Employing Exoglycosidase And Transferase Enzymes in Capillary Nanogel Electrophoresis for the Determination of N-glycan Linkages and Enzyme Michaelis-Menten Constants.

Lloyd Bwanali
West Virginia University, llbwanali@mix.wvu.edu

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Part of the Analytical Chemistry Commons

Recommended Citation
https://researchrepository.wvu.edu/etd/8273

This Dissertation is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Dissertation has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.
Employing Exoglycosidase And Transferase Enzymes in Capillary Nanogel Electrophoresis for the Determination of N-glycan Linkages and Enzyme Michaelis-Menten Constants.

Lloyd Bwanali

Dissertation submitted to the Eberly College of Arts and Sciences at West Virginia University in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry

Lisa A. Holland, Ph.D., Committee Chair
Kathleen Brundage, Ph.D.
Harry O. Finklea, Ph.D.
Peng Li, Ph.D.
Stephen Valentine, Ph.D.

Department of Chemistry

Morgantown, West Virginia
2021

Keywords: N-glycans, Enzymes, Exoglycosidase, Transferase, Capillary Electrophoresis, Sialic Acids, Michaelis-Menten

Copyright 2021 Lloyd Bwanali
Abstract

Employing Exoglycosidase And Transferase Enzymes in Capillary Nanogel Electrophoresis for the Determination of N-glycan Linkages and Enzyme Michaelis-Menten Constants.

Lloyd Bwanali

As a post translational modification protein glycosylation plays a crucial role in protein signaling, binding, kinetics and folding. In disease diagnosis and prognosis, monitoring glycosylation has been identified as a biomarker. Sialylation and sialic acid linkage in N-glycans are markers of cancers including liver, pancreatic and kidney cancer. Quantification of sialic acid linkage is analytically challenging because of the diverse linkages and the presence of heterogenous branching. A capillary electrophoresis method is reported that integrates a unique combination of enzymes and lectins to modify sialylated asparagine-linked glycans (N-glycans) in real time in the capillary so that N-glycan structures containing α2–6-linked sialic acid are easily separated, detected, and quantified. N-glycans were sequentially cleaved by the enzymes at the head of the separation capillary so that the presence of α2–6-linked sialic acids corresponded to a shift in the analyte migration time in a manner that enabled interpretation of the N-glycan structure. Complex N-glycans from α-1-acid glycoprotein were analyzed using this approach, revealing that a limited number of α2–6-linked sialic acids were present with biantennary, triantennary, and tetraantennary N-glycans of α-1-acid glycoprotein (AGP) generally containing 0 or 1 α2–6-linked sialic acid. The capillary electrophoresis method quantified the sialic acid linkages using nanoliter volumes of enzyme and lectins. Conversion with enzyme was in real time and incubation and separation occurred in less than 40 minutes.

In biotherapeutics glycosylation of IgG is a critical attribute, and modification of the glycosylation will influence the IgG efficacy. Glycosyltransferases have been employed to modify this glycosylation. Capillary nanogel electrophoresis was utilized to create discrete regions for an online galactosyltransferase reaction and subsequent separation of substrate and product. The β1-4 galactosyltransferase enzyme, donor, and co-factor were patterned in the capillary. The substrate was driven through these zones and converted to galactosylated products which were separated and identified. The degree of glycosylation was discernable. The method was applied in establishing the Michaelis-Menten value, $K_M$ of the enzyme. Additionally, the method was adapted to transfer galactose residues to protein. The applicability of the method for real-time online modification of whole protein was demonstrated with the Herceptin glycoprotein. The method demonstrated the applicability of capillary electrophoresis to characterize glycosyltransferases. This method is compatible with low enzyme or substrate volumes and is fast and automated.
Acknowledgements

I would like to acknowledge my research advisor, Dr. Lisa A. Holland for the outstanding mentoring during my Ph.D. studies. I will always be grateful for the opportunities she afforded me during the graduate studies, which will continue to be an invaluable asset in my career. Her belief that we can always go one step further, will always push me on. Thank you, Dr. Holland, for helping to give me the necessary acumen for my professional development. It is my hope and prayer that you remain steadfast in your commitment to furthering chemistry graduate research at WVU.

I would like to thank the rest of my graduate committee: Dr. Harry O. Finklea, Dr. Stephen J. Valentine, Dr. Peng Li, and Dr. Kathleen Brundage for your advising efforts during my graduate career. Your investment in terms of time, patience, and insight into my dissertation is greatly appreciated.

I would acknowledge and thank the National Institute of Health who provided funding through grant number R01GM114330.

Lastly, I would like to acknowledge my lab mates (past and present): Cassandra Crihfield, Srikanth Gattu, Gayatri Gautam, Vincent Nyakubaya, Courtney Kristoff, Lindsay Veltri, Grace Lu, Tyler Davis, Patrick Russo, Ebenezer Newton and Marriah Ellington. Thank you for the assistance, support, and teamwork. You made my graduate career more navigable.
Dedication

"We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours." John of Salisbury

To my parents Fredrick and Alice Bwanali. You always believed we can “see more, and things that are more distant.” To Chrissylee Bwanali for the faith and strength. I also dedicate this dissertation to my brothers and sisters, Memory, Wellington, Regina, Taonga and Aaron. You were a great source of inspiration, support, love, and motivation.

To Alexander Matidaise Bwanali and Alden Munashe Bwanali for telling me: “Dad you do the coolest stuff in the world. When we grow up, we want to do what you do.”
# Table of Contents

Abstract ............................................................................................................................ ii
Acknowledgements ......................................................................................................... iii
Dedication ....................................................................................................................... iv
Table of Contents .......................................................................................................... v
List of Figures................................................................................................................ viii
List of Tables .................................................................................................................. xi
List of Symbols/Nomenclature .................................................................................... xiii
Chapter 1 Introduction and Background on Glycosylation Modification and Analysis with Capillary Electrophoresis ................................................................. 1
  1.1 Introduction ............................................................................................................ 2
    1.1.1 Glycosylation significance. ........................................................................... 2
    1.1.2 Advantages of capillary electrophoresis in glycosylation analysis. ............... 2
    1.1.3 Dissertation overview .................................................................................. 3
  1.2 Background ........................................................................................................... 3
    1.2.1 From monosaccharides to glycans. ............................................................... 3
    1.2.2 Capillary electrophoresis as a tool for glycan analysis. ................................ 7
    1.2.3 Sialic acid detection and quantification ....................................................... 19
    1.2.3 Biological reagents for asparagine-linked glycan analysis in capillary electrophoresis .............................................................................................................. 26
  1.3 Conclusions ......................................................................................................... 33
  1.3 Acknowledgements .............................................................................................. 33
  1.4 References ........................................................................................................... 34
Chapter 2 : Quantification of the α2-6 Sialic Acid Linkage in Branched Asparagine-Linked Glycan Structures with Capillary Nanogel Electrophoresis .............................................................................. 45
  2.1 Introduction ......................................................................................................... 46
  2.2 Materials and methods ...................................................................................... 49
    2.2.1 Chemicals and reagents ............................................................................. 49
    2.2.2 Preparation and derivatization of standards .............................................. 50
List of Figures

Figure 1.1 Six representative structures of D conformer monosaccharides ....................... 4
Figure 1.2 N-glycan core structure and main classes ............................................................. 5
Figure 1.3 Diversity in asparagine linked glycan structures due to different monomers, linkages or branching ......................................................................................................................... 6
Figure 1.4 Illustration of with main components of capillary electrophoresis ....................... 7
Figure 1.5 Illustration of charge distribution in the capillary when the pH is above 4 .......... 9
Figure 1.6 Conceptual diagram of separation of mixture of analytes with capillary electrophoresis ........................................................................................................................................... 10
Figure 1.7 Electropherograms of Immunoglobulin G asparagine-linked glycans obtained with nanogel ............................................................................................................................................... 14
Figure 1.8 Schematic of the reaction for the reductive amination labeling of asparagine-linked glycans ........................................................................................................................................ 16
Figure 1.9 Effects of 2-aminobenzoic acid reaction conditions on the peak area .............. 17
Figure 1.10 Electropherogram showing the separation of α2-3 and α2-6 linked sialic acids in asparagine-linked glycans ................................................................................................................................. 23
Figure 1.11 Electropherogram of asparagine-linked glycans with α2-3 and α2-6 linked sialic acids from human plasma .................................................................................................................. 24
Figure 1.12 Illustration of exoglycosidase enzymes and the glycosidic bond they cleave ....................................................................................................................................................... 27
Figure 1.13 Illustration of a glycosyltransferase reaction where monomer is transferred from donor to acceptor ..................................................................................................................................... 29
Figure 2.1 Conceptual diagram of quantification of α2-6 sialic acids in a sample with both α2-3 and α2-6 sialic acids ............................................................................................................. 48
Figure 2.2 Illustration of in capillary reaction showing that presence of α2–6-linked sialic acid is related to galactose residues following processing with serial enzymes ............ 53
Figure 2.3 Concentration range of α2-3 sialidase for triantennary asparagine-linked glycan ........................................................................................................................................... 61
Figure 2.4 Online serial enzyme sequencing of α-1-acid glycoprotein sample 1 ............ 65
Figure 2.5 Establishing the working range of α2-3 sialidase enzyme concentration for α-1-acid glycoprotein sample 1 ................................................................................................................... 70
Figure 2.6 Establishing the working range of α2-3 sialidase enzyme concentration for α-1-acid glycoprotein sample 2 .......................................................................................................................... 74
Figure 2.7 Analyses of α-1-acid glycoprotein sample 2 asparagine-linked glycans ...... 78
Figure 2.8 Identification of glycan and contaminant peaks in Figure 2.7 .................... 79
Figure 3.1 Conceptual diagram illustrating the online reaction where the transferase enzyme adds a monomer to an acceptor biantennary glycan, pentasaccharide, that has been end labeled with aminobenzoic acid ........................................................................................................ 95
Figure 3.2 Before and after cleanup samples of 2-aminobenzoic acid labeling reaction. ........................................................................................................................................... 99
Figure 3.3 Impact of manganese zone on biantennary N-glycan conversion ............. 102
Figure 3.4 The process of in capillary galactosyltransferase enzyme reaction .......... 106
Figure 3.5 Determination of the Michaelis-Menten for the galactosyltransferase enzyme. ........................................................................................................................................ 111
Figure 3.6 Calibration curve used to establish relationship between concentration and area ................................................................................................................................................... 112
Figure 3.7 The Michaelis-Menten curves showing reproducibility of method. ........ 114
Figure 3.8 Online modification of glycans recovered from Herceptin ..................... 117
Figure 3.9 Confirmation of online modification of glycans recovered from Herceptin with lectin ......................................................................................................................................................... 119
Figure 3.10 Flow injection analysis done by pushing APTS with 10% nanogel ........ 121
Figure 3.11 Online modification of glycan on intact IgG1 glycoprotein .................... 124
Figure 3.12 Establishing the amount of modification at different enzyme to substrate ratio as evidenced by lectin retardation of modified Herceptin ......................... 126
Figure 3.13 Confirmation that presence of enzyme in capillary caused shifting of Herceptin peaks ........................................................................................................................................ 128
Figure 4.1 Degradation of sialic acids due to heat ................................................... 140
Figure 4.2 Online reaction employing α2-3 sialyl transferase to modify standard biantennary asparagine-linked glycan ..................................................................................................................... 144
Figure 4.3 Online modification of standard agalacto biantennary asparagine-linked glycan in 3-(N-morpholino)-propanesulfonic acid pH 7. .......................................................... 148
Figure 4.4 Online modification of standard agalacto biantennary glycan in 2-(N-Morpholino)ethanesulfonic acid pH 6.5. ............................................................ 149
Figure 4.5 Online modification of Herceptin asparagine-linked glycans in 2-(N-Morpholino)ethanesulfonic acid pH 6.5. ............................................................ 150
Figure 4.6 Modification of intact Herceptin glycoprotein when enzymatic conversion pH is different to separation pH. ....................................................... 152
Figure 4.7 Separation of Alexaflour 488 labeled Protein G. ..................................... 154
Figure 4.8 Online labeling of Herceptin with Alexaflour 488 tagged Protein G. ....... 156
List of Tables

Table 1.1 Some glycosyltransferases and the monomers they add. ................................. 29
Table 1.2 Some lectins used in N-glycan analysis. ............................................................... 32
Table 2.1 Normalized areas for Figure 2.2B employed for establishing α2-6 linked sialic acid in triantennary standard using multiple enzymes ................................................. 57
Table 2.2 Raw areas for Figure 2.2B employed for establishing α2-6 linked sialic acid in triantennary standard using multiple enzymes ...................................................... 57
Table 2.3 Normalized areas for Figure 2.2C used to validate α2-6 linked sialic acid in triantennary standard using α2-3 sialidase ................................................................. 59
Table 2.4 Raw areas for Figure 2.2C used to validate α2-6 linked sialic acid in triantennary standard using α2-3 sialidase ................................................................. 59
Table 2.5 Comparison of triantennary glycan to internal standard areas across instruments for each enzyme condition ................................................................. 60
Table 2.6 Figure 2.3 normalized areas of α2-3 sialidase working concentration range for triantennary standard ................................................................. 62
Table 2.7 Figure 2.3 and Table 2.6 raw areas of α2-3 sialidase working concentration range for triantennary standard ................................................................. 63
Table 2.8 Peak area of α2-6 linked sialic acid from α-1-acid glycoprotein glycans ............. 66
Table 2.9 Figure 2.4 normalized peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 1 ................................. 67
Table 2.10 Figure 2.4 raw peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 1 ................................. 68
Table 2.11 Figure 2.5 normalized areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 1 ................................................................. 71
Table 2.12 Figure 2.5 raw areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 1 ................................................................. 72
Table 2.13 Comparison of α-1-acid glycoprotein sample 1 glycan area to internal standard area across instruments for each enzyme condition ............................. 72
Table 2.14 Figure 2.6 normalized areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 2 ................................................................. 75
Table 2.15 Figure 2.6 raw areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 2. .......................................................... 76
Table 2.16 Comparison of α-1-acid glycoprotein sample 2 glycan area to internal standard area across instruments for each enzyme condition ........................................ 76
Table 2.17 Peak area of α2-6 linked sialic from α-1-acid glycoprotein sample 2 glycans. ............................................................................................................. 80
Table 2.18 Figure 2.7 normalized peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 2. ........................................... 81
Table 2.19 Figure 2.7 raw peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 2. .................................................. 82
Table 3.1 : Impact of manganese zone on biantennary N-glycan conversion ............ 102
Table 3.2 Line equations and errors for 3 sets of data used to achieve the calibration curve. .................................................................................................................. 112
Table 3.3 Areas used to achieve the Calibration Curve. ............................................. 113
Table 3.4 Reproducibility in substrate peaks for n=3 runs used for K_M. ................. 115
Table 3.5 Reproducibility in product peaks for n=3 runs used for K_M ..................... 115
Table 3.6 Area distribution of substrate and product with changing enzyme concentration ........................................................................................................... 118
Table 3.7 % distribution of substrate and product with changing enzyme concentration. ............................................................................................................. 118
Table 4.1 Relative abundance by area of α2-6 sialic acid of heat treated α-1-acid glycoprotein asparagine-linked glycans ......................................................... 141
## List of Symbols/Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>EPH</td>
<td>Electrophoretic mobility</td>
</tr>
<tr>
<td>AGP</td>
<td>α-1-acid glycoprotein</td>
</tr>
<tr>
<td>2AB</td>
<td>2-Aminobenzoic acid</td>
</tr>
<tr>
<td>ECL</td>
<td><em>Erythrina Cristagalli Lectin</em></td>
</tr>
<tr>
<td>AAL</td>
<td><em>Aleuria Aurantia Lectin</em></td>
</tr>
<tr>
<td>MAL</td>
<td><em>Maackia Amurensis Lectin</em></td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus Nigra Agglutinin</em></td>
</tr>
<tr>
<td>GAL T</td>
<td>β1-4 Galactosyltransferase</td>
</tr>
<tr>
<td>N-glycan</td>
<td>Asparagine-linked glycan</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction and Background on Glycosylation Modification and Analysis with Capillary Electrophoresis

1.1 Introduction

1.1.1 Glycosylation significance.
Glycosylation is a protein post translational modification which plays a crucial role in protein signaling, binding\textsuperscript{1}, kinetics and folding.\textsuperscript{2} Glycosylation therefore plays an important role in many physiological processes.\textsuperscript{1, 3} It's importance has been demonstrated as monoclonal antibody glycosylation impacts efficacy\textsuperscript{4, 5} and acute phase protein glycosylation is employed as a biomarker for disease diagnosis and prognosis.\textsuperscript{3, 6} In biotherapeutics, research has focused on how a particular type of glycosylation impacts purification before monitoring efficacy\textsuperscript{7}, how designer glycosylation can be achieved\textsuperscript{4, 8} and the motoring of batch to batch glycosylation.\textsuperscript{5} In disease diagnosis and prognosis, glycosylation research has focused on monitoring changes in the glycosylation with goal to detect and quantify these aberrations thereby linking them to disease states.\textsuperscript{3, 9, 10} New tools are being developed to detect, quantify or modify glycosylation. Advances have been made with capillary electrophoresis to meet these needs.

1.1.2 Advantages of capillary electrophoresis in glycosylation analysis.
Capillary electrophoresis (CE) offers several advantages which makes it an attractive analytical technique for asparagine-linked glycan (N-glycan) analysis and modification. High separation efficiency and high peak capacity is realized in CE making it ideally suited for complex N-glycan analysis.\textsuperscript{11} Investigations in CE are usually fast and multiple capillaries can be employed in a single instruments to increase throughput.\textsuperscript{12} Capillary electrophoresis can be integrated with other techniques like MS\textsuperscript{12-14} thereby increasing the information that is achieved. Capillary electrophoresis is adaptable to carry out real
time reactions and analysis with minimal user intervention. Easy of automation and consuming picolitre to nanoliter volumes of sample. Capillary electrophoresis is suited for analysis with limited biological reagents.

1.1.3 Dissertation overview.
This dissertation is composed of a literature review of status of N-glycan analysis and two manuscripts submitted to the Analytical Chemistry journal. Chapter 1 is a literature review on the fundamentals and importance of glycosylation. Furthermore, background information on capillary electrophoresis, its use in N-glycan analysis and competitive techniques is discussed. The chapter concludes by focusing on enzymes and lectins for glycan identification and modification. Chapter 2 focuses on a cost effective in capillary method that integrates a unique combination of enzymes and lectins to modify sialylated N-glycans in real time. This approach enables separation, detection and quantification of N-glycan structures containing α 2-6 sialic acids. Chapter 3 covers the employment of a thermally reversible nanogel in capillary electrophoresis to create discrete regions for a galactosyltransferase reaction to modify glycans and subsequent separation of substrate and product.

1.2 Background
1.2.1 From monosaccharides to glycans.
Glycans are compounds that have multiple monosaccharide monomers are linked. A monosaccharide is the simplest sugar molecule and cannot be hydrolyzed further into a simpler sugar. Many monosaccharides exist, with the common ones existing as hexose
(six carbon sugar), deoxy-hexose (a hydroxyl group at the 6-position or the 2-position of the hexose) or acetyl or amino substituted hexose. The structures of the common monosaccharides are shown in Figure 1.1. For easier representation, the Consortium of Functional Glycomics has developed nomenclatures to represent monosaccharides. For the 6 monosaccharides illustrated in Figure 1.1, this nomenclature is shown next to the monosaccharide name.

Figure 1.1 Six representative structures of D conformer monosaccharides Glucose, mannose, and galactose are common unsubstituted hexose saccharides. Fucose has one less hydroxyl group and is termed a deoxy-hexose sugar. N-Acetylglucosamine and N-acetyleneuraminic acid represent substituted hexose saccharides.
Monosaccharides link together through the glycosidic bond, and when several of them are connected they form glycans. These glycans can be covalently linked to a variety of biological molecules including proteins and lipids. When a protein has a glycan attached to one of its amino acids, it is referred to as a glycoprotein. Linkage of glycans to protein can be on an asparagine amino acid (N-glycans) or threonine/serine amino acid (O glycans). N-glycan linkage is through the N-acetylglucosamine (GlcNAc) attaching to the nitrogen atom on the asparagine through a β-1N linkage. The common feature of N-glycans is the mannose core as shown in Figure 1.2A. Attachment of other monomers to the mannose core give rise to the complexity observed in N-glycans. Three main classes based on these attachments are high mannose, complex and hybrid as depicted in Figure 1.2B.

A. N-glycan core structure

B. N-glycan classification

Figure 1.2 . N-glycan core structure and main classes.
Complexity and structural diversity of N-glycans is further demonstrated by the presence or absence of monomers, the linkage of these monomers, or the position. Linkage can either be α or β, and which arm has the monomer can be different. Although a variety of N-glycan structures can occur, the biosynthetic pathways of these molecules limit the diversity of structures that are physiologically relevant. Figure 1.3A illustrates the assortment that can be found in complex N-glycans with the addition of different monomers. Figure 1.3B illustrates the two common positional isomers found for sialic acids. The diversity introduced by branching is depicted in Figure 1.3C.

Figure 1.3 Diversity in asparagine linked glycan structures due to different monomers, linkages or branching. A shows variability by the presence or absence of monomers. B shows variability introduced by linkage position of sialic acids. C shows variability due to branching.
1.2.2 Capillary electrophoresis as a tool for glycan analysis.

1.2.2.1 Basics of capillary electrophoresis.

Capillary electrophoresis separates analytes in a capillary based on their charge to size ratio which is a result of two transport mechanisms. The main components of a capillary electrophoresis system are the high voltage power supply, the capillary, the buffer vials, the detector, and a signal processor as depicted in Figure 1.4. The high voltage power source is used to drive analytes in the capillary past the detection window. The most

Figure 1.4 Illustration of with main components of capillary electrophoresis. These components are the high voltage power supply, the capillary, the buffer vials, the detector, and a signal processor.
common modes of optical detection are UV-absorbance and fluorescence, though hyphenated techniques like CE-MS exist. Transport in the capillary is either due electrophoretic mobility or electroosmotic flow. Electrophoretic mobility is based on the migration of the cations towards the cathode and the migration of the anions towards the anode, under the influence of applied voltage. This migration of charged particle is defined by the equation 1.1, where $\mu$ is the electrophoretic mobility, $q$ the analyte charge, $\eta$ is the viscosity of the background electrolyte and $r$ is the analyte radius.

$$\mu = q/(6\pi\eta r)$$

Equation 1.1 shows that highly charged analytes will have greater electrophoretic mobility if they have the same radius with analytes that have a lower charge. In contrast, for analytes with the same charge, lower radius analytes will have a greater electrophoretic mobility compared to those with a larger radius.

The second mode of transport is the electroosmotic flow, which is the bulk flow of the liquid inside capillary. At a pH above 4, the capillary surface is deprotonated and will have a net negative charge. This will result in positively charged ions forming a layer on the capillary surface as shown in Figure1.5. In turn some anions in the bulk solution will form a layer on these cations. When a voltage is applied these cations migrate towards the cathode dragging with them counter ions in solution. This phenomenon leads to the entire buffer solution moving towards the cathode. Electroosmotic flow is pH dependent, and higher pH lead to higher EOF, as a consequence of the capillary surface being more deprotonated.
Apparent mobility in the capillary is the sum of the electroosmotic flow and the electrophoretic mobility. When a separation is carried out in an uncoated capillary the analytes pass the detection region in the order of cations first, followed by neutrals and then anions. For analytes of similar size, the mobility is summarized by vectors as shown in Figure 1.6.

Figure 1.5 Illustration of charge distribution in the capillary when the pH is above 4. The density of the positive charges formed in the ionic layer decreases exponentially with distance from capillary surface.
Figure 1.6 Conceptual diagram of separation of mixture of analytes with capillary electrophoresis. Each analyte is represented to show the corresponding vector for electro-osmotic flow, electrophoretic and the net mobility. The sample mixture consists has five different analytes of cations, neutral and anions with different charge to mass ratio. Reproduced with permission from: Gattu, S.; Crihfield, C. L.; Lu, G.; Bwanali, L.; Veltri, L. M.; Holland, L. A., Advances in enzyme substrate analysis with capillary electrophoresis. *Methods* 2018, *146*, 93-106. Copyright © 2021 Elsevier B.V.
1.2.2.2 Suppressing the electroosmotic flow.

Capillary zone electrophoresis is when the separation is carried out in an unmodified capillary and the two modes of transport detect analyte migration. Challenges with capillary zone electrophoresis include analyte adsorption to the capillary surface, and everything injected in the capillary ends up migrating towards the detector. Negatively charged silanol groups on the capillary surface have been shown to interact with cations and proteins through charge-charge interaction. This poses a challenge for in-capillary reactions where analytes need to be passed through zones of enzymes or lectins. At appropriate pH with suppressed EOF stationary reactions zones can be created in the capillary allowing for real time modification of analytes. In addition with suppressed EOF only analytes of interested, i.e. either cations or anions, are migrated towards the detector simplifying interpretation of the electropherogram. This is advantageous especially in UV-Vis absorbance where most analytes would absorb at the 200 or 214 nm wavelength commonly used for monitoring peptides.

