillary Preparation

A 70 cm length of 365 µm o.d., 50 µm i.d. capillary was used for separation. The inner wall of the capillary was coated with a 5% aqueous hydroxypropyl cellulose (HPC) solution to render the inner surface neutrally-charged.82

To coat the inner wall of the separation capillary, the capillary was rinsed with solutions using the CE instrument as per Table 2.1 below.
Once the inner wall of the capillary was coated with the HPC solution, the capillary was placed into a GC oven and baked for 20 min at 60 °C with a temperature ramp to 140 °C at 5°C/min, and then held at 140 °C for 2 h before returning to 20 °C. While being baked, nitrogen (99.998%, Praxair, Mississauga, ON, Canada) was flushed through the capillary at 20 psi.

Following removal from the GC oven, the capillary was flushed with water at 20 psi for 20 min and stored with both ends of the capillary immersed in water.

### 2.2.4 Small Volume Injection Vial Preparation

Vials with a narrow tip suitable for injecting small volumes were produced by thermally sealing the end of 10 µL micropipette tips. The modified micropipette tip was placed in a modified PCR tube used as an adaptor for standard CE sample vials. A spring was placed under the PCR tube to ensure consistent sample height and position. As little as 1 µL of sample was placed into the modified micropipette tip vial and centrifuged at 1000 rpm for 30 sec to draw the sample down to the end of the vial. A 1 µL volume of mineral oil was layered over the sample to prevent evaporation over the course of sample preparation and injection.
2.2.5 Equipment and Instrumentation

Capillary electrophoresis was performed using a Beckman Coulter (Brea, CA, USA) PA800 Plus automated instrument coupled to a mass spectrometer via a flow-through microvial ESI interface. The instrument was controlled using 32 Karat version 9.1 software. Unless otherwise stated, a separation voltage of -30 kV was applied across the separation capillary, with an additional 0.3 psi of forward pressure to drive bulk flow in the neutrally-coated capillary. The analyte was injected hydrodynamically into the separation capillary using a positive pressure of 1.0 psi for 10.0 sec, resulting in the injection of approximately 17 nL of analyte solution (estimated based on the Poiseuille equation).

Modifier solution was delivered to the ESI interface using a Harvard Apparatus Pump 11 (Holliston, MA, USA) at a flow rate of 0.3 µL/min.

The mass spectrometer used for Neu5Gc calibration was a Thermo Scientific (Waltham, MA, USA) Orbitrap Fusion Lumos Tribrid. The flow-through microvial ESI interface was positioned approximately 1 cm away from the MS orifice and an electrospray voltage of -3.7 kV was applied. The ion transfer tube maintained at 300 °C. MS² was performed with a precursor of 324 m/z, an isolation width of 2 m/z, and a collision induced dissociation energy setting of 22%. The linear ion trap (LIT) detector was used at a turbo scan rate over a mass range of 150-400 m/z, with an automatic gain control of 2x10⁵. Ion collection was limited to 30 msec.

The mass spectrometer used for Neu5Ac acidic/basic modifier experiments and Neu5Ac calibration, sialic acid reduction, and small volume sample injection experiments was an Applied Biosystems/MDS Sciex (Framingham, MA, USA) API 4000 Triple Quadrupole. Analyst software version 1.4.2 was used to control the instrument. The flow-through microvial ESI interface was positioned approximately 1 cm away from the MS orifice, approximately 5 mm offset from the
centre, with the beveled point of the interface needle oriented towards the orifice. An ionization voltage of -3.7 kV was applied. Single reaction monitoring (SRM) scans were collected for single analytes. Initial MS conditions for sialic acid detection were a collisional activated dissociation flow rate of 4, a declustering potential of -35 V, an entrance potential of -10 V, a collision energy of -30 V, and a collisional cell exit potential of -15 V. Optimized SRM conditions for each sialic acid are given in the table below.

Table 2.2: Optimized conditions for SRM of Neu5Ac and Neu5Gc

<table>
<thead>
<tr>
<th>Sialic Acid</th>
<th>Precursor ion [M-H]^-, (m/z)</th>
<th>Declustering potential (V)</th>
<th>Entrance potential (V)</th>
<th>Collision energy (V)</th>
<th>Collisinal cell exit potential (V)</th>
<th>Collisional activated dissociation (CAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac</td>
<td>308.1 170.0</td>
<td>-55</td>
<td>-13</td>
<td>-19</td>
<td>-7</td>
<td>4</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>324.1 116.0</td>
<td>-60</td>
<td>-8</td>
<td>-20</td>
<td>-10</td>
<td>4</td>
</tr>
</tbody>
</table>

2.3 Results and Discussion

2.3.1 Neu5Gc LOD and LOQ Determination

A set of serially diluted Neu5Gc solutions were prepared and injected into the CE-MS instrument described in section 2.2.5 to estimate the LOD and LOQ values for the instrumental measurement. Each sample of Neu5Gc was prepared in the BGE. The amounts of Neu5Gc injected are shown in Table 2.3 below.
Table 2.3: Neu5Gc standards analyzed using CE-MS

<table>
<thead>
<tr>
<th>Sample Concentration (µM)</th>
<th>Volume Injected (nL)</th>
<th>Amount Injected (picograms)</th>
<th>Amount Injected (femtomoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>120.0</strong></td>
<td>17</td>
<td>664</td>
<td>2040</td>
</tr>
<tr>
<td><strong>12.0</strong></td>
<td>17</td>
<td>66.4</td>
<td>204</td>
</tr>
<tr>
<td><strong>1.2</strong></td>
<td>17</td>
<td>6.64</td>
<td>20.4</td>
</tr>
</tbody>
</table>

The peak heights, or intensities, of Neu5Gc detected were plotted against concentration to generate a calibration curve to determine sensitivity (Fig. 2.1).

Figure 2.1: Calibration curve of Neu5Gc

The signal-to-noise ratio (S/N) calculated at 1.2 µM of Neu5Gc was 3.4, which is approximately the S/N at the LOD. Therefore, the estimated instrument LOD of Neu5Gc was 1.2 µM. The LOQ was estimated as the concentration giving a S/N of 10, so the estimated LOQ for Neu5Gc is 4 µM. The maximum concentration of Neu5Gc attributable to EPO that could be found
in serum is 7 ng/L (assuming that all of the possible sialic acid residues found on each EPO molecule were Neu5Gc rather than Neu5Ac). Thus, the instrumental sensitivity for Neu5Gc was insufficient to detect Neu5Gc extracted from serum without at least 100,000-fold concentration or significant instrumental sensitivity enhancement.

2.3.2 Post-Column Environment Modification

One possible strategy to optimize the detection of analytes is to adjust solution properties to enhance ionization efficiency following separation. The separation of Neu5Ac using CE-MS was performed in an acidic BGE. The use of an acidic BGE for the separation of sialylated N-linked glycans allows for greater selectivity based on subtle differences in sialic acid pKₐ. Since the pKₐ of Neu5Ac is 2.6, the use of an acidic BGE gives the analyte a partial negative charge dependent on its pKₐ value(s). The partial negative charge on different glycans increases the dispersion of electrophoretic mobilities, enhancing separation selectivity.

An acidic BGE is typically paired with a modifier solution containing an aqueous component that is “matched” to the BGE, ensuring that the same counter-ions are present in each solution to prevent mismatches when mixing the two solutions just prior to electrospray. Avoiding mismatches in BGE and modifier prevents the formation of moving ionic boundaries. However, when performing negative-mode ESI, basic solutions are generally used to enhance ionization efficiency and spray stability. The measurement of Neu5Ac using an acidic modifier resulted in a noisy peak (see Fig. 2.2A).

This poor peak shape could not be attributed to spray instability. A comparison of the Neu5Ac signal and background signal over time indicated that a consistent amount of material entered the mass spectrometer. However, different amounts of Neu5Ac were detected second by second, suggesting that the ionization efficiency of Neu5Ac was poor. Ionization efficiency is
affected by various parameters, including flow rate, solution characteristics, and analyte characteristics.\textsuperscript{154} Sialylated glycans are known to exhibit poor ionization efficiency.\textsuperscript{155}

To enhance the sensitivity of sialic acid detection, the effect of modifier solution conditions on the ionization efficiency of Neu5Ac was investigated. The proton-accepting nature of basic solutions can improve the stability and sensitivity of negative-mode ESI by promoting ionization via deprotonation.\textsuperscript{156} The detection of Neu5Ac using modifier solutions with acidic or basic pH values were tested at ionic strengths of 10 mM. The use of a basic modifier solution resulted in a more symmetrical peak and significantly more stable signal for Neu5Ac, as seen in Figure 2.2B.
Figure 2.2: Schematic of the flow-through microvial interface, where (A) shows the Neu5Ac signal with an acidic modifier, and (B) shows the signal with a basic modifier. The extracted ion electropherograms shown have been attenuated on the x-axis (time) to only depict the peak shape. Reprinted from Risley, J.M. and Chen, D.D.Y. (2017) Improved sensitivity by post-column chemical environment modification of CE-ESI-MS using a flow-through microvial interface. Electrophoresis. 38(12):1644-1648. with permission from Wiley. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

When augmented with a basic modifier condition (pH 9), Neu5Ac signals were qualitatively sharper, with a smoother shape, while the signals collected using an acidic modifier were noisier, broader, and more skewed. To quantify the resulting change in performance, calibration curves ranging from 10 to 500 nM Neu5Ac were constructed for both acidic and basic modifier conditions by plotting Neu5Ac peak height against concentration. The slope of the basic modifier was 1.4-fold greater, indicating an improvement in sensitivity (Fig. 2.3).
Figure 2.3: Calibration curves of peak height are shown for Neu5Ac analysis using acidic or basic modifier conditions. The LOD, average S/N at 100 nM, and peak asymmetry factor range at 100 nM for each condition are listed in the table below the curves. Reprinted from Risley, J.M. and Chen, D.D.Y. (2017) Improved sensitivity by post-column chemical environment modification of CE-ESI-MS using a flow-through microvial interface. Electrophoresis. 38(12):1644-1648. with permission from Wiley. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

A more detailed comparison between acidic and basic modifier conditions using the LOD, S/N, and peak symmetry confirmed that a basic modifier was superior. The LOD for the basic
modifier was lower than the acidic modifier by a factor of approximately nine (see Fig. 2.3). Considering that the injection volume was in the low nanolitres and analyte concentrations were in nanomolar (nM), femtograms of Neu5Ac were successfully detected. S/N calculated for each modifier at 100 nM Neu5Ac using peak height as the definition of signal, and the noise as the standard deviation of the baseline showed a more than double improvement for the basic modifier over the acidic modifier. Peak asymmetry was calculated for signals at 100 nM at 10% of the maximum peak height. The peak asymmetry factor was calculated by dividing the distance from the midpoint of the signal to the tailing edge by the distance from the midpoint to the fronting edge at 10% of peak height. A peak asymmetry factor of 1.0 indicates perfect symmetry, while factors greater than 1.0 indicate peak tailing and factors less than 1.0 indicate peak fronting. The asymmetry factor for an acidic modifier had a broader range and deviation with both peak fronting and tailing, while the basic modifier peak asymmetry factor range was narrow and close to 1.0 with a small amount of peak tailing attributable to the parabolic flow profile generated by the addition of pressure during separation.

To corroborate the effect of acidic and basic modifier conditions on signals, Neu5Gc and SiaLac were also injected into CE-MS using an acidic and a basic modifier. As can be seen in Figure 2.4, the signals for Neu5Ac, Neu5Gc, and SiaLac are all improved when a basic modifier is used.
Figure 2.4: Extracted ion electropherograms and mass spectra are shown for two additional example analytes. 100 µM N-glycolyneuraminic acid (Neu5Gc), which has a molar mass of 325 g/mol, is shown when analyzed using an acidic (A) or basic (C) modifier, with the corresponding mass spectra for each shown in (B) and (D). 50 µM 3'-Sialyllactose (SiaLac), which has a molar mass of 633 g/mol, is shown when analyzed using an acidic (E) or basic (G) modifier, with the corresponding mass spectra for each shown in (F) and (H). The ions shown are deprotonated \([M – H]\). Reprinted from Risley, J.M. and Chen, D.D.Y. (2017) Improved sensitivity by post-column chemical environment modification of CE-ESI-MS using a flow-through microvial interface. Electrophoresis. 38(12):1644-1648. with permission from Wiley. Copyright © 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
2.3.3 Refinement of BGE and Modifier Solution pH Conditions for Separation and Sensitivity

Based on the experiments from section 2.3.2, the basic modifier solution was the selected condition moving forward. The pH of the BGE was refined in the acidic range to determine the BGE pH resulting in the best resolution between Nue5Ac, Neu5Gc, and SiaLac. The modifier solution pH was then refined using the BGE with the optimal pH.

As was stated in the previous section, an acidic BGE was chosen to allow for better selectivity between sialylated glycans. Therefore, the pH values of 2.5, 2.8, and 3.1 were chosen to determine the optimal pH for the BGE. The pKₐ of Neu5Ac is 2.60,¹⁵³ Neu5Gc is 2.92,¹⁵⁷ and SiaLac is 2.89.¹⁵⁸ At a pH of 2.5, all three analytes are semi-protonated and are more neutral to a degree. At pH 2.8, Neu5Ac is expected to be slightly more deprotonated than Neu5Gc and SiaLac. At pH 3.1, all three analytes would be partially negatively charged.

The voltage applied to the separation capillary was -30 kV, which would repel any negatively charged analytes away from the inlet and towards the mass spectrometer. The addition of 0.3 psi to the separation allowed neutral analytes to be detected. Because a bare-fused silica capillary was used for the optimization of BGE pH, the pH of the BGE affected the charge of the silanol groups of the inner capillary wall. At pH 2.5 and 2.8, the walls of the capillary were fully protonated, whereas at pH 3.1, the silanol groups were slightly deprotonated, resulting in a small bulk EOF towards the inlet. The EOF towards the inlet at pH 3.1 caused the analytes to be detected later than when a BGE at pH 2.5 or 2.8 was used.
Table 2.4: Comparison of migration times, peak areas, peak heights, and peak widths of Neu5Ac, Neu5Gc, and SiaLac under different BGE pH conditions

<table>
<thead>
<tr>
<th>BGE pH</th>
<th>Analyte</th>
<th>Migration time (min)</th>
<th>Peak Area (cps)</th>
<th>Peak Height (cps)</th>
<th>Peak Width (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Neu5Ac</td>
<td>14.84</td>
<td>5.01e7</td>
<td>4.90e6</td>
<td>0.3488</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>14.85</td>
<td>4.36e7</td>
<td>4.11e6</td>
<td>0.3489</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>14.24</td>
<td>1.06e8</td>
<td>9.13e6</td>
<td>0.4964</td>
</tr>
<tr>
<td>2.8</td>
<td>Neu5Ac</td>
<td>16.18</td>
<td>5.62e7</td>
<td>5.29e6</td>
<td>0.4696</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>16.22</td>
<td>4.42e7</td>
<td>4.18e6</td>
<td>0.2818</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>16.44</td>
<td>8.97e7</td>
<td>8.07e6</td>
<td>0.5233</td>
</tr>
<tr>
<td>3.1</td>
<td>Neu5Ac</td>
<td>17.72</td>
<td>3.64e8</td>
<td>3.79e6</td>
<td>0.3354</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>17.79</td>
<td>4.36e7</td>
<td>3.11e6</td>
<td>0.4830</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>19.19</td>
<td>8.22e7</td>
<td>8.11e6</td>
<td>0.3891</td>
</tr>
</tbody>
</table>

As shown in Table 2.4, there is little difference between the peak areas, peak height, or peak widths determined for each analyte in all three conditions. As the separation between Neu5Ac and Neu5Gc based on migration time was barely discernable at pH 2.5, so only pH 2.8 and 3.1 were more seriously considered. Using a BGE with a pH of 3.1 not only gave the greatest separation between Neu5Ac and Neu5Gc, it also gave the greatest resolution between the sialic acids and SiaLac. The peak shapes were also qualitatively better. Therefore, a pH of 3.1 was selected as the optimal pH for the BGE.

The modifier solution pH was optimized using pH 3.1 BGE, and the pH values for the modifier chosen to test were pH 7, 8, and 9.
Table 2.5: Comparison of migration times, peak areas, peak heights, and peak widths of Neu5Ac, Neu5Gc, and SiaLac under different modifier pH conditions

<table>
<thead>
<tr>
<th>BGE pH</th>
<th>Analyte</th>
<th>Migration time (min)</th>
<th>Peak Area (cps)</th>
<th>Peak Height (cps)</th>
<th>Peak Width (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>Neu5Ac</td>
<td>No peaks</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>detected</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8.0</td>
<td>Neu5Ac</td>
<td>17.12</td>
<td>1.40e7</td>
<td>1.48e6</td>
<td>0.3757</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>17.08</td>
<td>1.03e7</td>
<td>1.03e6</td>
<td>0.3489</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>18.53</td>
<td>1.90e7</td>
<td>2.16e6</td>
<td>0.3354</td>
</tr>
<tr>
<td>9.0</td>
<td>Neu5Ac</td>
<td>16.85</td>
<td>3.96e7</td>
<td>4.15e6</td>
<td>0.3488</td>
</tr>
<tr>
<td></td>
<td>Neu5Gc</td>
<td>16.91</td>
<td>3.09e7</td>
<td>3.04e6</td>
<td>0.3623</td>
</tr>
<tr>
<td></td>
<td>SiaLac</td>
<td>18.05</td>
<td>7.80e7</td>
<td>8.92e6</td>
<td>0.3623</td>
</tr>
</tbody>
</table>

Slightly larger peak areas and heights were detected at a modifier pH of 9, but peak widths were similar between modifier pH values (Table 2.5). To select a modifier pH, the degree of separation between the sialic acids based on migration time was used. Since a modifier of pH 9 gave the largest difference in migration time between all three analytes, that value was chosen.

### 2.3.4 Small Volume Sample Injections

Given the consistently small sample volume injected into a capillary, the precise and reproducible injection of a sample taken from the smallest volume possible creates an opportunity for greater concentration of the analytes. This is particularly important when sample size is limited,
as when working with human plasma. Small sample volumes present significant challenges in reproducibility. At a very low volume, evaporation over the course of the experiment becomes a significant concern. Reliable repeat injections are also difficult, especially considering the configuration of the injection system of a Beckman Coulter PA800 Plus capillary electrophoresis instrument. Another challenge to reducing the sample volume is the shape of the sample vial, as a broadly rounded vial tip results in a low volume height which does not always allow the capillary tip to be fully submerged for injection.

To address the challenges of injecting from a small volume in a Beckman Coulter PA800 Plus CE system, a vial was produced using a 10 µL micropipette tip, part of a PCR tube, a spring, and a CE vial. The modified vial can be seen in Figure A.1 (Appendix A).

Secondly, a small volume of mineral oil was applied to the top of the sample to minimize evaporation. Finally, the configuration of the capillary injection end and electrode were adjusted to ensure that the capillary end extended past the electrode end to allow the capillary end to be submerged into the sample solution during injection.

The minimal volume from which reliable injections could be made was determined by quantifying the peak areas of Neu5Ac, Neu5Gc, and SiaLac in a sample mix at a concentration of 100 µM. The lowest possible sample volume for reliable injections was previously considered to be 10 µL. In this experiment, injections were tested from sample volumes of 0.5 and 1.0 µL using mineral oil to prevent evaporation. When 0.5 µL of sample was used, the three analytes could not be detected, suggesting a failure to inject sample into the capillary. However, at 1.0 µL, analytes were detected and the results can be seen in Table 2.6.
Table 2.6: Results for injections from sample volumes of 1.0 µL with and without mineral oil

<table>
<thead>
<tr>
<th></th>
<th>Without Mineral Oil</th>
<th>With Mineral Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neu5Ac (counts, n=3)</td>
<td>Neu5Gc (counts, n=3)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>2.85 x 10^7</td>
<td>3.93 x 10^7</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>4.18 x 10^6</td>
<td>3.76 x 10^6</td>
</tr>
<tr>
<td><strong>RSD (%)</strong></td>
<td>14.7</td>
<td>9.56</td>
</tr>
</tbody>
</table>

The effect of the presence or absence of mineral oil on the precision of the analyte peak area was determined (Table 2.6). The absence of mineral oil resulted in higher relative standard deviation (RSD) values, meaning that the results were less precise. There was a 1.8-fold improvement in precision for Neu5Ac peak area when mineral oil was used, a 1.3-fold improvement for Neu5Gc, and a 2.1-fold improvement for SiaLac. Therefore, mineral oil was used to prevent evaporation.

With the ability to reliably inject from 1 µL of sample, sialic acid solutions could be highly concentrated. Assuming a maximum concentration of sialic acid in serum of 7 ng/L, 0.014 ng of sialic acid could be extracted from 2 mL of serum. In a total volume of 1 µL, the concentration of the sample would be approximately 1.4x10^-5 g/L, which is 43 nM of Neu5Gc. At these levels, it would be possible to detect sialic acids extracted from serum.
2.4 Concluding Remarks

It is feasible to use the modifier solution to alter the post-column chemical environment of analytes, facilitating compound separations under optimal for analyte selectivity and resolution, and detection under conditions which enhance sensitivity. Previously, the modifier has been used to simply reconcile the differences in volumetric flow rate requirements and to provide a source of organic solvent to improve desolvation during the ESI process, and the nature of the modifier solution has not been carefully considered for its impact on the actual detection of analytes. The results of this experiment show the importance of developing an optimized modifier solution in relation to the BGE and characteristics of the analyte(s).

Differentiating between exogenous and endogenous erythropoietin in human serum is challenging due to the incredibly similarity between the two glycoproteins and the extremely low levels at which they are present. Using a custom-built CE-MS interface to successfully modify the post-column chemical environment of analysis and using modifications to the CE system to allow for reliable injections from 1 µL of sample, the detection limits necessary to detect sialic acids released from EPO N-glycans has been shown to be possible.
Chapter 3: Separation of Exogenous and Endogenous Human Erythropoietin using Capillary Isoelectric Focusing with UV Absorption and Laser Induced Fluorescence

3.1 Introduction

Erythropoietin (EPO) analysis is largely accomplished using electrophoresis. Pharmaceutical analysis of EPO by organizations such as the European Pharmacopeia, which produces an official biological reference preparation of recombinant EPO, uses capillary zone electrophoresis to assess EPO glycoforms. Anti-doping regulatory authorities, such as the World Anti-Doping Agency (WADA), rely on isoelectric focusing (IEF) to detect and identify EPO, as EPO glycoforms may be separated based on isoelectric point (pI).

All proteins are amphoteric. Amphoteric compounds have acidic and basic groups that can be ionized and therefore these molecules can be positively or negatively charged depending on the pH of the surrounding environment. The pI of an amphoteric analyte is the pH at which the analyte is neutral. Due to differences in the degree of sialylation of N-linked glycans, EPO glycoforms are detected as charge ladders, which are a series of proteins or glycoproteins that differ from one another solely on the degree of charge, leading to a small variation in pI. Sialic acids contribute negative charges to the total charge of each EPO glycoform, and variance in the sialic acid content as well as other modifications to glycans from the producing cell line affect the pH range of the EPO pI.

Recombinant EPO produced in CHO cells tends to have pI values of 4.4 – 5.1, although Cifuentes et al. reported pI values of 3.78 - 4.69 for recombinant EPO, calculated using cIEF-UV. There is also an isoelectric difference that can be observed in the detection of hEPO extracted from serum (pI range of 4.1-4.9) or from urine (pI range of 3.8-4.7).
IEF is a separation technique that resolves analytes (often peptides or proteins) based on pI.\textsuperscript{163,164} During isoelectric focusing, analytes are focused into bands in a viscous material (to prevent convection) within a pH gradient.\textsuperscript{163,164} An acidic electrolyte solution called the anolyte is placed at the anode (+) while at the cathode (-), there is a basic electrolyte solution called the catholyte. Between the anode and cathode is a solution of carrier ampholytes, which are small (200-1000 g/mol) organic amphoteric molecules. A mixture of carrier ampholytes that cover a range of isoelectric points is used. When an electric field is applied, a current flows, which causes hydroxide ions to move towards the cathode and hydronium ions to move towards the anode. Carrier ampholytes also migrate according to their net charge until the net charge becomes zero and the carrier ampholytes become immobile. Once the carrier ampholytes have migrated into position, with the most acidic ampholytes nearest the anode and the most basic nearest the cathode, a pH gradient is formed. When amphoteric analytes have been added to the mix, they behave like the carrier ampholytes, migrating until they reach their pI.

Traditional IEF is performed on a slab-gel, which is time-consuming and labour-intensive.\textsuperscript{38,39} Relatively large volumes of electrolyte solution and carrier ampholyte solutions are required for slab-gel IEF. IEF can be performed rapidly and with less sample in a capillary. Capillary isoelectric focusing (cIEF) is automated and small-scale; the diameter of the capillary allows for better heat dissipation so higher currents can be used, reducing the separation time required. Detection methods for slab-gel IEF can also be limited in sensitivity, and are challenging to perform reproducibly. Using cIEF, small volumes of dilute sample can be concentrated on-column and detected, and automated capillary re-conditioning steps between analyses can significantly improve reproducibility.
To detect the focused bands of analytes, one-step or two-step cIEF is used. In one-step cIEF, the capillary is imaged much like a gel. In two-step cIEF, the focused bands are mobilized so that they pass through a detection point. To mobilize the bands, hydrodynamic (pressure) mobilization or chemical mobilization is used. During hydrodynamic mobilization, a small pressure is applied to the anode side of the capillary, generating a bulk flow through the capillary towards the detector. While this technique is simpler and readily adaptable to most commercial CE systems, parabolic flow through the capillary causes some band broadening. This is minimized by maintaining the electric field during mobilization, but presents a performance limitation nonetheless. One way to avoid the issue of parabolic flow is to use the electric field to generate the bulk flow. This is known as chemical mobilization. The capillary end in contact with the catholyte is transferred to a solution of a lower pH or higher ionic strength. When a lower pH solution is used, the counter-ion (for example, acetate when acetic acid is used) enters the capillary from the cathode end while hydronium ions from the anolyte enter the anode end. The pH gradient is disrupted, and the overwhelming excess of protons give the analytes a positive charge which causes them to migrate towards the cathode where the detector is located.