Several methods, including nanogel coating, have been used to passivate the capillary surface and suppress the EOF. Cationic, anionic, and neutral coatings have been reported and some are available commercially. The phospholipid nanogel coating developed in the Holland lab offers several advantages. The nanogel coating is composed of 25 mM 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 50 mM 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) dissolved in the relevant buffer. To make the nanogel, DHPC and DMPC are weighed into a vial and buffer added to get the required concentration. The solution is vortexed until the DMPC and DHPC are
completed dissolved. This is followed by three freeze thaws to ensure the DHPC and DMPC as adequately mixed. The solution is vortexed and aliquoted into vials. The aliquoted nanogels are frozen and have a lifetime of 10 days. The coating is neutral, which makes it versatile for separation of anionic analytes in reverse polarity or cationic analytes in normal polarity. The coating can easily be regenerated and is cost effective, costing less than 5 cents per separation.\textsuperscript{11}

### 1.2.2.3 Phospholipid Nanogel to Enhance Separation

Phospholipid nanogel fills are employed to enhance N-glycan separations.\textsuperscript{11, 19, 20} Phospholipids nanogel are thermally responsive allowing patterning in the capillary to improve separations. The nanogels increase viscosity which translates to an increase in frictional drag.\textsuperscript{20} Equation 1.1 demonstrates that when the viscosity of the separation medium increases, the mobility decreases. The change in mobility allows for the separation of n-glycans with subtle differences in structure. Employing phospholipid nanogels therefore allows for the separation of N-glycans. The morphology of the phospholipid assembly is temperature dependent. At low temperatures phospholipid exist as nano disks and have low viscosity enabling introduction in capillary. When temperature is increased nanoribbons are formed forming a gel like matrix. Phospholipid nanogel have previously been used to improve the separation efficiency N-glycans and resolve comigrating isoforms,\textsuperscript{11} as they increase the separation medium viscosity (which translates to decrease in mobility). In one study, by changing the % composition of the phospholipid nanogel used IgG N-glycans were resolved as demonstrated in Figure 1.7.

The goal of this research was to distinguish the bisected N-glycans which are known to
increase the antibody-dependent cell-mediated cytotoxicity.\textsuperscript{11} Three phospholipid concentrations, 10, 15, and 20 \%, were selected to improve separation resolution of co-migrating peaks 6 and 7. Peak 6 is not bisected and peak 7 is bisected. At lower nanogel concentration these two peaks were comigrating which made quantifying peak 7 challenging. It was demonstrated that with increase in the nanogel composition resolution is improved.
Figure 1.7 Electropherograms of Immunoglobulin G asparagine-linked glycans obtained with nanogel. Separations in A are done at 10% nanogel, B at 15% nanogel and C at 20% nanogel. Separations were obtained using a 25 μm inner diameter capillary at 17 °C with an effective length of 52 cm and a total length of 62 cm. The electric field was 387 V/cm in reversed polarity. Sample was injected at 10 kV for 4 s. Reproduced with permission from: Lu, G.; Holland, L. A., Profiling the N-Glycan Composition of IgG with Lectins and Capillary Nanogel Electrophoresis. Analytical Chemistry 2019, 91 (2), 1375-1383.
1.2.2.4 Glycan derivatization for Capillary

Derivatization of N-glycans is critical for CE analyses to facilitate detection. This labeling process also imparts charge on the neutral N-glycans rendering CE separation possible. CE is a charged based separation, but in N-glycans only the sialylated glycans are charged, with others being neutral. When separations are done in the neutral capillaries, uncharged glycans will have a net mobility of zero, and will not be detected. In an uncoated capillary the uncharged N-glycans will not resolve as the mobility will be the same. Employing charged tags by derivatizing the glycans imparts a negative charge on them, allowing the separation to happen based on the size of the glycan. The choice of the tag added on the N-glycan is based on the method of detection targeted. Laser-induced fluorescence (LIF) and UV-Vis detection has been utilized to detect and identify N-glycans by using fluorophores or chromophores, respectively.\(^{21, 22}\) Common derivatization is done on the reducing end of the N-glycan, and labeling is 1:1, i.e. one tag is added on each N-glycan. Traditionally, this derivatization is done through reductive amination\(^{21-23}\) as shown by the reaction in Figure 1.8.
UV-Vis Derivatization of N-glycans

In UV-Vis absorbance detection, N-glycans are traditionally labeled with 2-aminobenzoic acid (2AB) also called anthranilic acid, at the reducing end of the structure using reductive amination. This labeling method imparts a negative 1 charge on the N-glycans, which allows for the migration of neutral N-glycans under the influence of an electric field. Commercially available filters are used to monitor the absorption of 2AB either at 214 or 254 nm. Several alternatives to 2AB have been reported in literature. 2AB is MS compatible and has been shown to improve sensitivity and has cleaner spectra due to lower interferences, as compared to other UV-Vis tags.
There are several practical considerations for the 2AB labeling strategy. Figure 1.9 summarizes some of these considerations to be noted when carrying out a 2AB reaction. Even though 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labeling reaction are normally carried out in tetrahydrofuran, this solvent significantly decreases labeling efficiency for 2AB labeling. 2AB reactions are therefore normally carried out in methanol. A temperature of 65 °C achieves optimum labeling reaction in less than 2 hrs, with no benefit realized by longer reaction times. When choosing the reaction pH, it has been reported

![Figure 1.9 Effects of 2-aminobenzoic acid reaction conditions on the peak area. A shows the impact of reaction solvent and B shows the impact of pH. C and D shows the impact of temperature, with reactions in c carried out at 65C and reactions in d carried out at 40C. Adapted with permission from: Sato, K.; Sato, K.; Okubo, A.; Yamazaki, S., Optimization of Derivatization with 2-Aminobenzoic Acid for Determination of Monosaccharide Composition by Capillary Electrophoresis. Analytical Biochemistry 1998, 262 (2), 195-197.](image-url)
that very acid or basic pH reduces the reaction efficiency.\textsuperscript{26} A ratio of 7 2AB : 1 N-glycan has been demonstrated to achieve 100\% labeling at these conditions.\textsuperscript{19, 25, 26}

**Fluorescent derivatization of N-glycans**

To enable fluorescence detection, free N-glycans are traditionally labeled with 8-aminopyrene-1,4,6-trisulfonic acid (APTS),\textsuperscript{22, 23} at the reducing end of the structure using reductive amination, although alternatives to this dye are commercially available.\textsuperscript{27} This labeling strategy imparts negative three charges on the N-glycan and facilitate migration in an electric field. The excitation maximum of APTS dye matches the output of an argon ion laser. Several labels have been developed to enable fluorescence detection in CE and mass characterization in MS for CE-MS analysis.

There are several practical considerations to be noted when carrying out the APTS reactions. Incubations are commonly carried out at 37 °C or 65 °C\textsuperscript{11, 19, 28, 29} depending on the type of N-glycan to be labeled. The higher temperature is more desirable as reaction reaches maximum in less than 2 hrs.\textsuperscript{28} This conditions is normally used for desialylated glycans, as when sialylated N-glycans are labeled at this temperature there is loss of sialic acids.\textsuperscript{28} Sialylated N-glycans are normally incubated at 37 °C. Acetic acid at a composition of 20 \% is normally included in the reaction as it has been shown to improve labeling efficiency.\textsuperscript{28} When higher ratios of APTS to N-glycan are utilized, labeling efficiency is improved, but more cleanup to remove excess dye are required after the reaction.\textsuperscript{28}
Though APTS labeling strategies for CE-LIF are well established, improvements upon the reaction continue to be reported. A new strategy addresses the practice of carrying out the reactions in volumes of ≤ 5 mL in closed PCR vials. While increasing concentration of acetic acid leads to more glycan labeling, lower reaction volumes consistently result in more efficient N-glycan labeling. A report demonstrated that reactions subject to evaporation during labeling had higher product yield. Reactions incubated at 50 °C in a 3 mL volume versus a 13 mL volume supplemented with tetrahydrofuran had a 2-fold increase in total area, which was attributed to improved mixing. Reactions incubated at 50°C in a 3 mL volume performed in a closed versus an open vial which had fully evaporated revealed a 4.5-fold improvement in labeling efficiency, with < 5% relative standard deviation in N-glycan peaks measured using CE-LIF.

**1.2.3 Sialic acid detection and quantification**

N-glycans sialylation is related to several diseases, with the α2-3 vs α2-6 sialic acid linkage identified as biomarker for diseases diagnosis and prognosis. However, linkage analysis of sialic acids presents several challenges. Increases in α2-6 have been reported in cancer. The acute phase protein α-1-acid glycoprotein (AGP) sialic acids have been shown as potential biomarkers for various cancers. Complexity in analyzing the distribution of α2-3 vs α2-6 sialic acid is compounded by the heterogenous branching in most samples. Sialic acids are labile and can be lost due to handling or storage. These factors present a challenge in differentiating α2-3 vs α2-6 sialic sialylation. Clinically, sialic acid is quantified by isoelectric focusing, which separates the protein into
bands depending on number of sialic acids present. This current technique cannot differentiate α2-3 vs α2-6 sialylation.

1.2.3.1 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) has been employed for the N-glycan separation. The most common mode of HPLC employed for N-glycan fractionation and enrichment is hydrophilic interaction chromatography (HILIC). Advances in HILIC has enabled the method to be used to separate α2-3 vs α2-6 linked sialic acids. In order to differentiate the isomers this chromatographic technique is sometimes hyphenated with MS. There is need to ensure that the salts from HILIC buffers do not interfere with MS analysis. An alternative approach employs specific exoglycosidases enzymes offline before analysis with HILIC. This chromatographic requires large buffer volumes, is labor insensitive and may be time consuming.

1.2.3.2 Mass spectrometry

Mass spectrometry is routinely used for N-glycan analysis but requires derivatization to identify sialic acid linkages. The m/z of α2-3 vs α2-6 linked sialylated analytes the same, which makes structural assignment ambiguous. Several strategies have been developed to distinguish these isomers in MS. The most common strategy utilizes chemical derivatization like ethyl esterification, methylamidation or sialic acid linkage specific alkylamidation. In some of these derivatization techniques these is formation of byproducts, and they convert the α 2-3 linked sialic acid to a lactone, which is prone to hydrolysis. A different strategy has been to employ tandem MS but this may not be
readily available. In separate studies, the exoglycosidases are initially used to remove α2-3 linked sialic acids before analysis in MS.\textsuperscript{43, 46, 47}

### 1.2.3.3 Capillary electrophoresis

The high separation efficiency and peak capacity of capillary electrophoresis make it suited for separation of sialylated N-glycans.\textsuperscript{16} Separations have been done to differentiate α2-3 vs α2-6 sialic acids, in reverse polarity under suppressed EOF.\textsuperscript{19} The α2-6 sialic acids are arranged in a more compact structure with the N-glycan, forming a more compact structure with the N-glycan as compared to the α2-3 linked sialic acids.\textsuperscript{48} This leads to subtle differences in size of the N-glycan when there is α2-3 linked sialic acids vs α2-6 linked sialic acids. This yields a difference in the charge to size ratio of the two molecules and this variation has been employed in capillary nanogel electrophoresis, with the help of a single exoglycosidase, to distinguish α2-3 linked sialic acids vs α2-6 linked sialic acids in a sample with homogenous branching.\textsuperscript{19} The migration time of the products is predictable as loss is sialic acid leads to a change in both charge and size, which is observed as a shift to the right. For complex N-glycans containing α2–3 and α2–6 sialylation, a different approach is required. A sample with mixed branching will yield products with unpredictable migration time after the single enzyme treatment because the products will have unpredictable charge to size ratio which makes interpretation difficult. This confounds the interpretation of the electropherogram without the use of expensive glycan standards.
1.2.3.4 Other Approaches to distinguish sialic acids in complex samples with CE

Several approaches have been taken to distinguish the sialic acids in CE. In one approach, the separation of sialic acid isomers was achieved following derivatization of the sialic acid moieties and analysis using microfluidic CE laser-induced fluorescence. As shown in Figure 1.10, a total of 17 different glycans were separated and detected as single peaks in less than 3 min. The carboxylic acids on sialic acids were neutralized by converting them to methyl amines. Resolution was achieved due to subtle differences in the hydrodynamic radius of different glycans containing α2-3 vs α2-6 linked sialic acids. Microfluidic electrophoresis-laser-induced fluorescence enabled quantification of the structures that contained each type of sialic acid linkage. To correctly assign the structures for microfluidic CE-laser-induced fluorescence, other complementary techniques were used, including CE-MS, LC-MS, and MALDI-MS. Sialic acid linkage-specific alkylamidation, termed SALSA in many literature reports, was used to identify linkage according to the mass shift of α2-6 linked sialic acids (+41.063 Da) as compared to α2-3-linked sialic acids (+13.032 Da). Further, structural assignments were independently confirmed by cleaving the N-glycan mixture with a linkage specific enzyme, α2-3 sialidase, and analyzing the cleaved N-glycan products.

---

An alternative strategy to distinguish sialic acid linkage using derivatization chemistry and CE-MS was also reported. For this research, free N-glycans were end-labeled using a cationic moiety to facilitate electrophoretic separation and improve electrospray ionization (ESI) in positive mode. The results are shown in Figure 1.11 where the extracted ion electropherograms are displayed according to abundance. The upper trace is of peaks at high abundance (>2%). The middle trace is of N-glycans at intermediate abundance (from 0.5% to 1%). The lower trace is of N-glycans at low abundance (<0.25%). Differentiation of the α2-3 vs α2-6 linked sialic acid isomers was achieved through a two-
step derivatization process that resulted in ethyl esterification of α2-6 linked sialic acid (+319 Da) and amidation of α2-3 sialic acids (+290 Da). The CE-MS separation conditions were modified to be compatible with the MS analyses with a commercial CE instrument. Although these N-glycans were not fully resolved by the CE, the N-glycans within peaks were distinguished by the MS. In addition, structures of even low abundant peaks were identified. The separations obtained with the commercial instrument were longer than those reported with the microfluidic device; however, the CE-MS method did not need complementary techniques for structural identification.

Figure 1.11 Electropherogram of asparagine-linked glycans with α2-3 and α2-6 linked sialic acids from human plasma. The traces are obtained using CE-MS. The upper trace (A) is of N-glycans at high abundance (>2%). The middle trace (B) is of intermediate abundance (from 0.5% to 1%). The lower trace (C) is of N-glycans at low abundance (<0.25%). Adapted from Macmillan Publishers Ltd.: Nature Communications, Lageveen-Kammeijer, G. S. M.; de Haan, N.; Mohaupt, P.; Wagt, S.; Filius, M.; Nouta, J.; Falck, D.; Wuhrer, M. Highly sensitive CE-ESI-MS analysis of N-glycans from complex biological samples. Nature Communications 2019, 10 (1), 2137. Copyright 2019. https://www.nature.com/articles/s41467-019-09910-7.
Approaches to leverage both the separation efficiency of CE and structural identification of MS have also been described. CE separations of N-glycans were further analyzed using drift tube ion mobility-MS.\textsuperscript{46} This approach identified isomeric α2-3 vs α2-6 sialic acid linkages due to differences in drift time. CE-MS analysis of the distribution of all N-glycan features was reported for different therapeutic antibodies.\textsuperscript{49} This middle-up approach involving cleavage at the antibody hinge region identified 32 charge variants in the Fc region, including sialylation. In a further effort to expand the use of CE-MS for structural analyses of glycoprotein therapeutics, a validated method was reported to characterize and quantify glycosylation 1 of 10 monoclonal antibodies.\textsuperscript{50} Glycopeptides from each antibody were analyzed with both CE-ESI-MS and HILIC separations, producing similar results as established through a comparison of the abundance of different N-glycan structures.

Quantification of N-glycans in mass spectrometry can be impacted by differences in ionization efficiencies. Instrumental modifications have been reported to address this by simultaneously using laser-induced fluorescence detection with CE-ESI-MS. Laser-induced fluorescence was used to quantify glycans within the ESI Taylor cone obtained with a commercially sourced porous sheathless tip.\textsuperscript{51} Peak areas of N-glycans had intra- and inter-day reproducibility of 4% and 7%, respectively, when measured with laser-induced fluorescence. In order to compare the laser-induced fluorescence response to the mass spectrometry signal, the percent area obtained for each N-glycan peak relative to the total N-glycan area was determined using both detectors. The percent area distribution obtained with MS revealed a significant decrease in signal for slower migrating
peaks in comparison to the peak areas obtained with laser-induced fluorescence. An alternative approach to N-glycan quantification with laser-induced fluorescence prior to mass analysis adapted the cartridge of a commercial capillary instrument to interface the capillary with a commercial microvial ESI source. The capillary was configured outside of the commercial CE instrument by extending the length to 45 cm, making it possible to insert the capillary outlet into the ion source using sheath flow.

1.2.3 Biological reagents for asparagine-linked glycan analysis in capillary electrophoresis

1.2.3.1 Exoglycosidase for glycan characterization

Exoglycosidase enzymes have been employed in capillary electrophoresis to identify specific N-glycans linkages and quantify them. This class of enzyme, hydrolyzes the glycosidic linkage of N-glycans from the non-reducing end. Enzymes are monomer and linkage specific and will only hydrolyze the glycosidic linkage if the enzyme specify matches the terminal monomer on the N-glycan. Figure 1.12 summarizes the enzymes commercially available and the monomers they target. Analysis employing these enzymes can performed either offline or online. Offline reactions involve enzymatic digestion done in vial and when reaction is complete, the analytes are injected into the capillary for analysis. Online reactions involve introducing the enzyme in the capillary, injecting the sample to carry out enzymatic conversion, followed by separation.
Offline reactions for glycan identification and quantification have been reported\textsuperscript{46, 54, 55}. Reactions are typically done overnight in a separate vial, and are quenched and purified before injection and analysis in CE. This approach is time consuming, is more hands-on and consumes higher volumes of enzyme. Online enzyme digestion of N-glycans has also been reported\textsuperscript{19, 29, 53}. This approach integrates the enzymatic reaction and the separation, without the need for quenching or purification. The ability of nanogels to precisely pattern and control the enzyme was demonstrated\textsuperscript{19}. The nanogels have previously been used to pattern enzyme and lectins zones in capillary electrophoresis for the analysis of N-glycans.\textsuperscript{19, 53, 56} The use of phospholipid enabled the creating of stationary enzyme zones and was shown to improve enzyme stability.\textsuperscript{19, 53} Low nanomolar volumes of enzyme were consumed per each analysis and the study were automated.\textsuperscript{19, 29} These analysis showed the attractiveness of incorporating the enzyme reaction with separation in CE.

Figure 1.12 Illustration of exoglycosidase enzymes and the glycosidic bond they cleave.
Identifying the N-glycan is based on migration time shifts after the enzyme reaction. When the N-glycan passes through the enzyme zone and the enzyme specificity matches the terminal monomer, this monomer will be cleaved. This leads to a migration time shift which can be compared to an electropherogram achieved without the enzyme, enabling identification of the monomer cleaved. In the analysis of neutral N-glycans, only a loss in size is observed which leads to an increase in the charge to size ratio. This is observed in the electropherogram as peaks shifting to the left. In analysis of sialylated N-glycans, the loss in sialic acids leads to a change in both the size and charge, resulting in a shift to the left. This approach has been successfully used in capillary for single sialylated N-glycans and the purpose of the research in Chapter 3 was to extend it to more complex mixtures.

1.2.3.2 Transferase Enzymes for N-glycan Modification
Glycosyltransferases enzymes are enzymes responsible for the transfer of sugar monomers from one compound to another. Unlike exoglycosidase enzymes which cleave glycosidic bonds, the glycosyltransferases are responsible for forming the glycosidic bonds. This enzyme class cleaves a monomer from a donor, usually nucleoside mono/diphosphate, and adds it to an acceptor molecule. Figure 1.13 illustrates the reaction of a glycosyltransferase. Table 1.1 list some glycosyltransferase and the glycosidic bond they generate. Wild type transferase enzymes are mainly concentrated in the Golgi complex where they are responsible for N-glycan synthesis. In biological
therapeutics, glycosyltransferases have been employed to tailor glycosylation of therapeutic antibodies\textsuperscript{4,59} thereby modulating activity.\textsuperscript{4}

Table 1.1 Some glycosyltransferases and the monomers they add.

<table>
<thead>
<tr>
<th>Glycosyltransferase</th>
<th>Monomer added</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$2-3 Sialyltransferase</td>
<td>$\alpha$2-3 Sialic acid</td>
</tr>
<tr>
<td>$\alpha$2-6 Sialyltransferase</td>
<td>$\alpha$2-6 Sialic acid</td>
</tr>
<tr>
<td>$\beta$1-4 Galactosyltransferase</td>
<td>Gal$\beta$1-4</td>
</tr>
<tr>
<td>Fucosyltransferase</td>
<td>$\alpha$1-6 fucose</td>
</tr>
</tbody>
</table>

Figure 1.13 Illustration of a glycosyltransferase reaction where monomer is transferred from donor to acceptor.
Even though therapeutic IgG N-glycans play an important role in immune response, control of glycosylation poses challenge, and efforts have used mutant and wild type transferase to tailor the regio- and stereochemistry. IgG glycosylation during synthesis is non template. In order to control this glycosylation several strategies including chemical and chemoenzymatic processing have been utilized. Chemoenzymatic synthesis is desired because it has been demonstrated to be economically efficient and generates specific glycosylation. Over 65 glycosyltransferases have been identified and characterized for chemoenzymatic synthesis and new ones are in development. Challenges still exist in understanding glycosyltransferase reaction mechanism, including kinetics and inhibition.

Several assays are available for glycosyltransferase assays and they generally really on monitoring radiolabeled sugar nucleotides or chromophore tagged nucleotides. In a radiolabeling assay, the transferred monomer is radiolabeled allowing for the monitoring of the product. These assays are routinely utilized as they are sensitive and can be used with low enzyme volumes. A drawback of these assays includes the need to quench the reaction and remove the unreacted donor. Special licenses may be required for the handling of radioactive materials.

Spectrophotometry assays have been reported with some commercially available kits. They rely on the product being detectable in the presence of optically inactive reactants. These methods are limited by their requirement of high enzyme volumes, as µL or mL reaction volumes are utilized. High throughput assays utilizing
immunoassays have been developed. The acceptor is immobilized and the enzyme and donor are then added for the reaction to occur. Kinetics studies are difficult since limited acceptor is immobilized. In most available methods reactions are offline, large enzyme volumes are consumed and multiple analysis have to be done. New strategies should utilize low enzyme and substrate amounts, be fast and easily automated.

1.2.3.3 Lectin in glycans characterization

Lectins are glycan binding proteins that recognize specific sugars moieties. These sugar binding proteins are used to identify the presence of a particular glycosylation. Table 1.1 illustrates some lectins utilized in glycan analysis, and the sugars that they bind. The specificity of lectins has led to their employment in a variety of assays. High throughput lectin arrays are commercially available and have been used in the analysis of glycoproteins. Lectins have also been employed in affinity chromatography to fractionate and enrich glycan samples. A competing sugar is used to elute the bound fractions after capture.
Lectins have been employed in capillary electrophoresis to identify N-glycans.\textsuperscript{11, 53, 56} The lectins are either patterned as stationary zones\textsuperscript{11, 53, 56} or the whole capillary is filled with the lectin.\textsuperscript{69} It is more desirable to have stationary zones of lectins as enzymes can also be integrated as separate zones to enrich the information gained.\textsuperscript{53} Phospholipid nanogels are compatible with lectins and allow for the patterning of these discrete zones.\textsuperscript{11, 53} Once an analysis is done the lectin zone is expelled and a different lectin patterned. This approach was employed in a previous study to identify bisected N-glycans, sialylated, fucosylated and galactosylated N-glycans from IgG.\textsuperscript{11} Identification of the N-glycans with lectins was based on disappearance of peak from electropherogram when lectin is present compared to an electropherogram without lectin.

Table 1.2 Some lectins used in N-glycan analysis.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Primary Specific Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Maackia Amurensis Lectin</em> (SNA)</td>
<td>α2-3 Sialic acid</td>
</tr>
<tr>
<td><em>Sambucus Nigra Agglutinin</em> (SNA)</td>
<td>α2-6 Sialic acid</td>
</tr>
<tr>
<td><em>Erythrina Cristagalli Lectin</em> (ECL)</td>
<td>Terminal Galβ1-4</td>
</tr>
<tr>
<td><em>Aleuria Aurantia Lectin</em> (AAL)</td>
<td>α 1-6 fucose</td>
</tr>
<tr>
<td><em>Ulex Europaeus Agglutinin</em> (UAE)</td>
<td>α fucose</td>
</tr>
</tbody>
</table>
1.3 Conclusions

The chapter outlined the background information which is important for the studies done in Chapter 2 and 3 and for the future directions outlined in Chapter 4. Fundamentals of capillary electrophoresis separations were discussed. Relevant information on phospholipid nanogels and how they have been employed in the analysis of N-glycans was also provided. Material on strategies for labeling N-glycans for optical detection in CE was then provided. Furthermore, the Chapter outlined the current status of analysis of sialic acids, with a comparison done between CE and other cutting-edge techniques. Important information about use of enzymes and lectins was outlined. This information is pertinent to the research done in Chapter 2 and 3.