The use of chemical mobilization rather than pressure mobilization has been shown to increase resolution, though is necessitates the use of protein solubilizers such as urea. Because bands of protein are focused into narrow zones, the protein becomes more concentrated during the analysis. Once focused, the proteins are also neutrally-charged. Thus, the proteins often become less soluble and may precipitate or aggregate in the capillary. The addition of urea acts to improve the solubility of the proteins, as can the addition of glycerol. Both glycerol and urea also favourably increase the viscosity of the BGE. Glycerol acts as the main anti-convection viscous medium to limit band broadening due to Joule heating from current flow, molecular diffusion, and
EOF. Coating the walls of the capillary with a neutral coating also helps to suppress EOF to limit band broadening and the disruption of focused pI zones, increasing resolution and sensitivity.

This chapter presents a sensitive cIEF method that is capable of detecting hEPO at the naturally low levels found in serum (about 30-170 ng/L\textsuperscript{145}). The developed method should have sufficient resolution so as to differentiate between EPO glycoforms and between recombinant and endogenous EPO. To the knowledge of the author, no cIEF method exists yet in the literature that separates rhEPO from endogenous serum hEPO. In this thesis, the EPO extracted from human serum is the focus, as serum EPO has been less well studied than urine EPO. The use of cIEF would be an improvement on detection methods currently in use by regulatory agencies such as WADA, as cIEF is an automated technique suitable for parallelization and providing high sensitivity. To develop a method, model proteins and glycoproteins were used to optimize sensitivity and resolution using two different optical detection techniques (UV absorption and LIF). The method was tested using rhEPO from CHO cells, and endogenous hEPO extracted and purified from pooled human serum.

3.2 Materials and Methods

3.2.1 Materials

A polyvinyl alcohol (PVA)-coated CE-MS capillary (50 µm i.d., 125 cm effective length) was purchased from Agilent (Santa Clara, CA, USA) and was cut in half before use. A hydroxypropyl cellulose (HPC)-coated capillary was prepared in-house, as described below. Hydroxypropyl cellulose (100 000 mw) was purchased from Scientific Polymer Products, Inc. (Ontario, NY, USA).

Urea, glycerol, ammonia, Pharmalytes pH range 2.5-5.0, Pharmalytes pH range 3-10, Pharmalytes pH range 4-6.5, Pharmalytes pH range 5-8, sodium bicarbonate, sodium chloride,
tricine, putrescine, ovalbumin from chicken egg white, and beta-lactoglobulin A from bovine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Recombinant human EPO for physicochemical tests (Y0001725) and Biological Reference Preparation (BRP) recombinant human EPO (E1515000) produced in CHO cells was obtained from the European Pharmacopeia (EDQM, European Pharmacopeia, Council of Europe, Strasbourg, France).

Formic acid, methanol (HPLC grade), acetic acid (glacial), and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA).

A pi peptide marker kit was purchased from Sciex (Concord, ON, Canada), and Chromeo P503 labelling reagent was supplied by Cedarlane for Active Motif Inc. (Burlington, ON, Canada).

One batch of pooled normal human serum (50 mL) was purchased from Innovative Research (Novi, MI, USA).

An EPO Purification Kit (Art. No. 1390) was purchased from MAIIA Diagnostics (Uppsala, Sweden). Amicon Ultra-0.5 mL Centrifugal Filters (10K) and Millex HPF Nylon Syringe Filters (0.45 µm pore size, 25 mm diameter) were purchased from Millipore-Sigma (Etobicoke, ON, Canada).

3.2.2 Solution Preparation

A capillary electrophoresis background electrolyte (BGE) for CZE-UV of EPO was prepared using 21 g urea, 0.125 mL 1 M putrescine, 5 mL of salt concentrate, and 25 mL water. The pH of the solution was adjusted to 5.5 using 2 M acetic acid. The salt concentrate solution was prepared using 0.582 g sodium chloride, 1.793 g tricine, and 0.820 g sodium acetate in 100 mL water.
Anolyte solution was prepared using 125 mM formic acid containing a percentage of glycerol (10, 15, and 20% were used). The mobilizer solution was the same solution as the anolyte. The catholyte solution was 100 mM ammonia, containing the same percentage of glycerol as found in the anolyte solution.

A stock solution of 8 M urea was prepared in water, along with a stock of 80% glycerol.

A stock bicarbonate buffer was prepared at 50 mM using sodium bicarbonate adjusted to pH 8.5 using sodium hydroxide.

A Chromeo P503 labelling reagent stock was prepared by adding 100 µL of methanol to the 1 mg of labelling reagent powder to make a solution with a concentration of 25.4 mM. The labelling reagent stock was stored in an aluminum foil-covered PCR tube at 4 °C.

Stocks of ovalbumin and beta-lactoglobulin A were prepared in water. Ovalbumin was prepared at a concentration of 10 mg/mL, while beta-lactoglobulin A was prepared at a concentration of 4 mg/mL.

3.2.3 HPC-Coated Separation Capillary Preparation

A 50 cm capillary (50 µm i.d.) was coated with 5% hydroxypropyl cellulose (HPC) to render the inner surface neutral. To coat the inner wall of the capillary, the capillary was rinsed with solutions using the CE instrument as per Table 3.1 below.
Table 3.1: Conditioning and application of HPC to bare fused silica capillary for neutral coating

<table>
<thead>
<tr>
<th>Event</th>
<th>Value (psi)</th>
<th>Duration (min)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>5</td>
<td>Methanol</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>5</td>
<td>Water</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>20</td>
<td>0.1 M NaOH</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>5</td>
<td>Water</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>20</td>
<td>0.1 M HCl</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>5</td>
<td>Water</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>20</td>
<td>5</td>
<td>Methanol</td>
</tr>
<tr>
<td>Rinse - pressure</td>
<td>30</td>
<td>10</td>
<td>Air</td>
</tr>
<tr>
<td>Separate - pressure</td>
<td>50</td>
<td>20</td>
<td>HPC</td>
</tr>
<tr>
<td>Separate - pressure</td>
<td>50</td>
<td>60</td>
<td>Air</td>
</tr>
</tbody>
</table>

After coating the inner wall of the capillary with HPC solution, the capillary baked in a GC oven 20 min at 60 ℃, ramped to 140 ℃ at 5℃/min, and then held at 140 ℃ for 2 h before returning to 20 ℃. Nitrogen (99.998%, Praxair, Mississauga, ON, Canada) was flushed continuously through the capillary at 20 psi.

After baking in the GC oven, the coated capillary was flushed with water at 20 psi for 20 min and stored with both ends of the capillary immersed in water.

3.2.4 Sample Preparation

cIEF samples were prepared fresh daily. Each sample contained urea, glycerol, ampholytes, proteins or peptides, and water. Samples containing 4 M, 5 M, and 6 M urea were prepared, as well as sample containing 10, 15, and 20 % glycerol. Samples containing 0.5, 1.0, and 1.5 % ampholytes were also prepared. To further improve resolution, mixed-range ampholyte percentage in the sample of 1, 2, 3, and 4 % were prepared. Added protein/peptide concentrations varied, but were generally 1 mg/mL of each protein in each sample. The total volume of each sample prepared was 0.1 mL, so ultrapure water was added to make up the sample to that total volume as necessary.
3.2.5 **EPO Extraction and Desalting**

EPO was extracted from pooled human serum using a MAIIA Diagnostics EPO Purification Kit. Serum samples were prepared by mixing 2 mL of serum and 18 mL water in 50 mL conical tubes. Then 1 mL of serum buffer and 1 mL of exposure aid were added to the sample, mixed, and left at room temperature for 10 min. The solution was filtered using 0.45 µm HPF filters. Prepared samples were passed through anti-EPO columns at a flow rate of 1 mL/min under vacuum. After the sample had passed through the column, 1 mL of washing buffer was passed through the column. The columns were dried by centrifugation in a microtube at 2000 g for 1 min. Each collection tube had 5 µL of adjustment buffer added prior to the collection of EPO from the sample. The EPO was collected from the column by adding 50 µL of desorption buffer before centrifuging the column at 2000 g for 1 min. The collected EPO was vortex-mixed.

EPO extracted from human serum and purchased recombinant EPO was desalted using Amicon Ultra-0.5 mL (10K) centrifugal filters. Purchased EPO was received as freeze-dried solid powder, and prior to desalting was made into an aqueous solution by adding an appropriate volume of water such that the concentration of EPO was approximately 1 mg/mL. EPO extracted from serum was desalted from the desorption buffer. The filters were conditioned with 0.25 mL of Milli-Q water by centrifuging at 13 000 g for 10 min. Both the retentate and eluent were discarded. EPO was added to the sample reservoir and centrifuged at 13 000 g for 10 min. The EPO was desalted using 4 x 0.25 mL water and centrifuged at 13 000 g for 10 min each. The flowthrough was discarded after each round of centrifugation. The desalted EPO was recovered by centrifuging the upside-down sample reservoir in a new vial at 2 000 g for 2 min. To increase EPO recovery, the recovery step was repeated after adding 2 µL of water to the sample reservoir and centrifuging at 2 000 g for 2 min.
3.2.6 Ampholyte Photobleaching for cIEF-LIF

Ampholyte solutions were photobleached\textsuperscript{167} using blue LED strips (wavelength 476-490 nm) purchased online from ChiChin Lighting. Each LED module contained three Samsung 5630 chips and emitted 120-154 lumens per module.

Ampholyte solutions were left to photobleach with air cooling in a cylinder lined with the LED modules for up to 5 h.

3.2.7 Protein Labelling with Chromeo P503 Labelling Reagent

A fluorogenic labelling reagent called Chromeo P503\textsuperscript{168,169} was used to label protein samples for LIF detection. To each 100 µL total volume of reaction solution, 2 µL of labelling reagent stock was added. The other 98 µL of the reaction solution were composed of the protein to be labelled, typically to a final concentration of 1 mg/mL depending on protein stock concentration, and 60 µL of bicarbonate buffer stock for a final buffer concentration of 30 mM. This labelled protein stock was then further diluted during preparation as a cIEF sample.

After mixing the labelling reaction components together, the reaction solution was left to react in an aluminum foil-covered PCR tube at room temperature for 1 hr. The labelled protein stock was stored in the reaction tube at 4 °C.

All labelling steps were performed in a dark room to limit exposure of the labelling reagent to light.

3.2.8 Instrumentation and Instrumental Analysis

3.2.8.1 CZE-UV for Desalted EPO Detection

The capillary electrophoresis instrument used was a Beckman Coulter (Brea, CA, USA) P/ACE MDQ with a UV absorption detector set to collect at a wavelength of 214 nm. The
separation capillary was conditioned using 0.1 M sodium hydroxide at 20 psi for 60 min, followed by the BGE at 20 psi for 60 min. The purified EPO was injected at 0.7 psi for 20 sec. Separation was performed for 70 min at 15.7 kV.

3.2.8.2 cIEF-UV

The capillary electrophoresis instrument used was a Beckman Coulter (Brea, CA, USA) P/ACE MDQ CE system with UV absorption detection. A wavelength of 280 nm was used for cIEF-UV experiments. A representative cIEF method is shown in Table 3.2 below.

Table 3.2: Typical cIEF method used in EPO method development tests

<table>
<thead>
<tr>
<th>Summary</th>
<th>Time (min)</th>
<th>Event</th>
<th>Value</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea rinse</td>
<td></td>
<td>Rinse – pressure</td>
<td>20.0 psi</td>
<td>5.00 min</td>
</tr>
<tr>
<td>Water rinse</td>
<td></td>
<td>Rinse – pressure</td>
<td>20.0 psi</td>
<td>5.00 min</td>
</tr>
<tr>
<td>Sample injection</td>
<td></td>
<td>Rinse – pressure</td>
<td>10.0 psi</td>
<td>5.00 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wait</td>
<td></td>
<td>0.1 min</td>
</tr>
<tr>
<td>Anolyte injection</td>
<td>0.00</td>
<td>Inject – pressure</td>
<td>8.0 psi</td>
<td>20.0 sec</td>
</tr>
<tr>
<td>Focusing step</td>
<td>30.00</td>
<td>Separate – voltage</td>
<td>30.0 kV</td>
<td>30.00 min</td>
</tr>
<tr>
<td>Mobilization step</td>
<td>30.00</td>
<td>Separate – voltage +</td>
<td>30.0 kV + 0.3 psi</td>
<td>60.00 min</td>
</tr>
<tr>
<td></td>
<td>30.00</td>
<td>Autozero</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.8.3 cIEF-LIF

The same capillary electrophoresis instrument used for UV absorption detection experiments was used for LIF detection experiments.

A Beckman Coulter LIF detector with a 488 nm laser light source was used. A 600 nm bandpass emission filter (FWHM 40 nm, 12.5 mm diameter) from Andover Corporation (Salem, NH, USA) was used. The dynamic range was set to 10 relative fluorescence units (RFU), with high sensitivity filter settings.
3.3 Results and Discussion

3.3.1 Optimization of Voltage, Pressure, and Time for cIEF Focusing and Mobilization Steps

The two steps of capillary isoelectric focusing are focusing and mobilization, and both steps are driven by the application of a voltage across the capillary over a set period of time (when using chemical mobilization). Focusing is complete when peptides or proteins have reached their isoelectric points, and have stopped migrating through the capillary. The cessation of migration is generally indicated by reaching a stable current minimum as the ampholytes and analytes become neutral and focus into their pI bands. Mobilization can be aided by the application of a pressure across the capillary and is complete when all of the proteins have passed through the detection window of the capillary.

To determine the optimal voltage for the focusing step and the length of time for which focusing should be conducted, cIEF-UV analyses of peptide pI markers (pI 4.1, 5.5, and 7.0) were run at different focusing voltages over different periods of time. Focusing voltages of 20, 25, and 30 kV were tested, and focusing times of 15, 30, and 45 min were tested. Mobilization voltages of 20, 25, and 30 kV were tested, and mobilization times of 30, 60, and 90 min were tested. The impact on resolution of an applied pressure to assist mobilization was tested at 0.1, 0.3, 0.5, and 1.0 psi.
Figure 3.1: Electropherograms (left) and current profiles (right) of the optimized conditions of cIEF-UV. The conditions were kept constant at 30 kV for 30 min for focusing with 30 kV for 60 min with no added pressure for mobilization while one set of conditions was varied. The pI markers 4.1, 5.5, and 7.0 were diluted by a factor of 50 from the purchased stock solution, in the sample with 3 M urea, 15 % glycerol, and 1.5 % ampholytes (pH range 3-10). A 60 cm long, 50 µm id PVA-coated capillary was used, with a detection wavelength of 280 nm. (A) Electropherogram and current profile using a focusing voltage of 30 kV, (B) Electropherogram and current profile for 30 min focusing, (C) Electropherogram and current profile for mobilization at 30 kV, (D) Electropherogram and current profile for mobilization for 60 min (E) Electropherogram and current profile for added pressure of 0.3 psi during mobilization.

Comparing the focusing time, qualitative resolution of the three pI markers and the overall shape of the current profile of each run, the focusing voltage selected was 30 kV (Fig. 3.1A) for a period of 30 min (Fig. 3.1B). Comparing the qualitative resolution of the three pI markers and the migration time of the last eluting pI marker, a chemical mobilization voltage of 30 kV (Fig. 3.1C) for a period of 60 min (Fig. 3.1D) was selected. Based on the qualitative resolution and peak shape of the pI markers, 0.3 psi (Fig. 3.1E) was selected for pressure-assisted chemical mobilization.

### 3.3.2 Comparison of HPC and PVA Coated Capillaries

Both hydroxypropyl cellulose (HPC) and polyvinyl alcohol (PVA) have been previously used to suppress EOF and limit band broadening during cIEF. The two capillary coatings were compared here to determine which one showed better sensitivity and resolution for glycoproteins. A blank sample was prepared along with a sample containing beta-lactoglobulin A and ovalbumin. Each coated capillary was used for cIEF-UV analyses of the blank in duplicate and of the mixed sample in triplicate. Both capillaries were 50 cm in length, with an inner diameter of 50 µm.

Table 3.3: Comparison of PVA and HPC capillaries for sensitive and well-resolved cIEF-UV of proteins and glycoproteins
The full width half maximum (FWHM) and signal-to-noise ratio (S/N) of beta-lactoglobulin were used to estimate a measure of sensitivity and the resolution between the glycoforms of ovalbumin was determined as key factors to compare the PVA and HPC capillaries. A narrower FWHM has the potential to achieve greater sensitivity and improved resolution and the HPC capillary showed a narrower FWHM for beta-lactoglobulin (see Table 3.3). However, the PVA capillary gave better S/N for beta-lactoglobulin. A higher resolution was determined between ovalbumin glycoforms using the PVA capillary as well, so PVA was selected as the capillary coating for the cIEF studies.

### 3.3.3 Sample Solution Optimization for Glycoprotein Separation

To optimize the sensitivity and resolution of the method, the glycerol, urea, and ampholyte content of the sample solution and the composition of the ampholytes used were optimized. The ampholytes were also photobleached to reduce background signal and enhance sensitivity. Beta-lactoglobulin A and ovalbumin were selected as model proteins for method development and optimization as both have isoelectric points similar to EPO (see Appendix B Table B.1).
3.3.3.1 Glycerol Content of Sample and Solutions

As glycerol is used to increase the viscosity of the BGE and reduce EOF, the percentage of the glycerol component of the sample and the anolyte, catholyte, and mobilizer is a factor that affects the resolution of the method. The objective of this experiment was to determine the percentage of glycerol that yields the best resolution between proteins (ovalbumin and beta-lactoglobulin), and between glycoforms of the glycoprotein ovalbumin.

Figure 3.2: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 10%, (B) 15%, and (C) 20% glycerol in the sample, anolyte, catholyte, and mobilizer. Traces offset for clarity.
Table 3.4: Comparison of resolution and glycerol content for cIEF-UV of proteins and glycoproteins

<table>
<thead>
<tr>
<th>Glycerol content (%)</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>Resolution between the glycoforms of ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10.2</td>
<td>0.846</td>
</tr>
<tr>
<td>15</td>
<td>14.9</td>
<td>1.07</td>
</tr>
<tr>
<td>20</td>
<td>22.1</td>
<td>2.40</td>
</tr>
</tbody>
</table>

Based on the resolution calculated between beta-lactoglobulin A and ovalbumin as well as the resolution calculated between ovalbumin glycoforms, 20% glycerol yields the greatest resolution (see Table 3.4). However, when including a visual inspection of the appearance of the ovalbumin glycoforms (see Figure 3.2), the best subjective separation of glycoforms and of proteins occurs when 15% glycerol is used. Therefore, 15% glycerol was used for subsequent cIEF analyses.

3.3.3.2 Urea Concentration of Sample

cIEF performance is affected by protein precipitation and aggregation as proteins focus into regions of high concentration at their pI values. Urea is commonly used to aid in protein solubility by preventing inter-molecular hydrogen bond formations, and is also used as a rinse between analyses to maintain separation performance and reproducibility by removing proteins adsorbed to the capillary wall. The effects of urea in cIEF samples on resolution and sensitivity were determined by performing separations at different urea concentrations.
Figure 3.3: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 4 M, (B) 5 M, and (C) 6 M urea in the sample. Traces offset for clarity.

Table 3.5: Comparison of urea concentration for sensitive and well-resolved cIEF-UV of proteins and glycoproteins

<table>
<thead>
<tr>
<th>Urea concentration (M)</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>FWHM beta-lactoglobulin A (min)</th>
<th>S/N beta-lactoglobulin A</th>
<th>beta-lactoglobulin A and ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>25.8</td>
<td>1.97</td>
<td>0.553</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>14.6</td>
<td>0.936</td>
<td>0.449</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16.7</td>
<td>0.380</td>
<td>0.559</td>
<td>15.8</td>
<td></td>
</tr>
</tbody>
</table>

A urea concentration of 6 M resulted in the least well-resolved and sensitive signals for beta-lactoglobulin A and ovalbumin (see Figure 3.3). Concentrations of 4 M and 5 M urea were
better resolved and more sensitive, with 4 M resulting in greater resolution but 5 M resulting in a narrower FWHM for beta-lactoglobulin A (see Table 3.5). As a compromise between resolution and sensitivity, and to ensure protein solubility if higher protein concentrations were used, 5 M urea was chosen for cIEF samples.

3.3.3.3 Ampholyte Content of Sample

A pH gradient is established across the capillary during the focusing step of cIEF by the migration of the ampholyte molecules through the capillary in response to the electric field. Higher concentrations of ampholytes can lead to higher resolution but can also compromise sensitivity. The effects of broad range (pH 3-10) ampholyte content in cIEF samples on sensitivity and resolution was studied.

![cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 0.5%, (B) 1.0%, and (C) 1.5% broad range (pH 3-10) ampholytes in the sample. Traces offset for clarity.](image)

Figure 3.4: cIEF-UV electropherogram traces of beta-lactoglobulin A (1) and ovalbumin (2) using (A) 0.5%, (B) 1.0%, and (C) 1.5% broad range (pH 3-10) ampholytes in the sample. Traces offset for clarity.
Table 3.6: Comparison of ampholyte content for sensitive and well-resolved cIEF-UV of proteins and glycoproteins

<table>
<thead>
<tr>
<th>Ampholyte content (%)</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>Resolution between the glycoforms of ovlobumin</th>
<th>FWHM beta-lactoglobulin A (min)</th>
<th>S/N beta-lactoglobulin A</th>
<th>beta-lactoglobulin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>10.9</td>
<td>1.05</td>
<td>0.372</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>12.0</td>
<td>1.08</td>
<td>0.375</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>14.0</td>
<td>1.07</td>
<td>0.449</td>
<td>22.0</td>
<td></td>
</tr>
</tbody>
</table>

Resolution between ovalbumin glycoforms was similar for each level of ampholyte content used (see Figure 3.4). The best sensitivity and the best resolution between glycoforms were obtained for 1.0% ampholytes, while 0.5% ampholytes resulted in acceptable sensitivity and resolution between proteins and between glycoforms (see Table 3.6). An ampholyte content of 1.0% was selected for cIEF samples.

3.3.3.4 Comparison of Broad and Narrow Range Ampholytes

The pH range of the ampholytes used in cIEF samples can also affect the resolution and sensitivity of the separation. Narrow range ampholytes result in smaller pH increments which can give greater resolution, while broad range ampholytes provide a steeper pH gradient, resulting in sharper peaks and increased sensitivity. Because the pI of EPO is known to be in the region of 3.7 to 5.1, a narrow range ampholyte of pH 2.5 to 5 was selected and compared to a broad range ampholyte of pH 3 to 10.
The pI of beta-lactoglobulin A is 5.1 and the pI range of ovalbumin is 4.43-4.66, both of which are on the catholytic edge of the narrow range ampholyte (pH 2.5-5). This is likely why neither protein is detected when only the narrow range ampholyte was used; the two proteins would have focused at the outlet end of the capillary as the pH gradient formed across the capillary, and the location of the focused band was likely beyond the detector window, causing the proteins to remain undetected.
Table 3.7: Comparison of ampholyte pH range for sensitive and well-resolved cIEF-UV of proteins and glycoproteins

<table>
<thead>
<tr>
<th>Ampholyte pH range</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>Resolution between the glycoforms of ovalbumin</th>
<th>FWHM beta-lactoglobulin A (min)</th>
<th>S/N beta-lactoglobulin A</th>
<th>S/N beta-lactoglobulin A (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad (3-10)</td>
<td>10.1</td>
<td>ND</td>
<td>0.325</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>44.0</td>
<td>4.91</td>
<td>1.40</td>
<td>6.19</td>
<td></td>
</tr>
<tr>
<td>Broad:Narrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Broad:Narrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:1</td>
<td>18.7</td>
<td>2.22</td>
<td>0.409</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>Broad:Narrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Narrow (2.5-5)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

However, when both the broad and narrow range ampholytes were combined, a visual evaluation of the electropherograms of broad and narrow pH range ampholytes (Figure 3.5) showed that using only the broad range (Fig. 3.5A) or the 1:1 and 2:1 mix of broad to narrow range (Fig. 3.5D) ampholytes gave the best signals for beta-lactoglobulin A and ovalbumin. It is likely that the narrow range ampholytes condition did not result in improved resolution due to unoptimized catholyte conditions resulting in the formation of a poor pH gradient. The 1:1 mix of broad to narrow range ampholytes gave better resolution between proteins and between glycoforms for ovalbumin, which was confirmed by comparing resolution and sensitivity figures.
of merit for each of the ampholyte analyses (see Table 3.7). However, this gave a split peak for beta-lactoglobulin. The 2:1 mix of broad to narrow range ampholytes improved resolution between proteins, but, as expected, gave slightly less sensitivity than the broad range ampholytes alone.

3.3.3.5 Comparisons of Ratios of Complex Ampholyte Mixtures

Properties of a variety of complex ampholyte mixtures have been researched and published by Righetti et al.,\textsuperscript{172–178} who found that combining ampholytes of differing pH ranges can increase resolution. To determine the optimal mixture of ampholytes needed for the greatest resolution in the pH range of EPO, different ratios of ampholytes with varied pH ranges were prepared and tested. The ampholytes tested were pH 3-10, pH 2.5-5, pH 4-6.5, and pH 5-8, and were at 1% w/v in the sample.
Figure 3.6: cIEF-UV electropherograms of beta-lactoglobulin A and ovalbumin using (1) a range of different ampholyte ratios with three highlighted analyses of pH range 3-10:2.5-5:4-6.5:5-8 in ratios of (A) 0:0:1:1 (B) 1:0:1:0 and (C) 1:0:1:1. The three highlighted analyses are enlarged in (2). Traces offset for clarity.