1.3 Acknowledgements

The section “Other Approaches to Distinguish Sialic acids in Complex Samples with CE” is adapted with permission from Kristoff, C. J.; Bwanali, L.; Veltri, L. M.; Gautam, G. P.; Rutto, P. K.; Newton, E. O.; Holland, L. A., Challenging Bioanalyses with Capillary Electrophoresis. Anal Chem 2020, 92 (1), 49-66. Copyright © 2019 American Chemical Society. This section is part of the overall contribution by Lloyd Bwanali to the publication. Kristoff, C. J contributions to the publication were on the original draft preparation for the sections on Proteomics, Coatings, Cyclodextrins, Ionic Liquids, Metabolomics, CE-MS Interfaces, and the TOC diagram. Further contributions were on writing, reviewing, and editing the manuscript. Bwanali, L. contributions to the publication were on the original draft preparation for the sections on Glycosylation, Kₘ, Inhibitors and Mixing, Enzymes Analyses and Heparins. Further contributions were on writing, reviewing, and editing the
manuscript. Veltri, L. M contributions to the publication were on the original draft preparation for the sections on Immobilization of Enzymes, Single Cell Analysis, Figure Captions, and Cyclodextrins. Further contributions were on writing, reviewing, and editing the manuscript. Gautam, G. P. contributions to the publication were on the original draft preparation for the sections on DNA, FFE, 3D Printing, and Cyclodextrins. Further contributions were on writing, reviewing, and editing the manuscript. Newton, E. O contributions to the publication were on the original draft preparation for the sections on Biotherapeutics and Ionic Liquids. Further contributions were on writing, reviewing and editing the manuscript. Rutto, P. K. contributions to the publication were on the original draft preparation for the sections on Biotherapeutics and Ionic Liquids. Further contributions were on writing, reviewing, and editing the manuscript. Holland, L. A. contributions to the publication were on the original draft preparation for the Introduction and Protein sections. Further contributions were on writing, reviewing, and editing the manuscript, project supervision, project administration, and funding acquisition

1.4 References


27. Khan, S.; Liu, J.; Szabo, Z.; Kunnunmal, B.; Han, X.; Ouyang, Y.; Linhardt, R. J.; Xia, Q., On-line Capillary Electrophoresis/Laser-Induced Fluorescence/Mass


34. Sarrats, A.; Saldova, R.; Pla, E.; Fort, E.; Harvey, D. J.; Struwe, W. B.; de Llorens, R.; Rudd, P. M.; Peracaula, R., Glycosylation of Liver Acute-Phase Proteins in


53. Holland, L. A.; Gattu, S.; Crihfield, C. L.; Bwanali, L., Capillary Electrophoresis with Stationary Nanogel Zones of Galactosidase and *Erythrina Cristagalli* Lectin for the


We Control It? Annual Review of Chemical and Biomolecular Engineering 2020, 11 (1), 311-338.


64. Danby, P. M.; Withers, S. G., Advances in Enzymatic Glycoside Synthesis. ACS Chemical Biology 2016, 11 (7), 1784-1794.


Chapter 2: Quantification of the α2-6 Sialic Acid Linkage in Branched Asparagine-Linked Glycan Structures with Capillary Nanogel Electrophoresis

## 2.1 Introduction

Asparagine-linked carbohydrates, or N-glycans, are a type of post-translational protein modification that impacts physiological function. N-linked glycans have a common core structure which can be modified such that the non-reducing terminus is capped with sialic acid that is α2-3 or α2-6 linked to galactose residues. Sialic acid linkage is a direct measure of altered synthesis, metabolism, and regulation because the processes that affect sialic acid linkage are controlled by glycosyltransferases, glycosidases, and a variety of feedback mechanisms. Increases in the amount of α2-6 linked sialic acids in serum proteins is reported in cancer. For example, the sialic acid linkages of alpha-1-acid glycoprotein (AGP) in serum are associated with liver, pancreatic, and kidney cancer.

Analyses of N-glycan isomers with different sialic acid linkages are challenging. Sialylation can be heterogeneous and sialic acid residues are labile. Currently the measurement of sialic acid linkage is achieved utilizing chemical derivatization or tandem MS. Moreover, the linkage specific enzyme α2-3 sialidase is used to further verify the linkage composition. Because the distribution of N-glycan structures is complex, glycan samples must be fractionated prior to analyses. Promising strategies for fractionating complex N-glycan samples include HILIC, ion mobility spectrometry, and microfluidic or capillary electrophoresis.

Capillary electrophoresis has high separation efficiency and high peak capacity, making it ideally suited for complex N-glycan samples. Because capillary electrophoresis
separations are based on differences in charge-to-size ratio, they are affected by the highly charged nature of sialylated N-glycans. Thus, sialic acids are best resolved using reverse polarity and suppressed electroosmotic flow. Under these conditions, the anionic glycans are driven to the detector via electrophoretic transport. If a high viscosity media is also included in the background electrolyte, additional separation enhancement is observed as subtle differences in shape (i.e. sialic acid linkage isomers) are intensified by an increase in the frictional drag.

Capillary nanogel electrophoresis has been used to simultaneously separate and identify glycan structures. In this approach a stationary zone of exoglycosidase enzyme was included near the injection end of the separation capillary. The enzyme in the capillary cleaved specific terminal N-glycan monomers. For neutral residues such as galactose or N-acetylglucosamine, this resulted in a change in the size of the glycans, but not the charge, when monomers were cleaved. This created a migration time shift of product from that of the substrate allowing identification and quantification. This approach was effective for analyzing mixtures of asialylated N-glycans, or a single sialylated N-glycan for sialic acid linkage. Sequencing complex mixtures containing sialic acids with capillary electrophoresis and exoglycosidases is more difficult because removing the sialic acid changes both charge and size. For complex N-glycans containing both α2-3 vs α2-6 linked sialylation, the products do not have predictable charge-to-size ratio confounding interpretation of the electropherogram without the use of expensive glycan standards.
In this study, enzymes were patterned in an order that simplified the mass shift interpretation, enabling the analysis of complex mixtures of α2-3 and α2-6 linked sialylation (Figure 1.1). The approach was used for the analysis of N-glycans with different branching, and consumed only nanoliter volumes of enzyme, sample, and phospholipid. The N-glycans injected into the capillary were passed through three enzyme zones. The first enzyme zone contained α2-3 sialidase, which specifically
cleaved α2-3 linked sialic acids (Figure 1.1). The modified N-glycan then migrated through the second zone that contained β1-3,4 galactosidase, which cleaved terminal galactose. Employing α2-3,6,8 sialidase then removed the α2-6 linked sialic acids. With this process of in-capillary enzyme modification and separation, shifts in migration time reflected the α2-6 linked sialic acid composition and the amount of α2-6 linked sialic acid correlated directly to the number of galactose residues on the N-glycan (Figure 1.1). This enzyme-based approach provided quantitative information about the amount of α2-6 linked sialic acid for biantennary, triantennary, and tetraantennary N-glycans. The multiple enzyme approach was validated with a sialylated N-glycan standard and used together with lectins to quantify α2-6 linked sialic acid in AGP which is a heavily sialylated positive acute phase protein which serves as a marker of disease.

2.2 Materials and methods

2.2.1 Chemicals and reagents

The sialylated triantennary complex N-glycan (GKC-335300), Human AGP N-Linked Glycan Library (GKLB-001), α2-3 sialidase (GK80021 Sialidase S,) and the β1-3,4 galactosidase (GKX-5013) were from Agilent (Santa Clara, CA, formerly Prozyme Hayward, CA). Acetic acid was from Fisher Scientific (Pittsburgh, PA). Sodium phosphate, 8-aminopyrene-1,3,6-trisulfonic acid, triethylamine, AGP, acetonitrile, sodium hydroxide, methanol, sodium acetate, α2-3,6,8 sialidase (N2876-6UN) and sodium cyanoborohydride (dissolved in tetrahydrofuran) were from Sigma-Aldrich (St. Louis, MO). *Aleuria aurantia* lectin (AAL), *Erythrina Cristagalli* lectin (ECL), and *Maackia Amurensis* lectin (MAL) were from Vector Labs (Burlingame, CA). PNGase F was
purchased from New England Biolabs (Ipswich, MA). Deionized water was from an Elga Purelab ultra water system (Lowell, MA). The phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), were from Avanti Polar Lipids (Alabaster, AL). Nanogels were prepared by weighing phospholipids and combining them such that \([\text{DMPC}]/[\text{DHPC}] = 2.5\). The phospholipids were dissolved in an aqueous solution of 50 mM sodium acetate buffered to pH of 5.0 to achieve a concentration of 10, 20, or 40% weight/volume. The process to reconstitute phospholipids has been described previously.\(^{19-21}\) Each preparation was then aliquoted and stored at \(-20 \, ^\circ\text{C}\)

### 2.2.2 Preparation and derivatization of standards.

The glycans were labeled with a fluorescent chromophore as previously described.\(^{19, 20, 22}\) The sample 1 glycans were cleaved from AGP as described previously.\(^{20}\) Glycan samples were purified using DPA-6s columns and stored at \(-20 \, ^\circ\text{C}\) as described previously.\(^{22}\) For further analysis glycan samples were diluted at least 50-fold in 1 mM sodium acetate buffered to pH 5.

### 2.2.3 Preparation of enzymes.

All enzymes were reconstituted so that the appropriate concentration of enzyme was in 20% phospholipid buffered at pH 5 with 50 mM sodium acetate. The separation and enzyme zones were buffered at pH 5, which was compatible with enzyme conversion\(^{23-25}\) and was near the isoelectric point of the enzymes. At this pH the enzymes will be essentially stationary in the capillary in the absence of electroosmotic flow. The \(\alpha 2-3,6,8\)
Sialidase powder was reconstituted to a concentration of 250 mUnits/µL with 50 mM sodium acetate buffered to pH 5. The appropriate volume of master stock (1 µL) was diluted with 20% nanogel to a final enzyme concentration of 5 mUnits/µL. For the α2-3 sialidase, 1 µL of enzyme was reconstituted in 20% nanogel in sodium acetate phospholipid buffered at pH 5 to a concentration of 2 mUnits/µL. The appropriate volume of master stock was diluted with 20% nanogel to make concentrations between 5 µUnits/µL and 750 µUnits/µL. For analysis using β1-3,4 galactosidase, this enzyme was desalted and concentrated to 20 mUnits/µL using a 10 kDa molecular weight cut-off filter (UFC01025 EMD Millipore, Burlington, MA). The 20 mUnits/µL preparation was mixed with 40% nanogel to achieve a solution with final concentrations of 10 mUnits/µL β1-3,4 galactosidase in 20% nanogel buffered to pH 5 with sodium acetate.

### 2.2.4 Preparation of lectins.

Lectins (i.e. AAL, ECL, MAL) were desalted prior to use. The salts were removed using a 10 kDa molecular weight cut-off filter. The lectins were rinsed with a solution 50 mM sodium phosphate buffered to pH 6, and each lectin was then collected from the molecular weight cut-off filter. Each lectin sample was then mixed 1:1 with a 40% nanogel preparation that contained 50 mM sodium acetate buffered to pH 5. The final pH was approximately 5.5. The final concentration of the lectin preparation was 250 µM AAL, 150 µM ECL, or 100 µM MAL in 20% nanogel.
2.2.5 Capillary electrophoresis.

Analyses were performed using a SCIEX MDQ Plus (Sciex, Redwood City, CA) configured by the manufacturer with laser induced fluorescence detection and appropriate filters (3 mW solid state laser with $\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm). Silica capillaries with a 25 μm internal diameter and 360 μm outer diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ) were used for the separations. Each day capillaries were prepared as previously described. The electrophoresis was performed using reversed polarity to separate the negatively-charged APTS-labeled glycans (anodic reservoir near detector, cathodic reservoir near the site of injection). The background electrolyte was 50 mM sodium acetate buffered to a pH of 5. Prior to each run the capillary was held at 15 °C and the 40% nanogel was introduced in the capillary. Ambient thermal control of the room and instrument was maintained using a portable air conditioner as was described previously. This temperature was maintained between 14 to 16 °C. Large deviations in the ambient temperature resulted in irreproducible nanogel introduction and plug patterning.

For enzyme patterning, the zones were introduced in the opposite order they are in contact with the glycan (see Figure 2.2). Patterning in the capillary was an automated process performed by the capillary electrophoresis instrument in multiple steps.
Figure 2.2 Illustration of in capillary reaction showing that presence of α2–6-linked sialic acid is related to galactose residues following processing with serial enzymes. (A) Saccharides are represented with nomenclature defined by the Consortium for Functional Glycomics. (B) Electropherogram showing migration time shifts based on the number of terminal galactose residues present. (C) Verification of the α2–6-linked sialic acid distribution following treatment with α2–3 sialidase only. Areas labeled in the traces are normalized to the internal standard (IS). Y axis for trace C is normalized to the internal standard in trace B. Runs were performed in triplicate. See Tables 2.1, 2.2, 2.3, and 2.4 for raw and normalized areas. Separations performed at 23 °C in a 25 μm i.d. capillary with an effective length of 50 cm and E = 450 V/cm (reverse polarity). Preseparation at 4 kV for 9 min to drive substrate past enzyme plug. All enzymes are suspended in 20% nanogel with 50 mM sodium acetate buffered to pH 5. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. Analytical Chemistry. 2020, 92 (1), 1518-1524. Copyright © 2019 American Chemical Society.
First, the capillary was filled with 40% nanogel. Second, the α2-3,6,8 sialidase enzyme zone was introduced at 20.7 kPa (3 psi) for 14 s which was calculated to 1.6 mm. For the calculation, a plug of APTS was pushed with 20% nanogel at 3 psi. It took the APTS 14.8 min to cover 10.2 cm (102 mm), therefore 8.75 s introduction of the nanogel plugs covers 1 mm. The α2-3,6,8 sialidase enzyme zone was followed by injection of background electrolyte at 3.4 kPa (0.5 psi) for 5 s. The 3 psi 14 s α2-3,6,8 sialidase enzyme zone was calculated to be 1.6 cm. The purpose of the 5 s introduction of background electrolyte was to clean the outer surface of the capillary. A 10% lipid spacer at 20.7 kPa (3 psi) 20 s was then introduced to prevent contamination. Third, the β1-3,4 galactosidase was introduced at 20.7 kPa (3 psi) for 42 s (calculated to be 4.8 mm) followed by injection of background electrolyte at 3.4 kPa (0.5 psi) for 5 s, and then a 10% lipid spacer at 20.7 kPa (3 psi) 20 s. Fourth, the α2-3 sialidase was then introduced at 20.7 kPa (3 psi) for 14 s (1.6 mm) followed by injection of background electrolyte at 3.4 kPa (0.5 psi) for 5 s, and then a 10% lipid spacer at 20.7 kPa (3 psi) 20 s. Finally, the AAL zone was patterned into the capillary at 20.7 kPa (3 psi) for 28 s (2 mm) followed injection of 10% nanogel at 13.8 kPa (2 psi) for 23 s and then a background electrolyte pre-plug of 13.8 kPa (2 psi) for 20 s. After the capillary was filled, the temperature of the separation was increased to 23 °C for the sample injection, incubation, and separation. Sample injections were at 8 kV 5 s followed by a background electrolyte post-plug of 13.8 kPa (0.5 psi) for 5s. After a run was complete, a 276 kPa (40 psi) background electrolyte flush for 15 min was applied in the reverse direction to push out any remaining protein toward the site of injection. Data collection and analyses were performed using 32 Karat Software version
10.2. Reducing the velocity while passing through the enzyme zone maximized enzyme conversion.\(^{17}\) Therefore, a pre-separation at 4 kV for 9 minutes in the beginning of the run was performed to drive substrate through the enzyme zones.

2.2.6 Statistical analysis.

Significance testing was used to compare peak areas for optimization of residue cleavage from N-glycans for different enzymatic methods. The samples were considered to be normally distributed, assuming only random error existed in the measurement, and independent. Levene’s test was used to remove bias from the statistical analysis when choosing between a pooled \(t\)-test and a Welch test. In every case, at the 0.01 significance level, the results of Levene’s Test did not support a significant difference in sample (group) variance, indicating a 2-sided pooled \(t\)-test be conducted for all comparisons.

2.3 Results and discussion

2.3.1 Serial enzyme processing to determine \(\alpha_2\)-6 linked sialic acid.

The amount of \(\alpha_2\)-6 linked sialic acid was quantified by modifying the N-glycan so that the number of \(\alpha_2\)-6 linked sialic acids was related to the number of galactose residues remaining after sequential in-capillary digestion. As depicted in Figure 2.2, the separation capillary was patterned so that the APTS labeled N-glycan passed through three successive zones which were \(\alpha_2\)-3 sialidase, \(\beta_1\)-3,4 galactosidase, and \(\alpha_2\)-3,6,8 sialidase. Migration through the first two zones resulted in cleavage of any \(\alpha_2\)-3 linked sialic acid (zone 1) and the penultimate galactose (zone 2) attached to the cleaved \(\alpha_2\)-3 linked sialic acid (Figure 2.2A first arrow). Migration through the third zone cleaved the \(\alpha_2\)-6 linked sialic acid (Figure 2.2A second arrow). As a result, the galactose residues
remaining on the products were those that were linked to the α2-6 linked sialic acids. A unique advantage of this method is that it can be applied to fully or partially sialylated N-glycans because the lack of a sialic acid does not impact the quantification of the α2-6 linked sialic acid residues. It was established in the literature\textsuperscript{27} that α2-3 linked sialic acids are more readily cleaved than α2-6 linked sialic acids because of structural differences. As α2-3 linked sialic acid is more susceptible to loss associated with sample handling, derivatization, or storage, quantification of α2-6 linked sialic acid is a viable strategy for linkage analyses of physiological samples.

2.3.2 Quantifying α2-6 linked sialic acid in a triantennary asparagine-linked glycan standard.

A sialylated N-glycan standard was enzymatically processed in-capillary to demonstrate the feasibility of enzyme-based quantification of α2-6 linked sialic acid. The top trace of Figure 2.2B shows the stepwise shift in migration that occurred with each additional loss of a galactose residue. The structures were identified by migration order to determine the number of α2-6 linked sialic acid residues, which was measured as area. The α2-6 linked sialic acid distribution on the triantennary N-glycans summarized in Figure 2.2B was similar to that reported by the vendor,\textsuperscript{28} which for the triantennary structures containing 0, 1, 2, and 3 α2-6 linked sialic acids was reported to be 4%, 51%, 36%, and 5%, with 3% of the composition ambiguous. The normalized areas used to achieve this distribution are shown in Table 2.1 and the raw areas in Table 2.2. As shown in Figure 2.2B, split peaks were observed for the mono- (i.e. 23.1, 23.4 min) and disialylated (i.e. 24.7, 25.1 min)
structures. This peak splitting was due to the asymmetry of the structure emanating from the α1-3 and α1-6 mannose at the N-glycan core.\textsuperscript{29}

| Table 2.1 Normalized areas for Figure 2.2B employed for establishing α2-6 linked sialic acid in triantennary standard using multiple enzymes. |
|----------------------------------|------------------|-----------------|----------------|-----------------|
| I.S. | 1 | 2 | 3 | 4 | Total |
| SEQUENCE | NORM | 1 | 2 | 3 | 4 | Total |
| 850 | 33373 | 558459 | 323558 | 13807 | 929197 |
| 853 | 32223 | 530390 | 339330 | 15797 | 917739 |
| 869 | 39277 | 545098 | 326597 | 14430 | 925402 |
| AVERAGE | 34957 | 544649 | 329828 | 14678 | 924113 |
| RSD | 11 | 3 | 3 | 7 | 1 |

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.

| Table 2.2 Raw areas for Figure 2.2B employed for establishing α2-6 linked sialic acid in triantennary standard using multiple enzymes. |
|----------------------------------|------------------|-----------------|----------------|-----------------|
| I.S. | 1 | 2 | 3 | 4 | Total |
| SEQUENCE | RAW | 1 | 2 | 3 | 4 | Total |
| 850 | 71184 | 33373 | 558459 | 323558 | 13807 | 929197 |
| 853 | 70783 | 32041 | 527402 | 337418 | 15708 | 912569 |
| 869 | 68085 | 37567 | 521367 | 312379 | 13802 | 885115 |
| AVERAGE | 70017 | 34327 | 535743 | 324452 | 14439 | 908960 |
| RSD | 2 | 8 | 4 | 4 | 8 | 2 |
2.3.3 Verification of serial enzyme processing

The fidelity of three zone enzyme processing was verified by analyzing the migration order and areas obtained for the triantennary N-glycan standard using only the first zone of α2-3 sialidase (Figure 2.2C). If the peak assignment is correct, then when enzyme conversion is as expected the areas of peaks associated with structures of the same sialic linkage composition will be the same. The order of migration of the peaks obtained with a single α2-3 sialidase treatment was reversed when compared to the order obtained with the 3 enzyme processing. This was due to the difference in mobility associated with the sialic acid charge. Another notable difference was that the peaks obtained with the single enzyme treatment were not symmetrically spaced because the sialic acid residue was charged. The peak splitting of the mono-galactosylated and di-galactosylated structures observed in trace B and in other reports\textsuperscript{22, 30} for asialylated peaks was not observed in the partially sialylated glycans. This was attributed to the conformation of sialylated residues forming structures with similar hydrodynamic radius. In fully sialylated glycans the α2-6 linked sialic acids were reported to adopt a more compact structure with the penultimate galactose residues.\textsuperscript{27} In another report fully sialylated N-glycans were reported to adopt structures that allowed the sialic acid to fold against the N-acetylglucosamine residues at the reducing terminus of the structure.\textsuperscript{29} With the peak identities associated with each structure assigned, the areas obtained with the 3 enzyme versus the single enzyme treatment were compared. No significant difference was observed in the areas obtained between the single enzyme in Figure 2.2C (normalized areas in Table 2.3 and raw area in Table 2.4) and the three-enzyme analysis in Figure
2.2B (normalized areas in Table 2.1 and raw areas in Table 2.2) at a 0.01 significance level, when compared using a two-sided pooled T test.

Table 2.3 Normalized areas for Figure 2.2C used to validate α2-6 linked sialic acid in triantennary standard using α2-3 sialidase.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-3 Sialidase enzyme Run #918</td>
<td>20742</td>
<td>294943</td>
<td>513660</td>
<td>43468</td>
<td>872813</td>
</tr>
<tr>
<td>α2-3 Sialidase enzyme Run #921</td>
<td>22333</td>
<td>315809</td>
<td>550077</td>
<td>47430</td>
<td>935649</td>
</tr>
<tr>
<td>α2-3 Sialidase enzyme Run #924</td>
<td>21045</td>
<td>311713</td>
<td>539305</td>
<td>46202</td>
<td>918266</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>21373</td>
<td>307489</td>
<td>534348</td>
<td>45700</td>
<td>908909</td>
</tr>
<tr>
<td>RSD</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.

Table 2.4 Raw areas for Figure 2.2C used to validate α2-6 linked sialic acid in triantennary standard using α2-3 sialidase.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-3 Sialidase enzyme Run #918</td>
<td>69410</td>
<td>19327</td>
<td>274827</td>
<td>478627</td>
<td>40503</td>
</tr>
<tr>
<td>α2-3 Sialidase enzyme Run #921</td>
<td>69684</td>
<td>20055</td>
<td>283600</td>
<td>493975</td>
<td>42593</td>
</tr>
<tr>
<td>α2-3 Sialidase enzyme Run #924</td>
<td>67886</td>
<td>18899</td>
<td>279922</td>
<td>484302</td>
<td>41490</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>68993</td>
<td>19427</td>
<td>279450</td>
<td>485635</td>
<td>41529</td>
</tr>
<tr>
<td>RSD</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
2.3.4 Working range of specific sialidase.

Although α2-3 sialidase was specific for α2-3 linked sialic acid, at sufficiently high concentration it will cleave α2-6 linked sialic acid at a slower velocity. The concentration of 250 µUnits/µL α2-3 sialidase used for the analyses in Figure 2.2 was verified to ensure that the amount of α2-3 sialidase was sufficient to fully convert α2-3 linked sialic acids, but did not cleave α2-6 sialic acid. The same concentration of triantennary standard that was used to quantify the α2-6 linked sialic acid in Figure 2.2B was used to evaluate the working concentration range of the specific sialidase. A different instrument was used for this study. Although the response of these instruments differs the ratio of the peak area of the N-glycan to that of the internal standard is similar (Table 2.5).

<table>
<thead>
<tr>
<th>Table 2.5 Comparison of triantennary glycan to internal standard areas across instruments for each enzyme condition.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triantennary standard</strong></td>
</tr>
<tr>
<td>Specific enzyme range study</td>
</tr>
<tr>
<td>Multiple enzyme sequencing study</td>
</tr>
<tr>
<td>Internal standard (I.S.)</td>
</tr>
<tr>
<td>Glycan area</td>
</tr>
</tbody>
</table>

Internal standard (I.S.) and glycan areas are averages of 3 replicates performed on each instrument. The total glycan area was divided by total standard area.