All of the electropherograms of the different ratios of mixed ampholytes are shown in Figure 3.6, and the three that showed the best resolution and peak shape were selected. The three selected mixed ampholytes were compared in terms of resolution between proteins and between
glycoforms, as well as in FWHM and S/N of beta-lactoglobulin A.

Table 3.8: Comparison of sensitivity and resolution for analyses of glycoproteins using different ampholyte ratios in cIEF-UV

<table>
<thead>
<tr>
<th>Ampholyte ratio of 3-10:2.5-5:4-6.5:5-8</th>
<th>Resolution between beta-lactoglobulin A and ovalbumin</th>
<th>Resolution between glycoforms of ovalbumin (min)</th>
<th>FWHM beta-lactoglobulin A</th>
<th>S/N beta-lactoglobulin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:0:1:1</td>
<td>10.2</td>
<td>0.95</td>
<td>0.217</td>
<td>16.3</td>
</tr>
<tr>
<td>1:0:1:0</td>
<td>14.0</td>
<td>1.54</td>
<td>0.296</td>
<td>12.2</td>
</tr>
<tr>
<td>1:0:1:1</td>
<td>10.4</td>
<td>1.19</td>
<td>0.251</td>
<td>9.54</td>
</tr>
</tbody>
</table>

The highest resolution was seen between proteins and between the glycoforms of ovalbumin for the ratio of 1:1 for 3-10:4-6.5 mixed ampholytes, which also gave the second best S/N for beta-lactoglobulin A. Since that ratio of mixed ampholytes have the best results, it was chosen as the ratio of mixed ampholytes for subsequent experiments.

To ensure that the optimal sensitivity and resolution were obtained for cIEF separation using a mixture of ampholytes, the percent content of ampholytes was again optimized using the chosen ratio. Ampholyte content percentages of 1, 2, 3, and 4% were used.
Figure 3.7: cIEF-UV electropherograms of beta-lactoglobulin A and ovalbumin samples containing (A) 1%, (B) 2%, (C) 3%, and (D) 4% ampholytes. Traces offset for clarity.

The peak shapes and qualitative resolution for beta-lactoglobulin A and ovalbumin were all quite similar for each of the ampholyte content percentages used (Fig. 3.7). To avoid compromising sensitivity, 2% ampholytes was chosen for subsequent analyses.

3.3.3.6 Ampholyte Photobleaching for Reduced Background Signal using LIF Detection

Ampholytes have been shown to fluoresce slightly due to impurities found within the ampholyte solution.\(^{167}\) To reduce the background signal caused by ampholyte fluorescence, the ampholyte mixture was photobleached. By reducing the background signal, sensitivity is increased as the signal-to-noise ratio is increased. The optimized ampholyte mixture with a ratio of 1:1 of the 3-10:4-6.5 pH range ampholytes was photobleached. Ampholytes were photobleached using a homemade apparatus (see Appendix B Figure B.1) for 0, 1, 2, 3, 4, and 5 hours to optimize photobleaching time. At each time point, an aliquot of 100 µL was collected for testing with cIEF-LIF.
Figure 3.8: cIEF-LIF electropherogram of baseline signal from 15 – 25 min (after focusing but before protein detection) for (A) no photobleaching, (B) 1 hour of photobleaching, (C) 2 hours of photobleaching, (D) 3 hours of photobleaching, (E) 4 hours of photobleaching, and (F) 5 hours of photobleaching. Traces offset for clarity.

The blue LEDs used to photobleach the ampholyte mixture emit light in the 476-490 nm wavelength range, which bleach the ampholyte solution in the region of the wavelength of the excitation laser used by the LIF detector. By comparing the electropherogram that used ampholytes that had not been photobleached (Fig. 3.8A) with each time point of photobleaching, it can be seen that effective photobleaching occurred at 5 hours (Fig 3.8F). No photobleaching resulted in some random spikes in the baseline, while 1 and 2 hours of photobleaching showed a less stable baseline. Photobleaching for 3 and 4 hours showed some plateau regions in the baseline signal. Photobleaching for 5 hours gave a straight baseline with minimal deviations. Overall, there was no significant difference in baseline for each photobleaching timepoint, but out of caution, ampholytes that had been photobleached for 5 hours were used to prepare subsequent samples.
3.3.4 Sensitivity and Glycoform Resolution Determination of Developed cIEF-LIF Method using Ovalbumin

Ovalbumin was chosen as a substitute for EPO during method development and optimization not only because both glycoproteins have similar pI values, but also because both have similar lysine residue content (see Appendix B Table B.1). Having similar lysine residue content is significant because the protein label developed by Wolfbeis, Chromeo P503,\textsuperscript{168} works by reacting with the primary amine group of lysine.\textsuperscript{179} It was observed that when the dye solid is dissolved in methanol, it is a deep blue colour, but when it reacts with lysine residues, the solution becomes a red colour, as has been previously described.\textsuperscript{168,169} The conjugated label fluoresces when excited with wavelengths between 470 and 530 nm,\textsuperscript{168} which is compatible with the 488 nm laser of the LIF detector.

Serial dilutions of ovalbumin were analyzed using cIEF-LIF, and an LOD (at S/N = 3) was estimated to be 1.2 nM and an LOQ (at S/N = 10) was estimated to be 4.0 nM. The concentration of EPO estimated to be found in human serum is 30 - 170 ng/L\textsuperscript{145} (equivalent to approximately 1 - 6 pM EPO, assuming an EPO molecular weight of 30 kDa\textsuperscript{17,18}). Sample preparation of EPO from serum involved concentrating the extracted EPO from a serum volume of 2 mL into 50 µL of desorption buffer (a 40-fold concentration) which was followed by a desalting step that did not result in any dilution or concentration. The samples of EPO extracted from serum were expected to contain approximately 1.2 - 6.8 µg/L (0.04 - 0.23 nM). Despite sensitivity optimization efforts, based on the estimated LOD for ovalbumin, the method was not expected to be sufficient to detect EPO extracted from human serum.

To overcome the lack of sufficient sensitivity, the protein labelling reaction was optimized to ensure maximum labelling prior to analysis (data not shown). By optimizing the labelling
reaction parameters of the initial and final concentrations of both the label and the glycoprotein, and allowing the reaction to occur overnight, the S/N of the ovalbumin signal was increased by 2.5-fold, closing the gap between the LOD and the expected concentration of extracted EPO. The dynamic range used for cIEF-LIF was also optimized to further improve the S/N.

3.3.5 Detection of rhEPO and hEPO by cIEF-LIF

Samples of endogenous EPO were extracted from pooled human serum and analyzed using cIEF-LIF alone and combined with recombinant EPO. The resolution of the optimized method was determined for the combined sample of recombinant and endogenous EPO to ensure that the two types can be separated and differentiated. The isoelectric points of each type of EPO were estimated.

3.3.5.1 Detection of Desalted EPO

The Biological Reference Preparation (BRP) of recombinant EPO from the European Pharmacopeia contains 0.25 mg of EPO and a mix of salts (0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine, 4.5 mg of NaCl, and 3.5 mg of Na$_2$HPO$_4$) and is typically desalted via centrifugal filtration prior to analysis for better resolution of EPO glycoforms. To confirm that the EPO was not lost during desalting and that the desalting procedure had successfully removed the salts, an aliquot of desalted BRP EPO was tested using CZE-UV as described in section 3.2.8.1 using the BGE containing putrescine.$^{180}$ The electropherogram was expected to contain seven to eight glycoforms of EPO.
As shown in Fig. 3.9, seven glycoforms were resolved and clearly detected using CZE-UV. The detection of seven separate glycoforms rather than four or five indicated that the majority of the salts had been removed. The desalted rhEPO was diluted by a factor of 10, and was easily detected after desalting, indicating that minimal loss occurring during desalting.

3.3.5.2 cIEF-LIF Profiles of rhEPO and Serum hEPO

Samples of both recombinant EPO and serum-extracted EPO were labelled with Chromeo P503 and added to the cIEF sample mixture described in section 3.2.4. The samples were then analyzed using the cIEF-LIF method developed in this chapter. Samples contained 5 M urea, 15% glycerol, and 2% ampholytes in a 1:1 ratio of pH ranges 3-10:4-6.5. The anolyte, catholyte, and mobilizer were as described in section 3.2.2, and the cIEF instrumental method used was described in sections 3.2.8.2 and 3.2.8.3.
The cIEF-LIF electropherogram of the biological reference preparation of recombinant EPO showed six glycoforms of EPO with some resolution and good sensitivity (Fig. 3.10). Since endogenous EPO was extracted from pooled serum, a “smear” of glycoforms is seen in the electropherogram (Fig. 3.11) where the glycoforms have less dispersed charge states, resulting in
poor resolution. The lack of resolution may be due to the pooled nature of the sample, in that the EPO from a large number of individuals would be present, each exhibiting a slightly different pI range for EPO, resulting in one large signal composed of many different glycoforms. However, the endogenous EPO peak appears sharper and better defined than the recombinant EPO, so the sialylation state of the endogenous EPO may be more consistent than the recombinant EPO which shows greater micrometerogeneity.

Figure 3.12: cIEF-LIF electropherogram of (A) EPO extracted from pooled human serum, (B) beta-lactoglobulin A, (C) physicochemical test recombinant EPO, and (D) a pI 3.6 marker peptide.

When both the recombinant and serum-extracted EPO were run together, along with beta-lactoglobulin A and a pI 3.6 marker peptide, each protein can be distinguished, although there is no baseline separation between serum EPO and beta-lactoglobulin A (Fig. 3.12). The recombinant EPO, which was diluted by a factor of 10 to minimize the risk of overwhelming the serum-extracted EPO signal, shows less resolution of each glycoform, although some separation of
glycoforms can be seen. There is good separation between the recombinant and endogenous EPO using this cIEF-LIF method.

### 3.3.5.3 Determination of Isoelectric Points of Recombinant Human EPO Glycoforms by cIEF-LIF

A pI calibration curve was constructed by determining the migration times of three proteins or peptides with well-known isoelectric points. Beta-lactoglobulin A has a pI of 5.1, and ovalbumin has an average pI of 4.5. A peptide pI marker with a pI of 3.6 was also used to construct the curve, which is seen in Figure 3.13.

![Figure 3.13: Calibration curve of isoelectric point using pI markers.](image-url)
The pI range of recombinant EPO produced in CHO cells has been reported as 4.4 – 5.1 previously. In this experiment, the pI values of recombinant EPO produced in CHO cells were calculated as 3.7 – 4.4 (Fig. 3.14), which agrees with the results of rhEPO pI values of 3.78 - 4.69 obtained by Cifuentes et al. The pI of human serum EPO was determined to be 4.3, which is within the reported range of 4.1-4.9.

3.4 Conclusions

Using model proteins and glycoproteins, a sensitive method was developed and optimized to resolve and differentiate between recombinant EPO and human serum EPO. Sample and solution conditions including glycerol content, urea concentration, ampholyte content, and ampholyte ratios were investigated to optimize the resolution and sensitivity of the cIEF method. Ampholytes were photobleached to reduce background signal and further increase the sensitivity of the method. The sensitivity of the method was estimated to be 1.2 nM using ovalbumin as a model glycoprotein. Both recombinant EPO and endogenous EPO extracted from human serum

---

**Figure 3.14: Electropherogram of recombinant EPO with the pI of each glycoform labelled.**

The pI range of recombinant EPO produced in CHO cells has been reported as 4.4 – 5.1 previously. In this experiment, the pI values of recombinant EPO produced in CHO cells were calculated as 3.7 – 4.4 (Fig. 3.14), which agrees with the results of rhEPO pI values of 3.78 - 4.69 obtained by Cifuentes et al. The pI of human serum EPO was determined to be 4.3, which is within the reported range of 4.1-4.9.