In-capillary digestions were performed at α2-3 sialidase concentrations of 250 µUnits/µL (Figure 2.3B) and 750 µUnits/µL (Figure 2.3A) and the distribution of desialylated peaks compared. The peaks produced by these in-line enzyme reactions had reproducible migration times and areas, with the relative standard deviation less than 1% and less than 10%, respectively. The areas of the product peaks obtained at these two concentrations
(normalized areas Table 2.6 and raw areas Table 2.7) were not significantly different at a 0.01 significance level when compared using a two-sided pooled T test. When lectin with high specificity to α2-3 sialic acids (MAL) was included in the capillary (Figure 2.3C), no difference was observed in the trace, which further confirmed that the α2-3 sialic acids were completely cleaved from the sample. A lower concentration of α2-3 sialidase (5 μUnits/μL) generated incomplete cleavage of α2-3 linked sialic acid (Figure 2.3D).

Figure 2.3 Concentration range of α2-3 sialidase for triantennary asparagine-linked glycan. The concentration range of α2-3 sialidase enzyme suitable for complete conversion of α2-3 linked sialic acid, but not α2-6 linked sialic acid is established for triantennary standard. Electropherograms obtained at 250 μUnits/μL (B) and 750 μUnits/μL (C) enzyme are not significantly different. When MAL lectin specific for α2-3 linked sialic acid is included with 250 μUnits/μL enzyme (C) no change is observed confirming complete removal of α2-3 linked sialic acid. At concentrations lower than this working range (D) partial conversion is achieved (5 μUnits/μL). Separations condition are similar to Figure 2.2. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. Analytical Chemistry. 2020, 92 (1), 1518-1524. Copyright © 2019 American Chemical Society.
Table 2.6 Figure 2.3 normalized areas of α2-3 sialidase working concentration range for triantennary standard.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>750 μUnits/μL α2-3 Sialidase enzyme Run #707</td>
<td>11408</td>
<td>143357</td>
<td>246066</td>
<td>20340</td>
<td>421171</td>
</tr>
<tr>
<td>750 μUnits/μL α2-3 Sialidase enzyme Run #710</td>
<td>10300</td>
<td>141073</td>
<td>241576</td>
<td>19763</td>
<td>412711</td>
</tr>
<tr>
<td>750 μUnits/μL α2-3 Sialidase enzyme Run #713</td>
<td>10067</td>
<td>139303</td>
<td>238550</td>
<td>19270</td>
<td>407190</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>10592</td>
<td>141244</td>
<td>242064</td>
<td>19791</td>
<td>413691</td>
</tr>
<tr>
<td>RSD</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 μUnits/μL α2-3 Sialidase enzyme Run #671</td>
<td>9868</td>
<td>139788</td>
<td>227833</td>
<td>21253</td>
<td>398741</td>
</tr>
<tr>
<td>250 μUnits/μL α2-3 Sialidase enzyme Run #675</td>
<td>10874</td>
<td>135152</td>
<td>240907</td>
<td>23125</td>
<td>410059</td>
</tr>
<tr>
<td>250 μUnits/μL α2-3 Sialidase enzyme Run #720</td>
<td>10112</td>
<td>136564</td>
<td>223189</td>
<td>19518</td>
<td>389383</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>10285</td>
<td>137168</td>
<td>230643</td>
<td>21299</td>
<td>399394</td>
</tr>
<tr>
<td>RSD</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 μUnits/μL α2-3 Sialidase + MAL Run #726</td>
<td>10719</td>
<td>128733</td>
<td>224207</td>
<td>17721</td>
<td>381379</td>
</tr>
<tr>
<td>5 μUnits/μL α2-3 Sialidase enzyme Run #638</td>
<td>28313</td>
<td>134837</td>
<td>141121</td>
<td>19471</td>
<td>—</td>
</tr>
<tr>
<td>5 μUnits/μL α2-3 Sialidase enzyme Run #641</td>
<td>25619</td>
<td>132488</td>
<td>146714</td>
<td>21617</td>
<td>—</td>
</tr>
<tr>
<td>5 μUnits/μL α2-3 Sialidase enzyme Run #644</td>
<td>26186</td>
<td>142744</td>
<td>140036</td>
<td>19135</td>
<td>—</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>26706</td>
<td>136690</td>
<td>142624</td>
<td>20074</td>
<td>326094</td>
</tr>
<tr>
<td>RSD</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.
Table 2.7 Figure 2.3 and Table 2.6 raw areas of α2-3 sialidase working concentration range for triantennary standard.

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>750 µUnits/µL α2-3 Sialidase enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run #707</td>
<td>28521</td>
<td>9737</td>
<td>122361</td>
<td>210027</td>
<td>17361</td>
</tr>
<tr>
<td>Run #710</td>
<td>28715</td>
<td>8851</td>
<td>121230</td>
<td>207597</td>
<td>16983</td>
</tr>
<tr>
<td>Run #713</td>
<td>28768</td>
<td>8667</td>
<td>119930</td>
<td>205375</td>
<td>16590</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td>28668</td>
<td>9085</td>
<td>121174</td>
<td>207666</td>
<td>16978</td>
</tr>
<tr>
<td><strong>RSD</strong></td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>250 µUnits/µL α2-3 Sialidase enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run #671</td>
<td>28444</td>
<td>8400</td>
<td>118992</td>
<td>193939</td>
<td>18091</td>
</tr>
<tr>
<td>Run #675</td>
<td>27840</td>
<td>9060</td>
<td>112603</td>
<td>200714</td>
<td>19267</td>
</tr>
<tr>
<td>Run #720</td>
<td>28270</td>
<td>8555</td>
<td>115537</td>
<td>188824</td>
<td>16513</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td>28185</td>
<td>8672</td>
<td>115711</td>
<td>194492</td>
<td>17957</td>
</tr>
<tr>
<td><strong>RSD</strong></td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td><strong>250 µUnits/µL α2-3 Sialidase + MAL Run #726</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22692</td>
<td>7279</td>
<td>87422</td>
<td>152258</td>
<td>12034</td>
</tr>
<tr>
<td><strong>5 µUnits/µL α2-3 Sialidase enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run #638</td>
<td>33415</td>
<td>28313</td>
<td>134837</td>
<td>141121</td>
<td>19471</td>
</tr>
<tr>
<td>Run #641</td>
<td>33392</td>
<td>25601</td>
<td>132397</td>
<td>146613</td>
<td>21602</td>
</tr>
<tr>
<td>Run #644</td>
<td>32387</td>
<td>25380</td>
<td>138353</td>
<td>135728</td>
<td>18546</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td>33065</td>
<td>26431</td>
<td>135196</td>
<td>141154</td>
<td>0</td>
</tr>
<tr>
<td><strong>RSD</strong></td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
For this study it was unnecessary to establish a working range for the β1-3,4 galactosidase or the α2-3,6,8 sialidase because stepwise sequencing was only dependent upon these enzymes being above a minimum threshold for complete digestion. Concentrations of 10 mUnits/µL and 5 mUnits/µL, were used for β1-3,4 galactosidase or the α2-3,6,8 sialidase, respectively. These concentrations were selected as working at higher concentrations increased the potential to foul the capillary surface, which required more substantial flushes in-between the separations to maintain reproducible peak migration times and areas. These experiments confirmed that the enzyme processing strategy effectively quantified the abundance of sialic acid linkages for a single N-glycan standard; however, in order to apply the approach to sialylated glycoproteins it was evaluated with mixtures of N-glycans.

2.3.5 Measuring the α2-6 sialic acid content simultaneously in a mixture of asparagine linked glycans.

To analyze glycan mixtures, sample migration windows and branching distribution were established from reference traces shown in Figure 2.4A for AGP, which contains bi-, tri-, and tetraantennary N-glycans. The N-glycans were cleaved from AGP and labeled with APTS prior to in-capillary desialylation and separation. In-capillary enzyme processing cleaved the N-glycans to the terminal N-acetyl glucosamine (Figure 2.4A blue trace) or galactose residues (Figure 2.4A black trace). A lectin specific for fucose (AAL) was used to remove fucose containing structures. This produced three peaks (Figure 2.4A) for which the migration times delineated the range of the stepwise shift for each branched structure. The area of each branched structure obtained from triplicate runs (Table 2.8
Table 2.9 and Table 2.10) was used to assist in the analyses and were normalized to a mannose trisaccharide internal standard. The peak overlap of the fully galactosylated biantennary and the agalactosylated tetraantennary structures was addressed in α2-6 sialic acid determinations that followed by including a lectin with specificity to galactose residues (ECL) in processing. This removed the biantennary structure which allowed for quantification of the tetraantennary structure (Figure 2.4B blue trace).

Figure 2.4 Online serial enzyme sequencing of α-1-acid glycoprotein sample 1. (A) reference traces establish the area associated with biantennary, triantennary, and tetraantennary glycans. (B) the α2-6 linked sialic acid area of each branched structure is established through serial enzyme processing in the absence of ECL (black trace). Presence of ECL (blue trace) identifies agalactosylated glycans in co-migrating peaks. Separation conditions are as specified in Figure 2.2. The asterisk denotes contaminant peaks. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. *Analytical Chemistry.* 2020, 92 (1), 1518-1524. Copyright © 2019 American Chemical Society.
Table 2.8 Peak area of α2-6 linked sialic from α-1-acid glycoprotein glycans.

<table>
<thead>
<tr>
<th>Branch</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25(10)</td>
<td>89(7)</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>114(12)</td>
</tr>
<tr>
<td></td>
<td>104(5)</td>
<td>336(8)</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>440(9)</td>
</tr>
<tr>
<td></td>
<td>94(7)</td>
<td>265(3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>359(8)</td>
</tr>
</tbody>
</table>

Areas and relative standard deviation (RSD) of 2, 3, and 4 branch N-glycans from normalized alignment traces are: 107 (4), 433 (1), and 374 (2), respectively. Normalization is based on the highest quantified area for the internal standard within the alignment traces, sequencing traces, and sequencing traces with ECL (i.e. 35,090 RFU). Solid lines in the table indicate that the structure not possible. Separation conditions are as stated Figure 2.1.
Table 2.9 Figure 2.4 normalized peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 1.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2ECL</th>
<th>4ECL</th>
<th>Branch</th>
<th>Reference all gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQUENCE-ECL Run #584</td>
<td>27247</td>
<td>20180</td>
<td>129442</td>
<td>107138</td>
<td>107297</td>
</tr>
<tr>
<td>SEQUENCE-ECL Run #587</td>
<td>22347</td>
<td>18177</td>
<td>132156</td>
<td>439370</td>
<td>432599</td>
</tr>
<tr>
<td>SEQUENCE-ECL Run #590</td>
<td>24964</td>
<td>194471</td>
<td>128371</td>
<td>383185</td>
<td>373672</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>24853</td>
<td>192706</td>
<td>129990</td>
<td>383185</td>
<td>373672</td>
</tr>
<tr>
<td>STDEV</td>
<td>2452</td>
<td>10162</td>
<td>1951</td>
<td>9897</td>
<td>7753</td>
</tr>
<tr>
<td>RSD</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2ECL</th>
<th>4ECL</th>
<th>2-1</th>
<th>3+4-4ECL</th>
<th>4ECL</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQUENCE+ECL Run #593</td>
<td>24306</td>
<td>108384</td>
<td>100027</td>
<td>24853</td>
<td>113890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEQUENCE+ECL Run #596</td>
<td>23956</td>
<td>97361</td>
<td>86918</td>
<td>89037</td>
<td>113890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEQUENCE+ECL Run #600</td>
<td>27048</td>
<td>105262</td>
<td>95561</td>
<td>103669</td>
<td>113890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVERAGE</td>
<td>25103</td>
<td>103669</td>
<td>94169</td>
<td>113890</td>
<td>113890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV</td>
<td>1693</td>
<td>5682</td>
<td>6665</td>
<td>103669</td>
<td>103669</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSD</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>103669</td>
<td>103669</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.
Table 2.10 Figure 2.4 raw peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 1.

<table>
<thead>
<tr>
<th></th>
<th>I.S.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SEQUENCE -ECL Run #584</strong></td>
<td></td>
<td>3463126891</td>
<td>199229</td>
<td>127749</td>
<td>306687</td>
<td>270631</td>
</tr>
<tr>
<td><strong>SEQUENCE -ECL Run #587</strong></td>
<td></td>
<td>3509022347</td>
<td>181777</td>
<td>132156</td>
<td>291294</td>
<td>261339</td>
</tr>
<tr>
<td><strong>SEQUENCE -ECL Run #590</strong></td>
<td></td>
<td>3345123798</td>
<td>185388</td>
<td>122375</td>
<td>283961</td>
<td>248143</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td></td>
<td>3439124345</td>
<td>188798</td>
<td>127427</td>
<td>293981</td>
<td>260038</td>
</tr>
<tr>
<td><strong>STDEV</strong></td>
<td></td>
<td>846</td>
<td>2321</td>
<td>9212</td>
<td>4898</td>
<td>11599</td>
</tr>
<tr>
<td><strong>RSD</strong></td>
<td></td>
<td>2</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1ECL</th>
<th>2ECL</th>
<th>4ECL</th>
<th>Branch Reference all gal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SEQUENCE +ECL Run #593</strong></td>
<td></td>
<td>3209122229</td>
<td>99121</td>
<td>91478</td>
</tr>
<tr>
<td><strong>SEQUENCE +ECL Run #596</strong></td>
<td></td>
<td>3024620649</td>
<td>83921</td>
<td>74919</td>
</tr>
<tr>
<td><strong>SEQUENCE +ECL Run #600</strong></td>
<td></td>
<td>2779421424</td>
<td>83376</td>
<td>75692</td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
<td></td>
<td>3004421434</td>
<td>88806</td>
<td>80696</td>
</tr>
<tr>
<td><strong>STDEV</strong></td>
<td></td>
<td>2156</td>
<td>790</td>
<td>8937</td>
</tr>
<tr>
<td><strong>RSD</strong></td>
<td></td>
<td>7</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>
2.3.6 Working range of specific sialidase for an asparagine linked glycan mixture.

Although the AGP was a commercial standard, the amount of N-glycan present in the sample was reported as total N-glycan mass, but the concentration of each branched structure was not specified by the manufacturer. The bands of different N-glycans in the AGP sample, which passed through the enzyme zones at different velocities and times, were adjusted to maintain the enzyme working range established for the triantennary standard. The AGP sample was diluted so that the total peak area of triantennary N-glycan of the AGP sample matched the peak area of the triantennary standard used in Figure 2.2. While the areas of the tetraantennary and triantennary AGP N-glycans were similar, the area of biantennary N-glycan was 4-fold lower. As a result, the applicability of the working range for the AGP sample was validated.

The working range of α2-3 sialidase was established as described for the triantennary standard. Three replicates of in-capillary digestions were performed at α2-3 sialidase concentrations of 750 μUnits/μL and 250 μUnits/μL (Figure 2.5 and normalized areas in Table 2.11 and raw areas in Table 2.12) and the distribution of desialylated peaks compared. The peaks produced by these in-line enzyme reactions had reproducible migration times and areas, with the relative standard deviation less than 1% and less than 10%, respectively. The areas of the product peaks obtained at these two concentrations were not significantly different at a 0.01 significance level when compared using a two-sided pooled T test. When lectin with high specificity to α2-3 sialic acids (MAL) was included in the capillary no statistical difference in areas, which further confirmed that the α2-3 sialic acids were completely cleaved from the sample. A lower concentration of α2-
3 sialidase, 50 μUnits/μL, showed incomplete cleavage of α2-3 linked sialic acid. Two different instruments were used for the sialic acid analysis and the range study. However, the ratio of N-glycan to internal standard was the same because the same concentrations were used (Table 2.13).

Figure 2.5 Establishing the working range of α2-3 sialidase enzyme concentration for α-1-acid glycoprotein sample 1. This is defined by complete conversion of α2-3 sialic acids in-capillary and averting enzyme specificity for α2-6 sialic acids. Separations performed at 23 °C in a 25μm i.d. capillary, with an effective of 50cm and E=450V/cm (reverse polarity). Pre-separation done at 4 kV for 9 min to drive substrate past enzyme plug. The α2-3 sialidase is suspended in 20% nanogel buffered with 50mM pH5 sodium acetate. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. *Analytical Chemistry*. 2020, 92 (1), 1518-1524. Copyright © 2019 American Chemical Society.
Table 2.11 Figure 2.5 normalized areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 1.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>750 µUnits/µL Run #1071</td>
<td>0</td>
<td>68027</td>
<td>407593</td>
<td>121126</td>
<td>23371</td>
<td>96239</td>
<td>130271</td>
<td>43141</td>
<td>109180</td>
<td>54227</td>
<td>26541</td>
<td>34474</td>
<td>9</td>
<td>107522</td>
</tr>
<tr>
<td>750 µUnits/µL Run #1074</td>
<td>0</td>
<td>65741</td>
<td>404379</td>
<td>118955</td>
<td>23429</td>
<td>94472</td>
<td>127878</td>
<td>42543</td>
<td>107962</td>
<td>53814</td>
<td>25638</td>
<td>33971</td>
<td>10</td>
<td>105471</td>
</tr>
<tr>
<td>750 µUnits/µL Run #077</td>
<td>0</td>
<td>66208</td>
<td>407140</td>
<td>119798</td>
<td>23651</td>
<td>94748</td>
<td>129568</td>
<td>42215</td>
<td>108624</td>
<td>53392</td>
<td>26189</td>
<td>342045</td>
<td>10</td>
<td>103975</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>0</td>
<td>66659</td>
<td>406371</td>
<td>119960</td>
<td>23483</td>
<td>95153</td>
<td>129239</td>
<td>42633</td>
<td>108589</td>
<td>53811</td>
<td>26122</td>
<td>342170</td>
<td>10</td>
<td>106130</td>
</tr>
<tr>
<td>RSD</td>
<td>0</td>
<td>6900</td>
<td>40509</td>
<td>119998</td>
<td>23452</td>
<td>95087</td>
<td>129264</td>
<td>42675</td>
<td>108627</td>
<td>53856</td>
<td>26166</td>
<td>342281</td>
<td>10</td>
<td>106130</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 µUnits/µL Run #1062</td>
<td>0</td>
<td>67988</td>
<td>407005</td>
<td>121080</td>
<td>22759</td>
<td>95035</td>
<td>130043</td>
<td>41894</td>
<td>108123</td>
<td>48608</td>
<td>24841</td>
<td>307259</td>
<td>106679</td>
<td>1481312</td>
</tr>
<tr>
<td>250 µUnits/µL Run #1065</td>
<td>0</td>
<td>67881</td>
<td>405572</td>
<td>120146</td>
<td>22363</td>
<td>94302</td>
<td>128824</td>
<td>42388</td>
<td>108678</td>
<td>49215</td>
<td>24362</td>
<td>336612</td>
<td>103797</td>
<td>1504142</td>
</tr>
<tr>
<td>250 µUnits/µL Run #1068</td>
<td>0</td>
<td>68088</td>
<td>408465</td>
<td>120779</td>
<td>22907</td>
<td>94715</td>
<td>129721</td>
<td>42244</td>
<td>109521</td>
<td>50061</td>
<td>25095</td>
<td>338845</td>
<td>103975</td>
<td>1514416</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>0</td>
<td>67985</td>
<td>407014</td>
<td>120668</td>
<td>22676</td>
<td>94684</td>
<td>129529</td>
<td>42175</td>
<td>108774</td>
<td>49295</td>
<td>24766</td>
<td>327572</td>
<td>104817</td>
<td>1499957</td>
</tr>
<tr>
<td>RSD</td>
<td>0</td>
<td>6900</td>
<td>40509</td>
<td>120668</td>
<td>22676</td>
<td>94684</td>
<td>129529</td>
<td>42175</td>
<td>108774</td>
<td>49295</td>
<td>24766</td>
<td>327572</td>
<td>104817</td>
<td>1499957</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 µUnits/µL MAL Run #1043</td>
<td>0</td>
<td>64753</td>
<td>416838</td>
<td>122607</td>
<td>21539</td>
<td>97298</td>
<td>130189</td>
<td>41510</td>
<td>111004</td>
<td>45528</td>
<td>23926</td>
<td>344927</td>
<td>105494</td>
<td>1525612</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µUnits/µL Run #1047</td>
<td>54462</td>
<td>59894</td>
<td>423343</td>
<td>79404</td>
<td>20551</td>
<td>90541</td>
<td>127230</td>
<td>24409</td>
<td>138843</td>
<td>45595</td>
<td>10585</td>
<td>299277</td>
<td>66739</td>
<td>1440871</td>
</tr>
</tbody>
</table>

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.
Table 2.12 Figure 2.5 raw areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 1.

<table>
<thead>
<tr>
<th>Glycan area</th>
<th>I.S.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>750 µUnits/µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run #1071</td>
<td>59897</td>
<td>0</td>
<td>67982</td>
<td>407321</td>
<td>121045</td>
<td>23355</td>
<td>96175</td>
<td>130184</td>
<td>43112</td>
<td>109107</td>
<td>54191</td>
<td>26523</td>
<td>344519</td>
<td>107450</td>
<td>1530964</td>
</tr>
<tr>
<td>Run #1074</td>
<td>59937</td>
<td>0</td>
<td>65741</td>
<td>404379</td>
<td>119855</td>
<td>23429</td>
<td>94472</td>
<td>127878</td>
<td>42543</td>
<td>107962</td>
<td>53814</td>
<td>25638</td>
<td>339717</td>
<td>105471</td>
<td>1509999</td>
</tr>
<tr>
<td>Run #1077</td>
<td>58622</td>
<td>0</td>
<td>64755</td>
<td>398207</td>
<td>117170</td>
<td>23355</td>
<td>96175</td>
<td>130184</td>
<td>42315</td>
<td>107770</td>
<td>53409</td>
<td>25925</td>
<td>339592</td>
<td>105335</td>
<td>1508871</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>59485</td>
<td>0</td>
<td>66159</td>
<td>403302</td>
<td>119057</td>
<td>23305</td>
<td>94439</td>
<td>128262</td>
<td>42315</td>
<td>107770</td>
<td>53409</td>
<td>25925</td>
<td>339592</td>
<td>105335</td>
<td>1508871</td>
</tr>
<tr>
<td>RSD</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>250 µUnits/µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run #1062</td>
<td>59851</td>
<td>0</td>
<td>67890</td>
<td>406421</td>
<td>120906</td>
<td>22726</td>
<td>94899</td>
<td>129856</td>
<td>41834</td>
<td>107968</td>
<td>48538</td>
<td>24805</td>
<td>306818</td>
<td>106526</td>
<td>1479187</td>
</tr>
<tr>
<td>Run #1065</td>
<td>59870</td>
<td>0</td>
<td>67805</td>
<td>405119</td>
<td>120012</td>
<td>22338</td>
<td>94197</td>
<td>128680</td>
<td>42341</td>
<td>108557</td>
<td>49160</td>
<td>24335</td>
<td>336236</td>
<td>103681</td>
<td>1502461</td>
</tr>
<tr>
<td>Run #1068</td>
<td>59350</td>
<td>0</td>
<td>67421</td>
<td>404465</td>
<td>119596</td>
<td>22683</td>
<td>93787</td>
<td>128451</td>
<td>41830</td>
<td>108448</td>
<td>49571</td>
<td>24849</td>
<td>335526</td>
<td>102957</td>
<td>1501584</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>59690</td>
<td>0</td>
<td>67705</td>
<td>405335</td>
<td>120171</td>
<td>22582</td>
<td>94294</td>
<td>128996</td>
<td>42002</td>
<td>108324</td>
<td>49090</td>
<td>24663</td>
<td>326193</td>
<td>104388</td>
<td>1493744</td>
</tr>
<tr>
<td>RSD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>250 µUnits/µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAL RAW 1043</td>
<td>59846</td>
<td>0</td>
<td>64655</td>
<td>416205</td>
<td>122421</td>
<td>21506</td>
<td>97150</td>
<td>129991</td>
<td>41447</td>
<td>110835</td>
<td>45459</td>
<td>23890</td>
<td>344403</td>
<td>105334</td>
<td>1523296</td>
</tr>
<tr>
<td>50 µUnits/µL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAW 1047</td>
<td>5950154066</td>
<td>59458</td>
<td>420263</td>
<td>78826</td>
<td>20402</td>
<td>89882</td>
<td>126304</td>
<td>24231</td>
<td>137833</td>
<td>45263</td>
<td>10508</td>
<td>297100</td>
<td>66254</td>
<td>1430390</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.13 Comparison of α-1-acid glycoprotein sample 1 glycan area to internal standard area across instruments for each enzyme condition.

<table>
<thead>
<tr>
<th>I.S. area</th>
<th>Glycan area</th>
<th>Ratio glycan area to IS</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGP sample 1</td>
<td>a2-3 sialidase range study</td>
<td>34000</td>
<td>895000</td>
</tr>
<tr>
<td></td>
<td>Multiple enzyme sequencing study</td>
<td>60000</td>
<td>1490000</td>
</tr>
</tbody>
</table>

Internal standard (I.S.) and glycan areas are averages of 3 replicates performed on each instrument. The total glycan area was divided by total standard area.
2.3.7 Enzyme processing.

Serial enzyme processing was then applied to the AGP N-glycans to identify the α2-6 linked sialic acid distribution. The migration windows for the bi-, tri- and tetraantennary peaks with different numbers of galactose residues are emphasized with arrows in Figure 2.4A. The enzyme processing was performed in the absence (Figure 2.4B black trace) and presence (Figure 2.4B blue trace) of lectin with affinity to galactose bearing structures (i.e. ECL) to distinguish any comigrating peaks. From these runs areas were determined by assigning the biantennary structures to peaks until the summed area for biantennary peaks equaled the corresponding area established from the reference traces in Figure 2.4A. This process was repeated for the triantennary and then for the tetraantennary structures. Peak splitting observed with the triantennary standard (Figure 2.4B) due to differences in the α1-3 and α1-6 arm mannose linkages in the core were also observed in the AGP mixture (Figure 2.4B, black trace) for the mono-galactosylated structures that are biantennary (19.6 min, 19.9 min), triantennary (20.8 min, 21.1 min), and tetraantennary (22.1 min, 22.4 min). The agalactosylated biantennary peak (18.6 min) is broad because some of the biantennary N-glycans were partially desialylated and differences in mobility of the fully and partially sialylated structure induced this broadening.

2.3.8 Comparison of the α2-6 sialic acid content from different sources of α-1 acid glycoprotein.

A second sample, which was a commercially available source of free N-glycans cleaved from AGP, was analyzed with the same method to determine α2-6 sialic acid composition.
The second AGP sample was diluted so that the total peak area of triantennary N-glycan of the AGP sample matched the peak area of the triantennary standard used in Figure 2.2. The areas of the biantennary and tetraantennary N-glycan peaks were 1.4-fold and 2.3-fold lower than that of the triantennary N-glycan. The working range of α2-3 sialidase was confirmed as shown in Figure 2.6 and the area of each branched structure was normalized to the internal standard (Figure 2.6 and Table 2.14 for normalized areas and Table 2.15 for raw areas). Two different instruments were used for the sialic acid analysis and the range study. However, the ratio of N-glycan to internal standard was the same because the same concentrations were used, as shown in Table 2.16.

![Figure 2.6 Establishing the working range of α2-3 sialidase enzyme concentration for α-1-acid glycoprotein sample 2. This is defined by complete conversion of α2-3 linked sialic acids in capillary with no cleavage of α2-6 linked sialic acids. Separation conditions as described in Figure 2.4. Structures cannot be assigned with of α2-3 sialidase only. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. *Analytical Chemistry*. **2020**, *92* (1), 1518-1524. Copyright © 2019 American Chemical Society.](image-url)
Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.

<table>
<thead>
<tr>
<th>Table 2.14 Figure 2.6 normalized areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 2.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table</strong></td>
</tr>
<tr>
<td><strong>Run</strong></td>
</tr>
<tr>
<td><strong>750 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#448</strong></td>
</tr>
<tr>
<td><strong>750 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#451</strong></td>
</tr>
<tr>
<td><strong>750 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#454</strong></td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
</tr>
<tr>
<td><strong>RSD</strong></td>
</tr>
<tr>
<td><strong>250 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#436</strong></td>
</tr>
<tr>
<td><strong>250 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#442</strong></td>
</tr>
<tr>
<td><strong>250 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#445</strong></td>
</tr>
<tr>
<td><strong>AVERAGE</strong></td>
</tr>
<tr>
<td><strong>RSD</strong></td>
</tr>
<tr>
<td><strong>250 µUnits/µL MAL Run</strong></td>
</tr>
<tr>
<td><strong>#439</strong></td>
</tr>
<tr>
<td><strong>50 µUnits/µL Run</strong></td>
</tr>
<tr>
<td><strong>#298</strong></td>
</tr>
</tbody>
</table>

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.
<table>
<thead>
<tr>
<th>Run</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>750 µUnits/µL Run #448</td>
<td>35305</td>
<td>0</td>
<td>25440</td>
<td>169558</td>
<td>81714</td>
<td>29727</td>
<td>38611</td>
<td>18397</td>
<td>28805</td>
<td>12359</td>
<td>168593</td>
<td>44736</td>
</tr>
<tr>
<td>750 µUnits/µL Run #451</td>
<td>33526</td>
<td>0</td>
<td>24841</td>
<td>166277</td>
<td>80901</td>
<td>28602</td>
<td>37273</td>
<td>18722</td>
<td>28320</td>
<td>12185</td>
<td>159722</td>
<td>43078</td>
</tr>
<tr>
<td>750 µUnits/µL Run #454</td>
<td>34677</td>
<td>0</td>
<td>23812</td>
<td>163606</td>
<td>77570</td>
<td>29690</td>
<td>39624</td>
<td>20381</td>
<td>28486</td>
<td>170204</td>
<td>42719</td>
<td>608915</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>34503</td>
<td>0</td>
<td>24698</td>
<td>166480</td>
<td>80062</td>
<td>29340</td>
<td>38503</td>
<td>19167</td>
<td>28537</td>
<td>166173</td>
<td>43511</td>
<td>608925</td>
</tr>
<tr>
<td>RSD</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>250 µUnits/µL Run #436</td>
<td>37125</td>
<td>0</td>
<td>26846</td>
<td>182214</td>
<td>84946</td>
<td>30977</td>
<td>40624</td>
<td>19631</td>
<td>31476</td>
<td>13897</td>
<td>169694</td>
<td>44762</td>
</tr>
<tr>
<td>250 µUnits/µL Run #442</td>
<td>36215</td>
<td>0</td>
<td>25395</td>
<td>175164</td>
<td>79918</td>
<td>30052</td>
<td>39308</td>
<td>18056</td>
<td>32084</td>
<td>13740</td>
<td>166671</td>
<td>42460</td>
</tr>
<tr>
<td>250 µUnits/µL Run #445</td>
<td>29323</td>
<td>0</td>
<td>21040</td>
<td>137685</td>
<td>64385</td>
<td>25007</td>
<td>32394</td>
<td>14405</td>
<td>27570</td>
<td>10652</td>
<td>137828</td>
<td>32403</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>34221</td>
<td>0</td>
<td>24427</td>
<td>165021</td>
<td>76416</td>
<td>28679</td>
<td>37442</td>
<td>17364</td>
<td>30377</td>
<td>12763</td>
<td>158064</td>
<td>39875</td>
</tr>
<tr>
<td>RSD</td>
<td>12</td>
<td>0</td>
<td>12</td>
<td>15</td>
<td>14</td>
<td>11</td>
<td>12</td>
<td>15</td>
<td>8</td>
<td>14</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>250 µUnits/µL MAL Run #439</td>
<td>36287</td>
<td>0</td>
<td>24627</td>
<td>175597</td>
<td>81775</td>
<td>29565</td>
<td>39508</td>
<td>17974</td>
<td>32587</td>
<td>10485</td>
<td>163512</td>
<td>44001</td>
</tr>
<tr>
<td>50 µUnits/µL Run #298</td>
<td>38838</td>
<td>42296</td>
<td>19904</td>
<td>195582</td>
<td>53183</td>
<td>31606</td>
<td>41956</td>
<td>12870</td>
<td>44278</td>
<td>10904</td>
<td>176514</td>
<td>28898</td>
</tr>
</tbody>
</table>

Table 2.15 Figure 2.6 raw areas of α2-3 sialidase working concentration range for α-1-acid glycoprotein sample 2.

Table 2.16 Comparison of α-1-acid glycoprotein sample 2 glycan area to internal standard area across instruments for each enzyme condition.
<table>
<thead>
<tr>
<th></th>
<th>I.S. area</th>
<th>Glycan area</th>
<th>Ratio glycan area to IS</th>
<th>Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGP Sample 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2-3 sialidase range study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>79000</td>
<td>1750000</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Multiple enzyme sequencing study</td>
<td>34000</td>
<td>677000</td>
<td>20</td>
<td>2</td>
</tr>
</tbody>
</table>

Internal standard (I.S.) and glycan areas are averages of 3 replicates performed on each instrument. The total glycan area was divided by total standard area.

The second sample contained more unreacted APTS than sample 1. Additionally, the total glycan area of the second sample was approximately 2.4 times higher than that of sample 1, shown in Figure 2.7. As a result, more contaminant peaks, (which are labeled with an asterisk in Figures 2.7), were evident in the second sample. The contaminant peaks were distinguishable from the N-glycan peaks by comparing the signal obtained from serial enzyme processing performed in the absence and presence of ECL (Figure 2.8).
Figure 2.7 Analyses of α-1-acid glycoprotein sample 2 asparagine-linked glycans. The glycans were obtained from a different source than that shown for Figure 2.3. (A) reference traces establish the area associated with biantennary, triantennary, and tetraantennary glycans. (B) The α2-6 linked sialic acid area of each branched structure is established through serial enzyme processing in the absence of ECL (black trace). Presence of ECL (blue trace) identifies agalactosylated glycans in co-migrating peaks. Separation conditions are as specified in Figure 2.2. The asterisk denotes contaminant peaks. Reproduced with permission from: Bwanali, L.; Newton, E.; Crihfield, C. L.; Zeger, V.; Gattu, S.; Holland, L. A., Quantification of the α2-6 sialic acid linkage in branched N-glycan structures with capillary nanogel electrophoresis. *Analytical Chemistry*. **2020**, *92* (1), 1518-1524. Copyright © 2019 American Chemical Society.
Although different branching ratios were observed for sample 1 as compared to sample 2 for the biantennary (13% vs 21%), triantennary (51% vs 47%), and tetraantennary (36% vs 31%) structure, these branching distributions were within the ranges reported in the literature.\(^6,\)\(^{31,\)\(^{32}\) Interestingly, the predominant form of α2-6 sialic acid in each branched structure contained only a single α2-6 sialic acid. Small amounts of tetraantennary N-glycan containing two to four α2-6 sialic acids were observed (Table 2.17). The normalized and raw areas are shown in Table 2.18 and 2.19). This was expected as α2-3 sialic acids adopt an extended linear conformation; whereas, α2-6 sialic acids are oriented toward the mannose core forming a more compact glycan structure.\(^{27}\) Although

![Identification of glycan and contaminant peaks](image-url)
quantification of α2-6 linked sialylation in AGP had not previously been reported, AGP has been reported to contain monogalactosylated, as well as di- and tri-galactosylated tetraantennary structures.

Table 2.17 Peak area of α2-6 linked sialic from α-1-acid glycoprotein sample 2 glycans.

<table>
<thead>
<tr>
<th>Branch</th>
<th>α2-6 linked sialic acid ( area x 10^3 (%RSD)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56 (1) 339 (3) 0 — — 395 (3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>226 (3) 649 (6) 0 0 — 876 (7)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>216 (5) 339 (6) 19 (10) 52 (7) 19 (10) 645 (6)</td>
<td></td>
</tr>
</tbody>
</table>

Areas are reported in units of 10^3 RFU. The areas and percent relative standard deviation (RSD) of 2, 3, and 4 branch N-glycans from alignment trace normalized to internal standard are: 380 (2), 890 (4), and 630 (4), respectively. Normalization is based on the highest quantified area for the internal standard within the alignment traces, sequencing traces, and sequencing traces with ECL (i.e. 81,675 RFU). Solid lines in the table indicate that the structure not possible. Separation conditions are as stated Figure 2.2.
Table 2.18 Figure 2.7 normalized peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 2.

Areas normalized to internal standard (I.S.) within the data set. A correction factor was achieved by dividing the I.S. area in each run by highest I.S. within data set. The glycan area was then multiplied by corresponding this correction factor.
Table 2.19 Figure 2.7 raw peak areas for α2-6 sialic acid structural assignments using multiple enzyme in α-1-acid glycoprotein sample 2.

<table>
<thead>
<tr>
<th>I.S.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1</td>
<td>79148</td>
<td>54708</td>
<td>540649</td>
<td>279975</td>
<td>542181</td>
<td>309233</td>
<td>16483</td>
<td>49731</td>
</tr>
<tr>
<td>RUN 2</td>
<td>77574</td>
<td>52695</td>
<td>540649</td>
<td>286292</td>
<td>555643</td>
<td>338438</td>
<td>19868</td>
<td>52336</td>
</tr>
<tr>
<td>RUN 3</td>
<td>78756</td>
<td>53010</td>
<td>540649</td>
<td>286665</td>
<td>543191</td>
<td>328235</td>
<td>18419</td>
<td>46590</td>
</tr>
</tbody>
</table>

AVERAGE 78493 53471 543839 284311 547005 325302 18257 49552 18358

STDEV 819 1083 3999 3759 7498 14822 1698 2877 1609

RSD 1 2 1 1 1 5 9 6 9

2.4 Conclusions and future directions

Quantification of the amount of α2-6 sialic acid in N-glycan structures was achieved using an enzyme based method of glycan modification. With the use of nanogel, enzymatic conversion of N-glycans was achieved in real-time and consumed only nanoliter volumes of enzymes or lectins for each analysis. Capillary electrophoresis analyses of mixtures of sialylated N-glycans are complex because of the charge on the sialic acids. Additionally, N-glycans can be desialylated when exposed to heat and/or to different
solutions used in conventional sample processing. Moreover, differences in the stability of α2-6 vs α2-3 linked sialic acids are found in the literature. By incorporating the enzymes as outlined in this report, a predictable mobility shift based on a change in the charge-to-size ratio of the N-glycan was achieved. With this method, the α2-6 linked sialic acid linkage of N-glycans was determined for a pure standard as well as for mixtures of biantennary, triantennary, and tetraantennary structures derived from AGP. Moreover, the exact site of α2-6 linked sialylation in biantennary and potentially triantennary N-glycans can be inferred based on the difference in hydrodynamic radius for the α1-3 mannose, which is more extended, creating a larger hydrodynamic radius and a slower electrophoretic migration in the electric field observed in capillary electrophoresis separations of N-glycans. Determination of sialic acid linkage is critical to validating ongoing research on the use of α2-6 linked sialic acid as a biomarker of disease. Future work centers on the application of this technology to glycoproteins as well as glycan standards to authenticate the degree of sialylation and sialic acid linkage.

2.5 Acknowledgements
This material is based upon work supported by NIH Grant No. R01GM114330. CLC acknowledges a National Science Foundation IGERT fellowship, DGE #1144676. Victoria R. Zeger contributed to the statistical analysis of all data. Ebenezer O. Newton contributed to the data collection for Figure 2.3. Srikanth Gattu contributed to the experimental design of Figure 2.2. Cassandra L. Crihfield contributed to the experimental design, data analysis and interpretation of Figure 2.4 and 2.7. Lloyd Bwanali contributed in the data collection for all Figures, experimental design, manuscript preparation, data analysis and data
interpretation for all aspects of this work. Lisa Holland contributed to manuscript preparation, experimental design, data analysis and data interpretation for all aspects of this work. Lisa Holland was also responsible for acquiring project funding and supervising

2.6 References


6. Sarrats, A.; Saldova, R.; Pla, E.; Fort, E.; Harvey, D. J.; Struwe, W. B.; de Llorens, R.; Rudd, P. M.; Peracaula, R., Glycosylation of Liver Acute-Phase Proteins in


12. Jooß, K.; Meckelmann, S. W.; Klein, J.; Schmitz, O. J.; Neusüß, C., Capillary Zone Electrophoresis Coupled to Drift Tube Ion Mobility-Mass Spectrometry for the


Chapter 3 : Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein

Reproduced from

3.1 Introduction

Protein-based drugs comprise a new generation of pharmaceuticals that improve human health. Prior to the 2019 pandemic, the therapeutic antibody market was projected to reach an annual value of 300 billion USD in 2025. However, this projection is subject to change given the dramatic development and use of monoclonal antibody therapeutics to treat COVID19. IgG antibodies contain asparagine-linked glycans (N-glycans) in the C\textsubscript{H}2 domain, and these N-glycans modulate receptor interactions integral to immune response. Glycosylation, which is a critical quality attribute of protein therapeutics, is a non-templat ed post-translational modification controlled by enzymes. Modifications to IgG glycosylation influence the antibody effector function as well as pharmacokinetics. Several strategies have been developed to control enzymatic processing through engineered cell lines, and post-expression chemoenzymatic processing. Enzymatic modifications of glycoproteins have been demonstrated with wild type and mutant enzymes, and a variety of designer enzymes have been customized to tailor glycosylation. For example, one β-1,4-galactosyltransferase, GalT(Y289), enables custom antibody modification through click chemistry. Although enzymes can be developed through structurally guided point mutations, they must ultimately be screened and characterized to quantify the performance of the enzyme-substrate system.

Galactosyltransferase assays require a means to detect the products, and generally rely on modifying either the donor or the acceptor with a radiochemical label or chromophore. Because both the reactants and products are detectable they must be
distinguished prior to quantification. This has been accomplished with techniques such as chromatography or mass spectrometry. Commercial kits are available and for some kits a secondary reaction occurs which renders the products detectable in the presence of optically inactive reactants. Enzyme assays have been adapted for higher throughput platforms, for example through microarrays, multi-well plates, or through analyses in series. A consideration for these methods is the amount of enzyme consumed for each determination when multiple analyses are conducted. Ultimately, strategies to characterize transferase enzymes require technologies that are both fast and automated. Additionally, they should be compatible with low substrate and enzyme volumes given the effort required for enzyme expression and the cost of different substrate-donor pairs.

Capillary electrophoresis is an easily automated microscale method of analysis that consumes picoliter to nanoliter sample volumes. Moreover, capillary electrophoresis is amenable to in-capillary analyses that integrate the enzyme assay with a separation-based detection for product quantification. Several strategies have been reported to immobilize enzymes in the separation capillary,\(^{18, 19}\) including covalent immobilization methods based on the use of monoliths\(^{20}\) or particles,\(^{21}\) which generate high surface area to load enzymes. Alternatively, enzymes can be noncovalently immobilized directly onto unmodified\(^ {22}\) or surface-modified capillaries.\(^ {23}\) The ease of noncovalent immobilization and the advantage of high loading capacity has been realized through the use of noncovalently immobilized enzyme reactors based on self-assembled materials for entrapment, such as hydrogels\(^{24}\) and nanogels.\(^{25-27}\)
Nanogels are non-Newtonian fluids composed of phospholipids that self-assemble to form different morphologies with a temperature-dependence.\textsuperscript{28, 29} Nanogels have a low viscosity at low temperature (i.e., 19 °C) and a gel-like viscosity at temperatures of 25 °C and higher, allowing for introduction in capillary at low temperature. Once the nanogel is loaded into the separation capillary, the temperature is then raised to create a gel-like viscosity, to enhance the enzyme reaction and the separation. Separations of complex samples of N-glycans have been described with the use of nanogels as a viscous additive in capillary electrophoresis.\textsuperscript{30, 31} Nanogels have been used to pattern biological reagents in the capillary for N-glycan sequencing and for structural identification.\textsuperscript{25-27, 30} When the nanogel separation is complete easily the nanogel is expelled and then fresh nanogel are repatterned by dropping the temperature, after and the capillary is patterned with fresh biological reagents for a new run to be completed. Nanogels have been used to pattern enzyme reactions in-capillary prior to the separations, enabling the determination of Michaelis-Menten values,\textsuperscript{27} structural identification of N-glycans,\textsuperscript{32, 33} and oligosaccharide linkage analyses.\textsuperscript{25, 26} There are several advantages to using nanogels for enzyme reactions including enhanced enzyme stability,\textsuperscript{27} precise control of the size and position of the enzyme zone,\textsuperscript{25, 27} as well as flexibility in the reaction zone conditions.\textsuperscript{25, 27} The use of nanogels in enzyme analyses has been limited to exoglycosidase trimming of different N-glycan substrates.\textsuperscript{25-27, 32, 33} Transferase reactions, which are more complex, require the presence of a donor, acceptor, and often a cofactor. Nanogel patterning offers an opportunity to pattern and integrate a more complex reaction system in an automated microscale format.
To date, most capillary electrophoresis analyses of galactosyltransferase (GalT) have been used for off-line enzyme assays performed as benchtop reactions.\textsuperscript{34-37} In this study, nanogels were used to pattern the uridine-5’-diphosphogalactose (UDP-gal) donor, GalT and manganese cofactor to transfer galactose to oligosaccharides as shown in Figure 3.1. A labeled glycan substrate was driven electrophoretically through the reaction zone which was located within the thermally regulated portion of the capillary. Once the enzyme-catalyzed conversion was complete, the reaction products were separated and quantified. A pentasaccharide end-labeled with 2-aminobenzoic acid was used to evaluate the system as this molecule was representative of the biantennary structure of an N-glycan. With the ability to distinguish reaction products for which either one or two galactose residues were transferred (see Figure 3.1), the capillary nanogel electrophoresis system was used to determine the Michaelis-Menten value, $K_M$. The Michaelis-Menten constant for the addition of a single galactose residue was derived by plotting the velocity of product formation against the concentration of pentasaccharide introduced into the capillary. For the β1-4 galactosyltransferase, the $K_M$ value obtained for a pentasaccharide substrate was 1.23 ± 0.08 mM. Once the $K_M$ was established, the enzyme-to-substrate ratio was evaluated to add a single galactose residue, or to fully galactosylate a biantennary N-glycan. Fluorescently labeled biantennary N-glycans were used to determine the relationship between the enzyme-to-substrate ratio and the amount of galactose transferred. By utilizing fluorescence detection, a lower concentration of substrate was observed, which facilitated experiments with ratios of enzyme:substrate. Ratios of enzyme:substrate were successively increased in the reaction zone until a change in the distribution of galactosylation of the reaction products was observed.
Finally, the integrated nanogel separation was modified to transfer galactose residues to an intact glycoprotein. The applicability of the method for real-time online modification of whole protein was demonstrated with the Herceptin glycoprotein. Complete retardation by Erythrina Cristagalli lectin after enzymatic modification confirmed the addition of galactose residues to the Herceptin. This demonstrated the potential of the method to be used for online modification of other glycoproteins.

Figure 3.1 Conceptual diagram illustrating the online reaction where the transferase enzyme adds a monomer to an acceptor biantennary glycan, pentasaccharide, that has been end labeled with aminobenzoic acid. The figure shows how the patterning in the capillary is achieved, together with a concept trace showing how different degrees of galactosylation are identified. After identifying the galactosylation the concept is used to achieve the $K_M$ of the pentasaccharide with the enzyme $\beta_1$-$\beta_4$ galactosyltransferase. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the $\beta_1$-$\beta_4$ Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. Analytical Chemistry. Submitted June 2021.
3.2 Materials and methods

3.2.1 Chemicals and reagents.

The 8-aminopyrene-1,3,6-trisulfonic acid (APTS), β-1,4-galactosyltransferase (SAE0093), 2-aminobenzoic acid (118-92-3), UDP-α-D-galactose, disodium salt (670111-10MG-M), methanol, ethanol, 3-(N-morpholino)-propanesulfonic acid (MOPS), sodium cyanoborohydride (either 296813, 1.0 M dissolved in tetrahydrofuran or 156159), sodium acetate and sodium hydroxide were from Sigma-Aldrich (St. Louis, MO). Acetic acid was from Fisher Scientific (Pittsburgh, PA). Manganese (II) chloride tetrahydrate (13446-34-9) was obtained from Alfa Aesar (Haverhill, MA). Erythrina Cristagalli lectin (ECL), was from Vector Labs (Burlingame, CA). PNGase F was purchased from New England Biolabs (Ipswich, MA). 18.2 MΩ-cm deionized water produced from an Elga Purelab ultra water system (Lowell, MA) was utilized. The biantennary N-linked core pentasaccharide was from Dextra Laboratories Ltd (Bristol, UK). The phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), were from Avanti Polar Lipids (Alabaster, AL). Nanogels were prepared by weighing phospholipids and combining them such that [DMPC]/[DHPC] = 2.5. The phospholipids were dissolved in an aqueous solution of 100 mM MOPS buffered to pH of 7.0 to achieve concentrations of 10, 20 and 30% preparations. The process to reconstitute phospholipids has been described previously. Once made, each preparation was then aliquoted and stored at −20 °C. for up to 10 days.
3.2.2 Preparation and derivatization of standards.

The glycans were labeled with a fluorescent or UV absorbing chromophore as previously described.\textsuperscript{27, 30, 32, 33} Prior to labeling, the biantennary N-glycans were cleaved from the glycoprotein as described previously.\textsuperscript{32} Briefly, the labeling reaction was performed at 37°C overnight with a ratio of 500 nanomoles 8-aminopyrene-1,3,6-trisulfonic acid (APTS): 10 nanomoles biantennary N-glycans by reacting 2.5 μl volume of 4 mM biantennary N-glycans dissolved in 1 M sodium cyanoborohydride in tetrahydrofuran with 2.5 μl volume of 0.2 M APTS dissolved in 20% acetic acid. Once the reaction was completed the reaction mixture was evaporated to dryness using a Savant SpeedVac concentrator (ThermoScientific, Waltham, MA) and excess APTS was removed from the labelling reaction using a Discovery DPA-6S solid phase extraction cartridge (52624-U 50 mg packing material, Supelco, Bellefonte, PA) as previously described.\textsuperscript{30} Prior to analysis glycan samples were diluted in 1 mM MOPS buffered to pH 7.