3.4 Conclusions

Using model proteins and glycoproteins, a sensitive method was developed and optimized to resolve and differentiate between recombinant EPO and human serum EPO. Sample and solution conditions including glycerol content, urea concentration, ampholyte content, and ampholyte ratios were investigated to optimize the resolution and sensitivity of the cIEF method. Ampholytes were photobleached to reduce background signal and further increase the sensitivity of the method. The sensitivity of the method was estimated to be 1.2 nM using ovalbumin as a model glycoprotein. Both recombinant EPO and endogenous EPO extracted from human serum
were successfully detected. Isoelectric points of the glycoforms of recombinant EPO were
determined to be 3.7 – 4.4 using this method, while the isoelectric point of endogenous EPO
extracted from serum was found to be 4.3. The cIEF-LIF method developed in this chapter was
able to resolve recombinant EPO and endogenous serum EPO, and based on the difference in
average isoelectric points determined for each type of EPO, this method was able to distinguish
recombinant EPO from endogenous serum EPO.
Chapter 4: Theoretical Glycoprotein Desalting and N-Linked Glycan Purification Using the Electro-Fluid-Dynamic Microfluidic Device

4.1 Introduction

A microfluidic device, called the electro-fluid-dynamic (EFD) device, was developed for the continuous separation of analytes using both electric and hydrodynamic forces.\textsuperscript{181–183} Analytes move along a main channel due to an applied electric field or an applied pressure, or a combination of the two, and are distributed into side channels. This device was developed based on principles used in techniques such as flow counterbalanced capillary electrophoresis (FCCE)\textsuperscript{184} and continuous free flow electrophoresis (cFFE).\textsuperscript{185}

FCCE and cFFE inspired the development of the electro-fluid-dynamic (EFD) device. FCCE uses a hydrodynamic pressure applied in the opposite direction of EOF in order to increase resolution by keeping all but the most mobile analyte in the sample vial.\textsuperscript{181,184,186} This technique can separate and purify a small number of highly electrophoretically mobile analytes from a complex mixture. However, only the analyte with the highest electrophoretic mobility can migrate to the outlet to be collected in pure form, making this a slow technique.

cFFE allows for continuous purification by flowing buffer from one direction across a small chamber, while simultaneously applying an electric field perpendicular to the direction of buffer flow.\textsuperscript{185} Analytes are separated by electrophoretic mobility due to the electric field and collected from the end of the separation chamber into a series of channels, which can lead to incomplete resolution due to limited fractionation channel density and imperfectly focused collection.
A previous member of the Chen group, Dr. Chang Liu, designed the initial concept of the EFD device inspired by the concepts of FCCE and cFFE while minimizing several of the detrimental aspects associated with continuous analyte separation and purification using these techniques.\textsuperscript{181–183} Theoretically, by moving from a one dimensional capillary to a multi-branched network of microfluidic channels, multiple analytes can be collected simultaneously while still maintaining the “infinite resolution” of FCCE.\textsuperscript{181} Resolution may be infinite if one analyte may be held stationary or made to migrate in a direction opposite to that of another analyte.\textsuperscript{187} The application of a specific flow counter to the direction of analyte migration is one method by which an analyte may be held stationary or forced to migrate in the opposite direction with respect to another analyte.

The EFD device operates by the application of a potential at the sample inlet and each collection channel outlet as well as a flow of BGE applied counter to the direction of the electric field (Fig. 4.1).\textsuperscript{181–183} Ground is located near the end of the main channel. Analyte migration is governed by bulk flow generated by EOF, as well as the electrophoretic mobility of the analyte, and by the flow velocity in the channels due to the applied BGE flow. Ideally, the EOF is eliminated through channel coatings to limit flow velocity complications. At each junction in the device, the particle may migrate into the collection channel, or continue down the main channel, depending on the balance between the electrophoretic mobility of the analyte and the force exerted on it due to the applied BGE flow.
Figure 4.1: Schematic of a multi-branched EFD device with a sample inlet, I, at the top of the main channel, pressure P applied at the bottom of the main channel, and analyte outlets, O, at the ends of the collection channels. A positive potential is applied to the sample inlet and each collection outlet, while another side channel is grounded. Adapted with permission from Anal. Chem. 2014, 86, 22, 11380–11386. Copyright (2014) American Chemical Society

Dr. Liu conducted simulations and proof-of-concept experiments by applying varied electrical potentials and counter-flow to this device for the separation of fluorescent dyes, to show the ability of the device to distribute analytes into different channels according to their charge and size properties.\textsuperscript{181–183} The EFD microfluidic device is a tool that can be used to continuously separate and purify a specific analyte or set of analytes, with theoretically infinite resolution. The device requires very small volumes of both sample and solvent for analyte separation. As a microfluidic device, EFD chips have design flexibility, and the device has the capacity for automation. However, there are several areas which require further development or improvement to develop a fully functional EFD chip.
Further improvement of the device include the reduction of water electrolysis and Joule heating caused by high current flow through the chip. Electrolysis of water generates oxygen gas at the anode during oxidation and hydrogen gas during reduction at the cathode, which can create bubbles in the chip and interrupt current flow.\textsuperscript{188,189} Electrolysis can also cause pH changes through the formation of hydroxide ions at the cathode and protons at the anode, which can affect the EOF, analyte charge, and migration. Joule heating, which increases the temperature in the channels, can change the BGE viscosity, alter analyte migration times, or damage the sample. Joule heating can also limit the maximum voltage that may be applied to the system due to reduction in electrical resistance caused by heating.\textsuperscript{149} Reducing current flow across the device will limit electrolysis and Joule heating, and can be accomplished through BGE optimization and design optimization, which will involve channel dimension manipulation in terms of width and length. Design optimization will also enable the streamlining of operational hardware by reducing the required number of power sources to one.

A significant hardware limitation for the device is the high-voltage power source. In the current configuration of the chip, different potentials are applied to the sample inlet and each collection channel in order to affect separation. As the number of analytes collected in parallel increases, the number of collection channels increases, and so does the number of power sources necessary to supply the applied potentials. Using the flexibility of microfluidic chip design, the dimensions and geometry of the chip channels can be manipulated to reduce the required hardware. It was hypothesized that an increase in channel width would cause no change in electric field while decreasing flow velocity in the collection channel. An increase in channel length was expected to result in both a lower electric field and lower flow velocity. These relationships were expected to hold true both within and between the collection channels. Therefore, it was thought that by
manipulating the collection channel dimensions, differences in electric field strength and flow velocity could be induced between collection channels, enabling the use of a single power source to drive separations on the chip.

By reducing the hardware requirements to a single power source, and enhancing the chip by eliminating EOF and reducing current flow, the EFD device will be capable of rapid separation with high resolution. To identify the separated analytes and evaluate purity, however, a suitably sensitive detector, such as mass spectrometry, is needed. A key sample preparation step prior to mass spectrometric detection is desalting, to which the design of the EFD device lends itself well.

An abundance of non-volatile salts within a MS ion source is detrimental to evaporation during ionization, causing less stable spray, ion suppression, poor peak shape, and decreased signal-to-noise ratio.¹⁹⁰,¹⁹¹ Several techniques exist by which samples may be desalted, including dialysis,¹⁹⁰ solid phase extraction,¹⁹² chromatography, or microchannel devices that, for example, use laminar flow in two layers to salt exchange through diffusion.¹⁹¹ These techniques either exchange ions or extract them, and can include sample concentration or separation, but generally provide limited purification and often result in sample dilution. The coupling of CE and MS provides advantages including an additional mode of separation with high resolving power, and low sample volume requirements compared to other coupled separation methods.¹⁹³ However, CE-MS is no more capable of desalting samples while separating analytes than any other coupled separation technique. The theoretically infinite resolution provided by the EFD during purification of analytes along with separation driven by charged analyte interaction with an applied electric field suggest that the EFD device could be used to desalt, or remove highly mobile ions from analytes of interest, for MS analysis. Coupling the EFD device to MS would yield a device capable
of on-line sample desalting, separation, and detection, making the EFD device a valuable component of a micro total analysis system.

The objectives of this chapter are to further develop the EFD device design to enhance the theoretical separation performance and reproducibility for N-linked glycan purification, and to determine the feasibility of using the device for performing sample preparation functions such as glycoprotein desalting prior to MS detection. Power supply requirements will be minimized through channel dimension manipulation and optimization of the overall channel geometry of the chip, allowing for the selective detection of targeted compounds extracted from complex mixtures.

4.2 Materials and Methods

4.2.1 Instrumentation and Software

Simulations were performed on COMSOL Multiphysics (version 4.3b) (COMSOL Inc., Burlington, MA). Glass chip masks were designed using Adobe FreeHand 10 (Adobe Systems Inc., San Jose, CA) and AutoCAD 2014 (Autodesk, Inc., San Rafael, CA).

UV lithography was performed using a URE-2000/35 mask aligner with a UV lamp. A DZF-6030A vacuum drying oven was used, as well as a Thermo Scientific F47900 series Thermolyne muffle furnace, for bonding glass pieces together during chip fabrication.

A Spellman CZE1000R high voltage power source, along with a Harvard Apparatus 11 syringe pump and a SZM series Stereo microscope were used for the testing of fabricated EFD chips. A 1000 μL Hamilton Gastight syringe (model 1001; Hamilton Company, Reno, NV) was used to deliver BGE via syringe pump. Fused silica capillaries (I.D. 75 μm; Polymicro Technologies, Phoenix, AZ) were used to connect the syringe pump to the EFD chip, along with nanoports purchased from IDEX Health & Science (Oak Harbor, WA).
4.2.2 Materials

For chip fabrication at the Dalian Institute of Chemical Physics (DICP) in China, sodium hydroxide pellets, ammonium cerium (IV) nitrate, glacial acetic acid, acetone, hydrofluoric acid, nitric acid, sulphuric acid, and hydrogen peroxide were obtained from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). SG-2506 borosilicate glass pieces from Changsha Shaoguang Chrome Blank Co., Ltd. (Changsha, China) with 145 nm thick chromium film and 570 nm thick S-1805 positive photoresist were used.

Methanol was LC-MS grade purchased from JT Baker (Avantor Performance Materials, Center Valley, PA, USA), sulphuric acid was purchased from BDH Chemicals (VWR International, Edmonton, Alberta, Canada), and sodium hydroxide was purchased from Spectrum Chemicals and Laboratory Products (Gardena, CA, USA). Sodium tetraborate was purchased from Sigma (Oakville, Ontario, Canada). Phenol red, hydrochloric acid, and isopropanol were purchased from Fisher Scientific (Ottawa, Ontario, Canada).

Milli-Q (18 MΩ) water was used, and all aqueous solutions were filtered through 0.22 μm nylon filters prior to introduction into the EFD device.

4.2.3 Simulation Parameters and Models

Simulations were performed using COMSOL Multiphysics software in two dimensions using the Electrostatics model (using the Poisson equation, equation 5-1), the Laminar Flow model (using the simplified Navier-Stokes equation, equation 5-2), and the Transport of Diluted Species model (using the Mass Transport equation, equation 5-3).

\[(5-1) \nabla \cdot \nabla V = -\frac{\rho_v}{\varepsilon}\]

\[(5-2) \rho = -\nabla p + \eta \nabla^2 u + F_b\]
(5-3) \( N_i = -D_i \nabla c_i - z_i u_i F c_i \nabla V + u c_i \)

where \( V \) is the applied potential, \( \rho_v \) is charge density, \( \varepsilon \) is the dielectric constant of the medium, \( \rho \) is the fluid density, \( u \) is the fluid velocity, \( p \) is pressure, \( \eta \) is dynamic viscosity, \( F_b \) represents other forces acting on the fluid, \( N_i \) is the flux of the analyte, \( D_i \) is the diffusion coefficient of the analyte, \( c_i \) is the concentration of the analyte at a specific point along a channel, \( z_i \) is the charge of the analyte, \( u_i \) is the mobility of the analyte, and \( F \) is the Faraday constant. Together, these models are used to simulate the electric field distribution and fluid flow in the EFD chip, as well as analyte concentration within the device channels. The specific parameters used in the simulation models are shown in Table 4.1. The simulations were solved using the finite element method (FEM) using a Physics-controlled mesh (an unstructured tetrahedral mesh) with an extremely fine element size.

Table 4.1: COMSOL Multiphysics simulation parameters used for the geometry and models

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Materials</strong></td>
<td></td>
</tr>
<tr>
<td>Boundaries</td>
<td>Borosilicate</td>
</tr>
<tr>
<td>Domain</td>
<td>2.48 S/m water</td>
</tr>
<tr>
<td><strong>Electrostatics</strong></td>
<td></td>
</tr>
<tr>
<td>Potential (between sample inlet and ground)</td>
<td>1000 V</td>
</tr>
<tr>
<td>Potential (between collection channels and ground)</td>
<td>( \leq 1000 ) V</td>
</tr>
<tr>
<td><strong>Laminar Flow</strong></td>
<td></td>
</tr>
<tr>
<td>Wall</td>
<td>No slip</td>
</tr>
<tr>
<td>Inlet Flow Rate</td>
<td>( 9 \times 10^{-12} ) m(^3)/s</td>
</tr>
<tr>
<td><strong>Transport of Diluted Species</strong></td>
<td></td>
</tr>
<tr>
<td>Charge</td>
<td>+1 or -1</td>
</tr>
<tr>
<td>Mobility</td>
<td>( 5 \times 10^{-14} ) to ( 9 \times 10^{-13} ) s·mol/kg</td>
</tr>
<tr>
<td>Inflow Concentration</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Borosilicate glass was chosen as the boundaries because it is the same glass type as the fabricated chips, and water with a modified conductivity of 2.48 S/m was chosen as the domain in order to more closely simulate a BGE solution. The potential at the sample inlet was 1000 V.
because it allows for rapid migration, and the collection channel potentials were set lower so that analytes would migrate toward them selectively. The wall boundary was set to ‘no slip’ because it was of interest to simulate situations without EOF. The inlet flow rate was set at 9x10^{-12} \text{ m}^3/\text{s} for separation simulations because that volumetric flow rate allowed for analyte separation at the potentials chosen. Analytes were positively or negatively charged; no neutral analytes were simulated. Inflow concentration of analytes was 1 \text{ mM} because it is a reasonable analyte concentration based on concentrations of BGE that will be used. Mobility values were selected to be from 5x10^{-14} \text{ s} \cdot \text{mol/kg} to 9x10^{-13} \text{ s} \cdot \text{mol/kg} those values represent a good approximation range for proteins to small cations or anions.