The determination of the $K_M$ in the mM range required that the substrate was labeled with the 2-aminobenzoic acid chromophore detected by UV-visible absorbance. The substrate labeling procedure was previously developed and reported\textsuperscript{27} to ensure complete labeling of the substrate as residual unlabeled substrate would bias the $K_M$ measurement. Briefly, the labeling reaction was performed at a ratio of 1400 nanomoles 2-aminobenzoic acid: 200 nanomoles pentasaccharide by reacting 1 μl volume of 0.2 M saccharide dissolved in water with 1 μl volume of 1.39 M 2-aminobenzoic acid and 23 μl volume of 1 M sodium cyanoborohydride both of which were dissolved in 0.5 M acetic acid (obtained by diluting glacial acetic acid with methanol). The reaction proceeded at 65°C for 2 hours. Once
the reaction was completed the reaction mixture was evaporated to dryness using a Savant SpeedVac concentrator (ThermoScientific) and excess 2-aminobenzoic acid was removed from the labelling reaction using a Discovery DPA-6S solid phase extraction cartridge (52624-U Supelco) as previously described.27 Briefly, the saccharide solution was diluted to ensure a final composition of 95% acetonitrile prior to the purification process. The DPA-6S solid phase extraction cartridge tube was loaded with 1-mL of the sample diluted to contain 95:5 (v/v) acetonitrile:deionized water. The 1-mL flow-through captured during the initial sample loading was reloaded onto the cartridge twice. Once loaded in the extraction cartridge, the 2-aminobenzoic acid was eluted using 10-mL of a solution comprised of 95% acetonitrile, 5% deionized water that also contained 1 mM triethylamine. Next, the retained glycans were eluted from the cartridge using 3-mL of a solution of 25 mM triethylamine in deionized water. Following purification, the fraction containing labeled oligosaccharide was dried using a SpeedVac concentrator, reconstituted in 25 μL deionized water, and stored at -20 °C. Prior to injection, sample was diluted in 1 mM MOPS buffered to pH 7. The pentasaccharide was analyzed before and after purification and 100% recovery was demonstrated as shown in Figure 3.2
3.2.3 Preparation of enzyme and lectin.

The GalT enzyme was reconstituted so that the appropriate concentration of enzyme was in 10% phospholipid buffered at pH 7 with 100 mM MOPS. The enzyme zones were buffered at pH 7, which was compatible with enzyme conversion and was near the enzyme isoelectric point of 8.75. For the GalT enzyme 1 Unit was defined as the amount of enzyme required to transfer 1.0 nanomole of galactose from UDP-Gal to GlcNAc per minute at pH 7.5, 37 °C. The ECL Lectin was desalted prior to use. The salts were removed using a 10 kDa molecular weight cut off filter centrifuged at 14,000G and 19 °C. The ECL was rinsed by centrifuging 3 times each at 10 minutes with a 0.5 mL solution of 100 mM MOPS buffered to pH 7. Once desalted, the ECL was mixed 1:1 with a 20%
nanogel preparation that contained 100 mM MOPS buffered to pH 7. The final concentration of the ECL was 100 μM ECL in 10% nanogel.

3.2.4 Capillary electrophoresis.
Analyses were performed using a SCIEX MDQ Plus (Sciex, Redwood City, CA) configured by the manufacturer with laser induced fluorescence detection and appropriate filters (3 mW solid state laser with $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm) and a UV-visible absorbance detector (monitored at 214 nm). Silica capillaries with a 25 μm internal diameter and 360 μm outer diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ) were used for the separations. The electrophoresis was performed using reversed polarity to separate the negatively-charged amino benzoic acid-labeled, or APTS-labeled glycans (anodic reservoir near detector, cathodic reservoir near the site of injection). Ambient thermal control of the room and instrument was maintained using a portable air conditioner as was described previously. The electrophoresis was performed using reversed polarity in combination with a semi-permanent coating to suppress the electroosmotic flow to separate the negatively-charged amino benzoic acid-labeled, or APTS-labeled glycans (anodic reservoir near detector, cathodic reservoir near the site of injection). Each day capillaries were prepared as previously described. The capillaries were prepared by flushing with the following sequence: 1 M NaOH for 30 minutes at 170 kPa (25 psi), deionized water for 15 minutes at 170 kPa (25 psi), 15 minutes methanol at 170 kPa (25 psi), and deionized water for 15 minutes at 170 kPa (25 psi). The capillary was then coated with $q = 0.5$, 5% phospholipid by weight (with [DMPC]:[DHPC] = 0.5) containing 1.25 mM calcium for 20 minutes at 170 kPa (25 psi),
followed by a 3 minute 170 kPa (25 psi) rinse with MOPS. Prior to each run the capillary was held at 19 °C and the 10% nanogel was introduced in the capillary at 170 kPa (25 psi) for 8 minutes.

The amount of the cofactor manganese (II) chloride needed in the capillary for enzyme conversion was established in order to achieve optimum conversion as shown in Table 3.1 and Figure 3.3. When the 5 mM cofactor was present only in the enzyme plug, 24% of the substrate still remained with only one galactose added. When manganese (II) chloride was incorporate to the left of the enzyme plug the conversion increased, and only 9% remained with only one galactose added. No further improvement in conversion were realized when the enzyme plug was increased to 3 cm. Since this plug size did not negatively impact the separation it was chosen for all enzymatic reactions. Manganese (II) chloride at a higher concentration of 10 mM interacted with the lipid causing baseline disturbance
Figure 3.3 Impact of manganese zone on biantennary N-glycan conversion
5 mU/µL β1-3,4 galactosyltransferase is in nanogel was employed. When manganese (II) chloride was used a concentration of 5 mM was maintained. Pre-separation was done at 4 kV for 15 min to drive substrate past enzyme plug. The separations was then performed at 20 kV at 29 °C in a 25 um i/d. capillary with effective length of 50cm and applied voltage of 20 kV (reverse polarity)

Table 3.1: Impact of manganese zone on biantennary N-glycan conversion

<table>
<thead>
<tr>
<th></th>
<th>1 galactose area (SD)</th>
<th>2 galactose area (SD)</th>
<th>1 galactose area % (SD)</th>
<th>2 galactose area % (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 cm MnCl₂</td>
<td>35,000 (4,000)</td>
<td>110,000 (10,000)</td>
<td>24 (3)</td>
<td>76 (2)</td>
</tr>
<tr>
<td>1 cm MnCl₂</td>
<td>12,400 (600)</td>
<td>121,000 (1,000)</td>
<td>9 (0.3)</td>
<td>91 (0.1)</td>
</tr>
<tr>
<td>3 cm MnCl₂</td>
<td>11,400 (500)</td>
<td>(124,000) (4,000)</td>
<td>9 (0.1)</td>
<td>91 (0.1)</td>
</tr>
</tbody>
</table>
To convert the pentasaccharide in the online determination of the $K_M$, reactions zones were introduced in the capillary to achieve the patterning shown in Figure 3.4. Patterning in the capillary was an automated process performed by the capillary electrophoresis instrument in multiple steps. In the patterning for pentasaccharide analysis (data for Figure 3.4 and 3.5), the capillary was first filled with the 10% nanogel. Secondly, the MnCl$_2$ zone was introduced at 68.9 kPa (10 psi) for 13 s followed by injection of the enzyme plug at 68.9 kPa (10 psi) for 13 s. For the 40 cm capillary, it took a plug of 2 aminobenzoic acid 43.3 s to travel a distance of 10.2 cm when pushed at 10 psi by the 10% nanogel. A plug introduced for 4.25 s would be 1 cm long. Therefore, the MnCl$_2$ and enzyme zones were 3 cm long. The UDP-gal was then introduced at 68.9 kPa (10 psi) for 19.5 s to achieve a 4.5 cm zone. Finally, a background electrolyte pre-plug of 13.8 kPa (2 psi) for 7 s was introduced. After the capillary was filled, the temperature of the separation was increased to 29 °C for the sample injection, incubation, and separation. Sample injections were at 10 kV 10 s followed by a background electrolyte post-plug of 13.8 kPa (0.5 psi) for 5 s. After a run was complete, a 241 kPa (35 psi) background electrolyte flush for 15 min was applied in the reverse direction to push out any remaining enzyme toward the site of injection. Data collection and analyses were performed using 32 Karat Software version 10.2.

In the patterning for the Herceptin N-glycan analysis the capillary was first filled with 10% nanogel. The MnCl$_2$ zone was then introduced at 68.9 kPa (10 psi) for 21 s followed by injection of the enzyme plug at 68.9 kPa (10 psi) for 21 s. For the 60 cm capillary used in this analysis, it took a plug of APTS 70 s to travel a distance of 10.2 cm when pushed
at 10 psi with the 10% nanogel. A plug introduced for 6.9 s would be 1 cm long. Therefore, the MnCl$_2$ and enzyme zones were 3 cm long. The UDP-gal was then introduced at 68.9 kPa (10 psi) for 28 s (4 cm). Finally, a background electrolyte pre-plug of 13.8 kPa (2 psi) for 7 s was introduced. Sample injections were at 10 kV 10 s followed by a background electrolyte post-plug of 13.8 kPa (0.5 psi) for 5 s. All other conditions were the same as those used in the pentasaccharide analyses.

In the patterning for Herceptin glycoprotein analyses, the capillary was first with 10% nanogel buffered to Ph 7 with MOPS. An ECL plug was then introduced at 68.9 kPa (10 psi) for 7 s followed by an injection of 10% lipid spacer at 13.8 kPa (0.5 psi) for 7 s. For the 30 cm capillary employed in this analysis, it took a plug of 2 aminobenzoic acid 70 s to travel a distance of 20 cm when pushed at 10 psi by the 10% nanogel. A plug introduced for 3.5 s would be 1 cm long. Therefore, lectin zone was 2 cm long. The UDP-Gal zone was then introduced at 68.9 kPa (10 psi) for 14 s (4 cm) followed by injection of the enzyme plug at 68.9 kPa (10 psi) for 10.5 s (3 cm). A background electrolyte pre-plug of 13.8 kPa (2 psi) for 10 s was then introduced. The MnCl$_2$ was included in the anodic reservoir for the pre-separation step used to drive the substrate past the enzyme plug. Sample injections were at 5 kV 4 s followed by a background electrolyte post-plug of 13.8 kPa (0.5 psi) for 5 s. Reducing the velocity while passing through the enzyme zone maximized enzyme conversion.$^{25-27}$ Therefore, a pre-separation was performed, as noted in each figure caption, in the beginning of the run to drive substrate through the enzyme zone.
3.3 Results and discussion

3.3.1 In-capillary asparagine linked glycan processing and separation.

To achieve the real-time N-glycan modification, the capillary was patterned as shown in Figure 3.4A to ensure that both the donor and cofactor were present in the enzyme plug when substrate was driven through the reaction zone. The manganese (II) cofactor was incorporated in the enzyme plug and the plug on the anodic side of the enzyme zone. This ensured that the cationic manganese (II) cofactor, which was at a concentration of 5 mM, was present when substrate reached the enzyme plug. The anionic donor, UDP-galactose, was patterned at a concentration of 5 mM in the enzyme zone and in a nanogel zone on the cathodic side of the enzyme zone providing a constant supply of donor during conversion. In addition, during the pre-separation, the cathodic reservoir contained 5 mM UDP-galactose. The remainder of the capillary was filled with the 10% nanogel to facilitate the separation as previously described.25, 27, 30
Nanogel separations were adapted so that the in-capillary modification with β1-4 galactosyltransferase was carried out in 100 mM MOPS buffered to pH 7 which was within the working pH range of 6 to 9 recommended by the manufacturer. The enzyme, manganese zone and UDP-galactose zones were made in 10% nanogel. The enzyme zone was patterned to be in the thermostable region of the capillary where temperature was maintained at 29 °C. This temperature was used because the manufacturer recommended a reaction temperature of 30 °C, and nanogel morphology was reported to change above 29 °C. To improve conversion the substrate was driven through the

Figure 3.4 The process of in capillary galactosyltransferase enzyme reaction. (A) The in-capillary patterning of the reaction zones. The pentasaccharide residues are represented with nomenclature defined by the Consortium for Functional Glycomics. (B) Electropherogram showing the pentasaccharide peak in the absence of enzyme. (C) Electropherogram obtained in the presence of 2 mU/µL β1-4 galactosyltransferase resulting in the addition of one or two galactose residues. Separations performed at 29 °C in a 25 µm i.d. capillary, with an effective length of 30 cm. A voltage of 20 kV for 1.5 min was used to drive pentasaccharide to the beginning of the reaction zone followed by mixing by polarity cycling at 4 kV for 30 min (i.e. 6 min forward, 6 min reverse 6 min forward, 6 min reverse, 6 min forward). Separation was then done at 20 kV (reverse polarity). The reaction and separation zone were suspended in 10% nanogel with aqueous 100 mM MOPS buffered to pH 7. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. Analytical Chemistry. Submitted June 2021.
enzyme zone at a low voltage of 4 kV as lower velocities were previously demonstrated to produce a more reproducible reaction and to enhance the conversion.\textsuperscript{25-27} In addition, polarity switching was used in which the voltage was programmed to migrate the substrate in the forward and reverse directions for 5 passes through the 3 cm enzyme zone, which created a longer effective length of the reaction zone and an estimated reaction time of 30 min.

Pentasaccharide had two sites to which a galactose residue could have been transferred. The degree of galactosylation was differentiated when the transferase enzyme reaction was performed in-capillary and separated. The addition of a galactose monomer caused a change in the charge-to-size ratio, which resulted in a shift in the migration time. The pentasaccharide containing 0, 1, or 2 galactose residues migrated at 9.8, 10.8 and 11.6 minutes, respectively. The capacity of the transferase enzyme to modify the pentasaccharide substrate was observed by comparing the electropherograms obtained in the absence (Fig 3.4B) and presence (Fig 3.4C) of enzyme. The conversion occurred by repeatedly sweeping the substrate through a 3 cm zone of GalT at a concentration of 2 mU/µL. Notably, the area of the agalactosylated pentasaccharide (39,000) observed in the absence of enzyme (Fig 3.4B) was similar to the total peak area of the agalactosylated, monogalactosylated, and digalactosylated pentasaccharide substrate, which was 43,000 (Fig 3.4C). These separations revealed that the distribution of galactose residues was 39% agalactosylated, 49% monogalactosylated and 12% digalactosylated.
The mono and di-galactosylated peaks were broadened relative to the agalactosylated peaks as observed in the peak width at half height of the unmodified substrate, which was 0.14 minutes, in comparison to the products, which were 0.27 minutes. Previous studies have demonstrated the length of the reaction zone contributed to band broadening. The structural modification to the substrate changed the velocity in the electric field and the arrival of the converted product was the sum of the velocity prior to and following the enzymatic conversion. Therefore, substrate converted early in the enzyme zone had a slightly different velocity from substrate converted later in the enzyme zone, which caused peak broadening. These results demonstrated that the capillary electrophoresis method provided a means to investigate the enzyme performance with knowledge of the product formed.

3.3.2 $K_M$ determination of pentasaccharide glycan standard.

The power of the capillary electrophoresis method to both quantify and to distinguish enzyme products was used to determine the enzyme Michaelis-Menten constant, $K_M$, which was measured by plotting the velocity of product formation against the substrate concentration. For $K_M$ determinations the enzyme concentration was fixed for each substrate concentration. The $K_M$ constant was 50% of the half-maximal velocity, which required that the linear range of the detection method was compatible with product concentrations to be measured. For an accurate measurement of the $K_M$ at least three concentrations of substrate were analyzed at the point where velocity was changing significantly and also at two concentrations near saturation. Less than 10% of the substrate was converted to product. Unlike a benchtop reaction, where the velocity was
determined by quenching the reaction at a specific time and reporting the concentration of product formed per time; in an on-line system the velocity was a function of the measured product concentration per migration time through the reaction zone. A calibration curve was used to convert the area of the product formed into concentration. For the GalT enzyme-pentasaccharide reaction a prior report established the $K_M$ in the mM range; therefore, the pentasaccharide substrate was labeled on the reducing end with aminobenzoic acid which was detectable with UV absorbance. The concentration range of the substrate detected with a 10 kV 10 sec injection was 25 $\mu$M and 500 $\mu$M.

With these parameters, the GalT enzyme concentration of 1 mU/μL was chosen to limit the GalT modification to the addition of a single galactose residue. At this concentration the amount of the generated product remained within the linear range of the absorbance detection without requiring in-capillary mixing. The substrate was driven through the reaction zone at 4 kV and then separated in nanogel at 20 kV. No preliminary position step was used for the $K_M$ study. Although a 4 kV 15 min pre-separation drive through was used, the time for the reaction was 6 min based on the migration through a 3 cm zone of enzyme embedded in a 9.5 cm pre-separation zone. As the starting and ending position of substrate differed from the conditions used in Figure 3.4, the observed migration time of the substrate and product differed. In the electropherogram in Figure 3.5 the product peak had more band broadening than the substrate peak, as was also observed in the data in Figure 3.4. A range of pentasaccharide concentrations (i.e. 0.5, 0.75, 0.90, 1.23 mM) was analyzed as shown in Figure 3.5. Each substrate conversion was measured in triplicate and calibration curves were run before and after the $K_M$
determinations. The calibration curve is shown in Figure 3.6. For each data set employed in the calibration curve, the line equation and associated error are shown in Table 3.2, and the areas used to achieve each curve are shown in Table 3.3.
Figure 3.5 Determination of the Michaelis-Menten for the galactosyltransferase enzyme. (A) (B) (C) The Michaelis-Menten curve generated by plotting the substrate concentration versus the rate of product formation for pentasaccharide with 1 mU/μL β1-4 galactosyltransferase in nanogel. Substrate concentrations were 0.50 mM, 0.75 mM, 0.90 mM, 1.00 mM, 1.23 mM, 2.00 mM, 3.00 mM and 4.00 mM. Pre-separation at 4 kV for 15 min to drive substrate past enzyme plug, and the separations were then performed at 20 kV. Capillary conditions were a temperature of 29 °C in a 25 um i.d. capillary with effective length of 40 cm and E= 500 V/cm (reverse polarity). Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. Analytical Chemistry. Submitted June 2021.
Table 3.2  Line equations and errors for 3 sets of data used to achieve the calibration curve.

<table>
<thead>
<tr>
<th></th>
<th>Equation</th>
<th>Uncertainty in Slope</th>
<th>Uncertainty in Y intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>y = 16000x + 700</td>
<td>1000</td>
<td>300</td>
</tr>
<tr>
<td>Set 2</td>
<td>y = 15900x + 700</td>
<td>900</td>
<td>300</td>
</tr>
<tr>
<td>Set 3</td>
<td>y = 17900x + 400</td>
<td>700</td>
<td>200</td>
</tr>
</tbody>
</table>

Figure 3.6  Calibration curve used to establish relationship between concentration and area. Separations done without enzyme plugs. Separations conditions similar to Figure 3.2. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. Analytical Chemistry. Submitted June 2021.
Peak areas of the product achieved with enzyme sequencing were converted to concentration using this calibration curve constructed from an average of all measurements. The concentration was then converted to velocity with the known reaction time (i.e. 6 min). The curve of velocity versus substrate concentration was fit using nonlinear regression to obtain the $K_M$ (Fig 3.5). The $K_M$ value was determined as $1.26 \pm 0.08$ mM and $V_{MAX}$ as $0.073 \pm 0.01$ mM/min. The average $K_M$ and $V_{MAX}$ values were achieved from averaging the values of the triplicate analysis in Figure 3.7. Peaks were reproducible in area and time with relative standard deviations of 10% and 2% ($n = 3$), respectively (Table 3.4 and 3.5).

Table 3.3 Areas used to achieve the Calibration Curve.

<table>
<thead>
<tr>
<th></th>
<th>Set 1</th>
<th></th>
<th>Set 2</th>
<th></th>
<th>Set 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before $K_M$ curve data points</td>
<td>After $K_M$ curve data points</td>
<td>After $K_M$ curve data points</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025mM</td>
<td>806</td>
<td>723</td>
<td>663</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.050mM</td>
<td>1374</td>
<td>1279</td>
<td>1040</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.100mM</td>
<td>2860</td>
<td>2620</td>
<td>2395</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.200mM</td>
<td></td>
<td>4302</td>
<td>4262</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.500mM</td>
<td>8428</td>
<td>8433</td>
<td>9172</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The standard deviation for the three $K_M$ value was 0.08mM and for the $V_{MAX}$ was 0.001mM/min.
The $K_M$ value obtained with the in-line capillary electrophoresis was statistically similar to the reported reference value of 1.23 mM for pentasaccharide and $\beta$1-4 galactosyltransferase,\textsuperscript{42} as determined using a student’s t-test. Although the literature method did not describe the degree of galactosylation, the capillary electrophoresis method provided conclusive evidence that only a single galactose residue was transferred to the pentasaccharide. This demonstrated the validity of the capillary electrophoresis method and also drew attention to the ability of the method to confirm the product associated with the measurement. Furthermore, in comparison to other methods used

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Concentration & Time & Rsd & Area \\
\hline
µM & & & \\
500 & 11.2 & 1 & 9,500 & 8 \\
750 & 11.2 & 1 & 12,400 & 9 \\
900 & 11.2 & 1 & 13,800 & 9 \\
1230 & 11.4 & 1 & 18,900 & 10 \\
2000 & 11.4 & 1 & 26,400 & 8 \\
3000 & 11.5 & 1 & 35,600 & 10 \\
4000 & 11.5 & 1 & 47,600 & 6 \\
\hline
\end{tabular}
\caption{Reproducibility in substrate peaks for $n=3$ runs used for $K_M$.}
\end{table}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Concentration & Time & Rsd & Area \\
\hline
µM & & & \\
500 & 11.9 & 1 & 1,300 & 10 \\
750 & 12.0 & 1 & 1,800 & 4 \\
900 & 11.9 & 1 & 2,000 & 2 \\
1230 & 12.2 & 1 & 2,300 & 5 \\
2000 & 12.2 & 1 & 2,700 & 3 \\
3000 & 12.3 & 1 & 3,200 & 3 \\
4000 & 12.4 & 2 & 3,400 & 3 \\
\hline
\end{tabular}
\caption{Reproducibility in product peaks for $n=3$ runs used for $K_M$.}
\end{table}

\textsuperscript{42}
for $K_M$ determinations of GalT, the automated capillary electrophoresis method required less sample handling as there was no need for reaction quenching or purification prior to detection. In addition, the capillary electrophoresis method consumed only a 15 nanoliter volume of enzyme in the 3 cm reaction zone, injected from a 5 µL vial solution, making the method amenable to changing the in-line reaction to realize higher levels of galactosylation; thereby, gaining insight into the process of galactose transfer.

### 3.3.3 Evaluating the addition of galactose residues to asparagine linked glycan.

Galactose transfer is relevant to N-glycans to understanding and harnessing GalT. To demonstrate the mechanism of conversion relevant to glycoproteins, asparagine linked glycan structures (i.e. N-glycans) were used as a substrate. The N-glycans, which were from Herceptin, were analyzed at a higher enzyme:substrate ratio in order to observe the process of galactose addition to a biantennary N-glycan which has two sites that can be modified. In contrast to the pentasaccharide, which was labeled with aminobenzoic acid, the N-glycans were labeled with APTS. The APTS enabled fluorescent detection of N-glycan at nanomolar substrate concentrations. As previously reported the APTS-labeled Herceptin N-glycans are primarily agalactosylated with 77% of the N-glycans lacking galactose and 23% composed of a single galactose residue based on analysis of the peak areas in the electropherogram (see also Figure 3.8 and Tables 3.6 and 3.7). The degree of galactosylation was distinguished by time as the agalactosylated N-glycan peak (12.1 min, Fig 3.8A) had a larger charge-to-size ratio and migrated faster than the monogalactosylated N-glycan peaks (13.1 min, Fig 3.8A). The peak identities were
further confirmed by including the ECL lectin specific for galactose in the reaction zone (Figure 3.9) which confirmed when the peaks contained 0, 1, or 2 galactose residues.

Figure 3.8 Online modification of glycans recovered from Herceptin. Enzyme β1-4 galactosyltransferase is in nanogel. When enzyme is not in the capillary unmodified glycans are distributed as shown in first trace. Incorporating increasing enzyme concentration demonstrated conversion of the glycans. Separations performed in a 25 µm i.d. capillary, with an effective length of 50 cm and E=400 V/cm (reverse polarity). All other conditions similar to Figure 2. Differences in nanogel composition in enzyme zones caused slight shifts in runs. The migration time marker denoted by the Asterix was used to offset and align traces. The offsets are as follows Trace A 0 min, Trace B 0.046 min, Trace C -0.158 min, Trace D -0.449 min, Trace E 0.058 min and Trace F 0.099 min.

Table 3.6 Area distribution of substrate and product with changing enzyme concentration.

<table>
<thead>
<tr>
<th>mU Enzyme:</th>
<th>Agalacto biantennary</th>
<th>Mono galacto biantennary</th>
<th>Digalacto biantennary</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol Substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 : 1</td>
<td>89</td>
<td>27</td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>1.2 : 1</td>
<td>72</td>
<td>63</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>2.9 : 1</td>
<td>43</td>
<td>71</td>
<td>20</td>
<td>134</td>
</tr>
<tr>
<td>5.8 : 1</td>
<td>15</td>
<td>64</td>
<td>48</td>
<td>127</td>
</tr>
<tr>
<td>12 : 1</td>
<td>40</td>
<td>76</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>81 : 1</td>
<td>128</td>
<td>128</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7 % distribution of substrate and product with changing enzyme concentration.