4.2.4 Chip Design and Fabrication

EFD chips were designed such that the channel depth was 100 \text{ μm}, and the main channel was 44 \text{ mm} in length and 50 \text{ μm} in width. The side collection channel dimensions were varied. In one design, side channel dimensions equal for each channel, with lengths of 14.14 \text{ mm} and widths of 100 \text{ μm} (Figure 4.2A). A second chip was designed with side channel widths of 100 and 200 \text{ μm} and equal lengths of 14.14 \text{ mm} (Figure 4.2B). A third chip with different side channel lengths of 4.95, 10.60, and 19.80 \text{ mm} and equal widths of 100 \text{ μm} (Figure 4.2C) was also designed. Side channels connected with the main channel at a 45 degree angle. The grounded channel was 14.14 \text{ mm} in length and 100 \text{ μm} wide for each chip.
Glass EFD microfluidic chips were fabricated using standard lithography and wet-etching techniques. Briefly, glass pieces were exposed to UV radiation (approximately 150 mJ/cm²) for 10 sec through a polyester base transparency photolithography mask. The photoresist was then removed using sodium hydroxide (0.5 g in 100 mL deionized water), followed by chromium removal by ammonium cerium (IV) nitrate (100 g in 500 mL deionized water with 17.5 mL glacial acetic acid). Channels were wet etched using hydrofluoric acid and nitric acid (70 mL of each in 390 mL of deionized water) with agitation. Inlet holes were drilled with diamond-tipped bits and glass pieces were cut using a diamond-tipped glass cutter. The remaining photoresist was removed with acetone, followed by the removal of chromium as above. Glass pieces were washed using 3:1 (v/v) sulphuric acid to hydrogen peroxide (piranha solution) for an hour before being sonicated with deionized water and bonded first in a vacuum drying oven for an hour and then in a muffle furnace at 620 °C overnight (approximately 12 hours).
4.2.5 Chip Testing

Prior to testing, glass EFD chip inner channel surfaces were rehydrated by a 30 minute exposure to 1:1 (v/v) methanol to concentrated hydrochloric acid injected into the channels followed by a deionized water rinse. The glass chip was then cleaned for 30 min with concentrated sulphuric acid injected into the channels and thoroughly rinsed with deionized water again. Finally, the channel wall surfaces were pre-treated for 30 min with 1.0 M sodium hydroxide and then 0.1 M sodium hydroxide.

Platinum electrodes were taped down into the inlet and outlets of the EFD chip and connected to the voltage output of the Spellman power source using alligator clip wires. A syringe pump was connected to the chip using pieces of capillary and a nanoport attached to the EFD chip pressure inlet. The nanoport was attached by cleaning the glass surface using isopropanol, and then placing the gasket seal into the nanoport hub and aligning the epoxy ring underneath the hub around the chip inlet. The hub was clamped into place and heated in an oven at 170 °C for one hour.

Tests were conducted to determine appropriate BGE conductivities, applied potentials, and applied flow rates. BGE solutions of 50 and 200 mM borate (pH 9.1 and 9.0, respectively) and 0.1, 0.01, and 0.001 M sodium hydroxide (pH 11.9, 11.3, and 10.2, respectively) were used. Potentials of 100 – 2000 V were applied, and counter flows of 0.1 – 1 mL/hr (1.7 – 17 μL/min) were applied. Phenol red dye (8 mM) was introduced by pipetting a 2 μL aliquot into the sample inlet.

4.3 Results and Discussion

4.3.1 EFD Chip Design and Fabrication

Different glass microfluidic chips were designed and fabricated at DICP in China, and one set included a basic EFD device chip with side collection channels of the same width and length,
as well as two other chips with different widths or lengths (Figure 4.3) to test the effect of collection channel dimension manipulation. Wet etching with hydrofluoric acid is isotropic, meaning that etching occurs in all directions.\textsuperscript{195} HF etched channels are slightly rough, a characteristic which increases with etching time. Surface roughness can affect EOF, causing it to decrease as surface roughness increases.\textsuperscript{195,196} In the EFD chips fabricated, the surface became rough due to long etching times. Thus, this first iteration of chips are only suitable for initial testing, optimization of EOF suppression, optimization BGE composition and conductivity, and trial separations.

![Fabricated EFD chips with collection channels of (A) the same widths and lengths, (B) different widths, and (C) different lengths.](image)

**Figure 4.3: Fabricated EFD chips with collection channels of (A) the same widths and lengths, (B) different widths, and (C) different lengths.**

### 4.3.2 First Generation Chip Chemical Tests

EFD chips fabricated in China were tested at UBC using a high voltage power source to generate an electric field and syringe pump to supply a volumetric flow rate. BGE solutions were tested on the chip at different concentrations to find a BGE with a low conductivity to limit current flow from the power source. A pH indicator dye, phenol red, was used to determine appropriate applied potentials and flow rates needed to move the dye within the chip to specific collection
channels with minimal electrolysis. The application of potentials needed for collection showed evidence of pH changes due to electrolysis of water, along with gas bubble formation (Figure 4.4).

![Image](image.png)

**Figure 4.4:** A phenol red dye colour change of red to yellow as electrolysis occurs at high applied potential (2000 V) across the chip, and the pH of the BGE changes.

Different BGE solutions of various concentrations were tested to determine the effect of ion type and concentration on current readings. BGE solutions were tested with an applied potential of 1000 V, and it was found that for 50 mM and 200 mM borate, currents in excess of 300 μA were reached, which is the maximum current of the power source. The same result was found for 0.1 M and 0.01 M sodium hydroxide. However, current was between 40 and 50 μA at 1000 V for 0.001 M sodium hydroxide, so it was chosen as the BGE for the remainder of the experiments conducted thus far. While a low concentration of sodium hydroxide had low enough conductivity to limit current flow through the chip to below 300 μA, it does not have a good buffering capacity. BGE solutions with low conductivity as well as good buffering capacity (e.g. buffers such as MES or HEPES, which have relatively larger molecular weights and therefore lower mobilities resulting in lower conductivity⁻) will be tested in future chips.
Phenol red dye was prepared at a concentration of 8 mM in 0.001 M sodium hydroxide, and appropriate ranges of applied potential and flow rate were tested on the EFD device chip with collection channels of different lengths. Potentials of 200, 500, and 1000 V were applied at the sample inlet and collection channels along with flow rates of 0.1, 0.2, 0.5, and 1.0 mL/hr. It was not possible to reliably apply a potential of 200 V using the high voltage power source, but both 500 and 1000 V worked well to move the dye, with dye migrating more rapidly at 1000 V. A flow rate of 1.0 mL/hr was too high for the dye to overcome, and 0.1 mL/hr was too low to control the dye. A flow rate of 0.2 or 0.5 mL/hr was found to control dye movement sufficiently to direct it into a collection channel.

4.3.3 Theoretical Modelling of Glycoprotein Desalting by EFD Device

Simulations have been conducted to model the theoretical ability of one of the fabricated EFD chips to desalt proteins. A simulation was run to separate three analytes, one of which was negatively charged, while the other two were positively charged (Figure 4.5). Potential was applied at the sample inlet (1000 V) and each collection channel (< 1000 V), and BGE was introduced from the bottom of the main channel at a flow rate of \(9 \times 10^{-12} \text{ m}^3/\text{s}\). The negatively charged analyte remains at the sample inlet, which has a positive potential applied to it. The positively charged analyte with the lowest electrophoretic mobility \(5 \times 10^{-14} \text{ s} \cdot \text{mol/kg, to simulate the mobility of a glycoprotein}\) migrated to the first collection channel, while the positively charged analyte with the higher electrophoretic mobility \(9 \times 10^{-13} \text{ s} \cdot \text{mol/kg, to simulate a cation}\) did not enter the first collection channel and instead collected in the second channel. This simulation provides a base of theoretical support for the use of the EFD chip to act as a desalting device.
Figure 4.5: COMSOL simulation, with an applied potential of 1000 V and an applied flow rate of $9 \times 10^{-12}$ m$^3$/s, showing (A) the collection of a negatively charged analyte (electrophoretic mobility: $9 \times 10^{-13}$ s · mol/kg) at the sample inlet, (B) the collection of a positively charged analyte (electrophoretic mobility: $5 \times 10^{-14}$ s · mol/kg) in the first collection channel, (C) no collection of a second positively charged analyte (electrophoretic mobility: $9 \times 10^{-13}$ s · mol/kg) at first collection channel, and (D) the migration of the second positively charged analyte in the second collection channel and also down towards ground.

4.3.4 Collection Channel Dimension Manipulation Simulations

COMSOL Multiphysics software has been used to simulate several situations on the EFD device chip. The effects of collection channel width and length on the electric field and flow velocity in the chip have been simulated. The validity of manipulating channel dimensions in order to operate the EFD device using a single power source was tested. The trends obtained for the
effect of width and length on electric field and flow velocity were used to investigate analyte migration and selectivity in the chip, and to try to quantify electrophoretic selectivity for a separation of two analytes with different electrophoretic mobilities.

The electric field and flow velocity generated at the channel junctions for chips with different collection channel widths and lengths were simulated. The same potential was applied at the sample inlet and the collection channel to simulate the use of a single power source, and pressure was applied from the bottom of the main channel. Parametric sweeps were conducted to vary the width or length of a single collection channel. The resulting electric field or flow velocity values in the collection channel and in the main channel immediately preceding the junction were collected and plotted as a ratio between the two channels (Figures 4.6 and 4.7).
Figure 4.6: Plot of the relative electric field in the collection channel (EFCC) over the electric field in the main channel (EFMC) against collection channel width (left y axis) for an applied potential of 1000 V (at both the sample inlet and collection channel outlet), and a plot of the relative flow velocity in the collection channel \((vf_{CC})\) over the flow velocity in the main channel \((vf_{MC})\) against collection channel width (right y axis) for an applied flow rate of \(9 \times 10^{-12} \text{ m}^3/\text{s}\). Collection channel width varied from 10 to 500 \(\mu\text{m}\) in steps of 10 \(\mu\text{m}\), while the main channel width remained 100 \(\mu\text{m}\).
Figure 4.7: Plot of the relative electric field in the collection channel (EFCC) over the electric field in the main channel (EFMC) against collection channel length (left y axis) for an applied potential of 1000 V (at both the sample inlet and collection channel outlet), and a plot of the relative flow velocity in the collection channel (vf,CC) over the flow velocity in the main channel (vf,MC) against collection channel length (right y axis) for an applied flow rate of 9x10^{-12} m^3/s. Collection channel length varied from 3000 to 30,000 μm in steps of 1000 μm, while the main channel length from sample inlet to collection channel junction remained 14000 μm.

4.3.4.1 Manipulation of Channel Width Simulations

As seen in Figure 4.6, as width of the collection channel increases, the difference in electric field between the collection channel and main channel is small, while the difference in flow velocity between the two channels is larger. The migration of an analyte particle through the chip
is directed by its velocity. The velocity of an analyte particle is defined by the magnitude of the opposing driving forces of flow velocity, which is directly proportional to flow rate, and electrophoretic velocity, which is directly proportional to electric field. In order for an analyte to migrate towards the collection channel outlet, the particle velocity must be greater in the collection channel than in the main channel. The electrophoretic velocity ($\vec{u}_{ep}$) and flow velocity ($\vec{u}_f$) terms of particle velocity ($\vec{u}_p = \vec{u}_{ep} + \vec{u}_f$) are affected by changes in the electric field and flow velocity in the channels, and are therefore affected by changes in the collection channel width. For a practical microfluidic channel width range of 50 to 200 μm, the difference in electric field between the two channels is minimal. However, the difference in flow velocity between the two channels is large for this width range, and affects the particle velocity much more than electric field. Therefore, the applied flow rate can be used as the driving force in separations for collection channels with different widths.

### 4.3.4.2 Manipulation of Channel Length Simulations

Figure 4.7 shows the relationship between collection channel length and electric field or flow velocity. As length of the channel increases, both analyte particle driving forces (electric field and flow velocity) decrease with strong covariance, and do not affect the particle velocity direction. Therefore, the analyte separation can be affected by flow velocity only when a single potential is applied across the chip.