<table>
<thead>
<tr>
<th>mU Enzyme:</th>
<th>Agalacto biantennary</th>
<th>Mono galacto biantennary</th>
<th>Digalacto biantennary</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol Substrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 : 1</td>
<td>77</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>1.2 : 1</td>
<td>53</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>2.9 : 1</td>
<td>32</td>
<td>53</td>
<td>15</td>
</tr>
<tr>
<td>5.8 : 1</td>
<td>12</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td>12 : 1</td>
<td>0</td>
<td>34</td>
<td>66</td>
</tr>
<tr>
<td>81 : 1</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 3.9 Confirmation of online modification of glycans recovered from Herceptin with lectin. 5mU/µL β1-3,4 galactosyltransferase in nanogel was employed. When enzyme was not in the capillary unmodified glycans were distributed as shown in Trace A. Incorporating *Erythrina Cristagalli* galactosylated glycans were retarded (B). When enzyme was incorporated in the capillary all glycans were converted to two galactose residues (C) which was confirmed by *Erythrina Cristagalli* (D). Pre-separation was done at 4 kV for 15 min to drive substrate past enzyme plug. The separations were then performed at 29 °C in a 25 um i.d. capillary with effective length of 50 cm and applied voltage of 20 kV (reverse polarity). Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. *Analytical Chemistry*. Submitted June 2021.
The process of transferring galactose to N-glycan was observed by increasing the enzyme: substrate ratio in the reaction zone and quantifying the distribution of galactosylation on the reaction products. As the reaction was performed in-line, the actual amount of enzyme and substrate in the capillary was determined using the same units employed by the vendor to define activity. The enzyme:substrate ratio was changed by increasing the quantity of enzyme available for conversion while maintaining a fixed amount of substrate of 1.3 picomoles. This quantity of substrate in-capillary was calculated based on the voltage, time, and capillary dimensions used for the electrokinetic injection, with knowledge of the N-glycan mobility (i.e. $1.64 \times 10^{-4}$ m$^2$/Vs) which was measured in the nanogel. A 10 kV for 10s injection into a 25 µm i.d. capillary was performed from vial containing Herceptin N-glycans at a concentration of 100 nM. The quantity of enzyme in-capillary was calculated based on flow injection analysis of the velocity of the nanogel in Figure 3.10. A plug of APTS took 70 s when pushed with 10% nanogel (at 10 psi) to travel a distance of 10.2 cm. The velocity of the nanogel at these conditions is therefore 0.146 cm/s. Since the enzyme plug was pushed in for 21 s, the distance of capillary filled was 3 cm, which translates to a volume of 15 nL injected into the 25 µm i.d. capillary. To achieve five enzyme: substrate ratios, five injection vials with different concentrations of enzyme of 0.1 mU/µL, 0.25 mU/µL, 0.5 mU/µL, 1 mU/µL and 7 mU/µL were utilized. The amounts of enzyme injected from each vial were calculated to be 1.5 µU, 3.8 µU, 7.5 µU, 15 µU and 105 µU, leading to reaction enzyme:substrate ratios of 1.2:1, 2.9:1, 5.8:1, 12:1 and 81:1 mU:nmol.
The results of the in-line reactions performed at the five enzyme substrate ratios, shown in Figure 3.8 traces B to F, demonstrated that the enzyme preferentially transfers a galactose residue to one arm of the biantennary complex N-glycan. This preferential addition of a galactose that is β1-4 linked on the N-glycan arm containing the N-acetyl-D-glucosamine that is α1-2 linked to D-mannose which is α1-6 linked to the innermost mannose (Galβ1-4GlcNAcβ1-2Manα1-6Man) in the core structure has been reported previously. The peak denoted with the asterisk in Figure 3.6 was used as a migration time marker to align the traces. Shifting in the migration time of the products was attributed to differences in the nanogel composition of the enzyme zone. The peak areas,
summarized in Table 3.6, ranged from 120,000 to 130,000 total N-glycan peak area regardless of enzyme concentration. Only a single galactose residue was transferred until the enzyme was increased to 2.9 mU/1 nmol of substrate, and 15% of the N-glycan peak area was fully galactosylated (Table 3.7). The amount of fully galactosylated N-glycan increased as the enzyme concentration was increased. At a ratio of 12 mU/1 nmol, the substrate peak was absent, and the product contained a distribution of 34% mono and 66% di-galactosylated N-glycan. At a 81 mU/1 nmol ratio, the N-glycan was fully converted to the di-galactosylated product.

These area distributions indicated that a single galactose residue was preferentially added to the biantennary N-glycan. In a prior study, in the absence of the enzyme, capillary nanogel electrophoresis distinguished the presence of a galactose on the 1,2-1,6-arm or the 1,2-1,3-arm. However, in the presence of enzyme, mono-galactosylated peaks were obscured as seen in the traces in Figure 3.8. As previously noted, the migration time and peak broadening reflected differences in velocity related to the location within the enzyme zone that the conversion occurred. Additionally, transferase enzymes have been reported to also function as hydrolases further broadening the product peak if galactose residues were both trimmed and extended in the reaction zone. These studies confirmed that transferase enzymes could be integrated with capillary electrophoresis. With this proof-of-principle established the method was adapted to modify a glycoprotein.
3.3.4 Application to glycoprotein.

The capillary electrophoresis enzyme nanogel reaction zones were modified for the analysis of the Herceptin glycoprotein. The Herceptin was cationic, with an isoelectric point of 9,\textsuperscript{46} therefore the patterning and separation were performed under conditions of normal polarity. Thus, the order of patterning was reversed as compared to free N-glycans. The nanogel enzyme zone contained UDP-gal. As UDP galactose is anionic and will migrate towards the anode, more of the donor was patterned to the right of the enzyme zone as shown in Figure 3.11. The manganese is cationic and will migrate in a similar direction as Herceptin (towards the cathode). In order to ensure a constant supply of this cofactor during the modification, a concentration of 1 mM manganese (II) chloride was maintained in the anodic vial used during the enzymatic conversion. A galactose-specific lectin, ECL, was positioned after the reaction zone because protein glycoforms with different degrees of galactosylation did not have discernable mobilities.
In the presence of the lectin, the addition of galactose was detected when the ECL-bound Herceptin fraction was absent from the electropherogram. The ECL lectin was incompatible with the UDP galactose donor, and if patterned close together the galactose from the donor would have bound to the lectin. To ensure lectin was available to bind Herceptin glycoprotein only, and to reduce contamination with the donor, a nanogel spacer was incorporated between the lectin and the donor. The reaction zones and

![Figure 3.11 Online modification of glycan on intact IgG1 glycoprotein. In the absence of enzyme or lectin (A upper trace) Herceptin separated into its charge variants peaks with a the combined area of 590,000. Peak 1 is the basic variant of Herceptin, peak 2 is the main peak and peaks 3 to 5 are the acidic variants. When lectin is present, only the agalactosylated fraction of Herceptin is detected (lower trace, area = 330,000). When enzyme is in the capillary, but not lectin (B, upper trace) the total peak area is 560,000. When both enzyme and lectin are present, no Herceptin is detected (B lower trace) confirming the addition of galactose residues. The plug contained 5 mU/µL GalT and 1 mM UDP-Gal suspended in 10% nanogel with 100 mM MOPS buffered to pH 7. The lectin plug contained 100 µM ECL suspended in 10% nanogel. The 1 µM Herceptin was electrokinetically injected (5 kV 4 s) and detected with UV absorbance at 214 nm. Separations were performed in a 25 µm i.d. capillary, with an effective length of 10 cm and applied voltage of 12 kV (E=400 V/cm, normal polarity). A lower voltage was used for the reaction (pre-separation at 4 kV for 26 min). The separation and drive through were performed in 10% nanogel buffered with 100mM pH 7 MOPS maintained at a temperature of 20 °C. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. Analytical Chemistry. Submitted June 2021.
separation were maintained at a pH of 7 in the 10% nanogel buffered to pH 7 with 100 mM MOPS. At a separation temperature of 29 °C, which was used for the glycans, the Herceptin glycoprotein mobility was reduced significantly and was not observed within a 30-minute separation. Therefore, the separation temperature was lowered to 20 °C where the gel was less viscous, and the mobility of the Herceptin in nanogel was $2.7 \times 10^{-5}$ m$^2$/Vs. By reducing the 30 cm effective length that was used for glycans to a 10 cm effective length, the Herceptin separation was completed in 45 minutes if the field strength was maintained at 400 V/cm. A lower electric field strength was used (133 V/cm) to drive the Herceptin glycoprotein past the 3 cm enzyme plug. While no difference in lectin pull down was observed for Herceptin when the enzyme: substrate was 5.8 mU enzyme/1 nmole (Figure 3.12), no agalactosylated N-glycan remained when free N-glycan was subject to this ratio (Figure 3.8). To realize complete pull down of the Herceptin, which required the presence of only a single galactose residue on one of the two biantennary N-glycans in the hinge region, the amount of enzyme was increased above 12 mU enzyme/1 nmole (Figure 3.12). These experiments revealed that the enzymatic transfer of galactose was slower for intact glycoprotein. Therefore, to maximize the amount of galactose transferred to the Herceptin glycoprotein the highest concentration GalT that could be made, and the minimum concentration of Herceptin which was stable in solution were used. This resulted in a ratio of 42 mU enzyme/1 nmole Herceptin N-glycan.
The enzymatic transfer of galactose residues to Herceptin glycoprotein was evaluated in the presence and absence of enzyme and lectin. In the absence of enzyme and lectin (Fig 3.11A, upper trace), the total area was 590,000, with a charge variant distribution as previously reported. Peak one is the basic variant of Herceptin, peak 2 is the main peak.

Figure 3.12 Establishing the amount of modification at different enzyme to substrate ratio as evidenced by lectin retardation of modified Herceptin. Separation conditions are similar to Figure 3.8. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. *Analytical Chemistry*. **Submitted June 2021.**
and peaks 3 to 5 are the acidic variants.\textsuperscript{47} In the absence of enzyme but presence of lectin (Fig 3.11A, lower trace), the lectin zone led to a 44% decrease in the Herceptin peak area of 330,000 (i.e. 56% agalactosylated). This was anticipated as the Herceptin N-glycan distribution (Fig 3.8 and Table 3.7.) revealed that the amount of N-glycans that contained no galactose residues or one galactose residue, was 77% and 23%, respectively. Assuming a random distribution, 60% of the Herceptin was expected to be agalactosylated. If the GalT enzyme successfully transferred galactose to the glycoprotein, even more of the Herceptin would be retarded by the lectin zone. As shown in Figure 3.8B, in the presence of the enzyme and lectin, no Herceptin peak was observed, which confirmed that all Herceptin molecules had been modified with at least one galactose residue. The shift in the Herceptin peak migration time in the presence of enzyme was attributed to protein-protein interaction rather than a change in mobility associated with the addition of galactose residues because when the Herceptin separation was repeated in the presence of the enzyme, but with no UDP-gal donor or manganese cofactor (Fig 3.13) the migration shift persisted. These data demonstrated the utility of the capillary electrophoresis method for online enzyme modification of intact glycoprotein.
Figure 3.13 Confirmation that presence of enzyme in capillary caused shifting of Herceptin peaks. Separation conditions are similar to Figure 3.7. Reproduced with permission from: Bwanali, L.; Holland, L. A., Capillary Nanogel Electrophoresis for the Determination of the β1-4 Galactosyltransferase Michaelis-Menten Constant and Real-Time Addition of Galactose Residues to N-Glycan and Glycoprotein. *Analytical Chemistry*. Submitted June 2021.

3.4 Conclusions and future directions

Nanogels enabled control over positioning and zone length of reactions integrated with separations that were well suited for N-glycans and proteins. These nanogel zones were employed to demonstrate on-line enzymatic transferase modification. The in capillary
modification could distinguish the addition of one versus two galactose residues to pentasaccharide as well as to biantennary N-glycan. A Michaelis-Menten constant of 1.26 mM was obtained for β1-4 galactosyltransferase and pentasaccharide using the capillary electrophoresis method. This $K_M$ value matched literature reported value. Furthermore, the in-capillary enzymatic modification gave insight into the order of addition of galactose. Finally, on-line transferase reaction was adapted to Herceptin glycoprotein, demonstrating the utility of the method in modifying whole protein in real time.

Practical advantages of the capillary nanogel electrophoresis method include the low volume (i.e. 15 nL) of enzyme required to modify the substrate. Additionally, the capillary electrophoresis method was automated, and it did not require reaction quenching or purification associated with offline reactions. This reduced the hands-on time required. Finally, the time required to complete the enzyme reaction as well as the quantitative analysis was less than 45 minutes.

The data achieved and the advantages of the in capillary enzymatic modification opens avenues for further glycosyltransferase studies. With the successful modification of Herceptin glycoprotein, the approach will be expanded to other glycoproteins. Additional benefits of the capillary nanogel electrophoresis method can be realized by applying it to other transferase enzymes and other enzyme classes. Finally, nanogels have been shown to extend the lifetime of exoglycosidase enzymes. Additional studies will determine the role of nanogel as a stabilizing medium for transferase enzymes.
3.5 Acknowledgements

This material is based upon work supported by NIH Grant No. R01GM114330.

3.6 References


16. Mercer, N.; Ramakrishnan, B.; Boeggeman, E.; Verdi, L.; Qasba, P. K., Use of Novel Mutant Galactosyltransferase for the Bioconjugation of Terminal N-


23. Li, X.; Yin, Z.; Cui, X.; Yang, L., Capillary Electrophoresis-Integrated Immobilized Enzyme Microreactor With Graphene Oxide As Support: Immobilization Of Negatively


38. Agilent B1,4-galactosyltransferase.


40. SigmaAldrich Beta-1,4-Galactosyltransferase 1, human.


42. Ramasamy, V.; Ramakrishnan, B.; Boeggeman, E.; Ratner, D. M.; Seeberger, P. H.; Qasba, P. K., Oligosaccharide Preferences of β1,4-Galactosyltransferase-I: Crystal Structures of Met340His Mutant of Human β1,4-Galactosyltransferase-I with a Pentasaccharide and Trisaccharides of the N-Glycan Moiety. Journal of Molecular Biology 2005, 353 (1), 53-67.


Chapter 4 Conclusions and Future Directions

4.1 Introduction

Capillary electrophoresis is a powerful tool for glycan analysis and modification, which can be adapted to achieve real time complex reactions. In summary, this dissertation illustrates how capillary electrophoresis was employed to carry out complex exoglycosidase and transferase enzyme reactions to provide useful information about N-glycan sequence distribution and modification of glycoprotein online. Chapter 1 offered background information from literature about N-glycan structures and current methods of analysis, which include capillary electrophoresis. Fundamentals of capillary electrophoresis and how it is a versatile technique for N-glycans analysis was discussed. Considerations in labeling with either a chromophore or fluorophore were outlined. Analysis of N-glycans with enzymes and lectins in capillary electrophoresis for N-glycan structural modification, identification and quantification was then outlined.

The serial enzyme sequencing in Chapter 2 showed the distribution of α2-6 linked sialic acids in a tri-antennary standard and AGP samples. Phospholipid nanogel was used to pattern the enzymes at the head of the separation capillary and sequentially cleave the N-glycans, so that the presence of α2-6 linked sialic acids corresponded to a shift in the analyte migration time in a manner that enabled interpretation of the N-glycan structure. It was demonstrated that for the tri-antennary standard 0, 1, 2, and 3 α2-6 linked sialic acids were 4%, 59%, 36% and 2% which matched the vendor reported values. The method was used to distinguish differences in α2-6 linked sialic acids in two samples of AGP and provided new information of the distribution of these sialic acids on each branch.
The most dominant form of α2-6 linked sialic acids in the two samples was only a single α2-6 linked sialic acid on each branch. For the bi-antennary AGP sample one had 21% as zero α2-6 linked sialic acids and 78% as 1 α2-6 linked sialic acids. AGP sample two had 15% and 85% as 0 and 1 α2-6 linked sialic acids, respectively. For the tetra-antennary sample 1 had 26% with 0 α2-6 linked sialic acids and 74% with one α2-6 linked sialic acids. In contrast, sample two had 33%, 52%, 3% 8% and 3% as 0, 1, 2, 3, and 4 α2-6 linked sialic acids. correlated directly to the number of terminal galactose residues that remained. The method is able to quantify α2-6 linked sialic acids on each branch and identify differences in α2-6 linked sialic acids between samples.

Chapter 3 discussed how the thermally reversible nanogel is used in capillary electrophoresis to create discrete regions for saccharide modification by galactosyltransferase reaction and a subsequent separation of the substrate and product. The β1-4 galactosyltransferase enzyme, donor, and co-factor were patterned in the capillary, and pentasaccharide substrate was driven through these zones and converted to galactosylated products which were separated and identified. The degree of glycosylation was discernable for a pentasaccharide as well as for biantennary N-glycans. With the ability to distinguish reaction products for which galactose addition resulted in a single or two galactose residues, the capillary nanogel electrophoresis system was used to determine the Michaelis-Menten value, $K_M$. The $K_M$ for this β1-4 galactosyltransferase was established to be $1.23 \pm 0.08$ mM for a pentasaccharide substrate. Once the $K_M$ was established, the enzyme-to-substrate ratio was evaluated to add a single galactose residue, or to fully galactosylated a biantennary N-glycan. Additionally, the method was
adapted to transfer galactose residues to protein. The applicability of the method for real-time online modification of whole protein was demonstrated with the Herceptin glycoprotein. Complete retardation by *Erythrina Cristagalli* lectin after enzymatic modification confirmed addition of galactose to the Herceptin.

4.2 Future

4.2.1 Serial enzyme sequencing

Since sialic acids are labile and can be cleaved due to heat, storage or processing, the serial enzyme processing method will be adapted to monitor the changes in the α2-6 linked sialic due to these processes. In preliminary findings, a sample of AGP was analyzed for the change in the sialic acid distribution due to heat degradation. Three reactions were incubated in 20% acetic acid at 65 °C for 0, 3 and 6 hrs. The acetic acid was used to mimic the APTS labeling conditions employed for N-glycans. As shown in Figure 4.1, from this preliminary data, when the AGP N-glycans were analyzed without enzymes it demonstrated that peaks close to 18 minutes start to decrease, while partially desialylated peaks close to 20 minutes start to increase in intensity. When these samples were analyzed with the multiple enzyme sequencing, Table 4.1, there was no significant difference in the amount of the α2-6 linked sialic acid present for the 3 hrs compared to 0 hrs. This preliminary data suggests that the α2-3 linked sialic acid were degraded first, therefore no change observed with the multiple enzyme sequencing. This result agrees with literature findings that the α2-3 linked sialic acids are more readily cleaved than the α2-6 linked sialic acids because of structural differences. As the incubation time was increased to 6 hrs, lose in α2-6 linked sialic acid starts to be realized. This demonstrated
the utility of the method to be utilized in demonstrating the impact of longer incubation and storage.

Figure 4.1 Degradation of sialic acids due to heat. Electropherogram showing migration time shifts as more sialic acids are lost from prolonged incubation. Separations performed at 23 °C in a 25 µm i.d. capillary, with an effective length of 50 cm and E=450V/cm (reverse polarity). Separation is in 20% nanogel buffered with 50 mM pH 5 sodium acetate.
Table 4.1 Relative abundance by area of α2-6 sialic acid of heat treated α-1-acid glycoprotein asparagine-linked glycans.

<table>
<thead>
<tr>
<th>Branch</th>
<th>0 hrs</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi</td>
<td>76(8)</td>
<td>310(5)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>386(9)</td>
</tr>
<tr>
<td></td>
<td>80 (9)</td>
<td>290(7)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>370(11)</td>
</tr>
<tr>
<td></td>
<td>120(7)</td>
<td>260 (7)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>380(10)</td>
</tr>
<tr>
<td>Tri</td>
<td>230(4)</td>
<td>570(7)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>800(8)</td>
</tr>
<tr>
<td></td>
<td>250(5)</td>
<td>600(10)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>850(11)</td>
</tr>
<tr>
<td></td>
<td>300(6)</td>
<td>540(11)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>840(13)</td>
</tr>
<tr>
<td>Tetra</td>
<td>210(9)</td>
<td>320(3)</td>
<td>16(9)</td>
<td>41(6)</td>
<td>15(7)</td>
<td>603(13)</td>
</tr>
<tr>
<td></td>
<td>190(4)</td>
<td>340(6)</td>
<td>13(10)</td>
<td>43(5)</td>
<td>0</td>
<td>586(13)</td>
</tr>
<tr>
<td></td>
<td>210(6)</td>
<td>310(1)</td>
<td>25(4)</td>
<td>40(6)</td>
<td>32(1)</td>
<td>617(9)</td>
</tr>
</tbody>
</table>

Solid lines in the table indicate that the structure not possible

4.2.2 Real-time in-capillary transferase reactions.

4.2.2.1 Application to other transferase reactions.

With knowledge of the in-capillary transferase modification the system will be applied to other transferase enzymes. With approximately 200 human wild type transferase available\(^3\) and custom transferase enzymes being developed,\(^4,6\) this method is attractive in characterizing transferase enzymes, as it has less sample handling involved. In addition, the method employs nanoliter volumes of reagent which makes it adaptable to limited biological reagents. With this knowledge, the applicability of the in-capillary transferase modification was extended to obtain preliminary data with α2-3 sialyl transferase enzyme. Similar to β1-4 galactosyl transferase, the α2-3 sialyl transferase is also monomer specific and is used to attach α2-3 sialic acid to terminal galactose residues. The donor substrate, cytidine-5’-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac), is needed to supply monomer for the transferase reaction. Addition of the
neutral monomer galactose in the GalT reaction, caused a change in the size of the N-glycan, leading to product detected to the right of substrate. In contrast, sialic acids are charged, and addition will lead to a change in both the size and net charge of the resultant molecule. This is observed as a migration time shift to the left of the product in comparison to substrate.

**Capillary patterning and separation conditions.** Optimum working conditions for enzymes were achieved by using the manufacturer recommended enzyme working pH for separation, and by employing phospholipid nanogel to pattern the enzymes in the capillary. The α2-3 sialyl transferase enzyme has an optimum working pH range of 7.5 to 8.5. The separation conditions were therefore tailored to be within this optimum working range for the enzyme. The separation with and without enzyme modification, in the addition of sialic acids, was done using sodium phosphate buffered to a pH of 8. At lower pH than 7, the enzyme has been shown to act as a sialidase. The enzyme also has some partial α2-6 sialyl transferase activity between pH 4-7. Enzyme activity for the α2-3 sialyl transferase is not enhanced by the addition of divalent cofactors hence they were not included in the separation. For patterning, a 1 cm plug of the enzyme and CMP Neu5Ac in nanogel was pushed to the beginning of the thermostable region of the capillary using a nanogel plug filled with CMP Neu5Ac. The rest of the capillary had been filled with nanogel to enhance glycan separation. A temperature where the nanogel has maximum viscosity to enhance separation, of 29 °C, was chosen for the analysis.
The donor substrate migrates in a similar direction as the N-glycan and was therefore patterned to ensure constant supply in enzyme plug during N-glycan modification. The CMP Nue5Ac is anionic and at the separation condition it would migrate in the same direction as analyte, towards the detector. Patterning was therefore done to ensure there is donor substrate when the N-glycan reaches the enzyme plug. Neu5Ac was incorporated in the enzyme plug, in lipid push plug and in cathodic reservoir used when driving N-glycan through enzyme plug.

**Online sialic acid modification.** Sialic acids modification of standard glycan was achieved in real time using the sialyl transferase. As shown in Figure 4.2, the standard galactosylated biantennary N-glycan was analyzed in a capillary with and without the sialyl transferase enzyme. Without the enzyme in the capillary the standard galactosylated biantennary N-glycan peak migrates at 16.8 minutes. When sialyl transferase enzyme and CMP Neu5Ac were incorporated in the capillary α 2-3 sialic acids were added to N-glycan giving rise to two products. All the initial substrate was modified with α2-3 sialic acids with 30% (peak at 14.5 minutes) having one sialic acid and 70% (peak at 13.3 minutes) having two sialic acids. Increasing the sialyl transferase enzyme concentration could be used to ensure two sialic acids are added on every N-glycan. This demonstrates the utility of the method to modify N-glycans in real time using an alternate transferase enzyme.
Figure 4.2 Online reaction employing α2-3 sialyl transferase to modify standard biantennary asparagine-linked glycan. 
(A) Electropherogram showing the N-glycan peak in the absence of enzyme. 
(B) Electropherogram obtained in the presence of 1 mU/μL α2-3 sialyl transferase resulting in the addition of one or two sialic acid residues. 
Separations performed at 29 °C in a 25 μm i.d. capillary, with an effective length of 60 cm. A voltage of 4 kV 15 min was used to drive the substrate past the enzyme plug. Separation was then done at 20 kV (reverse polarity). The reaction and separation zone were suspended in 10% nanogel with aqueous 50 mM sodium phosphate buffered to pH 8.
Reproducibility in the runs confirmed absence of non-specific adsorption or capillary fouling by the enzyme. The total area was reproducible in the with and without enzyme, meaning injections were not impacted by the presence of enzyme in the capillary. The analysis without enzyme had an area of 40,000 (relative standard deviation of 9%) which matched analysis with enzyme, total area of 41,000 (relative standard deviation of 3%). The migration time relative standard deviation for the set without enzyme was 0.3% and when enzyme was present it was 0.2% for the first peak and 3% for second peak. Reproducibility in time showed that the enzyme was not fouling the capillary surface. Plate count for analysis without enzyme was 590,000 and when enzyme was incorporated it was 540,000 for first peak and 570,000 for second product peak. This confirmed that there wasn’t any peak broadening in the capillary because of mass transfer. Conversion for the sialyltransferase occurred over a shorter plug compared to the GalT, which contributed to less broadening in the former.