### 4.3.4.3 Theoretical Modelling of Analyte Selectivity

Having determined a mechanism by which to direct analyte migration, the degree of electrophoretic selectivity for analyte separation was simulated. A chip design using one collection channel with a width of 150 μm compared to a main channel width of 100 μm was drawn and a parametric sweep was performed for the potential applied to both the sample inlet and collection
A parametric sweep was also performed for the applied flow rate. Two analytes were simulated, one with a fixed electrophoretic mobility \((5 \times 10^{-14} \text{ s}\cdot\text{mol/kg})\) while the electrophoretic mobility for the other varied from the fixed analyte by a difference of 5, 20, and 100%. Concentration data was collected for the collection channel and plotted as a colored contour plot (Figure 4.8A), and then the difference between the concentration data for the analyte with an electrophoretic mobility of \(5 \times 10^{-14} \text{ s}\cdot\text{mol/kg}\) and the analyte at each of the other electrophoretic mobilities was also plotted (Figure 4.8B-D). The plot of absolute analyte concentration in the collection channel shows a trend of collection into the channel as both potential and flow rate increase, indicating the required balance between separation driving forces directing analyte migration. The difference plot shows the degree of accumulation in the collection channel between the two analyte concentrations, and the relative contour intensity gives an idea of the electrophoretic selectivity for one analyte over another based on differences in electrophoretic mobility for a set of potential and flow rate conditions. In plots B-D, a positive contour height indicates that the original analyte has collected preferentially into the collection channel. The more positive the contour height value, the less contaminated the analyte is by the presence of the second analyte in the collection channel, indicating the selectivity of the device at each given combination of applied potential and flow rate.
Figure 4.8: Coloured contour plots showing (A) the concentration in the collection channel of an analyte (electrophoretic mobility: $5 \times 10^{-14}$ s · mol/kg) as applied potential ranges from 200 to 1000 V in steps of 100 V, and applied flow rate ranges from $5 \times 10^{-13}$ to $1 \times 10^{-12}$ m$^3$/s in steps of $5 \times 10^{-14}$ m$^3$/s, (B) the difference plot of the concentration of the first analyte (electrophoretic mobility: $5 \times 10^{-14}$ s · mol/kg) and a second analyte (electrophoretic mobility: $5.25 \times 10^{-14}$ s · mol/kg) over the same applied potential and flow rate ranges, (C) the difference plot of the concentration of the first analyte and a second analyte (electrophoretic mobility: $6 \times 10^{-14}$ s · mol/kg) over the same applied potential and flow rate ranges, and (D) the difference plot of the concentration of the first analyte and a second analyte (electrophoretic mobility: $1 \times 10^{-13}$ s · mol/kg) over the same applied potential and flow rate ranges.
4.3.4.4 Theoretical Modelling of the Effects of Multiple Collection Channels on Electric Field Strength and Flow Velocity

It was expected that an increase in channel width would cause no change in electric field strength while decreasing flow velocity in the collection channel between collection channels. An increase in channel length was expected to result in both a lower electric field strength and lower flow velocity between collection channels. However, when an EFD chip with multiple collection channels of different dimensions positioned along the main channel was simulated, the results showed that the electric field strength was greater in collection channels with both wider and longer channels, and flow velocity was greater in both cases as well. When collection channels with different width or length dimensions were combined into one EFD chip, the potential drop across the channels including the main channel had not been taken into account. Proximity to the pressure source and surface interactions were not considered for flow velocity. Regardless of collection channel width or length, the electrical resistance of the main channel would cause a potential drop towards ground, and would decrease the potential at each junction further down the main channel. This arrangement results in a greater potential difference between the end of the collection channel and the junction, resulting in a greater electric field in collection channels positioned further down the main channel. In terms of flow velocity, channels that are closer to the pressure inlet experience more flow velocity at each junction as the fluid flow splits. The results of these simulations suggest that the distances between the collection channels as well as collection channel position along the main channel may play a more significant role in separations than the relative dimensions of the collection channels themselves.
4.4 Concluding Remarks

Initial operation of the fabricated glass EFD microfluidic chips with dyes has shown a need for electrolysis suppression. EOF should also be eliminated in order to separate analytes based solely on electrophoretic mobility. Simulations have suggested that the EFD device is theoretically capable of desalting samples prior to introduction into MS by directing the glycoprotein of interest into a specific collection channel, while directing anions and cations into different channels.

The effects of collection channel dimension manipulations to allow operation of the device using a single power source were also simulated and found to be limited to flow velocity control by collection channel width manipulation. Simulations have also been conducted to determine the ability of the device to selectively separate analytes based on electrophoretic mobility. The chip geometry in terms of collection channel position and spacing was also shown to be an important factor to optimizing chip performance. The combination of the results of each of these components of the project will yield a more functional EFD device, capable of achieving more meaningful separations.

Long-term device developments will take the form of new chip design iterations. By manipulating the width dimension of the collection channels, it may be possible to selectively separate analytes using a single power source for a flow velocity controlled regime. Further optimization of the chip in terms of main channel dimensions as well as chip geometry will improve the selective separation ability of the device. The pressure inlet will be re-positioned such that it is located closer to the collection channel than the junction for the ground channel. As new versions of the device are produced, electrophoretic selectivity will be enhanced.
Once the design of the device has been optimized, new chips can be fabricated to physically test glycoprotein desalting prior to mass spectrometric detection using a single power supply and the purification of N-linked glycans extracted from human serum EPO.
Chapter 5: Conclusion and Future Work

5.1 Concluding Remarks

The work presented in this thesis describes various techniques developed and implemented in the study of the therapeutic glycoprotein erythropoietin (EPO). Different strategic approaches have been identified to differentiate exogenous EPO from endogenous EPO that focus on slight differences found in the glycan component of the molecule. The goal of this thesis was to further develop techniques to separate and identify exogenous and endogenous EPO based on aspects EPO N-linked glycosylation.

Capillary electrophoresis coupled to mass spectrometry through an interface previously developed by our group was used to study the sialic acid residues of N-linked glycans, which comprise the majority of the glycosylation found on EPO. The effects of post-column chemical environment manipulation on the detection of sialic acids were studied to optimize a CE-ESI-MS method. The optimized method was found to have a LOD sufficient for the detection of the low abundance of N-glycolylneuraminic acid (Neu5Gc) found on exogenous EPO. The minimum sample volume necessary for reproducible and reliable injections for CE was determined to be 1 µL which further enhanced the LOD of the developed method.

Differences in the acidic nature of the glycans of EPO due to the source of the protein and the sialic residues present affect the isoelectric point of each glycoform of EPO, allowing capillary isoelectric focusing (cIEF) to be used as an effective technique for separating EPO from different sources. A cIEF with laser-induced fluorescence (LIF) detection method was developed to differentiate between EPO produced recombinantly from CHO cells and EPO extracted from human serum. The method was optimized for sensitivity, and resolution between proteins and between glycoforms. The method was used to determine the isoelectric points of six detected
glycoforms of exogenous EPO and the isoelectric point of endogenous EPO extracted from serum, and to separate EPO produced in CHO cells from serum-extracted EPO.

Sample preparation, such as protein desalting prior to mass spectrometric analysis, is a time-consuming task and often lacks automation. The development of microfluidic devices to tackle sample preparation steps in an automated fashion allows for a more streamlined approach to sample preparation. Simulations of the EFD device for desalting glycoproteins using a single power supply have shown that the device could be an effective tool in sample preparation for glycoprotein analysis.

Erythropoietin is a well-known and well-studied glycoprotein that is of both clinical and regulatory interest. The study of erythropoietin provides further insights into the characterization of therapeutic glycoproteins as well as techniques for protecting the integrity of sport and the health of athletes.

5.2 Future Research

5.2.1 Developing Internal Standards to Quantify Free Sialic Acids using CE-MS

A method to detect sialic acids using CE-ESI-MS was developed in Chapter 2, and could be used to quantify sialic acids. To quantify sialic acid residues using mass spectrometric detection, an internal standard is required to compensate for any variations in signal intensity due to the detector response and any matrix effects leading to ion suppression during ionization.\textsuperscript{198} An isotopically-labeled analyte is an effective internal standard as it differs from the analyte only in molecular mass, and otherwise behaves in the same way as the analyte. Using borohydride and borodeuteride to reduce sialic acids\textsuperscript{199,200} and add either a hydrogen atom or a deuterium atom, isotopically-labelled analytes and internal standards can be synthesized and used to accurately quantify Neu5Ac and Neu5Gc. This technique could then be used to determine an endogenous
threshold of Neu5Gc from serum-extracted EPO and differentiate between any exogenous EPO that may be present and endogenous EPO.

5.2.2 Developing a cIEF-ESI-MS Method for Human Serum EPO Detection and Quantification

In Chapter 3, a cIEF-LIF method was developed to separate exogenous and endogenous EPO. Further developing this method to allow mass spectrometric detection would provide an extra dimension of analysis, as the mass spectrometer could be used to provide secondary confirmation of the identity of the analytes. The cIEF-MS method would also allow for more accurate quantification of the EPO glycoproteins rather than qualitative detection only. cIEF coupled to MS has been shown to be a highly effective technique for protein analysis.201–203

5.2.3 Desalting Extracted EPO on the EFD Device

In Chapter 4, simulations were used to show that the EFD microfluidic device has the potential to be used for protein desalting prior to mass spectrometric detection. The most commonly used approach for EPO desalting currently is the use of centrifugal filters, which is time-consuming and cannot be integrated on-line into sample analysis. The EFD device operates in an automated fashion and could be used to desalt glycoproteins such as EPO prior to mass spectrometric analysis, as well as to purify and concentrate samples extracted from serum for further analysis.

5.2.4 Comparing the Glycoprofiles of Recombinant Human EPO Produced in Human Embryonic Kidney Cells against Endogenous Human EPO

EPO produced recombinantly in human cell lines, such as human embryonic kidney (HEK) cells is assumed to be identical to endogenous EPO. Certainly the amino acid sequence of the two glycoproteins is identical because the amino acid sequence is encoded in the recombinant DNA
used to produce the glycoprotein, but there is no data found in the literature to support the claim that the glycosylation patterns of the two differently sourced EPO is identical. I hypothesize that, due to differences in cell line culture conditions and the human body, there would be identifiable differences in the glycans found on EPO produced by HEK cells than EPO produced in the human body. Using a tool such as MALDI-TOF MS, the glycoprofiles of EPO from HEK cells and EPO extracted from human serum or urine could be analyzed and compared to determine if they statistically significant different. Individual glycans could be purified from each type of EPO using the EFD microfluidic device prior to MALDI-TOF MS analysis.
Bibliography


Accessed: 26 February 2020


46. Takegawa, Y., Ita, H., Keira, T., Deguchi, K., Nakagawa, H., & Nishimura, S. Profiling of N- and O-glycopeptides of erythropoietin by capillary zwitterionic type of hydrophilic


138. Lee, H. S., Qi, Y. & Im, W. Effects of N-glycosylation on protein conformation and
5, 8926 (2015).

139. Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological
Products: ICH. http://www.ich.org/products/guidelines/quality/quality-
single/article/specifications-test-procedures-and-acceptance-criteria-for-

140. Rohrer, J. S., Basumallick, L. & Hurum, D. High-performance anion-exchange
chromatography with pulsed amperometric detection for carbohydrate analysis of

141. Rohrer, J. S., Thayer, J., Weitzhandler, M. & Avdalovic, N. Analysis of the N-
acetylneuraminic acid and N-glycolylneuraminic acid contents of glycoproteins by high-pH
anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD).

142. Anumula, K. R. Rapid Quantitative Determination of Sialic Acids in Glycoproteins by
High-Performance Liquid Chromatography with a Sensitive Fluorescence Detection. Anal.

143. Van der Ham, M., Prinsen, B. Huijmans, J., Abeling, N., Dorland, B., Berger, R., Koning,
T., & Velden, M. Quantification of free and total sialic acid excretion by LC–MS/MS. J.

144. Matsuno, K. & Suzuki, S. Simple fluorimetric method for quantification of sialic acids in
145. Lasne, F., Martin, L., Martin, J. A. & de Ceaurriz, J. Isoelectric profiles of human 

146. Mozafari, M., Nachbar, M. & Deeb, S. E. Precise small volume sample handling for 

147. Šlampová, A. & Kubáň, P. Injections from sub-μL sample volumes in commercial capillary 


150. Klampfl, C. W. & Himmelsbach, M. Sheath Liquids in CE-MS: Role, Parameters, and 
Optimization. in *Capillary Electrophoresis - Mass Spectrometry (CE-MS): Principles and 

151. Foret, F., Thompson, T. J., Vouros, P., Karger, B. L., Gebauer, P., & Bocek, P. Liquid 
Sheath Effects on the Separation of Proteins in Capillary Electrophoresis/Electrospray Mass 

152. Jayo, R. G., Thaysen-Andersen, M., Lindenburg, P. W., Haselberg, R., Hankemeier, T., 


157. N-Glycolylneuraminic acid (HMDB0000833).


158. Sialyllactose (HMDB0006569). *Human Metabolome Database*


Appendices

Appendix A

Figure A.1: Example of a small volume sample vial used in Ch. 2.
Figure B.1: LED apparatus used to photobleach ampholytes in Ch. 3.
Table B.1: Characteristics of EPO, beta-lactoglobulin A, and ovalbumin for cIEF and labeling with Chromeo P503

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (kDa)</th>
<th>pI Value</th>
<th>Lysine residues</th>
<th>Total amino acids</th>
<th>Percentage of lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythropoietin</strong></td>
<td>30.4</td>
<td>4.4-5.2</td>
<td>8</td>
<td>165</td>
<td>4.8%</td>
</tr>
<tr>
<td><strong>Beta-lactoglobulin A</strong></td>
<td>18.4</td>
<td>5.1</td>
<td>15</td>
<td>162</td>
<td>9.3%</td>
</tr>
<tr>
<td><strong>Ovalbumin</strong></td>
<td>42.7</td>
<td>4.43-4.66</td>
<td>20</td>
<td>386</td>
<td>5.2%</td>
</tr>
</tbody>
</table>
Figure B.2: N-linked glycans with sialic acid structures of Neu5Ac (left) and Neu5Gc (right).