This preliminary online conversion with α2-3 sialyl transferase demonstrates the applicability of the online conversion with other transferase enzymes. In future work $K_M$ of sialic acid addition for the α2-3 sialyl transferase can be demonstrated by monitoring the kinetics of addition of the one sialic acid and two sialic acids. The method can further be extended to demonstrate the difference in kinetics for the addition of α2-6 sialic acids vs α2-3 sialic acids, with sialic acids of whole proteins also demonstrated online. With the demonstration of both neutral and charged monomer addition completed this paves a way for characterizing the addition of a different monomers in a single run. Future work can also focus on the action of both wild type and mutant transferase enzymes.
Adaptability of online transferase modification to different pH

Transferase enzymes have specific pH where activity is optimum\(^7\), \(^1\text{2}\) with activity decreasing or exoglycosidase activity observed at different pH values.\(^7\) The online transferase modification is adaptable to characterize the transferase enzyme at different pH values. If more than one transferase enzyme is to be patterned in the capillary, this may require the discrete enzyme zones to be patterned at pH values optimum for each individual enzyme. Having discontinuous pH zones in the capillary enables patterning the enzyme zone at conditions for optimum reaction and setting the separation conditions to maximize efficiency in enzyme catalysis.

To demonstrate the applicability of the online transferase modification to different pH values, the data in Figure 3.7 was first repeated with an agalactosylated standard biantennary N-glycan utilizing similar conditions as Figure 3.7. The results of this analysis are shown in Figure 4.3. When lectin was incorporated in the capillary, without enzyme, the migration time, and the area of biantennary N-glycan (Figure 4.2B) matched that of the blank (Figure 4.2A) confirming no galactose residues present added. When the enzyme was incorporated in the capillary, the N-glycan was modified, and a new peak appeared at 14.4 minutes (Fig 4.4C). The complete modification was confirmed by incorporating lectin in the capillary and the peak disappeared from the electropherogram. The same analysis was repeated at a different pH with a different buffer to confirm enzyme activity. Analysis was done with a 10% nanogel buffered with 100 mM MES pH 6.5 as shown in Figure 4.4. When the enzyme was present in the capillary the agalacto
bianterinary N-glycan was modified to two galactose residues. Herceptin N-glycans analyzed under these conditions also demonstrated complete conversion as shown in Figure 4.5. These preliminary data demonstrate that when the pH is within the working range of the enzyme complete modification is achieved. Future studies can focus on repeating the analysis at pH values outside the working range. This will open avenues for rapid method to characterize the optimum working range of the transferase enzymes.
Figure 4.3 Online modification of standard agalacto biantennary asparagine-linked glycan in 3-(N-morpholino)-propanesulfonic acid pH 7. 
Enzyme concentration is 2 mU/µL β1-3,4 galactosyltransferase in nanogel. When enzyme is not in the capillary unmodified glycans are distributed as shown in Trace A. No response is observed to *Erythrina Cristagalli* confirming no galactose residues present (B). When enzyme is incorporated in the capillary the N-glycan is converted to two galactose residues (C) which is confirmed by *Erythrina Cristagalli* (D). Separations condition similar to Figure 3.7. The Asterix denotes contaminant peaks. These contaminants did not respond to lectin or enzyme.
Figure 4.4 Online modification of standard agalacto biantennary glycan in 2-(N-Morpholino) ethanesulfonic acid pH 6.5. Enzyme concentration is 2 mU/µL β1-3,4 galactosyltransferase in nanogel. When enzyme is not in the capillary unmodified glycans are distributed as shown in upper trace. When enzyme is incorporated in the capillary the N-glycan is converted to two galactose residues as shown in lower Trace. Separation is in 10% nanogel buffered to pH 6.5 with 100mM MES. Separations are performed at 20 kV, 29 °C in a 25 µm i.d. capillary with effective length of 50 cm.
Figure 4.5 Online modification of Herceptin asparagine-linked glycans in 2-(N-Morpholino) ethanesulfonic acid pH 6.5. Enzyme concentration is 2 mU/µL β1-3,4 galactosyltransferase in nanogel. When enzyme is not in the capillary the unmodified glycans are distributed as shown in upper trace. When enzyme is incorporated in the capillary the N-glycans are converted to two galactose residues as shown in lower Trace. All other separations conditions similar to Figure 4.4.
Adaptability of online transferase modification when enzymatic conversion and separation pH are different.

To demonstrate the applicability of the method using discontinuous zones, the capillary electrophoresis enzyme nanogel reaction zones were modified for the analysis of the Herceptin glycoprotein. Patterning, as demonstrated in figure 4.6, was achieved to ensure the reaction occurs at optimum conditions for the enzyme but separation is done at conditions that improve efficiency. Since the Herceptin is cationic, the patterning and separation were maintained under conditions of normal polarity. Nanogel was employed only for the enzyme zone with the UDP-gal plug, manganese (II) chloride zone, lectin zone and separation zone made from aqueous buffers. The UDP galactose zone, manganese (II) chloride and the lectin zone were reconstituted in aqueous MOPS buffered to a pH of 7. The reaction zone was maintained at a pH of 7 in the 10% nanogel as had been demonstrated in Figure 3.7, but the separation was performed in an aqueous solution of 50 mM sodium acetate solution buffered to pH 5 because Herceptin mobility is higher at pH 5 (9.4 x10^{-5} \text{cm}^2/\text{vs}). This allowed the drive through and separation to be accomplished in a 30 cm effective length separation with similar analysis time as in Figure 3.8, which utilized 10 cm effective length.
Figure 4.6 Modification of intact Herceptin glycoprotein when enzymatic conversion pH is different to separation pH. Separation without enzyme or lectin shows the Herceptin peaks (A). In the absence of enzyme or lectin the protein charge variants are separated. When lectin is present only the galactosylated fraction is retarded (B). Incorporating enzyme in capillary modifies glycans on Herceptin (C) which is confirmed by complete retardation of Herceptin when both enzyme and lectin are in the capillary (D). Herceptin with a concentration of 1 µM was electrokinetically introduced in the capillary by a 2 kV 2 second injection. Detection was performed at 214nm. Separations performed in a 25 µm i.d. capillary, with an effective length of 20 cm and E=400 V/cm (normal polarity). Pre-separation at 2 kV for 12 min to drive substrate past enzyme plug. Enzyme concentration is 5 mU/µL β1-4 galactosyltransferase suspended in 10% nanogel with 100 mM MOPS buffered to pH 7. Separation BGE does not contain nanogel and is aqueous 50 mM sodium acetate buffered to pH 5.
An enzyme: substrate ratio of 67:1 was maintained for the modification. When enzyme was included in the reaction zone and galactose residues were transferred to the glycoprotein, no shift in the peak migration pattern was observed (Fig 4.6 A, B). This was because the relative increase in mass of the Herceptin (MW ~ 150 kDa) was negligible and had little effect on the charge-to-size ratio. The effectiveness of the enzymatic modification was observed by including the galactose specific ECL lectin in the reaction zone. In the absence of enzyme (Fig 4.6 C), the lectin zone led to a decrease in the Herceptin peak area of 50%. In the presence of the enzyme and lectin zones (Fig 4.6 D), no Herceptin peak was observed, which confirmed that all Herceptin molecules had been modified with at least one galactose residue. These data demonstrated the utility of the capillary electrophoresis method for online enzyme modification when enzymatic conversion pH is different to separation pH. This knowledge can be employed when the separation and the reaction conditions need to be different.

**Online Fluorescence labeling of Glycoprotein**

Fluorescence labeling of the glycoprotein will translate to lower amount utilized in the analysis. This is desirable as it will increase the enzyme to substrate ratio in the capillary. Furthermore, this approach is useful for limited glycoprotein samples quantities. Preliminary experiments have been done to label the Herceptin online using Alexaflour 488 tagged Protein G.
Protein G is a 22 KDa bacterial protein that binds to IgGs. The binding constant is in the picomolar level and the binding is optimum between pH of 4 and 5. The isoelectric point of Protein G is 4.5 and in a separation buffer at a pH of 5 Protein G will be anionic. A 30 cm total length capillary, with 10 cm effective length from the cathodic side to the detector was utilized to analyze Protein G by itself. For the separation and detection, the capillary was patterned as demonstrated in Figure 4.7. Protein G was injected from the cathodic side and the profile demonstrated in Figure 4.7 was observed.

![Diagram of Protein G separation](image)

Figure 4.7 Separation of Alexaflour 488 labeled Protein G. The protein G was injected on the cathodic side of the capillary. Injection is 2 kV for 2 s. Separation is performed in a 25 µm i.d. capillary, with an total length of 30 cm and 10 cm from the cathodic side to detector. E=400 V/cm (normal polarity). Separation is aqueous 50 mM sodium acetate buffered to pH 5.
To demonstrate the online labeling of Herceptin with Protein G the capillary was set up as shown in Figure 4.8. Sodium chloride was incorporated in the separation to reduce non-specific interaction of the proteins with the column surface. Aggregates were observed as spikes in the electropherogram when sodium chloride was absent in background electrolyte. A dual opposite injection was utilized to introduce Herceptin and Protein G simultaneously in the capillary. Since the Herceptin is cationic at pH of 5 it was injected from the anodic side. The Protein G is anionic and was injected from the cathodic side. The Protein G has a higher mobility and has shorter distance to pass the detector, therefore the unbound Protein G peak was observed as the first peak. After passing the detector the Protein G was bound to the Herceptin. The higher charge to size ratio of Herceptin compared to Protein G dictated that the mobility of the Herceptin dominated the mobility complex. The labeled Herceptin was detected as a peak at 10 minutes. The position of the complex formation, and therefore the time it appears in electropherogram, was changed by changing the time Herceptin is injected in the capillary. If Herceptin was injected when Protein G had been pre-separated in the capillary, the complex would shift to faster times. In order to achieve reproducible separations a more rigorous flush between runs was introduced to strip any adsorbed protein from the capillary surface. This flush included 7.5 minutes of methanol 5 minutes of water, 7.5 minutes of base, 7.5 minutes of buffer, 20 minutes of coat and 5 minutes of buffer. This demonstrated the utility of the method of fluorescently label the Herceptin online before the transferase reaction. The principle is also applicable to identifying IgGs in complex mixtures.
Figure 4.8 Online labeling of Herceptin with Alexaflour 488 tagged Protein G. The Protein G was injected on the cathodic side and the Herceptin on the anodic side of the capillary. Injection is done at 2 kV for 2 s. Separation is performed in a 25 µm i.d. capillary, with a total length of 30 cm and 10 cm from the cathodic side to detector. E=400 V/cm (normal polarity). Separation is aqueous 50 mM sodium acetate buffered to pH 5 containing 40 mM sodium chloride.
4.3 Future directions

The preliminary data for both the online serial enzyme sequencing and the transferase enzyme modification demonstrated the possibility of extending the work reported in Chapter 2 and 3. The preliminary data in analyzing heat degraded samples with serial enzyme digestion demonstrated the method can be used to analyze heat or age degraded samples. Initial findings with α2-3 sialyl transferase data demonstrated that the online transferase modification can be extended to other glycosyltransferase enzymes. Furthermore, data in this chapter showed adaptability of method to different pH values. Online labeling of Herceptin with Protein G was demonstrated which opens avenues for increasing the enzyme to substrate ratio for the transferase reactions.

4.4 References


Appendix

Figure A-1. Migration time shifts based on the number of terminal galactose residues present in a biantennary standard N-Glycan after serial enzyme processing. Data associated with Figure 2.2B.
Figure A-2. Verification of the serial enzyme processing for the biantennary standard N-Glycan using α2-3 sialidase. Data associated with Figure 2.2C.
Figure A-3. Electropherograms achieved with 750 µUnits/µL of α2-3 sialidase in the enzyme range study for the biantennary standard N-Glycan. Data associated with Figure 2.3A.
Figure A-4. Electropherograms achieved with 250 μUnits/μL of α2-3 sialidase in the enzyme range study for the biantennary standard N-Glycan. Data associated with Figure 2.3B.
Figure A-5. Electropherogram achieved with 250 µUnits/µL of α2-3 sialidase plus MAL lectin in the enzyme range study for the biantennary standard N-Glycan. Data associated with Figure 2.3C.
Figure A-6. Electropherograms achieved with 5 µUnits/µL of α2-3 sialidase in the enzyme range study for the biantennary standard N-Glycan. Data associated with Figure 2.3D.
Figure A-7. Serial enzyme sequencing reference trace with α2-3,6,8 sialidase for AGP sample 1. Data associated with Figure 2.4A black trace.
Figure A-8. Serial enzyme sequencing reference trace with α2-3,6,8 sialidase and β1-3,4 galactosidase for AGP sample 1. Data associated with Figure 2.4A blue trace.
Figure A-9. Serial enzyme sequencing of AGP sample 1 in the absence of ECL.

Data associated with Figure 2.4B black trace.
Figure A-10. Serial enzyme sequencing of AGP sample 1 in the presence of ECL.

Data associated with Figure 2.4B blue trace.
Figure A-11. Electropherograms achieved with 750 µUnits/µL of α2-3 sialidase in the enzyme range study for AGP sample 1. Data associated with Figure 2.5 first trace.
Figure A-12. Electropherograms achieved with 250 µUnits/µL of α2-3 sialidase in the enzyme range study for AGP sample 1. Data associated with Figure 2.5 second trace.
Figure A-13. Electropherograms achieved with 250 µUnits/µL of α2-3 sialidase plus MAL in the enzyme range study for AGP sample 1. Data associated with Figure 2.5 third trace.
Figure A-14. Electropherograms achieved with 50 μUnits/μL of α2-3 sialidase in the enzyme range study for AGP sample 1. Data associated with Figure 2.5 fourth trace.
**Figure A-15.** Electropherograms achieved with 750 μUnits/μL of α2-3 sialidase in the enzyme range study for AGP sample 2. Data associated with Figure 2.6 first trace.
Figure A-16. Electropherograms achieved with 250 µUnits/µL of α2-3 sialidase in the enzyme range study for AGP sample 2. Data associated with Figure 2.6 second trace.
Figure A-17. Electropherogram achieved with 250 µUnits/µL of α2-3 sialidase plus MAL in the enzyme range study for AGP sample 2. Data associated with Figure 2.6 third trace.
Figure A-18. Electropherogram achieved with 250 µUnits/µL of α2-3 sialidase plus MAL in the enzyme range study for AGP sample 2. Data associated with Figure 2.6 fourth trace.
Figure A-19. Serial enzyme sequencing reference trace with α2-3,6,8 sialidase for AGP sample 2. Data associated with Figure 2.7A black trace.
**Figure A-20.** Serial enzyme sequencing reference trace with α2-3,6,8 sialidase and β1-3,4 galactosidase for AGP sample 2. Data associated with Figure 2.7A blue trace.
Figure A-21. Serial enzyme sequencing of AGP sample 2 in the absence of ECL.

Data associated with Figure 2.7B black trace.
Figure A-22. Serial enzyme sequencing of AGP sample 2 in the presence of ECL.

Data associated with Figure 2.7B blue trace.
Figure A-23. Electropherograms of the pentasaccharide in the absence of GaIT enzyme. Data associated with Figure 3.4B
Figure A-24. Electropherograms of the pentasaccharide in the presence of 2 mU/µl GalT enzyme. Data associated with Figure 3.4C
Figure A-25. Set 1 of electropherograms of the pentasaccharide used to achieve $K_M$ curve. Data associated with Figure 3.5 and Figure 3.7
Figure A-26. Set 2 of electropherograms of the pentasaccharide used to achieve $K_M$ curve. Data associated with Figure 3.5 and Figure 3.7
Figure A-27. Set 3 of electropherograms of the pentasaccharide used to achieve $K_M$ curve. Data associated with Figure 3.5 and Figure 3.7
Figure A-28. Set 1 of electropherograms of the pentasaccharide used to achieve the calibration curve. Data associated with Figure 3.6
Figure A-29. Set 2 of electropherograms of the pentasaccharide used to achieve the calibration curve. Data associated with Figure 3.6.
Figure A-30. Set 3 of electropherograms of the pentasaccharide used to achieve the calibration curve. Data associated with Figure 3.6
Figure A-31. Electropherograms showing addition of galactose residues to N-glycans recovered from Herceptin. Data associated with Figure 3.8
Figure A-32. Electropherograms showing confirmation of GalT modification with ECL of N-glycans recovered from Herceptin. Set 1 of data associated with Figure 3.9
Figure A-33. Electropherograms showing confirmation of GaIT modification with ECL of N-glycans recovered from Herceptin. Set 2 of data associated with Figure 3.9
**Figure A-34.** Electropherograms showing confirmation of GaIT modification with ECL of N-glycans recovered from Herceptin. Set 3 of data associated with Figure 3.9
Figure A-35. Electropherograms showing GaIT modification Herceptin glycoprotein. Set 1 of data associated with Figure 3.11
Figure A-36. Electropherograms showing GalT modification Herceptin glycoprotein. Set 2 of data associated with Figure 3.11
Figure A-37. Electropherograms showing GalT modification Herceptin glycoprotein. Set 3 of data associated with Figure 3.11
Enzyme : substrate calculation for N-glycan

Flow injection 70 s to cover 10.2 cm
Velocity 0.146 cm per sec
Injection 21 s which translates to 3.1 cm

volume = πr²h
= [π x (12.5x10⁻⁶ m)² x 0.0031 m
= 15.2 10⁻¹² m
= 15.2 x10⁻⁹ L = 15 nL

For the lowest concentration 15 nL x 0.1 mU/µL
= 15 nL x 0.1 µU/nL
= 1.5 µL

<table>
<thead>
<tr>
<th>Enzyme concentration vial</th>
<th>Amount in capillary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mU/µL</td>
<td>1.5x10⁻⁶ U</td>
</tr>
<tr>
<td>0.25 mU/µL</td>
<td>3.8x10⁻⁶ U</td>
</tr>
<tr>
<td>0.5 mU/µL</td>
<td>7.5x10⁻⁶ U</td>
</tr>
<tr>
<td>1 mU/µL</td>
<td>15x10⁻⁶ U</td>
</tr>
<tr>
<td>7 mU/µL</td>
<td>105x10⁻⁶ U</td>
</tr>
</tbody>
</table>

\[ Q_{N\text{-glycan}} = \frac{\pi r^2 \mu \text{Vt/L}}{[\text{Ci}]} \]
\[ \mu = (60x50)/(15.3x60x20,000) = 1.64x10^{-4} \]
\[ = \pi x (12.5x10^{-6} \text{ m})^2 \times 10,000 \times 1.64x10^{-4} \times 10s / 6x10^{-1} \text{ m}][\text{Ci}] \]
\[ = 8.1 \times 10^{-9} / 6x10^{-1} \text{ m}][\text{Ci}] \]
\[ = 13.4 \times 10^{-9} \text{ m}^3 [\text{Ci}] \]
\[ = 13400 \times 10^{-12} \text{ m}^3 [\text{Ci}] \quad (13400 \times 10^{-9} \text{ L injected}) \]
\[ = 13400 \times 10^{-9} \text{ L} \times 100 \times 10^{-9} \text{ moles/L} \]
\[ = 1.34 \times 10^{-12} \text{ moles} = 1.34 \text{ picomoles} \]

<table>
<thead>
<tr>
<th>[Enzyme]</th>
<th>Amount in capillary</th>
<th>Ratio enzyme to substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mU/µL</td>
<td>1.5x10⁻⁶ U</td>
<td>1.2 x10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>0.25 mU/µL</td>
<td>3.8x10⁻⁶ U</td>
<td>2.9 x10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>0.5 mU/µL</td>
<td>7.5x10⁻⁶ U</td>
<td>5.8 x10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>1 mU/µL</td>
<td>15x10⁻⁶ U</td>
<td>12 x10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>7 mU/µL</td>
<td>105x10⁻⁶ U</td>
<td>81 x10⁶ U E: 1 mole S</td>
</tr>
</tbody>
</table>

197
Calculation of enzyme to substrate ratio for Herceptin glycoprotein

Flow injection 70 s to cover 20 cm
Velocity 0.286 cm per sec
Injection 10.5 s which translates to 3 cm

Volume = πr₂h
= [π x (12.5x10⁻⁶ m)² x 0.03 m
= 14.7 10⁻¹² m
= 14.7x10⁻⁹ L = 15 nL

Amount in capillary for lowest concentration = 15 nL x 0.345 mU/µL
= 15 nL x 0.345 µU/nL
= 0.345 µU = 5.2 µU

<table>
<thead>
<tr>
<th>Enzyme concentration vial</th>
<th>Amount in capillary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.345 mU/µL</td>
<td>5.2x10⁻⁶ U</td>
</tr>
<tr>
<td>0.690 mU/µL</td>
<td>10.4x10⁻⁶ U</td>
</tr>
<tr>
<td>1.13 mU/µL</td>
<td>17x10⁻⁶ U</td>
</tr>
<tr>
<td>1.38 mU/µL</td>
<td>21x10⁻⁶ U</td>
</tr>
<tr>
<td>5 mU/µL</td>
<td>75x10⁻⁶ U</td>
</tr>
</tbody>
</table>

Q_{herceptin} = [πr² μ_{app} V_{int} t_{int} / L] [C_i]
μ_{app} = (30x10) / (15.8 x 60 x 12,000) = 2.7 x 10⁻⁵ m²/Vs

= [π x (12.5x10⁻⁶ m)² x 5,000 x 2.7 x 10⁻⁵ x 4s / 3x10⁻¹ m] [C_i]

= [2.7 x 10⁻¹⁰ / 3x10⁻¹ m] [C_i]

= 9 x 10⁻¹⁰ m³ [C_i]

= 900 x 10⁻¹² m³ [C_i]

= 900 x 10⁹ l [1 x 10⁻⁶ moles/L]
= 9E-13 moles with 2 glycans per her 1.8 picomoles glycan

<table>
<thead>
<tr>
<th>[Enzyme]</th>
<th>Amount in capillary</th>
<th>Ratio enzyme to substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.345 mU/µL</td>
<td>5.2x10⁻⁶ U</td>
<td>2.9 x 10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>0.690 mU/µL</td>
<td>10.4x10⁻⁶ U</td>
<td>5.8 x 10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>1.13 mU/µL</td>
<td>17x10⁻⁶ U</td>
<td>9.5 x 10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>1.38 mU/µL</td>
<td>21x10⁻⁶ U</td>
<td>12 x 10⁶ U E: 1 mole S</td>
</tr>
<tr>
<td>5 mU/µL</td>
<td>75x10⁻⁶ U</td>
<td>42 x 10⁶ U E: 1 mole S</td>
</tr>
</tbody>
</table>
Figure A-38. Flow injection analysis done by pushing APTS with 10% nanogel. The 30 cm capillary used for Herceptin glycoprotein analysis was filled with 10% nanogel at 10 psi. A plug of 2AB was introduced using a 0.5 psi 5 s injection. The 2AB was then pushed a distance of 20 cm to pass the detection window. Push performed with 10% nanogel at 19 °C using a pressure of 10 psi.
Curriculum Vitae

Lloyd Bwanali
828 Idlewood Drive, Morgantown, WV, 26505
681-285-8324 llbwanali@mix.wvu.edu

Education
2015 – 2021: Ph.D. – Analytical Chemistry, WVU, Morgantown, WV, USA
Research focus: Employing Exoglycosidase And Transferase Enzymes in Capillary Nanogel Electrophoresis for the Determination of N-glycan Linkages and Enzyme Michaelis-Menten Constants, Advisor: Dr. L.A. Holland

2012 – 2014: Master in Business Administration –, NUST, Bulawayo, Zimbabwe

2004 – 2008: BSc. (Hons.) – Applied Chemistry, NUST, Bulawayo, Zimbabwe
Final year project topic: “Analysis, evaluation and monitoring of groundwater in industrial areas of Bulawayo, Advisor: Dr. C.T. Parekh

Publications


Presentations


Professional Experience

- 2015-2021 Graduate Research Assistant, West Virginia University, WV, USA
- 2015 Graduate Teaching Assistant, West Virginia University, WV, USA
- 2008-2015 Manager, Bestafoam Pvt Ltd, Harare, Zimbabwe

Positions and Awards

- 2017- current: Member of American Chemical Society
- August 2018: John R. Conard Scholarship in Chemistry, WVU
- October 2007: National University of Science and Technology Book Prize